The application of loop mediated isothermal amplification for the detection of the sexually transmitted pathogens *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and *Trichomonas vaginalis*, at the point of care

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Abbreviations

A_{260}	absorbance measured at the 260nm wavelength
A_{280}	absorbance measured at the 280nm wavelength
ATTC	American Type Culture Collection
ATP	adenosine triphosphate
BASHH	British Association of Sexual Health and HIV
BHI	brain heart infusion
BLAST	basic local alignment search tool
bp	base pairs
BiP	backwards inner primer
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
CL^-	chloride ions
Ct	cycle threshold
CT	*Chlamydia trachomatis*
Da	dalton
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EB	elementary body
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme- linked immunosorbent assay
ER	Endoplasmic reticulum
EtOH	ethanol
FDA	Food and Drug Administration
FiP	forward inner primer
FWD	forward
GC	gonnococcus (*Neisseria gonorrhoeae*)
GuCN	guanidine isothiocyante
GuHCl	guanidine hydrochloride
HIV	human immunodeficiency virus
HCl	hydrochloric acid
HPV	human papillomavirus

IPA	isopropanol
K^+	Potassium ions
LAMP	Loop-mediated isothermal amplification
LCR	ligase chain reaction
LJMU	Liverpool John Moores University
MG	*Mycoplamsa genitalium*
NA	nutrient agar
Na^+	Sodium ions
NAAT	nucleic acid amplication test
NASBA	nucleic acid sequence based detection
NCBI	National Centre for Biotechnology Information
OD_x	Optical density at wavelength of X nanometres
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHE	Public Health England
POC	point of care
PVP	polyvinylpyrrolidone
qPCR	quantitative PCR
RB	reticulate body
RFU	relative Fluorescence Units
RNA	ribonucleic acid
RNase	ribonuclease
RPA	recombinase polymerase amplification
rRNA	ribosomal RNA
RT	reverse transcriptase
rt-PCR	real-time PCR
RVS	reverse
SDA	strand displacement amplification
SEM	scanning electron microscope
STD	sexually transmitted disease
STI	sexually transmitted infection
Ta	Time to amplification

TAE	tris acetate EDTA buffer
TE	tris EDTA buffer
TM	transport media
TMA	transcription mediated amplification
Tris	tris(hydroxymethyl)aminomethane
TV	*Trichomonas vaginalis*
TVV	*Trichomonas vaginalis* virus
UoL	University of Liverpool
UV	ultra violet
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation

i) Abstract

The purpose of this multi-partnered project was the production of a fully integrated POC system, combining automated nucleic acid extraction in a centrifugally operated microfluidic disk (the LabDisk), with loop mediated isothermal amplification (LAMP) and optical detection, capable of detecting the sexually transmitted pathogens *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Trichomonas vaginalis* in clinical urine and swab samples. LAMP is a novel nucleic acid amplification method, designed to amplify target nucleic acid in a highly specific and rapid manner, under isothermal conditions.

The work detailed in this thesis presents the development of a rapid total nucleic acid extraction process, based on the capture of target nucleic acid by magnetic silica beads, optimised for use on the LabDisk platform. The extraction process was capable of the purification of target nucleic acid from a clinical sample within 5 minutes, and was robust when challenged with a range of inhibitory compounds potentially encountered in samples for STI testing. The system was capable of tolerating *N. gonorrhoeae* (1×10^5 CFU/ml) urine suspensions containing samples containing 50% total blood volume, 1×10^8 *E. coli* cells per ml, and 10mg/ml of BSA, without any effects on the downstream amplification time of the *N. gonorrhoeae* specific LAMP assay. A freeze dried lysis buffer pellet was developed, that was able to increase the sample volume, thereby decreasing the time to detection, whilst minimising the stored fluid volume on the LabDisk.

LAMP assays were designed for the detection *N. gonorrhoeae* and *M. genitalium*, and the limits of detection and specificity of the assays were evaluated. *The N. gonorrhoeae* ORF1 assay was able to detect a minimum of 20 copies of the *N. gonorrhoeae* genome per reaction, whilst the *M. genitalium pdhD* assay was capable of detecting 16 genome copies. The tolerance of the ORF1 LAMP assay to urea, and blood, was found to be 1.8M, and 20% reaction volume, respectively. The increased tolerance of the LAMP assay to these inhibitors in comparison to PCR demonstrates the suitability of LAMP when processing urine samples for STI's.

To our knowledge this is the first application of LAMP technology for the detection of these organisms, and the first attempt at commercialising a fully integrated molecular diagnostics system based on LAMP.

ii) Project overview

This work details our contribution to a multi partner, Technology Strategy Board (TSB) funded, project “Fighting Infection Through Detection”, which was carried out with the purpose of designing a point of care (POC) nucleic acid amplification testing (NAAT) system for the simultaneous detection of a number of sexually transmitted pathogens from urine and swab samples. The remit of the original grant call required the system to be fully automated , integrating every step of the assay process, including nucleic acid extraction, and target amplification and detection, in order to provide a “sample in, answer out” process. The target organisms were *Chlamydia trachomatis* and *Neisseria gonorrhoeae*; the most common bacterial sexually transmitted pathogens, and significant public health concerns. At a later stage the organisms *Mycoplasma genitalium* and *Trichomonas vaginalis* were added to the target s of the assays. The amplification step was carried out using loop mediated isothermal amplification (LAMP); a rapid isothermal DNA amplification technology, which provided a reaction fast enough to be utilised in a point of care setting, and without the requirement of expensive thermal cycling equipment.

At the end of the first year of the project, it was decided that the assay would be processed using LabDisk; a centrifugally operated microfluidic disk system designed by IMTEK (Freiburg, Germany). The rights to use a LabDisk designed for the processing of a 1ml sample using a magnetic silica bead based extraction method were purchased from IMTEK.

ii.i) Project partners

Mast Group Ltd, Liverpool, UK

Mast Group Ltd was the commercial project partner, and had originally applied for TSB funding for the project. Their responsibilities in the project involved determination of the machine requirements, manufacturing, technical consultation and development of the LAMP reaction mix.

Public Health England, South West Virology lab (PHE)

PHE were tasked with LAMP assay design, the testing of LAMP assays with clinical samples, and laboratory trials of the prototype and finished machines. They designed a number of LAMP assays utilised during the work produced in this thesis; the *C. trachomatis* 0332 LAMP assay and 16S rRNA

taqman assay, the *N. gonorrhoeae PorA* and *GroEL* LAMP assays, the *M. genitalium* 16S rRNA assay and the *T. vaginalis* DNA rpt assay.

University of Liverpool, Department of Electrical Engineering and Electronics (UoL)

UoL were responsible for the design of the electrical circuitry and control units of the device, software programming, and development of the optical detection system.

Liverpool John Moores University, School of Pharmacy and Biomolecular Sciences (LJMU); the research described in this thesis

During this work I developed a total nucleic acid extraction procedure, based around the magnetic silica solid phase extraction methodology used on the King Fisher Ml platform. The developed methodology will then be integrated into the microfluidic LabDisk platform by MAST Group Ltd. The development process involved optimising the component concentrations of the lysis and wash buffers, determining the required incubation times, and investigating the use of a heat shock during the lysis process to increase the speed of the procedure. I was then able to develop stable freeze dried lysis pellets to minimise stored fluid volumes on the LabDisk system. I carried out an inhibitor study, by challenging the system with a range of common inhibitory substances than may be expected in clinical STI samples, such as blood and protein, in order to demonstrate the robustness of the methodology. The suitability of the system to process all of the target organisms was demonstrated. Additionally I evaluated a range of swab types and transport media, in order to optimise the sample acquisition process.

My research also included the design and evaluation of two LAMP assays; the *N. gonorrhoeae* ORF1 LAMP assay, and *M. genitalium pdhD* LAMP assay. The *M. genitalium pdhD* LAMP assay was compared with two commonly used PCR assays from the literature, in a small sensitivity study. I then investigated the tolerance of the *N. gonorrhoeae* ORF1 LAMP assay to urea and blood, and demonstrated the ability of this assay to use heated urine lysates as a sample, without negatively impacting assay sensitivity. During the work, I also determined the limits of detection of all the LAMP assays produced by myself and the PHE during the course of the project.

Chapter 1

Sample preparation in a microfluidic device for the detection of sexually transmitted pathogens in urine and swab samples, at the point of care

1.1. Introduction

Point of care (POC) is an emerging field of medical diagnostics, involving the testing of patient samples at or near the patient site. Typically this ranges from tests performed directly at the patients bedsides, in a physician's office, operating theatres or outside a medical setting entirely; for example at the sites of accidents or in remote areas (Junker *et al.*, 2010). The primary advantage of this decentralization of diagnostic testing away from a dedicated regional or hospital laboratory is the dramatic reduction seen in time between sample acquisition and analysis (turnaround time). Performing diagnostic tests at the point of care typically reduces pre-analysis sample transport and processing and removes post analysis result validation, report transfer and data entry (St-Louis, 2000). This reduction in turnaround time enables a more rapid diagnosis, leading to improved patient management and the faster onset of an appropriate treatment programme (Bissonnette and Bergeron, 2010). In a hospital setting, this reduction in turnaround time can minimise length of hospital stay, reduce outlay of resources and ensure optimum use of professional time. POC tests are required to be as simple as possible, requiring minimal sample or reagent handling, ideally providing a 'sample in, answer out' system (Easley *et al.*, 2006). A key characteristic of POC testing is the removal of the need for skilled technical staff; the tests are generally carried out by healthcare professionals with minimal laboratory training, or in the case of a handful of home testing kits (e.g. blood glucose monitoring), the patient themselves (Holland and Kiechle, 2005). However, it worth noting that the effectiveness of POC tests depends to some extent on their correct application by the user, which depends on the ease of use of the system and also the provision of any required training.

POC testing can be particularly valuable in diagnosing conditions in which rapid treatment onset is greatly beneficial to clinical outcomes, such as sepsis (Kemmler *et al.*, 2009). In fact, POC testing is only of any benefit if the results produced will lead to immediate therapeutic decisions; otherwise less costly and, generally speaking, more accurate lab based diagnostics remain the optimum solution. For example, testing for the anticoagulation status of patients taking Heparin, an anticoagulant, was found to be more accurate when carried out in a centralised laboratory, compared to POC testing. However, POC testing dramatically reduced turnaround time and enabled clinicians to alter the treatment regime much more rapidly, improving patient care (Solomon *et al.*, 1998). Economic factors also play a part in determining an appropriate situation for POC testing. POC tests are typically more expensive on a result per patient basis than tests taking place in a centralised diagnostic laboratory (St-Louis, 2000). POC testing for glucose levels in diabetes patients has been shown to lie variably between 1.1 to 4.6 times the cost for the laboratory based

counterpart (Nosanchuk and Keefner, 1995). However, despite this, POC blood glucose measurement is now becoming widespread, due to the improvement in patient satisfaction and effective monitoring. The importance of determining the cost effectiveness of developing a POC programme before implementation has been recognised, and various studies have provided evaluations of prospective disease areas, such as HIV in Africa (Vickerman *et al.*, 2006). One of the most common class of POC diagnostic tests currently in use are lateral flow tests, the best known example of which is the home pregnancy test. These immunochromatographic assays rely on capillary action to draw the sample through a membrane or strip of paper, where it reacts with labelling and capture reagents in order to form a visible mark indicating a positive result (Chin *et al.*, 2012). Lateral flow tests are commonly used in developing countries for pathogen detection, due to their low cost and ease of use, for targets including malaria and HIV (Murray *et al.*, 2008; Vijayakumar *et al.*, 2005).

Currently, the most frequently employed individual POC test is the blood glucose test, used for diabetes screening and home blood glucose monitoring by diabetic or hypoglycaemic patients. Home blood glucose monitoring enables sufferers of type 1 diabetes to frequently assess their own glucose levels in order to determine an appropriate Insulin dose. This diagnostic tool provides them with the necessary information for effective self administration of medication, minimising the impact of the disease on the patient. These tests rely on invasive blood sampling, typically requiring a drop of fingerstick blood. A Biochemical reaction (reliant on either Glucose Oxidase, Glucose Dehydrogenase or Hexokinase activity) is then used to determine blood glucose (Kiechle and Main, 2000). The majority of POC tests for blood glucose measure plasma glucose levels rather than whole blood glucose levels, due to difficulties in lysing the red blood cells prior to the reaction. This can present problems in patients with an abnormally low or high proportion of red blood cells in their whole blood (haematocrit extremes), leading to an over or underestimation of glucose concentration (Kiechle and Main, 2000). Despite this reduced accuracy, POC monitoring is seen as the optimum disease management solution due to the benefits it brings to the life of the patient.

POC testing has also been applied to microbial diagnostics, with assay development focused on the detection of pathogens which cause disease of which the clinical outcome will greatly benefit from early diagnosis and treatment. For example, POC testing for the causative bacteria of respiratory infections, such as *Streptococcus pneumoniae*, *Legionella pneumophila* and *Haemophilus influenzae* is of great benefit towards patient outcomes (Charles and Grayson, 2007). This is due to both the difficulty in detection of the organisms by standard culture methods, rapid disease progression in absence of treatment, and rapid response to treatment if administered during early stages of

infection. A clinically used antigen test for legionella detection in urine samples has a sensitivity of 94%, and specificity of ~100%. This is compared with culture which has a specificity of between 10% and 80% (due to difficulty of culturing the organism), and takes 3 – 7 days (Stürenburg and Junker, 2009). Pneumonia caused by legionella can be fatal without an appropriate antimicrobial therapy, requiring antibiotic agents not indicated by other causes of pneumonia, and POC testing enables this to be administered much faster, significantly improving disease prognosis (Stürenburg and Junker, 2009). Sepsis, a bacterial infection of the blood, urine or skin, resulting in a whole body inflammatory state, is another medical condition in which rapid diagnosis is vital. A study of the outcome of 2154 sepsis patients (Kumar *et al.*, 2006) showed that the survival rate of 83% for patients treated within 1 hour of the onset of symptoms dropped by 7% for every hour after that. Although the need has been identified, difficulties have been encountered developing a rapid POC test for sepsis diagnosis, and the fastest option remains the clinical observation of systemic inflammatory response syndrome, confirmed via nucleic acid amplification tests (NAAT), such as PCR. However studies have been carried out to identify suitable biomarkers for POC testing, typically components of the inflammatory response system (Meybohm *et al.*, 2013).

The largest challenge facing the development of POC diagnostics for routine clinical use is the difficulty in coupling the characteristics which enable POC use, e.g. the lack of need for specially trained staff or expensive equipment, with the sensitivities required for the test to be a valuable tool clinically. Currently available rapid diagnostic tests carried out at point of care offer a poorer performance in both sensitivity and specificity compared with molecular diagnostic testing carried out by skilled practitioners in a dedicated laboratory environment (Bissonnette and Bergeron, 2010).

1.1.1 POC nucleic acid testing

Nucleic acid testing (NAT) is currently routinely carried out for the detection of infectious disease, most frequently in the form of PCR based tests. PCR is an enzyme driven process, allowing for the exponential amplification of short nucleotide sequences *in vitro* (Mullis and Faloona, 1987). The process requires a pair of oligonucleotide primers; short nucleic acid sequences designed to be complementary to the 3’ end of both the sense and antisense strands of the DNA target, in combination with a further source of deoxynucleotide triphoshpates (dNTPs) and a thermostable DNA polymerase. Thermocycling is employed to change the state of the reaction to allow for DNA melting, primer annealing, and subsequent extension, which results in a duplication of the original

target strand. As the reaction progresses the generated DNA fragments are also used as targets for amplification, resulting in an exponential increase of target DNA per cycle of extension. A successful amplification can be detected at the end point of the reaction, for example by separation of DNA fragments using agarose gel electrophoresis. The detection can also occur in real time, using intercalating dyes or specific oligonucleotide probes to generate a signal which is detected as the amplification proceeds, removing the need for post-PCR processing. This is known as Real-time PCR, which requires more complex equipment than standard PCR methods, as the detection system is integrated into the thermocycler.

A distinct advantage of PCR based diagnostic methods, when compared with non molecular options such as immunoassays, are the higher sensitivities achievable. The limit of detection of commercial Real-time PCR assays is typically ~10 copies per reaction (EGFR PCR kit, Qiagen; Herpes Simplex Virus LHSV PCR test, Mayo Medical Laboratories), which is in agreement with levels widely reported in the literature (Poon *et al.*, 2003; Welzel *et al.*, 2006). A recent comparison of a rapid antigen immunoassay (Direct Antigen 1-2-3 Group A Strep test kit, BD Diagnostic Systems, Sparks, Md.) and a Real time PCR assay (LightCycler Strep A assay, Roche Applied Science, Indiapolis, Ind) for the detection of Group A Streptococci (Uhl *et al.*, 2003), found the sensitivity of the PCR based method to be 93%, compared with 55% for the immunoassay. This difference in sensitivity is typical between the two assay types. Selection of a DNA target that occurs at more than one point in the target organisms genome, or a sequence included in a gene residing in a multi-copy plasmid, can increase the ratio of the detectable target to the pathogen itself, further increasing specificity (Roorda *et al.*, 2011).

Reverse transcription PCR (RT-PCR) is a PCR variant allowing for the detection of RNA, which is converted into a complementary DNA copy before amplification by the inclusion of a reverse transcriptase enzyme in the reaction tube (Bustin, 2002). As well as having a massive impact on the study of RNA and gene expression, this technique has also lead to improvements in NAT. Most metabolically active pathogenic organisms will contain high levels of specific mRNA transcripts, and by targeting these with specific primers during RT-PCR, a much higher starting copy number is achieved. This results in an increase in assay sensitivity and an improvement in the limit of detection. RT-PCR also allows for nucleic acid amplification from clinically relevant RNA viruses, such as HIV and Hepatitis B.

PCR based methods also allow for multiplexing, where multiple primer sets are included in each assay, allowing for simultaneous detection of multiple targets. Detection can be carried out at the reaction end point via agarose gel electrophoresis, as long as the product from each primer set is of

a different length. Alternatively, specific probes can be used to track the amplification of each primer set in real time, although this does limit the number of targets per assay. Multiplex PCR tests have been developed for the simultaneous detection of multiple pathogenic bacteria species from a single sample, including the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, Mycoplasma *genitalium*, *Ureaplasma urealyticum*, *Corynebacterium spp*. and *Pseudomonas aeruginosa* from urine samples (Lee *et al.*, 2007), and the detection of seven respiratory viruses in sputum samples (Hindiyeh *et al.*, 2001).

1.1.2. Isothermal methods

A major advancement in the field of NAAT and molecular diagnostics has been the development of novel isothermal amplification methodologies, capable of amplifying specific nucleic acid targets without the use of thermal cycling required by PCR. The ability of isothermal assays to operate at a single reaction temperature removes the need for expensive thermal cycling equipment, as the assays can be carried out in a water bath, or single temperature heating block. This increases the portability of the instrumentation required to process the assay, making isothermal reactions more amenable to POC testing. Isothermal assays are also typically quicker than standard PCR based assays, as a single reaction temperature allows the reaction to proceed at a maximum speed, without regular temperature changes (Gill and Ghaemi, 2008).

Recombinase polymerase amplification (RPA) is an isothermal amplification technology that relies on the actions of three proteins; a recombinase, a strand displacement polymerase, and a single-stranded DNA binding protein (SSB) (Euler *et al.*, 2012), and sold commercially by TwistDx (UK). The recombinase, polymerase and SSB form a complex, and catalyse the unwinding of the DNA template, recombinase-mediated primer annealing, and subsequent DNA synthesis mediated via the actions of the polymerase (Daher *et al.*, 2014). The reaction requires a set of two primers, upstream and downstream of the amplicon, which are easily designed, in a similar fashion to PCR primers (Euler *et al.*, 2013). The reaction can operate between 25^{o}C and 42^{o}C, meaning it has modest heating requirements for operation, and also withstands temperature fluctuations between these values. RPA can be monitored in real-time via the inclusion of Exo probes (TwistDx, UK), which generated a fluorescence signal during DNA synthesis, which can be monitored by a fluorometer. A real-time reverse transcription RPA assay for foot and mouth disease virus (FMDV) has been developed, with a sensitivity of 98% and specificity of 100% when testing clinical samples (Abd El Wahed *et al.*, 2013).

The study found that the assay would be easily transportable to quarantine stations or farms, providing the benefits of sensitive molecular testing, with a minimum waiting time enabling instant results.

The novel combination of an RPA assay, coupled with the visual detection of amplified product using a lateral flow dipstick, has shown promise as a POC assay. A test for malaria designed in this manner was shown to have a limit of detection of four malaria parasites per test, a 100% specificity and sensitivity in the small sample tested, and provided a result within 15 minutes (Kersting *et al.*, 2014). The test requires that the RPA reaction is carried out first as a standard tube assay, although by using freeze dried reagents the only pipetting step required is the introduction of the sample. After a 15 minute incubation, the sample is diluted in buffer, and the dipstick is added to the solution. The reaction is visualised by the binding of the labelled amplicon binding to polyclonal anti-carboxyfluorescein antibodies conjugated to gold nanoparticles, manifesting as a visible line on the dipstick (Kersting *et al.*, 2014).

Helicase-dependent amplification (HDA) is another isothermal amplification technology, and is modelled after the *in vivo* process of DNA replication, utilising a DNA helicase to separate double stranded DNA into single strands (Vincent *et al.*, 2004). A set of sequence specific oligonucleotide primers is then used to hybridise to the single stranded target, followed by DNA polymerase mediated DNA synthesis. The reaction is maintained at 37^{o}C, and requires no temperature changes. Like PCR, detection can either be carried out using end-point agarose gel electrophoresis, or in real-time via the inclusion of fluorescent probes or intercalating dyes such as SYBR green (Vincent *et al.*, 2004; Goldmeyer *et al.*, 2007). A HDA assay for toxigenic *Clostridium difficile* has been developed, with detection carried out using a Type 2 BESt cassettes (BioHelix, US), a lateral flow device enabling visual detection of the target and control amplicons. The test requires prior DNA extraction from fecal samples, which would prevent its use at the true POC, but same day results are available using the test (Chow *et al.*, 2008). The limit of detection of the assay was found to be 20 genome copies per reaction, which translated to a clinical sensitivity of 100%, and specificity of 100% compared with a PCR test. The combination of a HDA assay for *Mycobacterium tuberculosis* with a novel nanoparticle based detection method has shown potential as a near patient testing assay, making use of a portable handheld potentiostat (Torres-Chavolla and Alocilja, 2011). The integration of a HDA assay within a fully integrated microfuidic chip combining solid phase DNA extraction with HDA amplification and optical detection of fluorescence has been demonstrated (Mahalanabis *et al.*, 2010). The chip based assay was able to detect 10 CFU of *E. coli*, required no manipulation after

sample application, and the disposable nature of the closed chip removes the risk of amplicon contamination for subsequent assays.

Two of the earliest isothermal amplification methods to be developed were transcription mediated amplification (TMA), and nucleic acid sequence based amplification (NASBA), which both function via similar principles, and were originally designed for the amplification of RNA, but can be modified to target a DNA sequence (Guatelli *et al.*, 1990; Compton, 1991). In both reactions an initial thermal denaturation step separates double stranded RNA, allowing the annealing of an ssDNA primer which carries a 5' T7 polymerase promoter. The synthesis of a complementary DNA strand from the attached primer is carried out via the action of a reverse transcriptase, producing an RNA/DNA heterodimer. In the TMA reaction the reverse transcriptase enzyme hydrolyses the original RNA portion of the heterodimer, whilst in the NASBA reaction this occurs via the action of RNase H. The remaining ssDNA strand from the heterodimer then anneals to the second primer, with DNA synthesis occurring via the reverse transcriptase, producing a dsDNA strand tagged with the T7 polymerase promoter. The recognition of this promoter results in the T7 RNA polymerase enzyme producing multiple ssRNA copies from this dsDNA template, feeding back into the reaction. TMA has been appropriated by GenProbe (US) for use in their commercially available Aptima assays, which can be run on their high throughput automated systems such as the Tigris and Panther. Available FDA approved assays include the Aptima Combo 2, for the simultaneous detection of *C. trachomatis* and *N. gonnorhoeae* (Gaydos *et al.*, 2003), a HPV assay (Dockter *et al.*, 2009)and trichomoniasis assay (Andrea and Chapin, 2011). A number of research use only assays are available, including one for *M. genitalium* detection (Hardick *et al.*, 2006). The sensitivity and specificity of the Aptima assays are comparable to other NAATS, with sensitivity values typically ranging between 94% and 100% depending on the assay and sample type (Gaydos et al., 2003; Hardick et al., 2006; Andrea and Chapin, 2011).

Strand displacement amplification is another isothermal amplification technology, which utilises the activity of a restriction enzyme to create a "nick" in the dsDNA template, from which a polymerase enzyme can extend the 3' end of the nick, displacing the downstream strand (Walker *et al.*, 1992). The reaction product of the sense displacement becomes the template for the anti-sense equivalent, and vice versa, creating an exponential amplification of the target sequence. Becton Dickinson (US) produces a range of commercially available SDA assays for use in conjunction with their BD ProbeTec ET instrument, for a range of targets. The combined *C. trachomatis* and *N. gonorrhoeae* assay has sensitivities of 92% for *C. trachomatis* and 96.4% for *N. gonorrhoeae*, and the assay specificties were

96.1% and 99% for the *C. trachomatis* and the *N. gonorrhoeae* assays respectively (Cosentino *et al.*, 2003).

1.1.3. Loop mediated isothermal amplification

Loop-Mediated Isothermal Amplification (LAMP) is a novel nucleic acid amplification method, designed to amplify target nucleic acid in a highly specific and rapid manner, under isothermal conditions (Notomi *et al.*, 2000). A strand displacement DNA polymerase is used in conjunction with a specially designed set of four primers (forward primer: F3, backward primer: B3, Forward inner primer: FiP, Backward inner primer: BiP), specific to a total of 6 distinct regions of the target DNA sequence. Detection can be carried out in real time using a fluorescent intercalating dye in conjunction with a real time detection platform, such as the ESEquant (Qiagen, US), or by visual end point detection. Additionally, detection can be carried out by monitoring the reaction turbidity, due to the generation and subsequent precipitation of magnesium pyrophosphate, which increases during the reaction. LAMP can be used to amplify a DNA target, or amplify an RNA target via the addition of a reverse transcriptase to the reaction mix (Parida *et al.*, 2008).

The LAMP reaction is initiated with one of the F3 or B3 primers annealing to a complementary strand of double stranded DNA. The strand displacement polymerase then initiates DNA synthesis, displacing and releasing a single strand of DNA (Fig.1.2., 1). A DNA strand complementary to the template DNA is then synthesised from the 3’ end of the F2 region of the FIP (2). The F3 primer then anneals to the template DNA, just outside of the FiP. DNA synthesis occurs, displacing the FiP linked complementary strand, and forming a double strand from the F3 primer (3,4). The strand synthesised from the FiP is released and forms a stem loop structure at the 5’ end due to complementary F1c and F1 regions (5,6). This strand then forms the target for B3 and BiP strand displacement DNA synthesis, forming a double strand of DNA with a linear structure, ultimately resulting in the displacement of the BiP linked complementary strand (6,7). The strand forms a structure with stem loops on both ends, forming a characteristic barbell shape. This serves as the starting point for the amplification cycling in the LAMP reaction.

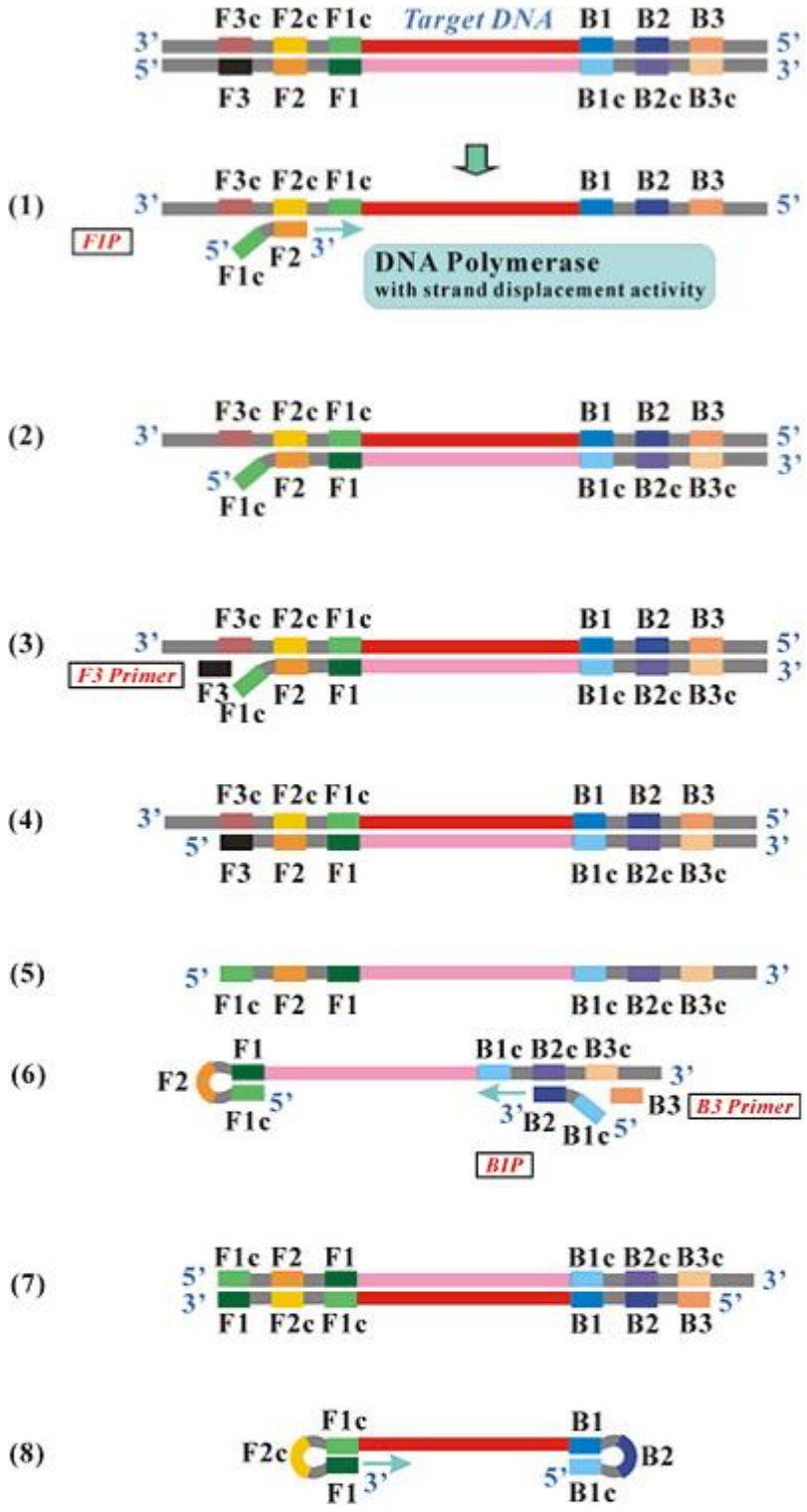

Fig 1.1. (1-8). Diagram showing progression of LAMP reaction from a DNA target. Images taken from the Eiken website: loopamp.eiken.co.jp, with permission from the author.

The dumbbell like starting structure is then converted into a stem loop DNA strand via self primed DNA synthesis. FiP strand displacement DNA synthesis releases the previously synthesised strand, which then forms a stem loop structure due to complementary B1c and B1 regions (9). The FIP linked complementary strand is then released due to further strand displacement, forming a dumbbell structure due to the complementary regions at both the 3' and 5' end (11). The result is a single stem loop structure, as at the start of the reaction, in addition to a stem loop structure which is twice as long (10). BiP and B3 mediated synthesis then act to create similar structures, and DNA synthesis continues by displacing double stranded DNA. As a result, various sized DNA amplicons are formed by alternately inverted repeats of the original target sequence.

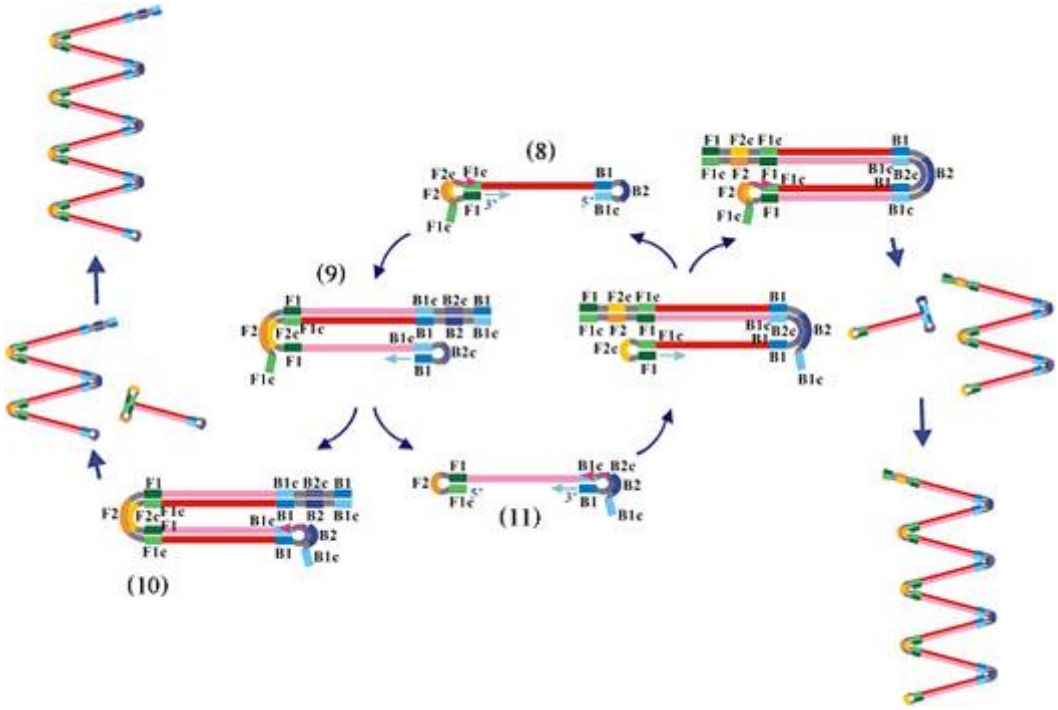

Fig.1.1. (8-19). Diagram showing progression of LAMP reaction from a DNA target. Images taken from the Eiken website: loopamp.eiken.co.jp

The reaction is capable of producing 10^9 copies of a DNA target from less than 10 original copies, in an hour (Notomi *et al.*, 2000). One of the biggest benefits of this method in comparison to standard PCR is the increase in specificity seen, due to the use of 4 primers specific to 6 separate regions of the target sequence. Previous studies have shown it to have a tenfold lower limit of detection than a PCR assay for the same target sequence, and also be less influenced by inhibitory compounds in clinical samples (Kaneko *et al.*, 2007). This makes the assay very well suited for pathogen detection

in positive samples with potentially low organism load. A LAMP assay for the detection of *Listeria monocytogenes* from food samples was determined to have a limit of detection of 6 CFU per reaction tube, lower than that of a standard PCR assay (Shan *et al.*, 2012). Both the sensitivity and specificity over 94 samples was found to be 100%. However, it is thought the sensitivity of the assay can differ from assay to assay, possibly due to primer sequence, as a minority of studies have shown sensitivity to be no greater than PCR (Jenkins *et al.*, 2011). LAMP assays have been described for a wide range of target pathogens including bacteria (Ueda and Kuwabara, 2009) DNA viruses (Enomoto *et al.*, 2005), RNA viruses (Parida *et al.*, 2008), and protozoa (Koloren *et al.*, 2010). During LAMP assay deisgn, reaction products can be confirmed as being specific to the intended target by digesting the amplicon with a suitable restriction endonucleases, and examining the product size using agarose gel electrophoresis, or sequencing the product.

Modifications of the original LAMP process have been described, involving the addition of extra primer sets. The most common of these are loop primers, which can be designed using the free LAMP primer design software package from Eiken. Loop primers hybridise to the stem-loops of the product of the initial phase of the LAMP reaction, generating a larger amplicon and hastening DNA synthesis (Nagamine *et al.*, 2002). The inclusion of these primer sets has been shown to reduce the time to detection in real time assays by up to 50% (Nagamine *et al.*, 2002). The ability to include these primers depends on the sequence of the stem loops, and their sequence length, as the primers must sit between the B2 and B1 sites, or F2 and F1 sites (Gandelman *et al.*, 2011).

In order to provide a more flexible method of accelerating reaction speeds, the use of stem primers has been suggested (Gandelman *et al.*, 2011). These primers can be used with or without loop primers, and provide a similar increase in amplification speed and generated product. Stem primers bind to the stem structures of the LAMP amplicon, can be designed in sense or antisense orientation, do not rely on the positioning of the B1/F1 or B2/F2 sites, and can be multiplexed, enabling greater flexibility in primer design that the more commonly used loop primers.

One significant advantage of LAMP over conventional PCR is the constant reaction temperature (typically between 63^{o}C and 67^{o}C). This removes the need for expensive thermocycling equipment as the reaction can be carried out in a thermal block or even a water bath. A recent innovation was the use of a disposable pocket warmer to provide the necessary reaction temperature for a LAMP assay for the detection of *Bacillus anthracis* (Hatano *et al.*, 2010). This test was designed for field use in developing countries or for use by the military, and requires no electricity to run. Identical limits of detection were seen when using the pocket warmer or a hot block. This provides a way of carrying

out sensitive NAAT in any environment, without the need for any more equipment than a simple pocket warmer and the components of the assay itself.

Multiplexing of LAMP reactions is difficult, as the inclusion of four primer sequences required per assay, at high concentration, causes a high likelihood of non-specific amplification if multiple sets are contained in the same assay. Multiplexing also removes the ability to either detect amplification via agarose gel electrophoresis, visual dyes, or intercalating agents, as these methods are not target specific. However, multiplexing of LAMP reactions has been reported via the use of DARQ probes; quencher labelled FiP or BiP primers, annealed to a fluorescently labelled complementary probe sequence, which is realeased during the incorporation of the quencher labelled primers in the LAMP reaction (Tanner *et al.*, 2012). The use of DARQ probes has been applied to the detection of four SNPs, using four labelled FiP primers of varying sequence. One drawback of this method is that it requires that there are no sequence variations in non labelled primers, and hence can only be used to detect differences in the target region corresponding to the labelled primers, and cannot be used as an internal control. The inability to include an internal control (IC) reaction, as is standard for real-time PCR assays, is one of the disadvantages of LAMP. However, the main purpose of including an IC is to detect reaction inhibition, and the increased robustness of the LAMP reaction to common sample inhibitors somewhat alleviates the need for an IC (Francois *et al*., 2011).

Quantitative real-time LAMP assays have been developed, which rely on the use of LAMP reactions containing known DNA or target gene concentrations to produce a standard curve of the CT value against time, which can then be compared with the ct value of LAMP reactions of unknown samples (Soleimani *et al*., 2013). This approach enables a quantification estimate, but is not as accurate as quantitative PCR, due to the continuous exponential amplification that occurs, rather than the ordered doubling of product per thermal cycle that occurs in a PCR reaction. However, the reliability for certain quantitative assays has been reported to be quite high, with a quantitative LAMP assay for HIV-1 reporting a standard curve with an R^2 of 0.991 (Zeng *et al*., 2014)

One issue with the use of LAMP tests in resource poor settings, where the lack of need for thermal cycling equipment has the greatest benefit, is the requirement for a cold chain transport network for the kits. Although studies have shown LAMP reagents are stable up to 37^{o}C (Thekisoe *et al.*, 2009), package inserts of the kits recommend -20^{o}C storage, and the effects of temperature fluctuations during transport on kit performance are unknown (Njiru, 2012). To address this, Eiken have developed kits containing lyophilised reagents, for the diagnosis of sleeping sickness (Mitashi *et al.*, 2013), tuberculosis (Boehme *et al.*, 2007) and malaria (Hopkins *et al.*, 2013); three diseases of immense importance in the developing world. These kits have been developed in collaboration

between Eiken, the World Health Organisation, and the Foundation of New Innovative Diagnostics (FIND), a charitable organisation dedicated to the development of novel diagnostic solutions in developing countries. The use of lyophilised reagents removes the need for a cold-chain, which when combined with the lack of specialised equipment required, increases the affordability of using the assays in low resource settings. An evaluation of the use of the tuberculosis assay in clinics in Peru, Bangladesh and Tanzania, found the sensitivity of the assay to be 97.7% in smear positive + culture positive specimens, and 48.7% in smear negative + culture positive specimens. The specificity of the assay was 99% (Boehme *et al.*, 2007). The specificity of the *Trypanosome brucei* specific sleeping sickness assay was found to be 87.3%, with a specificity of 92.8%, indicating that the LAMP assay performs equally well as 18S rRNA PCR, whilst being simpler to perform. A field evaluation of the malaria assay in Uganda found it to have a sensitivity of 89.5% and specificity of 95.9% compared with a three well nested PCR, giving it comparable performance to a single well nested PCR (Hopkins *et al.*, 2013). A larger evaluation of the malaria kit found it to have sensitivities and specificities of 98.4% and 98.1% respectively, providing the sensitivity of nested PCR but with a reduced time to result (Polley *et al.*, 2013). FIND recommends that these diagnostic kits are used in conjunction with the LoopAmp PURE (Procedure for Ultra Rapid Extraction) DNA extraction kit (Eiken, Japan), which is a simple tube based nucleic acid extraction method designed for use in resource poor environments, with minimum equipment requirements of access to a 75^{o}C waterbath and a pipette (Ou *et al.*, 2014). The extraction tube is a closed system, with the lysis of the cells carried out via the 75^{o}C heat treatment, and reagent mixing carried out by manually handling the tube, with the eluted DNA removed from the extraction tube via manually squeezing. This simple methodology was found to be effective at extracting pathogenic nucleic acid from sputum samples (Mitarai *et al.*, 2011), which can be challenging to extraction procedures due to their viscocity and abundance of amplification inhibitors (Aldous *et al.*, 2005). The application of LAMP in resource poor settings has been aided development of novel polymerases, such as the OmniAmp DNA polymerase, which has an improved tolerance for freeze drying and temperature changes than the *bst* polymerase, possesses intrinsic reverse transcriptase activity, and has an increased tolerance to inhibitors (Chander *et al.*, 2014).

A number of portable detection systems exist, enabling the real-time detection of LAMP amplification, without the need for expensive qPCR systems. The OptiGene Genie systems (OptiGene, UK) are portable fluorometers with heating elements capable of processing eight LAMP reactions simultaneously (Tomlinson *et al.*, 2010). The newest incarnation, the Genie III, has been developed for outdoor use, and has a battery capable of maintaining a days use. The Optigene system does not have integrated sample preparation, and requires the prior purification of nucleic acid, or a simple heat treatment of the sample prior to the assay step.

The LAMP reaction, due to its speed, lack of equipment requirements, and the opportunity for simple visual detection, seems well suited for use in POC testing, and recent studies have sought to exploit this. One study has demonstrated a fully integrated microfluidic platform for bacterial identification at the POC, using LAMP (Fang *et al.*, 2010). Nucleic acid was released by using an 85^{o}C heat shock to lyse the cells. DNA was retained in capture pockets in the chip, which were then filled with the LAMP reaction mix. Each well on the chip contained either a positive control, or either strain specific or negative control primer set. Calcein, an intercalating dye, was used to provide a visually detectable colour change at the end point of the reaction. The limits of detection were found to be 27 copies/µl (with limited reproducibility) or 270 copies/µl.

A recently described POC test has combined LAMP with a novel electrochemical detection method, and a flexible fluid handling cassette (Safavieh *et al.*, 2014). The device enabled the detection of 30 CFU/ml of *E. coli*, using either real-time or endpoint detection, and without any further fluid handling steps after sample application. The device does not include a nucleic acid purification step, and instead lyses cells via heat shock in the presence of the LAMP reagents. This relies on the high tolerance of LAMP to inhibitory compounds preventing amplification inhibition, and simplifies the assay processes. However, this would render the device unsuitable for certain sample types, which are inhibitory to LAMP in an unprocessed state, such as sputum samples. The heating required for lysis occurs at 65^{o}C, which is insufficient to denature the *bst* polymerase. The novel detection method operates via the inclusion of redox-reactive osmium complex in the LAMP reaction mix. During the amplification process, the redox molecule intercalates with the newly synthesised DNA, decreasing the free redox in solution, which can be monitored electrochemically. The device is simple to operate, and relatively high throughput, processing 12 LAMP reactions simultaneously.

Despite encouraging attempts such as this to produce a LAMP POC device for clinical use, the assay is only commercially available in kit form and requires pre extracted DNA before use. In addition to the Kits for malaria, tuberculosis and sleeping sickness diagnosis, kits are also available for norovirus strains (Eiken, Japan), in addition to DNA and RNA amplification kits, requiring self-designed primer sets (Eiken, Japan). In order to integrate LAMP into a true POC test, various issues must first be tackled, including efficient preparation of the raw sample, integrated nucleic acid extraction, and the minimisation of reagent handling.

1.1.4. POC systems

The Atlas Io system (Atlas Genetics, UK) is a fully automated POC NAAT platform, which utilises single use assay cartridges enabling automated nucleic acid extraction, amplification, and novel end-point electrochemical detection. The platform processes a single assay at a time, although stacks containing multiple cartridge entry ports are available as additions to the system, and takes 30 minutes to provide results. The cartridge contains a mixture of freeze dried and liquid reagents, housed in blister packs, and a solid phase silica DNA extraction is carried out, followed by a rapid PCR amplification, with detection of an amplified product occurring via end-point electrochemical detection. The first batch of tests are scheduled for release in 2014, encompassing an STI range including *C. trachomatis* and *T. vaginalis*, and a hospital acquired infection range, including MRSA. A small scale lab evaluation (90 samples) of the *T. vaginalis* assay found the assay to have a sensitivity and specificity of 95.5% and 95.7%, respectively (Pearce *et al.*, 2013). This is a marked improvement on current POC testing for this organism, which involves microscopy, which is insensitive. The assay performed as well as laboratory based NAAT testing for this organism, whilst enabling rapid diagnosis, providing the sensitivity of molecular based testing, whilst being amenable to use at the POC.

The most widespread commercially available POC system is the GeneXpert (Cepheid, USA), a platform for processing real-time PCR based assays with fully automated sample preparation, amplification and detection on disposable assay-specific cartridges (Helb *et al.*, 2010). The sample preparation is carried out using a patented microfluidic cartridge, which incorporates a filter, syringe drive, rotary drive and sonic horn. The sample (typically blood or urine) is entered into the cartridge where it is initially filtered to isolate any cells contained in the sample, which are then lysed by ultrasonic energy delivered by the sonic horn, resulting in the release of nucleic acid. The syringe and rotary drives move liquid between the cartridge chambers in order to wash, purify, and concentrate the nucleic acid. After the extraction is complete, the nucleic acid extract is moved into the cartridge reaction chamber. The PCR reagents, which are lyophilized and held in separate chambers inside the cartridge, are then released to the sample and a real time PCR reaction is carried out by the thermal cycler inside the core GeneXpert module. The whole process takes between 1hr to 2hrs, from the introduction of the raw sample to the generation of results. A number of experimental controls are in place in order to guarantee the accuracy of the results generated, including probe checks, pressure checks and sample-processing controls. Importantly, each test run has an internal control incorporated to ensure that the cartridge and associated reaction chemistry is functioning correctly.

The GeneXpert software also has features to enhance internal QC practices. The software tracks external controls during the assays and provides trend analysis of the QC data over time, which can then be used as evidence of external QC documentation (Marlowe and Wolk, 2008).

Currently there are FDA approved GenExpert assays available for *M. tuberculosis* (Marlowe *et al.*, 2011), *C. difficile* (Babady *et al.*, 2010) and a combined *C. trachomatis* and *N. gonorrhoeae* (CT/NG) assay (Tabrizi *et al.*, 2013), all of which have high sensitivities and specificities, and provide results within 90 minutes. Cepheid has announced that it plans to release a *T. vaginalis* assay in the 2014-2015 product range, enabling testing for trichomoniasis to be carried out using this platform.

The CT/NG assay enables the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae*, from both swab and urine samples. These pathogens are frequently tested for simultaneously, due to their overlapping symptoms and the high frequency of their co-infection. The assay has been shown to have a limit of detection of 10 copies per sample for both target organisms, and a challenge of the assay with 372 non-target bacterial strains found that the assay had 100% specificity (Tabrizi *et al.*, 2013). A large scale test of the assay, involving 1,722 female and 1,387 male samples found the Chlamydia assay to have sensitivities of 97.4%, 98.7%, 97.6% and 97.5%, from endocervical swabs, vaginal swabs, female urines, and male urines, respectively (Gaydos *et al.*, 2013). The GC assay had sensitivities of 100%, 100%, 95.6% and 98%, from endocervical swabs, vaginal swabs, female urines, and male urines, respectively. The specificity of either assay for any sample type exceeded 99.8%.

Although the CT/NG assay does not have regulatory approval for its use in diagnosing rectal infection from rectal swab samples, the test has been shown to have a sensitivities of 86% (CT) and 91.1% (GC) and specificity of 99.2% (CT) and 100% (GC), with this sample type, compared to the gold standard of the Aptima Combo 2 assay (Genprobe, US) (Goldenberg *et al.*, 2012). This level of performance was achieved despite the samples being diluted to 6% of their original concentration, as the samples used were in a Genprobe transport media, which interfered with the GeneXpert extraction process.

The GeneXpert MTB/RIF assay simultaneously detects the presence o f *M. tuberculosis*, and also mutations in the *rpoB* gene, present in 95% of rifampin resistant isolates (Zeka *et al.*, 2011). The test is most sensitive when processing pulmonary specimens, with sensitivities of 100% and 68.6% for smear positive and smear negative specimens, respectively. For extrapulmonary specimens the sensitivity was 100% for smear positive and 47.7% for smear negative samples. The assay also correctly identified rifampin resistant and susceptible isolates, as confirmed with MIC testing.

Field testing of the Cepheid GeneXpert *C. trachomatis* assay in South Africa has given promising results, demonstrating high clinical sensitivity in combination with being well suited for use outside of the traditional laboratory environment (Jenson *et al.*, 2013), and the GeneXpert *M. tuberculosis* assay is already being widely used in sub-Saharan Africa (Osman *et al.*, 2014; Lawn *et al.*, 2013). An analysis of the predicted impact of the instigation of the GeneXpert TB assay in South Africa found that both the number of cases identified and also the cost per test would be increased from normal levels, although this analysis did not work out the economic benefits of the increased diagnostic efficiency and reduction in transmission caused by the POC testing (Meyer-Rath *et al.*, 2012). The study predicted that the number of yearly TB cases diagnosed would be increased by 30%-37%, with 81% of cases being diagnosed at the first visit, compared with 46% using the current methods. The study found that the cost per diagnosis would increase 55%, and ultimately cost the country an initial outlay of 22 million USD, supplemented by 287-316 million USD over the next 6 years (Meyer-Rath *et al.*, 2012). Other cost/benefit studies that have included reductions in spending due to knock on effects such as reduced transmission have predicted that the use of the GeneXpert TB assay could actually reduce costs in relation to smear microscopy testing (Millman *et al.*, 2013).

There are some limitations of the GeneXpert system, which affect its suitability for meeting diagnostic needs in rural or underserved areas of developing countries, where certain diseases, such as TB, are especially problematic. The system needs a stable electrical supply, and ideally an internet link to provide QC data. It also requires a degree of temperature control, with the assay cartridges requiring storage at 2^{o}C-28^{o}c, and the machine requiring temperatures below 30^{o}C (Schito *et al.*, 2012). The machines and cartridges are also expensive; although a reduced pricing has been agreed for developing countries where TB is endemic, the machines retail for 15,700 USD for a 4-module instrument to 65,500 USD for the larger 16 module instrument (Nicol *et al.*, 2013).

One current focus of POC assay development is for the detection of *M. tuberculosis*, the etiological agent of the disease tuberculosis. Tuberculosis causes the highest number of deaths worldwide of any infectious disease, with 1.45 million deaths occurring in 2010 (WHO, 2011) and is most prevalent in developing countries, where access to sensitive NAAT diagnostics is limited. Additionally, the methods currently in use are cumbersome and time consuming, and rely upon the presentation of symptomatic individuals to healthcare facilities, which results in a high proportion of newly presenting cases being past the early and more easily treated stages of the disease. There is also a serious issue with patients neglecting to turn up for results, or further test of cure diagnosis, in large due to the lengthy turn-around time with current testing methods (Niemz and Boyle, 2012). This has led to the identification of tuberculosis as a disease that would greatly benefit from POC, and calls

for the development of POC tests for this disease have been made by the World Health Organisation (Schito *et al.*, 2012).

The Genedrive (Epistem, UK), is a POC NAAT system for the detection of *M. tuberculosis*, currently awaiting its commercial release. The system is capable processing sputum samples, using a novel filtration system, with a subsequent nucleic acid extraction followed by PCR based amplification and detection. Compared with culture, this system was found to have a sensitivity of 90.8% and specificity of 100% (Castan *et al.*, 2014). Assays in development include a cancer biomarker panel, and personalised medicine biomarkers for a range of conditions.

The Liat (lab-in-a-tube) Analyzer (IQuum, UK) is a recently released NAT system designed for POC diagnostics. FDA approved commercially released tests have included assays for the viruses H1N1, and HIV (Tanriverdi *et al.*, 2010), however IVD and biodefence applications are currently in development. The system uses PCR and real-time PCR detection and has a turnaround time (tat) of as little as 26 minutes. The Liat Analyzer system consists of an analyzer module coupled with a disposable flexible tube, which acts a sample vessel, and in which the entire assay takes place. All the necessary reagents for the assay are held in separate tube compartments and multiple sample processing actuators in the analyzer module compress the tube to selectively manipulate reagent release and sample movement. Sample preparation is based on conventional solid-phase extraction with magnetic particles. Magnetic beads are incubated with a sample for target enrichment, captured using magnets and then washed to remove potential inhibitory reagents. Nucleic acids are then eluted from the beads and transferred between tube segments for PCR and rt-PCR detection. Limits of detection of 57 copies per ml have been demonstrated for the HIV assay, giving the assay a similar analytical sensitivity to commercially available lab-based NAAT systems (Tanriverdi *et al.*, 2010).

1.1.5. Sample Preparation

One of the biggest challenges to overcome in the design of a point of care nucleic acid amplification testing (NAAT) system for pathogen diagnostics is the integration of the sample preparation process. The majority of commercially available NAAT based assays are complex (more than 15 steps) and time consuming (assay times of more than 4 hours), mainly due to the need for manual preparation of nucleic acid from a raw sample before the detection step (Dineva *et al.*, 2007). Currently only one

widely commercially available system, the Cepheid GenExpert, has fully integrated sample processing, whilst a handful of other fully integrated NAAT platforms are nearing release.

The advantages of full integration of the sample preparation process with the assay and detection steps are numerous. The automation of the process removes the need for skilled laboratory personnel to prepare the sample, enabling healthcare professionals such as clinicians or nurses to carry out the test directly in a clinical setting. This greatly reduces the time taken between taking the sample and obtaining the results of the test, allowing a true point of care testing service to be offered, with all the benefits that entails. It also massively reduces the amount of time required to operate the test, the user simply has to introduce the sample and initiate the test. The fact that no laboratory equipment is needed also makes the system suitable for use in areas without dedicated lab support, such as remote locations or developing countries. Integrated sample handling also reduces the contact the operator has with the sample; ideally the sample should be in a contained environment once introduced to the device. This is important for biosafety, especially when testing samples suspected positive for highly infectious pathogens such as *B. anthracis* or HIV.

In the proposed device, a urine or swab sample would need to be processed to the stage were a sample of the target nucleic acid can be introduced to the assay step. This would require isolation of any target organisms from the urine, lysis of the cells to remove nucleic acid, extraction and purification of the nucleic acid and finally washing of the nucleic acid to remove any potential inhibitors of the PCR reaction, which in urine include haemoglobin, urea and calcium ions (Betsou *et al.*, 2003). The nucleic acid may then need to be moved to a separate assay chamber but this will depend on the design of the device.

1.1.6. Sampling

Sampling for sexually transmitted pathogens is carried out either by taking a urine sample from the patient, or by taking a swab from a suitable genital site. Potential sites for swab sampling include the urethra, endocervix, and vagina in female patients, and the urethral meatus in males. Extragenital sites such as the rectum and pharynx are can also be sampled via swab for sexually transmitted infections, dependent on the patient's sexual history. The optimum site of swab sampling can vary between infections caused by different organisms; for example higher yields of *C. trachomatis* can be obtained from an endocervical swab than a vulvovaginal swab, whilst the opposite is true for *T. vaginalis*. The majority of data available relating to the variance between sampling sites for sexually

transmitted infections on organism load, and downstream assay sensitivity, is related to *C. trachomatis* and *N. gonorrhoeae*, as these organisms are the two most commonly tested for, and the only targets of national screening programmes in the UK and US.

The development of NAAT for sexually transmitted infections has allowed for the use of urine testing, as the improved sensitivities of the assay negate the typical reduction in organism load encountered in urine samples in comparison with swab samples (Michel *et al.*, 2007). Urine sampling is minimally invasive, and convenient for patients, who are more likely to present for testing for asymptomatic infection if they know invasive sampling will not be performed (Garland and Tabrizi, 2004). Urine sampling is currently carried out for *C. trachomatis* and *N. gonorrrhoeae* NAAT testing in the UK, with first void urine (FVU) being the preferred sample type. The use of FVU sampling stems from the improved sensitivities obtained when *C. trachomatis* culture is carried out from FVU, rather than midstream urine. However, the sensitivity of current NAAT tests are relatively equivalent for each sample type, due to the improved detection limits of NAAT testing compared with those of culture (Mangin *et al.*, 2012). The higher sensitivity of NAAT testing has also reduced the importance of the voiding interval on *C. trachomatis* testing, with a large study involving 1649 men finding no significant difference in positivity between men with a voiding interval of 2 hours or over, compared with those with a voiding interval of less than 2 hours (Manavi and Young, 2006). The two most commonly used sampling sites for detecting *C. trachomatis* in women in the community setting, or screening programmes, are first void urine and self-collected vulvovaginal swabs, due to their minimally invasive nature and high sensitivity of down-stream NAAT testing using these sample types. A study involving 146 paired vulvovaginal and urine samples from women with *C. trachomatis* infection found the sensitivity of the subsequent NAAT testing to be 97.3% and 91.8% for the swab and urine samples, respectively (Skidmore *et al.*, 2006). Urine sampling has also been shown to be as effective in detecting *C. trachomatis* as cervical swabs during NAAT testing, in some populations (Haugland *et al*., 2010).

A study aiming to determine the organism load obtained when sampling for *C. trachomatis* from a range of anatomical sites in both men and women found that the mean number of *C. trachomatis* elementary bodies (EB's) obtained from FVU and urethral swabs in men were 821 and 1,200 per 100µl of sample, respectively (Michel *et al.*, 2007). In female patients, the mean number of *C. trachomatis* EB's obtained from endocerivical swabs, self-collected vaginal swabs (SCVS), urethral swabs and FVU were 2,231, 773, 162, and 47 EBs per 100µl of sample, respectively. The high organism load found in male FVU samples make FVU an ideal, non-invasive sample type for testing males, without compromising assay sensitivity. In comparison, FVU in female samples yielded a

mean organism load of 47 cells per 100µl, only 5.7% of the yield found in male FVU. FVU serves as a urethral washout, releasing any bacterial cells in the urethra. Whilst genital infection in males always arises from inoculum of the urethra during sexual activity, this is not the case in females, when the primary site of infection is likely to be the cervix or epithelia of the vaginal canal, and infection of the urethra is rarely encountered in the absence of a cervical infection (Bradley *et al.*, 1985). This results in a lower organism load at this site in females, especially during the early stages of infection. The high organism load detected in the SCVS samples, which are less invasive than endocervical swabs, show that this sample site is effective in sampling for this organism.

The use of FVU in *N. gonorrhoea* testing in females is more reliable, with one large scale study finding the sensitivity of NAAT testing for this organism to be 88.6% when using this sample type, compared with 100% for SCVS, and 95.5% for endocervical swab samples (Fang *et al.*, 2008). In men, as with *C. trachomatis* testing, there is little discrepancy in sensitivity between the use of FVU samples and urethral swabs for NAAT testing. One study found the sensitivities obtained by a PCR assay using urethral swabs and FVU to be 97.3% and 94.4% respectively (Crotchfelt *et al.*, 1997).

Sampling for *T. vaginalis* from female patients is carried out using a vaginal swab or urine sample; T. vaginalis rarely infects the cervix and the organism load will be far higher in the vagina (Garber, 2005). The use of urine for *T. vaginalis* detection in females is not recommended due to the reduction in sensitivity seen when using this sample type, even with molecular methods. One study found the sensitivity of a *T. vaginalis* PCR assay to be 89% when vaginal swab samples were used, and 64% using urine samples (Lawing *et al.*, 2000). In men, the use of FVU samples has been shown to improve the sensitivity of subsequent PCR testing, compared with the use of urethral swabs (Schwebke and Lawing, 2002).

Testing for *M. genitalium* is carried out less regularly than for *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis* in the UK, due to its lower prevalence, mild or absent symptoms, and relative lack of serious disease sequelae. Testing is almost exclusively carried out by NAAT, from either swab or urine samples (Shipitsyna *et al.*, 2010). One study found the sensitivity of a home-brew PCR assay when used in combination with vaginal swab samples, endocervical swab samples, and urine samples to be 85.7%, 74.3%, 61.4% respectively (Lillis *et al.*, 2011). The use of FVU samples in males gives equivalent sensitivity as the use of urethral swab samples, 87% compared with 91% in one study (Mena *et al.*, 2002). FVU samples are regarded as a suitable sample type from males for all of the organisms used in our study.

An important factor in diagnosing STIs from swab samples is the structure and composition of the swab itself. Several variations in design are available, which differ in their ability to capture and then release bacteria present in the urogenital tract, and will have a big effect on the overall sensitivity of the process. A recent addition to the varieties of swabs approved for STI diagnosis are flocked swabs, commercialised by Copan as FLOQswabs. These swabs consist of a plastic tip, to which Nylon fibres are attached at right angles, in a process termed "flocking" which the electrochemical attachment of the fibred. The benefit of this is that it creates a highly absorbent open structure, and ensures that cells remain trapped on the surface of the swab rather than being absorbed into the core area, as happens with cotton or fibre wound swabs. The use of these swabs has been shown to improve the sensitivity of NAAT testing for *C. trachomatis* and *N. gonorrhoeae* (Chernesky *et al.*, 2006), and to perform equally as well as Dacron swabs for diagnosing *T. vaginalis* using a TMA assay (Jang *et al.*, 2012). The high performance of Copan FLOQswabs has been confirmed for the capture of *T. vaginalis*, *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* (http://www.copanitalia.com/KC012-Copan-Flock-Tech-Brochure.pdf); all of the target organisms of our developed diagnostic assay.

1.1.7. PCR inhibitors in urine

Several standard components of urine, and components of urine present due to disease or an underlying physiological condition, are known to be inhibitory to nucleic acid amplification by PCR. Generally inhibitors will exert their effects at one of three possible stages of the PCR process; the lysis of the target cells, nucleic acid degradation or capture, or the amplification of the target DNA by the polymerase enzyme. Reaction inhibition can be total or partial; its effect can range from a minor reduction in detection sensitivity to total reaction failure.

The most significant PCR inhibitor routinely found in urine samples is urea. It exerts its inhibitory effects via denaturing DNA polymerases, reducing enzymatic activity and therefore DNA amplification (Saulnier and Andremont, 1992). Urea has a similar solubility to DNA and therefore is not completely removed during classic extraction protocol, and will be present in the final DNA preparation, where it will then inhibit PCR (Moreira, 1998). Urine has been shown to inhibit PCR at a concentration of over 50mM (Khan *et al.*, 1991). The normal concentration of urea in urine in adults is 330mM although this is highly variable. Taq polymerase is a magnesium dependent enzyme and relies on the availability of Mg^{2+} ions in order to function. The concentration of Mg^{2+} ions has a large effect on the success and specificity of the PCR. Ca^{2+} ions, which are present in urine, can interfere

with the interaction of Mg^{2+} ions and Taq polymerase, and therefore can inhibit DNA amplification (Bickley *et al.*, 1996).

Blood present in the urine sample has been shown to have an inhibitory effect on DNA amplification. Blood is not an expected component of urine but may be present in patients who have recently experienced kidney trauma, or who have a urinary tract infection. Serum proteins in blood can act as blocking proteins, preventing polymerase enzymes from binding with target DNA, and haemoglobin is also known to inhibit PCR (Wilson, 1997). The anitocoagulant heparin, released by basophils and mast cells, is also known to inhibit PCR amplification. A haemoglobin concentration in the sample of 1 mg ml^{-1} and a heparin concentration of 13 mg ml^{-1} have been shown to completely inhibit the PCR reaction (Perch-Nielsen *et al.*, 2003). It has been shown that the level of whole blood in the PCR reaction volume should be kept at lower than 4% in order to enable effective DNA amplification (Wilson, 1997). However, some studies have found that the concentration of blood present in a urine sample has no inhibitory effect on PCR detection of *C. trachomatis* (Toye *et al.*, 1998).
A recent study carried out in order to investigate the sensitivity of PCR for the detection of *C. trachomatis* compared to culture methods (Pasternack *et al.*, 1996) found that PCR inhibition was seen in 15.4% of 39 samples positive for *C. trachomatis*, resulting in false negative result. This level of false negative results among true positive patients would be a major obstacle when considering use of PCR for diagnosis of *C. trachomatis* in a clinical setting. However, the same study found that when special precautions were taken to eliminate the inhibitors effect, 97.4% of 39 samples positive for *C. trachomatis* were correctly identified. This underlines the sensitivities that can be accomplished using PCR detection if steps are taken to remove the impact of PCR inhibitors from the system.

A key step in the sample preparation process will be the removal of compounds inhibitory to PCR prior to the detection assay. Filtering or washing by centrifugation can be used to remove inhibitory compounds which are extracellular or unbound to the cells. Although effective and easy to carry out in a laboratory setting, such methods are fairly labour intensive and are unsuited for incorporation into a microsystem. One of the simplest techniques for the removal of inhibitors is to fixate the target cells or target DNA (depending on which stage of the process the inhibitors will be removed), and then wash away and discard any other materials, including the inhibitors using wash buffers. The buffers can be housed on chip (Baier *et al.*, 2009) or added to their respective chambers present on a microfluidic chip prior to use by the operator (Xu *et al.*, 2010). The buffers could also be housed inside the actual machine itself, with the required amount being injected into the chip circuit via

buffer inlets on the chip. This basic process of inhibitor washing can be carried out in a number of different ways.

Previous chips for nucleic acid testing have successfully removed inhibitors by using solid phase extraction (SPE) to fix the DNA subsequent to target cell lysis. This process is followed by one or more washing steps, where a washing buffer is passed through the SPE chamber which removes all fluids and cellular debris, leaving only the DNA behind. The wash buffer is then disposed of via an outlet channel (Baier *et al.*, 2009). The composition of the washing buffers used can vary. One study (Baier *et al.*, 2009) used a two -step washing procedure to remove inhibitors from a 30µl SPE chamber; firstly 230µl of a washing buffer composed of 75% ethanol and 25% 3M Guanidinium isothiocyanate (GuCN) was used. This was followed by 120µl of 96% ethanol. The GuCN is present in the first wash in order to denature proteins, especially DNase enzymes which may be present in the chamber, and is also used as a binding salt during SPE. Some components of commercially available wash buffers, including GuCN, are thought to inhibit PCR reactions to a certain degree (Xu *et al.*, 2010), so a final wash of ethanol is used to remove any components introduced in the first wash.

It is also possible to remove inhibitors prior to cell lysis. One such method of accomplishing this in a microsystem uses dielectrophoresis (DEP) as a selective filter to withhold target cells whilst the inhibitory compounds are removed (Perch-Nielsen *et al.*, 2003). DEP is a force exerted on a dielectric particle when it is subjected to a non-uniform electric field. By using DEP it is possible to attract the target cells to electrodes in a microfluidic chamber, whilst molecules with different dielectric properties, such as any inhibitory compounds, are free to move through the system. Buffers can then be applied to wash away any inhibitors before the DEP force is removed, releasing the cells, and leaving a concentrated population of cells ready for the next stage of the sample preparation process.

1.1.8. Sample preparation using microfluidics

One of the most efficient ways to address the challenge of fully integrating the sample processing steps is to use microfluidic technologies to carry out these processes ‘on-chip’. Microfluidics involves the scaling of macroscopic processes down to the microscopic level, using the precise control of fluids and gases at very low volumes (typically at the nanolitre and picolitre level). By keeping sample and reagent volumes low, reaction times are shortened, and molecular processes

can be carried out much more rapidly, which is of upmost importance for molecular diagnostics (Mairhofer *et al.*, 2009). Single use microfluidic cartridges or chips can be designed to carry out all sample preparation steps to the raw sample, either after being pre loaded with reagents during manufacture, or loaded prior to use by the operator. These cartridges or chips can be made using polymers via injection moulding, an inexpensive process which results in disposable single-use chips being financially viable (Baier *et al.*, 2009). Disposable microfluidic cartridges are utilised in the GenExpert system (Cepheid). These contain syringe and rotary drives to move the reagents and sample throughout the microfluidic circuit and an ultrasonic horn to lyse the cells.

A variety of different methods for various stages of the extraction of DNA from organisms in a biological sample have been attempted on chip. These include filtration, magnetically activated cell sorting (MACS) and acoustophoresis for organism isolation and a variety of methods for cell lysis, including mechanical, thermal, chemical and electrical methods.

1.1.9. Sample Concentration

Initially the sample will consist of a minimum of 1ml of urine taken from a urine sample given by the patient. The majority of microfluidic systems would be unable to process a sample of this size, and it may be necessary to reduce the volume to a manage level for the microfluidic chip to process, whilst retaining the maximum amount of pathogenic material to enable sensitive and rapid detection. The sample can be concentrated by isolating the target organisms from the bulk liquid, which can then be ejected as waste. A number of methods to accomplish this have successfully been implemented 'on chip'.

1.1.10 Filtration

One of the easiest ways to concentrate the cells present in the sample would be to filter the bulk liquid, capturing the cells and enabling the remaining liquid to be discarded as waste. The cells could then be lysed at the site of the filter and their contents washed to the next phase of the process in order for the DNA content to be isolated. This method of cell isolation is utilised in the microfluidic cartridges of the commercially available GenExpert system (Cepheid). Conventional computer controlled (CNC) machining has been used to create various designs of microfilters for the

separation of cells from biological samples from glass-silicon, including comb shaped filters, weir type filters and torturous channels. The use of weir type filters to isolate white blood cells from whole blood has been demonstrated, and was able to achieve a capture efficiency of 7%, whilst eliminating >99% of red blood cells(Yuen *et al.*, 2001). Simple nylon filters capable of isolating human cells from the sample have also been incorporated into microfluidic sample preparation chips, with the cell lysis step being carried out directly on the filtrate (Baier *et al.*, 2009).

One major issue with this method of cell concentration is that the filter can become clogged by material in the bulk liquid or excess cells, reducing or preventing flow through the filter. A number of potential remedies to this problem have been developed. One such method is to reverse the pressure present in the system, back flushing the filter chamber to dislodge any debris (Wei *et al.*, 2011). This typically requires extra design requirements, such as valves or extra channels, and a pumping system able to reverse its flow. In order to detect filter clogging a pressure sensor is also required (Baier *et al.*, 2009). Another issue encountered when using filters in this way is the mechanical lysis of cells upon contact with the filter pores. The mechanical pressure exerted upon the cells, especially under high liquid velocity, can deform or lyse the cells, causing the lysed cell and its contents to be pushed through the filter along with the bulk liquid (Kuo *et al.*, 2010). However, It has been shown that by carefully considering the hydrodynamic environment housing the filter during system design this problem can be minimised (Kuo *et al.*, 2010).

Recent developments in the use of porous membranes for particle sorting have yielded the ability to create poly(dimethylsiloxane) (PDMS) membranes with accurately determined pore sizes down to size of 6.4 μm, and pores of multiple sizes, in predetermined arrangement (Wei *et al.*, 2011). A particle sorter made using this principle was able to separate polystyrene beads of various diameters with an efficiency greater than 99.9%. This can enable a much greater degree of selectivity in the filtration process, although there would be difficulties in implementing this technology in a device for the simultaneous detection of multiple organisms of differing sizes.

It has also been demonstrated that a filtration process could be used to isolate the DNA itself after lysis of the cells. Nanofilters can be used as molecular sieves, enabling the separation of DNA from a bulk liquid (Han *et al.*, 2008). This process is likely to be less reliable than a solid phase extraction method due to the possibility of the nanofilters being clogged by cellular debris or protein in the urine sample; however, it is very rapid and can operate in a continuous fashion (Han *et al.*, 2008).

1.1.11. Magnetically activated cell sorting (MACS)

Another potential method for isolating the cells from the sample is the use of magnetically activated cell sorting (MACS), or a variation of this technique. MACS relies on target specific antibodies attached to magnetic beads or nanoparticles (Schmitz *et al.*, 1994). These magnetic particles are then incubated with the sample, allowing the antibodies to bind to their specific antigens on the surface of the target cell, creating a strong attachment between the target cells and magnetic particles. The cell suspension is then transferred to a column placed in a strong magnetic field, where the beads and attached cells remain on the column, whilst the other components of the cell suspension are washed through and can be discarded as waste (Miltenyi *et al.*, 1990). The cells can then be eluted from the column and analysed. Variations of this technique are used during sample preparation in a variety of point of point of care diagnostic devices, including the Liat Analyzer (IQuum), Philips Magnotech technology (Philips & Biomerioux) and FL rapid PCR system (Enigma Diagnostics). The process relies on the presence of a specific surface antigen which is present on the target cell but no other components or cells in the sample. If any non-target cells also express the antigen then they will also be captured, reducing the selectivity of the system. A multi-target system would require antibody conjugated magnetic beads for each target. This process also requires the specific antibodies, which if commercially available are expensive, and if not, will require synthesis.

One commercially available MACS system is the Dynabeads system (Invitrogen, US). Dynabeads are superparamagnetic spherical polymer particles, available in several sizes, which can be pre-coupled with a large variety of ligands, including antibodies, antigens, proteins or DNA/RNA probes. Dynabeads have successfully been used for cell concentration during sample preparation on microfluidic chips for nucleic acid testing, in an experimental context (Liu *et al.*, 2004).

The main advantage of MACS is its high specificity, which is highly advantageous if a low number of target molecules are present in the sample in combination with a potentially large population of non target cells, as is the case when detecting low numbers of pathogenic cells in a clinical sample. The separation of astrocytes and microglia from a cell suspension using MACS has been demonstrated, with the use of magnetic beads coupled to microglia specific antibodies enabling the selection of a >99% pure population of microglia, completely free from astrocytes (Marek *et al.*, 2008). However, as in NAAT diagnostics the specificity is inherent in the amplification assay, a catch-all method of sample concentration may be the most efficient, and cheapest option.

A variation of MACS known as multi target MACS has been developed in a microfluidic chip to enable the capture of two distinct cell populations from the same sample in a continuous flow manner, and their collection from separate outlets (Adams *et al.*, 2008). Two cell populations were tagged with magnetic tags with differing magnetization and size. The device was engineered so that the combined effects of the hydrodynamic force produced flow within the microchannel and the magnetophoretic force produced from the design of the ferromagnetic structures within the microchannel result in the selective purification of the target cells into multiple independent outlets dependent on the characteristics of their tags. The design was capable of sorting multiple bacterial cell types with a purity of >90% and >500-fold enrichment at a throughput of 10^9 cells per hour. The drawbacks of this system include the expense of having two sets of magnetic tags coupled to different antibodies, and the slow flow rate of the system. The system is operated at a flow rate of 47ml/hr; however of this 47ml volume only 5ml is made up of the actual sample, meaning it can process 5ml of sample per hour. In order to process a 1ml sample the processing time would be 12 minutes, which is excessively long for this process in a diagnostic system intended for use at the POC.

1.1.12. Cell Lysis in a microfluidic system

One of the initial stages of an on-chip nucleic acid extraction procedure is the lysis of target cells in the sample, which is necessary in order to access the intracellular DNA, which will then need to be extracted and presented to the assay step for amplification. This step occurs directly after any pre-concentration step, if present, or can occur directly after sample application. The methods available for lysing cells in a microfluidic system fall into four broad categories; chemical, thermal, mechanical and electrical.

1.1.13. Chemical Lysis

Chemical lysis of bacterial cells involves the use of compounds capable of inducing outer cell wall permeability, creating holes in the cell membrane, and causing the cells intracellular contents to be released. Organic solvents (such as DMSO, methanol and benzene), chaotropic agents, antibiotics and chelating agents are all capable of inducing lysis in bacterial cells. Detergents can be used, but require a pretreatment step to degrade the cell wall before they can act on the cell membrane. Lysozymes can also be employed as an effective lytic agent when dealing with gram positive or gram

negative bacterial cells. These enzymes work by catalyzing hydrolysis of 1,4 beta linkages, which act to bind N-acetylmuramic acid and N-acetyl-D-glucosamine residues present in the peptidoglycan cell wall. However, lysozyme mediated lysis of Gram-negative bacteria has been shown to occur independently of the enzymes muramidase activity, indicating another pathway is responsible (Nash *et al.*, 2006). Chaotropic salts, such as guanidinium thiocyanate and guanidinium chloride, are useful for the lysis of cells for nucleic acid extractions. Not only do they disrupt bacterial cell membranes, they also denature RNAses, preventing the degradation of RNA by these enzymes.

An important consideration when utilising chemical lysis in a microsystem is that the inhibitory effect elicited on PCR reactions by many chemical lysis agents means that they need to be removed from the sample prior to the amplification stage. This typically means washing buffer needs to be introduced to the sample during DNA extraction, removing inhibitors and leaving the DNA ready for the assay step. This has an impact on the complexity of the device, with lysis agent and washing buffer needing to be stored on chip, typically in quite significant volumes. It also increases the time taken for the device to extract PCR ready DNA from the raw sample.

Bench-top DNA extraction kits, commonly used for DNA extraction from clinical samples for pathogen detection in laboratories, frequently employ the use of lysis buffers in order to lyse the cells, in order to release the DNA. Lysis buffers containing NH_4CL as their active ingredient are frequently used to lyse erythrocytes, although they are ineffective at lysing bacterial cells. Lysis occurs as NH_3 diffuses into the cells where it is converted into NH_4^+ ions in order to maintain the intracellular pH equilibrium. This results in a need for counter-ions, which are generated by the conversion of intracellular CO_2 into HCO_3^- ions, catalysed by the carbonic anhydrase enzyme. The accumulated HCO_3- ions are then exchanged for extracellular Cl^- ions, present due to the lysis buffer, through the band 3 anion translocator (Sethu *et al.*, 2004). The build up of Cl^- and NH_4^+ ions inside the cell leads to osmotic swelling, which results in cellular lysis. The use of an NH_4CL based lysis buffer has been demonstrated during the lysis of erythrocytes in a microfluidic device (Sethu *et al.*, 2004), which enabled almost complete lysis (99.4%) of the sample.

A significant issue with the use of lysis buffers which needs to be overcome before it can be used in a rapid POC diagnostics microsystem is the ratio of lysis buffer needed compared to the volume of sample. A typical extraction kit using a NH_4CL-based lysis buffer would recommend the application of 15ml of buffer to 1ml of sample and then a 5 minute incubation period (Sethu *et al.*, 2004). This would increase the sample volume to an unmanageable volume for a microsystem to rapidly process. However, a number of microfluidic chips have used chemical lysis in order to release the DNA from the cells prior to DNA extraction with relative success. One study addressed the problem

of high buffer to sample volume ratios by using a comprehensive computational fluid dynamics (CFD) simulation to determine the optimum channel dimensions and shape for buffer/sample mixing (Chen *et al.*, 2007). A T-type mixing model, sandwich type mixing model with lined channel and a sandwich type mixing model with coiled channel were all evaluated. The sandwich type mixing model with coiled channel was predicted to have a far greater degree of mixing, and therefore an improved rate of lysis. As the rate of lysis in the macroscale environment is diffusion limited (Sethu *et al.*, 2004), optimising the mixing of buffer and sample can lead to significant decreases in the time taken to lyse the cells. Mixing in a microfluidic chip is exclusively caused by diffusion due to the laminar flow through the channels, resulting in a slow rate of mixing.

Although this study was able to demonstrate a significant increase in the efficiency of chemical lysis on-chip, the fastest sample flow rate they were able to obtain whilst retaining lysis efficiency was 5µl/min, which would be far too slow for use in a microsystem which needs to achieve detection from an unprocessed sample in under 15 minutes. The lowest ration of lysis buffer to sample volume they were able to achieve was 5:1, which is an improvement on typical bench top extraction kits, but not by a large enough margin to be effective in a rapid POC system

1.1.14 Mechanical Lysis

Mechanical lysis relies on mechanical force applied directly to the target cells to puncture the cell membrane or wall. Mechanical methods are amongst the most simple to carry out in a microfluidic environment, many rely on internal static features which can be built into the microfluidic channels themselves. However, mechanical methods are associated with some disadvantages when used to lyse cells for DNA extraction. Mechanical lysis tends to cause bacterial cell membrane to disintegrate into very small fragments, making the isolation of DNA from the resulting mixture more difficult. The DNA needs to be purified from the complex mixture of all the released intracellular components, including membrane fragments and proteins.

1.1.15. Filtration

One of the simplest ways to mechanically lyse the cells is to pass them through a filter at high flow rates. The rate of lysis can be improved by considering the structure of the filter. Features such as

nanostructured barbs added to the filter structure increase cell lysis on contact (Carlo *et al.*, 2003). Whilst having many advantages, such as the simplification of the process, and reduction in the need of moving parts or heating elements, the process remains quite inefficient. The rate of lysis caused by a standard filter to red blood cells at a flow rate of 300µl per min lies between 1.9% and 3.2%, which can be increased to between 4.8% and 7.5% upon the addition of nanoscale barbs (Carlo *et al.*, 2003).

1.1.16. Ultrasonication

Ultrasonication is a method of mechanically lysing cells using high frequency ultrasound waves. Ultrasonic generators are used to create sonic pressure waves throughout the sample liquid, causing microbubbles which then implode, generating enough localized pressure to damage cell membranes, resulting in cell lysis. The ultrasonic energy is generated by piezoelectric generators made of lead zirconate titanate crystals. The energy generated is then transmitted down a metal horn attached to the chamber in which the ultrasonication is taking place, causing the chamber to resonate, typically between 15-25 kHz (Huang *et al.*, 2002). This method is employed in the GeneXpert system (Cepheid, US), which uses a generator in the main module to transmit ultrasonic energy down the ultrasonic horn present in each single use cartridge. This method is well suited to lysing robust bacterial species such as *M. tuberculosis*, which are difficult to break down due to the presence of a waxy cell wall (Verschoor *et al.*, 2012). This method does have its disadvantages however, as the ultrasonic processors generate considerable heat during their activation and also have a larger energy requirement than other methods of cell lysis, such as chemical methods and most other mechanical methods.

1.1.17. Thermal lysis

Thermal lysis relies on the generation of a sufficiently high temperature to break down the bacterial cell wall. This is one of the most rapid methods for cellular lysis and one of the most amenable to miniaturisation and inclusion in a microsystem. Due to the high temperatures the released intracellular proteins tend to become denatured, however the DNA is able to withstand comparatively higher temperatures and so can be extracted intact. Total lysis of *E. coli* in a glass microchip has been demonstrated by heating the chip to 94^{o}C for 4 minutes (Waters *et al.*, 1998).

The lysis of over 90% of *E.coli* cells in a microfluidic chip by exposure to 93°C for just 15 seconds has been reported (Privorotskaya *et al.*, 2010). This was achieved using silicon microcantilever heaters coated with a layer of 100nm thick electrically insulating ultrananocrystalline diamond layer, which were then electrically heated.

A comparison of the quantity of DNA released from *E.coli* via thermal lysis and ultasonication (considered one of the optimal methods of lysis for preserving DNA) carried out using real time PCR found comparable amounts between the two methods, indicating that the high temperature has minimal effect on the quality of DNA released (Baek *et al.*, 2010). The high power requirements for heating the sample to 90°C – 100°C are an important consideration when designing a portable device, which may be battery powered. Due to the small sample volumes present in a microsystem this power requirement to reach these temperatures are comparatively low when compared to standard lab heaters or thermal cyclers, but even a small addition to the systems power requirement can impact the amount of tests which can be processed before recharging.

1.1.18. Laser irradiated magnetic bead system (LIMBS)

LIMBS is a novel technique for the acceleration of thermal cellular lysis in biological samples in a microfluidic environment (Lee *et al.*, 2006). The sample is integrated with magnetic microbeads, and then an 808 nm, 1.0W laser is used to irradiate the sample for a period of 40 seconds. The microbeads facilitate lysis by dissipating the light energy of the laser uniformly throughout the sample as thermal energy, resulting in the thermal lysis of cells in the surrounding liquid. The 40 second irradiation is sufficient to lyse *E. coli* and gram negative bacteria in blood, and also the hepatitis B virus mixed with human serum.

Gold nanorods have also been used in microfluidic chambers to generate sufficient heat to lyse *E. Coli* cells following irradiation by a 808nm, 1.0W laser (Cheong *et al.*, 2008). The longitudinal resonance of the gold nanorods converts the near infra red energy into thermal energy, increasing the temperature in the 8µl chamber to 95°C after an irradiation period of 70 seconds. The gold nanoparticles where not inhibitory to the qPCR reaction to any degree, demonstrating that they do not interfere with PCR amplification. The chamber size used in the tests, 8µl, would require an extremely high level of sample concentration when dealing with a 1ml sample, in order to ensure all cells in the sample were present in the chamber prior to lysis. Alternatively a larger chamber could be used, but whether the thermal energy generated in this method would be sufficient in a larger

chamber remains to be seen. This could be rectified by using a more powerful laser, which has been shown to increase temperature when used in this manner (Cheong *et al.*, 2008).

1.1.19. Electrical lysis

Electrical lysis employs the use of electrical fields to increase membrane permeability or fully disrupt the cell wall/membrane. This method is well suited to incorporation into a microfluidic system as on chip systems can require as little as ~500V to generate a sufficient electric field to lyse bacterial cells, due to the small sizes involved minimising the distance between electrodes (Kim *et al*., 2009). This negates the need for a bulky generator and reduces the energy requirements needed, when compared to carrying the procedure out in the macroscale. It is a reagent free process, and both quicker and cheaper than chemical lysis. Electrical lysis can be achieved by exposing cells to high intensity pulsed electric fields (PEFs). The pulse length and electrical field strength required for lysis is dependent on the morphology of the target cell type, but the strength of the field needs to be sufficient to raise the cell membrane potential to ~1.1V (Kim *et al.*, 2009), typically between 500 V and 1000 V. Electrical lysis of red blood cells using a dc-biased ac electrical field has been demonstrated in a constriction microchannel (Church *et al.*, 2010). A fixed voltage of 160V was across the microchannel, producing 1600V/cm at the site of lysis. It was found that by increasing the ac component of the field the rate of lysis could also be increased. When 160 V pure dc was applied, very few cells were lysed. Partial lysis was seen at 80 V dc, 80 V ac, whilst complete lysis occurred upon the application of 40 V dc, 120 V ac. It was found that cells could be lysed as rapidly as 80ms after entering the electric field.

1.1.20. DNA extraction on a microfluidic chip

The next phase of the extraction process, subsequent to the lysis of cellular material in the sample, is the isolation and purification of the nucleic acid from the rest of the sample mixture, which includes numerous proteins, fragments of cell wall and cell membrane, and potentially any reagents used during the lysis process. The nucleic acid, once purified, requires transferring to the reaction phase in order to undergo amplification and detection. Maximisation of the efficiency of the extraction method, maximise the concentration of nucleic acid at the start of the amplification reaction, which leads to an improved sensitivity and reaction speed.

1.1.21. Solid Phase Extraction

Solid phase extraction (SPE) is the most frequently used technique for on-chip DNA extraction, and also the simplest. SPE utilises the affinity of solutes suspended in a liquid, such as the DNA in the sample, for a solid which the liquid is passed through. By passing the sample through a solid structure which DNA has a high affinity for, it is possible to retain the DNA whilst the rest of the sample can be removed. The DNA can then be eluted from the solid structure to which it is bound using a low ionic strength buffer, and then analysed.

1.1.22. Silica based SPE

A typical technique for SPE on chip involves silica based surface affinity, which utilises the ability of nucleic acid to bind with silica or glass in high ionic strength solutions due to decreases in the electrostatic repulsion (Kim *et al.*, 2009). The bound DNA is then washed with a non-polar solvent to remove any contaminating protein and then eluted using a low ionic strength buffer. A simple microfluidic chip housing an extraction channel containing only 1 nanogram of silica resin has been shown to be capable of extracting 70% of the DNA released from lysed white blood cells (Tian *et al.*, 2000), showing the efficiency of this method even in its most basic form.

A rapid and simple method for SPE on chip has been demonstrated using the chaotropic agent guanadinium thiocyanate, which facilitates the binding of the DNA to silica particles (Boom *et al.*, 1990). Guanadine salts are chaotropic agents, meaning that they can disrupt the bonds between water molecules in solution. This enables the formation of salt bridges between the negatively charged nucleic acid molecules, and negatively charged silica. These bonds form between the negatively charged oxygen atoms in the silica matrix and negatively charged oxygen atoms in the phosphate backbone of nucleic acids (Tan and Yiap, 2009).

Using this reagent has the added benefits of deactivating nucleases also released from the lysed bacterial cells. In this method guanadinium thiocyanate was also the reagent responsible for the lysis of the cells prior to SPE. Guanadine hydrochloride can also be used in this manner and poses far less of a health risk. This methodology has been adapted for use in a fully integrated microfluidic chip for the automated extraction of nucleic acid from human cells (Baier *et al.*, 2009). A silica filter material was used as the stationary phase in the SPE. Three layers of the silica filter were placed in a 30µl chamber, which the sample would flow through after cell lysis was completed. Any cellular debris

was then removed with an alcohol wash and the DNA was then eluted from the filters with a Tris-EDTA buffer.

The capture efficiency of silica based microfluidic devices is highly dependent on the surface area of silica available on which the DNA can bind. For example, in one study a silica chamber with a surface area 16% of that of another captured 78% less DNA (Christel *et al.*, 1999). By carefully designing silica containing SPE chambers to ensure maximum surface area, DNA capture efficiency can be increased. A recent novel microfluidic chip for DNA extraction utilised silica based monoliths to maximise DNA capture (Shaw *et al.*, 2009), which was found to be a robust and highly reproducible in a microfluidics device. One study (Chen *et al.*, 2007) detailed a fully integrated microfluidic device, including automated cell sorting, cell lysis and DNA purification. The DNA extraction step was carried out using a microfabricated porous silicon matrix, made by an anodizing technique using an ethanol/HF buffer electrolyte. A thermal oxidisation step was then carried out during manufacture to generate silanol groups on the surface, which act as nucleic acid absorption sites during the extraction. The silanol density was increased by the anodising and oxidation steps, increasing the absorption sites within the matrix. The chip was tested with raw blood samples and it was found that it could extract 37.5ng of purified genomic DNA from 1µl of blood sample. When compared to a bench top centrifugation based extraction kit, it was found the chip required 50 minutes running time to generate the same amount of DNA. Silica based SPE has been successfully integrated in a microfluidic chip in numerous studies, demonstrating great flexibility and reproducibility. The main disadvantages of this method of DNA extraction on chip is that the devices can be expensive and labour intense to fabricate, and do not lend themselves as easily to mass production as other DNA extraction methods such as polymer based SPE.

1.1.23. Polymer based SPE

Polymer based extraction chambers can be used as an alternative to silion based extraction chambers for SPE in a microfluidic chip. Materials such as poly(methyl methacrylate) (PMMA), or poly-carbonate (PC) have been used as a cheaper, more reproducible alternative to silicon as surfaces for DNA absorption on chip (Price *et al.*, 2009). Typically these techniques rely on electrostatic interactions between the negatively charged DNA and modified polymer surfaces.

The production of a novel microfluidic DNA extraction device composed of photoactivated polycarbonate (PPC), with its surface patterned using UV LiGA (a German acronym for Lithography,

electroplating and molding), a microfabrication technique capable of creating high aspect ratio surface features, has been described (Witek *et al.*, 2006). The extraction surface of the chip was covered with an ordered array of 5:1 aspect ratio posts, providing an increased surface area for nucleic acid binding. This surface was then exposed to UV radiation, creating carboxylate groups to assist in the absorbance of the nucleic acid. DNA is able to bind to carboxy-coated surfaces under a high concentration of poly (ethylene glycol) (PEG) and salts, which were present in the DNA extraction channel on chip (3% PEG, 0.4 M NaCl, 70% ethanol). Protein and debris were then removed by an ethanol wash which was applied through the chip and then DNA was then eluted. The chip demonstrated a high extraction efficiency, with 85% of a purified DNA sample retrieved. PCR amplifiable bacterial DNA was also extracted from a blood sample spiked with *E. coli* DNA. The whole process of extracting DNA took 25 minutes, which does not include the steps needed to remove the DNA from the cells, or originally concentrate the cells. However it is possible that this process could be optimised further.

DNA extraction chips utilising a surface coating of the linear polysaccharide chitosan have been developed (Cao *et al.*, 2006), which take advantage of the pH dependent binding of nucleic acid to chitosan. Chitosan has a cationic charge at a pH of 5, resulting in an electrostatic attraction to negatively charged DNA. However at pH 9 this charge is neutralised, meaning any bound DNA dissociates. A microfluidic chip containing an extraction chamber housing chitosan covered microbeads has been applied to the extraction of DNA from blood (Cao *et al.*, 2006). These beads bound DNA in a pH 5 solution, with the DNA then being eluted using a pH 9.1 Tris buffer. The recovery rate of human genomic DNA from a sample of lysed whole blood was found to be 68%, with a the purity of the extracted DNA being sufficient for PCR amplification.

1.1.24. Nanoporous membrane filtration

Nanoporous membranes have been used to selectively filter nucleic acid from bulk liquids by a combination of sieving and electrostatic interactions (Jungkyu *et al.*, 2006). Nanoporous aluminium oxide membranes (AOM) have been used to purify genomic DNA from lysed whole blood in a macrosystem (Elgort et al., 2004). AOM is commercially available under the trade names Anopore or Anodisc (Whatman inc, US), and can be obtained with pore sizes of 20nm, 100nm and 200nm. The membranes are 60µm thick and have a porosity of 50% which allows for high liquid flow rates. A study examining the optimisation of AOM filtration for DNA extraction on a microfluidic device

found that higher pH, salt concentration and anionic sample solutions all yielded higher capture efficiencies due to an increase in attraction between the AOM surface and DNA (Jungkyu *et al.*, 2006). It was also found that a nanopore diameter of 100nm was optimum when extracting DNA from complex biological samples.

1.1.25. LabDisk

The automation of laboratory processes using microfluidics, also known as lab-on-a-chip technology, enables complex, multi-step processes, which would normally be carried out manually by skilled technicians to be automated in a microfluidic environment. These devices typically use plastic chips or cassettes, complete with channels and chambers for the manipulation of small volumes of liquids and reagents, and various systems have been produced capable of automating DNA extraction (Baier *et al.*, 2009), blood fractionation (Amasia and Madou, 2010), immunoassays (Noroozi *et al.*, 2011), cell sorting (Burger *et al.*, 2012), and a range of other processes.

The movement of fluids in microfluidic systems can be achieved in a number of ways. Capillary forces can be used to passively draw liquid along a channel, without the application of any external forces, but this process is slow, and it is difficult to move large volumes in this manner. Micropumps and microvalves are commonly employed on microfluidic devices in order to manipulate fluid flow, although these are expensive and prone to breaking (Nguyen *et al.*, 2002). Additionally, microfluidic diagnostic devices are ideally self-contained and disposable, with all electrical equipment housed in the operating unit. Micropumps are too expensive to house on disposable chips, and the integration of pumps in the operating machine that could interact with each disk would be complex and expensive.

One novel method for addressing this issue is by the use of centrifugally operated microfluidic disks, which can be operated by spinning at varying speeds, in order to move and mix fluids using centrifugal, Euler and Coriolis forces (Ducrée *et al.*, 2007). The applications of centrifugally driven microfluidic systems have included haematocrit determination from blood samples (Ducrée *et al.*, 2007), magnetic bead based DNA purification (Strohmeier *et al.*, 2013), rt-PCR (Strohmeier *et al.*, 2014), and fully integrated multiplex immunoassays (Noroozi *et al.*, 2011).

The LabDisk, developed by IMTEK (Freiburg, Germany), is a microfluidic disk that can be operated by centrifugal control (Fig.1.3.), provided by a specially modified ESEQuant tube scanner (Qiagen, US), capable of applying the necessary centrifugal control to the LabDisk, and also housing an optical detection system (Fig.1.4.). The current control system is considered ill-suited for commercial use as a POC device as it can only handle a single LabDisk at a time, possesses single channel optics, suffers from temperature fluctuations, has no anti-contamination measures, and cannot integrate with existing medical software systems used in the UK.

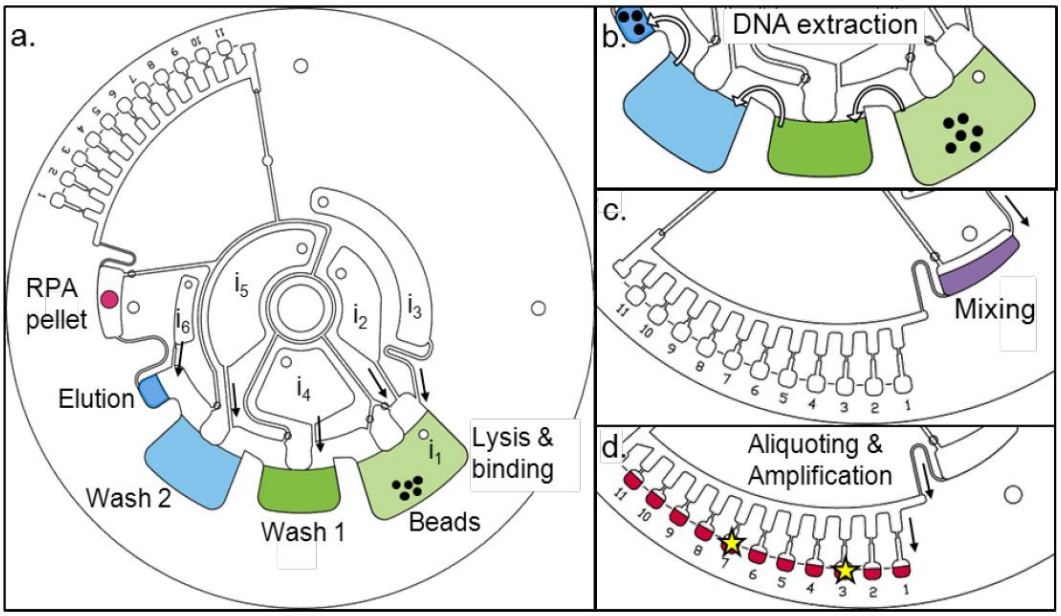

Fig.1.2. Schematic diagram of a LabDisk designed for the extraction of nucleic acid from a 200µl sample, with subsequent recombinase polymerase amplification (RPA) and fluorescence detection. (a) Chambers housing the sample and magnetic beads (i1), lysis buffer (i2), isopropanol (i3), wash 1 (i4), wash 2 (i5), and elution buffer (i6). (b) Magnetic silica bead based extraction is carried out via the movement of the beads from the lysis buffer to the elution buffer, via two wash buffer chambers, by holding the beads in position with a fixed magnet and then moving the disk around that point. (c) The eluted nucleic acid is then used to rehydrate the RPA reaction mix. (d) The eluted nucleic acid is then aliquoted into the reaction wells, rehydrating the primers dried into the wells, and the isothermal reaction begins by heating the disk to the required temperature. Detection is

carried out by the optical detection of fluorescence generated by the intercalating dye present in the RPA reaction mix. Image taken from Strohmeier *et al* (2012).

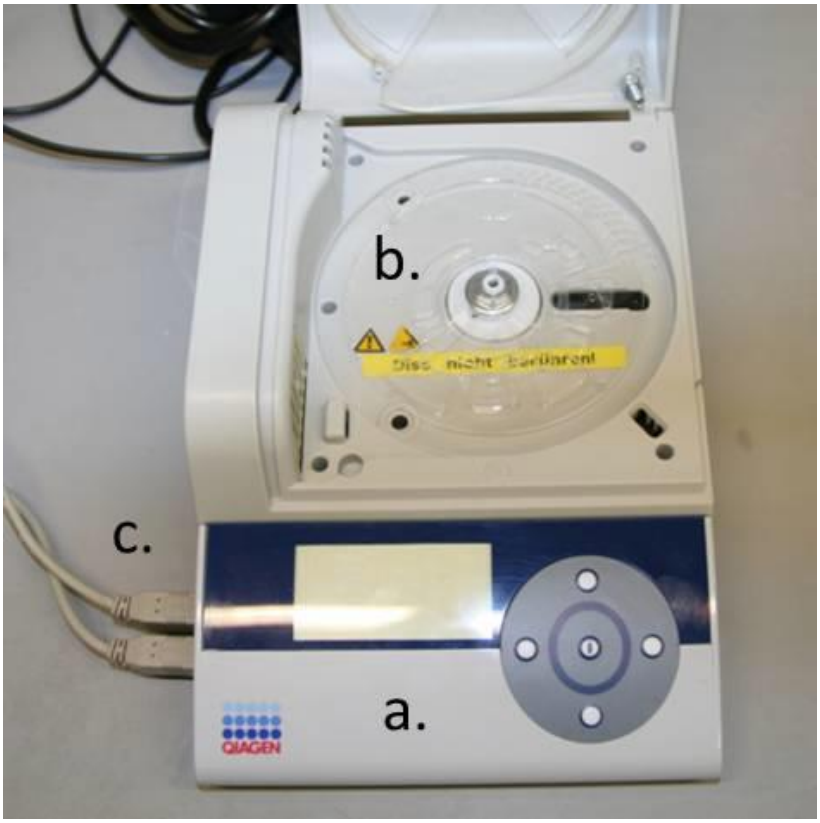

Fig.1.3. (a) The LabDisk control unit utilised by IMTEK, currently in development. (b) The plinth for the LabDisk. (c) USB connection between the control unit and operating PC. Image taken from Strohmeier *et al.*, (2012)

Aims

- To optimise the composition of the lysis buffer, and lysis process, in terms of speed and efficiency.
- To determine the tolerance of the extraction system, in combination with LAMP detection, for the presence of common amplification inhibitors found in clinical samples.
- To investigate the delivery of the lysis reagents in a dried format, in order to reduce on-disk fluid volumes.
- To optimise the swab acquisition process, determining the optimum swab type, transport media, and protocol for sample application

1.2. Materials and Methods

1.2.1. Culture of *N. gonorrhoeae*

N. gonorrhoeae (ATCC 19424) was cultured on chocolate agar (blood agar base, Sigma Aldrich, UK; defibrinated horse blood, TCS, UK) at 37^{o}C at 5% CO_2. Broth cultures were maintained under the same conditions, in brain heart infusion broth (Oxoid, UK).

1.2.2. Culture of *Escherichia coli*

E. coli K-12 (lab strain) was cultured on nutrient agar (Oxoid, UK) incubated at 37^{o}C. Broth cultures were maintained under the same conditions in nutrient broth (Oxoid, UK).

1.2.3. Use of human urine

For each experiment where human urine was used, the urine was a mix of urine taken from one individual over a 12 hour period. Urine was stored at 6^{o}C and used no later than 24 hours after excretion.

1.2.4. Public Health England (PHE) *N. gonorrhoeae* LAMP primer sets

Sequences were provided by PHE for *N. gonorrhoeae* LAMP primer sets, specific to the *GroEL* and *PorA* genes. The primer sequences are shown in Table 1.1.

LAMP Primer	Sequence	Length (bp)
N. gonorrhoeae GroEL F3	ACTTTATCAACGACGCGGAA	18
N. gonorrhoeae GroEL B3	ACGGTTTTCAGGATGCCG	19
N. gonorrhoeae GroEL FiP	AGGTCGCGGATGTTGCTGATTTAAATCGCCGGTCTGGACA	40
N. gonorrhoeae GroEL BiP	TGGAACAAGTGGCGAAAGCCAGACCAAAGTCGCCAAGGC	40
N. gonorrhoeae GroEL Loop F3	GTCGAACAGCAAAACAAACGG	21
N. gonorrhoeae GroEL Loop B2	CCCGCTGTTGATTATCGCTGAAG	23

Table 1.1. Sequences of the *N. gonorrhoeae GroEL* LAMP primer sets, designed by Public Health England Southwest.

1.2.5. LAMP Reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), as follows; 5µl five times LAMP reaction buffer, 12µl molecular grade water, 1µl intercalating dye (propriety dye, emission in FAM channel), 1µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 1µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers. Loop primers, if present, were included at a 25pmol/µl concentration in the primer mix. Finally 5µl of DNA sample or water was added to the reaction. Reactions were carried out in a ESEQuant Tube Scanner (Qiagen Inc., CA), at 63^{o}C for 60 minutes. Reactions were terminated by heating to 80^{o}C for 1 minute in order to denature the *Bst* polymerase.

1.2.6. Reaction Product Detection

Detection of reaction products from the PCR and LAMP reactions was carried out primarily by electrophoresis, using 1.5% w/v agarose gels with transillumination and photography provided by a

BioRad Gel/Chem Doc system (Bio-Rad Inc, UK). LAMP reactions were also monitored in real-time using the ESEQuant Tube Scanner (Qiagen Inc, UK), through the FAM channel. The "Tube Scanner Studio" software package was used to analyse the real time data and produce a manually set threshold line for the purposes of determining time to amplification. For experiments involving a larger number of simultaneously carried out reactions, an ABI 7500 real-time PCR system was used for the amplification and detection of product. The ABI software was used to manually set a threshold line for the determination of cycle threshold (ct) values.

1.2.7. Total nucleic acid extraction from *E. coli* culture using a magnetic silica based total nucleic acid extraction method.

A 10ml sample of an overnight culture of *E.coli* K-12 in nutrient broth was added to 100ml of nutrient broth in a shaking incubator at 37^{o}C. After 3 hours of incubation the OD_{600} of the culture was monitored every 30 minutes until an OD_{600} of 0.4 was reached, indicating the culture had entered growth phase and mRNA expression would subsequently be high.

The extractions were carried out using a Kingfisher Ml automated nucleic acid extraction platform. A single 5 well strip was added to the Kingfisher Ml tray for each of the 3 extractions to be carried out. Each well of the strips was then filled with the correct buffer, according to Table 1.2:

Well	Buffer	Volume (µl)	Time in Buffer
1	Lysis	750 + 10 Magnasil	10 mins
2	Wash 1	750	30 secs
3	Wash 2	750	30 secs
4	Wash 3	750	30 secs
5	H_20	75	10 mins

Table 1.2. Buffers, volumes and duration of each step of the extraction process automated by the KingFisher ML system.

The composition of the standard buffers are shown in Tables 1.3. – 1.6;

X1 Lysis Buffer (101.5ml)	
Guanidinium isothiocyanate	50g
0.1M Tris/HCl pH 6.4	42.5ml
0.2M EDTA pH 8.0	9ml
Isopropanol	50ml

Table.1.3. The composition of the standard lysis buffer

Wash Buffer 1: Isopropanol Wash (105ml)	
Isopropanol	25ml
Ethanol	25ml
H_2O	50ml
4M NaCl	5ml

Table.1.4. The composition of wash buffer 1

Wash Buffer 2: NEET Wash (105ml)	
Ethanol	50ml
H_2O	50ml
4M NaCl	5ml

Table.1.5. The composition of wash buffer 2.

Wash Buffer 3: 75% Etoh (100ml)	
Ethanol	75ml
H_2O	25ml

Table.1.6. The composition of wash buffer 3.

200µl of the sample to be processed was then added to well 1 of each strip. The correct extraction programme was selected, and started. After elution, the eluted nucleic acid was removed and used in downstream applications, or stored at -80^{o}C.

1.2.8. Agarose gel electrophoresis of nucleic acid

Extracted nucleic acid was examined by electrophoresis, using 1.5% w/v agarose gels, containing 1µl of 1omg/ml ethidium bromide solution per 100ml of gel. Transillumination and photography was provided by a Gel/Chem Doc system (Bio-Rad Inc, UK). Aliquots of 8µl of nucleic acid was added to 2µl of 5x DNA loading buffer (Bio-Rad Inc, UK). Electrophoresis was carried out at 80V for 90 minutes.

1.2.9. Quantification of nucleic acid

DNA was quantified spectrophotometrically using Spectrostar plate reader in conjunction with an LVIS plate (BMG Labtech, UK).

1.2.10. A comparison of thermal and chemical lysis methods for total nucleic acid extraction from *E. coli* culture

A 10ml sample of an overnight culture of *E. coli* K-12 in nutrient broth was added to 100ml of nutrient broth in a shaking incubator at 37°C. After three hours of incubation the OD_{600} of the culture was monitored every 30 minutes until an OD_{600} of 0.4 was reached. Three 1ml samples of this culture was then taken; Samples A, B and C. Total nucleic acid was extracted from the samples according to the methodology detailed in section 1.2.7, with sample A remaining in the lysis buffer for 10 minutes, sample B remaining in the lysis buffer for 30 seconds, and sample C remaining in the lysis buffer for 30 seconds, after being heated to 95°C for one minute in a waterbath. 50µl of extracted RNA was taken from each sample and visualised on a 2% agarose gel stained with ethidium bromide, in order to assess RNA integrity and relative quantity.

1.2.11. Initial evaluation of the effect of chaotropic salt on lysis buffer performance

Nucleic acid extractions were carried out from a cell suspension of *N. gonorrhoeae* (1×10^5 CFU/ml) in urine, using the Kingfisher ML system. Extractions were carried out using the standard guanidine

isothiocyanate lysis buffer, a lysis buffer containing an equal molarity of guanidine hydrochloride in place of the guanidine isothiocyante, and the guanidine hydrochloride lysis buffer with a 95°C pre heating step. The composition of the guanidine hydrochloride lysis buffer is shown in Table 1.7. Five replicates were carried out for each lysis buffer.

Component	Weight/Volume of components	
	GuCN buffer	GuHCl buffer
Guanadine isothiocyante	10g	
Guanadine hydrochloride		8.12g
0.1M Tris/HCl pH 6.4	8.5ml	8.5ml
0.2M EDTA pH 8	1.8ml	1.8ml
Isopropanol (IPA)	10ml	10ml
Guanadine molarity (buffer)	3.86 M	3.86 M
Guanidine molarity (final)	3.05 M	3.05 M

Table.1.7. Composition of the guanidine isothiocyante and guanidine hydrochloride based lysis buffers

The extracted nucleic acid from each extraction was then assayed for the presence of *N. gonorrhoeae* nucleic acid using the GC *GroEL* LAMP assay, and reactions were monitored in real-time using a Qiagen ESEQuant Tubescanner.

1.2.12. Effect of guanidine hydrochloride lysis buffer and thermal lysis on a range of dilutions of *N. gonorrhoeae* cell suspension in urine.

Nucleic extractions were carried out on 200µl aliquots of *N. gonorrhoeae* cell suspension in urine, with cell concentrations of 1 x 10^4, 1 x 10^3, 1 x 10^2 and 10 CFU per 200µl, using the guanidine isothiocyante based lysis buffer, and the guanidine hydrochloride buffer with a 95°C 30 second heat step either before or after the addition of the sample. These extractions were carried out in triplicate for each lysis method, from each dilution of cell suspension. Extracted nucleic acid was assayed for the presence of *N. gonorrhoeae* using the GC *GroEL* LAMP assay, which was monitored in real-time using an ABI-7500. The amplification times were compared to given an indirect measurement of the nucleic acid yield and integrity produced using each lysis method.

1.2.13. Effect of the duration of the 95°C heating step during lysis on the yield of nucleic acid and LAMP amplification time

Nucleic extractions were carried out on 200µl aliquots of *N. gonorrhoeae* cell suspension in urine, with cell concentrations of 1 x 10^4, 1 x 10^3, 1 x 10^2 and 10 CFU per 200µl, using the guanidine isothiocyante based lysis buffer, and the guanidine hydrochloride buffer with a 95°C 30 second heat step either before or after the addition of the sample. These extractions were carried out in triplicate for each lysis method, from each dilution of cell suspension. Extracted nucleic acid was assayed for the presence of *N. gonorrhoeae* using the GC *GroEL* LAMP assay, which was monitored in real-time. The amplification times were compared to given an indirect measurement of the nucleic acid yield and integrity produced using each lysis method.

1.2.14. Effect of GuHCl concentration on concentration and quality of extracted DNA

In order to determine the optimal guanidine hydrochloride concentration, nucleic acid extractions were carried out from a *N. gonorrhoeae* cell suspension in urine (1 x 10^6 CFU/ml) using lysis buffers containing guanidine hydrochloride concentrations of 3.5M, 4M, 4.5M, 5M, 5.5M, and 6M. The final concentrations after the addition of the sample are shown in Table 1.8.

GuHCl buffer concentration (M)	Final GuHCl concentration (M)
6	4.74
5.5	4.35
5	3.95
4.5	3.55
4	3.15
3.5	2.76

Table 1.8. The final guanidine hydrochloride concentration in the lysis reaction, corresponding to the buffer concentration.

The extracted nucleic acid was quantified spectrophotometrically, and the 260nm/280nm ratio was taken as a measure of purity.

1.2.15. Buffer component concentrations

In order to determine whether the EDTA or Tris/HCl concentrations in the lysis buffer could be reduced without negatively effecting the yield or purity of the extracted nucleic acid, lysis buffers containing reduced volumes of these components were tested. nucleic acid extractions were carried out in triplicate from a *N. gonorrhoeae* cell suspension in urine (1 x 10^6 CFU/ml) using lysis buffers containing 100%, 75%, 50% 25%, and 0% of the original volume of 0.2M EDTA (Table 4).

Component	**Weight/Volume of components**				
	100% EDTA	**75% EDTA**	**50% EDTA**	**25% EDTA**	**0% EDTA**
Guanadine hydrochloride	8.12g	8.12g	8.12g	8.12g	8.12g
0.1M Tris/HCl pH 6.4	8.5ml	8.5ml	8.5ml	8.5ml	8.5ml
0.2M EDTA pH 8	1.8ml	1.35ml	0.9ml	0.45ml	-
Isopropanol (IPA)	10ml	10ml	10ml	10ml	10ml
H_2O	-	0.45ml	0.9ml	1.35ml	1.8ml

Table1.9. Composition of buffers tested with varying concentrations of EDTA

Additionally, nucleic acid extractions were carried out in triplicate from a *N. gonorrhoeae* cell suspension in urine (1 x 10^6 CFU/ml) using lysis buffers containing 100%, 75%, 50% 25%, and 0% of the original volume of 0.1M Tris/HCl (Table 5).

Component	**Weight/Volume of components**				
	100% Tris/HCl	**75% Tris/HCl**	**50% Tris/HCl**	**25% Tris/HCl**	**0% Tris/HCl**
Guanadine hydrochloride	8.12g	8.12g	8.12g	8.12g	8.12g
0.1M Tris/HCl pH 6.4	8.5ml	6.38ml	4.25ml	2.13ml	-
0.2M EDTA pH 8	1.8ml	1.8mll	1.8ml	1.8ml	1.8ml
Isopropanol (IPA)	10ml	10ml	10ml	10ml	10ml
H_2O	-	2.13ml	4.25ml	6.38ml	8.5ml

Table 1.10. Composition of buffers tested with varying concentrations of Tris/HCl

Extracted nucleic acid was quantified spectrophotometrically, with the 260nm/280nm ratio used as a measure of purity.

1.2.16. Comparison of 1x and 2x lysis buffer for total nucleic acid recovery from clinical *C. trachomatis* samples, using a silica based total nucleic extraction method.

Nucleic acid extractions were carried out on 4 clinical *C. trachomatis* samples (2 positive urine samples, 1 positive endocervical swab sample, 1 negative urine sample), using the silica based total nucleic acid extraction method with both the standard 1x lysis buffer, and a 2x concentrated buffer. All extractions were tested for the presence of *C. trachomatis* nucleic acid with the CT-0332 LAMP assay.

1.2.17. Robustness of nucleic acid extraction method in the presence of inhibitory compounds

Nucleic acid extractions were carried out on 100µl aliquots of a suspension of *N. gonorrhoeae* cells in urine (1×10^5 CFU/ml), which were spiked with varying concentrations of whole blood, E. *Coli* culture, or bovine serum albumin (BSA) in 100µl volumes. The red blood cell content in whole defibrinated horse blood was quantified using an improved Neubauer haemocytometer. The extractions containing blood included 100µl of whole blood containing a concentration of red blood cells ranging from 5.5×10^7 to 5.5×10^3. The extractions containing E. coli cells included a range of 1:10 dilutions of E. coli cell suspension in nutrient broth, from 1×10^8 CFU to 1×10^5 CFU per 100µl. The suspension of *E. coli* was diluted to an OD_{600} of 1 (determined to be 1×10^9 CFU per ml by a previously calibrated growth curve). For the reactions containing BSA, a BSA solution of 20mg/ml was created using distilled water. Dilutions of this stock solution were used in 100µl volumes to provide final BSA concentrations of 10mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml and 0.1mg/ml per extraction. Extractions were carried out from each sample, and the nucleic acid was tested using the GC *GroEL* LAMP assay for the presence of the *N. gonorrhoeae* specific target. The reactions were monitored in real-time, and the speed of the reaction was used to examine for any inhibitory effects resulting from the inclusion of the potential inhibitory components in the extraction. Reactions were not carried out in duplicate or triplicate in order to conserve LAMP reagents.

1.2.18. Effect of lysis buffer lyophilisation on nucleic acid extraction

Four 375µl aliquots of 2x lysis buffer were lyophilised in a freeze drier for 6 hours. The freeze dried buffer aliquots were then each rehydrated with 375µl of H_2O. Magnetic silica based total nucleic acid extractions were then carried using the rehydrated lysis buffer from a sample of *N. gonorrhoeae* cell suspension in urine (2×10^6), in duplicate. Extractions were also carried out from the same samples using standard 2x lysis buffer, and from cell free water, using both lyophilised and liquid lysis buffer, as experimental controls. 5µl from each extracted nucleic acid sample was taken and tested for the presence of amplifiable *N. gonorrhoeae* nucleic acid using the GC *GroEL* LAMP assay, and the reactions were monitored in real-time to determine the presence of any inhibitory effect stemming from the use of the freeze dried buffer.

1.2.19. Tablet Manufacture

The lysis tablets were rehydrated with 1ml of sample, and in order to ensure the final GuHCl concentration was 3.6M, equal to that of the final concentration of GuHCl obtained using the 2x lysis buffer and 200µl sample, the water volume and GuHCl weight was adjusted. The components of the 2x lysis buffer, and composition of the buffer needed for the 1ml lysis tablets are shown in table 1.11 and table 1.12, respectively.

Component	Weight/Volume
GuHCl	8.3g
1M Tris/HCl pH 6.4	0.85ml
0.2M EDTA pH 8	0.9ml
H_2O	3.2ml
IPA	5.15ml
Total measured volume	15.75ml
GuHCl molarity	5.52M
GuHCl weight in 1ml	529.3mg
GuHCl weight in 375µl	197.7mg
GuHCl molarity in 375µl + 200µl sample	3.6M

Table 1.11. Composition of the 2x GuHCl lysis buffer

Component	Weight/Volume
GuHCl	8.12g
1M Tris/HCl pH 6.4	0.85ml
0.2M EDTA pH 8	0.9ml
H_2O	3.4ml
H_2O (IPA replacement)	5.15ml
Total measured volume	15.75ml
GuHCl molarity	5.4M
GuHCl weight in 1ml	515.8mg
GuHCl molarity in 1.5ml	3.6M

Table 1.12. Composition of the 2x GuHCl tablet lysis buffer

To produce lysis tablet 1, aliquots of 5ml of the GuHCl tablet lysis buffer were frozen at -80^{o}C for 24 hours, and then freeze dried. This freeze dried powder was then weighed into 516mg amounts, which were then individually compressed using an F3 single-station tablet press (Manesty, UK), using a 12.4mm diameter bevel edged punch and die set. The machine was manually operated by filling the die and manually turning the fly wheel through a full compression cycle. Tablets were individually stored in sealed plastic bags until use.

To produce lysis tablet 2, freeze dried GuHCl lysis buffer (Table 7) was taken and mixed with a range of excipients (Table 8). The excipients and ratios chosen are a basic mix that has been optimised by researchers in the School of Pharmacy.

This powder was weighed into 516mg amounts and then compressed into tablets using the same method as used during the manufacture of tablet 1. This produced a tablet with the same weight and size as tablet 1, but 50% of the active buffer component, meaning two tablets would be needed per extraction from a 1ml sample volume.

Component	**Weight**
Freeze dried 2x GuHCl tablet lysis buffer (active ingredient)	50%
Lactose (bulking agent)	46%
Explotab (disintegrant)	3%
Mag st (lubricant)	1%

Table 1.13. Composition of lysis tablet 2

1.2.20. Initial test of tablet performance

Nucleic acid extractions were carried out from a suspension of *N. gonorrhoea* in urine (2×10^6) and a reduced density cell suspension (2×10^2), using standard liquid lysis buffer, lyophilised lysis buffer, lysis tablet 1, and lysis tablet 2. Extractions using the liquid reagent were carried out as previously described.

During the extraction using the lysis tablets, a single lysis tablet 1, or two lysis tablet 2's were added to 1ml of the cell suspension, and 0.5ml of IPA, in a bijoux. The bijoux was gently agitated for 5 seconds in order to fully dissolve the tablet. A 1ml aliquot of this suspension was then added to the first well of a Kingfisher ml extraction strip, along with 10µl of MagnaSil beads. Although this method effectively reduced the volume of urine sample in the extraction from 1ml to 660µl, it is still preferable to attempting to carry out the extractions manually using a magnet, due to the unacceptably high loss of MagnaSil beads. The samples were then extracted using the KingFisher, as per the standard protocol. This methodology was also used for the lyophilised buffer. Extracted nucleic acid was assayed using the GC *GroEL* LAMP assay, and reactions were monitored in real-time.

1.2.21. Lysis buffer pellets

Component	Weight/Volume for 1ml tablet 1	Weight/Volume for 790µl pellet
GuHCl	8.12g	13g
1M Tris/HCl pH 6.4	0.85ml	1.65ml
0.2M EDTA pH 8	0.9ml	1.75ml
H_2O	3.4ml	3.4ml
H_2O (IPA replacement)	5.15ml	5.15ml
Total measured volume	15.75ml	20ml
GuHCl molarity	5.4M	6.8M
GuHCl weight in 1ml (700µl for tablet 2)	515.8mg	515.8mg
GuHCl molarity in 1.5ml	3.6M	3.6M

Table 1.14. Comparison of the composition of the 1ml lysis tablet and 790µl lysis pellet buffers

The 790µl lysis pellet buffer was formulated using the components shown in Table 1.14, in order to provide an equal final concentration of reagents as the 1ml lysis tablet, whilst reducing the volume of lysis buffer required, and the size of the pellet.

The lysis pellet buffer was frozen overnight at -80^{o}C in 700µl aliquots in plastic circular moulds (2.7cm diameter). These moulds were then placed in the cold chamber of a freeze drier, and freeze dried for 24 hours. Extractions were then carried out using the same methodology as that used with the lysis pellets, using one lysis pellet with a 1ml aliquot of sample and 0.5ml of IPA, on *N. gonorrhoeae* cell suspension with concentrations of 1 x 10^2, 1 x 10^3, 1 x 10^4, and 1x 10^5 CFU per ml. Extractions were also carried out on the same suspensions using standard liquid lysis buffer. Extractions were carried out for each dilution of *N. gonorrhoeae* in triplicate and then tested using the GC *GroEL* LAMP assay. Reactions were monitored in real-time using an ABI 7500 rather than the ESEQuant, in order to be able to run all assays simultaneously using the same reaction mix. The time to amplification of the assay was examined in order to determine any effect on the assay speed resulting from the use of the lysis pellets.

1.2.22. Lyoprotectants

Lysis buffer pellets were manufactured containing lyoprotectants at arrange of concentrations. The lyoprotectants were added as solutions during the manufacture of the liquid lysis buffer, and the volume of water added was reduced to account for this. Lysis buffers containing 0%, 1%, 2%, 4%, 8%, and 10% polyvinylpyrrolidone (PVP) were made, along with lysis buffers containing 5%, 10% and 15% Lyo B, and 5%, 10% and 15% Lyo D. Aliquots of 790µl of these buffers were frozen at -80^{o}C for 24 hours, in plastic cylindrical moulds with a 2.7cm diameter. The frozen buffer was then freeze dried in the temperature controlled chamber of a Lyotrap freeze drier (LTE) for 24 hours. Extractions were carried out using each pellet, following the same methodology as that used previously for the lysis pellets, using *N. gonorrhoeae* suspension in urine (1x 10^5 CFU per ml). Triplicate extractions were carried out for each variety of pellet. The GC *GroEL* LAMP assay was used to amplify target nucleic acid in the extracted samples. The time to amplification in each assay was monitored and compared, to test for any inhibitory effect of the various tablet compositions.

1.2.23. Lyoprotectants: PEG

Volumes of the lysis pellet buffer were produced, including varying concentrations of PEG 8000; 0%, 1%, 2%, 4%, 8%, 10%. The PEG 8000 was added as a solution, and the volume of water added to make the buffer up to the correct volume was reduced to account for this. The lysis buffers were frozen in individual 790µl aliquots in plastic cylindrical moulds with a 2.7cm diameter, at -80^{o}C for 24 hours. The frozen buffer was then freeze dried in the refrigerated chamber of a Lyotrap freeze drier (LTE) for 24 hours.

1.2.24. Swab performance

An evaluation of the ability of three swab types to recover *N. gonorrhoeae* cells from a cell suspension of varying concentration was carried out. The swabs used for the test were; flocked swabs, Sterilin cotton swabs, and Copan cotton swabs. A series of 1:10 dilutions of *N. gonorrhoeae* O/N culture (Brain heart infusion media) were carried out, and 10µl of each dilution was transferred

to chocolate agar (in triplicate), to enable quantification of viable cells using the Miles & Misra method. Volumes of 10ml were used for each dilution.

For each swab to be tested, 3x 1ml aliquots of each cell suspension were taken in eppendorf tubes. A swab was then placed into a tube and slowly rubbed up and down for 10 seconds. This swab was then removed, entered into a 1ml aliquot of sterile BHI media, and rubbed up and down for 10 seconds, before being disposed of. This media was then vortexed briefly, and 10µl of the media was transferred a chocolate agar plate (in triplicate) and left to air dry. The plates were then transferred to the incubator, at 37^{o}C and 5% CO_2. This process was repeated for all further dilutions of cell suspension. Colonies were then counted in order to work out the CFU/ml of the inoculated transport media, and then compared to the CFU/ml of the starting suspension in order to give the recovery rate.

1.2.25. Effect of Vortexing speed on swab recovery

An experiment was carried out in order to determine the effect of vortexing during the elution of the swab in transport media, on recovery rates. Copan flocked swabs were used to collect cells from 1ml aliquots of *N. gonorrhoeae* liquid culture (2x10^6 cells per ml). Total viable counts were performed using the initial cell suspension. The swabs were then placed in 1ml aliquots of Copan transport media, and vortexed at either 500rpm or 1000rpm for five seconds. Swabs that were left for five seconds without vortexing were used as a control. Total viable counts were then carried out using the transport media, in order to determine the overall recovery rate.

1.2.26. Swab buffer performance

An evaluation of three possible transport media were carried out, in order to determine which would give the optimum recovery rate from the Copan flocked swab. The three media tested were Copan Universal Transport Medium (UTM), PBS, and PBS tween (0.02% Tween 80). A *N. gonorrhoeae* cell suspension of 8.5 x 10^6 CFU/ml was serially diluted to 8.5 x 10^3 CFU/ml. Copan flocked swabs were used to collect cells from 1ml aliquots of each dilution, and then eluted into the various transport media, in triplicate. Total viable counts were then performed to determine the effect of

the transport media composition on organism elution, and overall recovery. Vortexing was not used to improve the capture rate, as the project partners were still undecided whether vortexing could be a viable option.

1.2.27. Evaluation of the use of lysis buffer as swab transport media

Copan flocked swabs were used to collect cells from 1ml aliquots of *N. gonorrhoeae* broth culture of three concentrations; 1 x 10^6 CFU per ml, 1 x 10^5 CFUper ml and 1 x 10^4 CFU per ml, in triplicate. The swabs were then taken and placed in either 1ml aliquots of 1x GuHCl lysis buffer or Copan transport media. Total nucleic acid extractions were carried out using 200µl of either the lysis buffer or transport media, using the KingFisher platform. The extracted nucleic acid from each sample was then assayed using the GC *GroEL* LAMP assay, monitored in real time using an ABI 7500.

1.2.28. Comparison of the effect of swab incubation time in lysis buffer and Copan transport media

Copan flocked swabs were used to collect cells from 1ml aliquots of *N. gonorrhoeae* liquid culture (2x10^6 cells per ml). The swabs were then taken and placed in either 1ml aliquots of lysis buffer or Copan transport media, and then incubated for variable time periods (0, 10, 20, or 30 minutes). Total nucleic acid extractions were carried out using 200µl of either the lysis buffer or transport media, using the KingFisher platform. The extracted nucleic acid from each sample was then assayed using the GC *GroEL* LAMP assay, monitored in real time using an ABI 7500.

1.3. Results

1.3.1. Total nucleic acid extraction from *E. coli* culture using a magnetic silica based total nucleic acid extraction method.

The nucleic acid extraction method initially under investigation was a magnetic silica based extraction system, capable of extracting and purifying both RNA and DNA from microbial cells. The process involved the addition of the sample to a lysis buffer containing guanidine salt, which simultaneously breaks open the cells and protects the released nucleic acid from nuclease activity via its reducing activity. Magnetic silica beads present in the buffer bind released nucleic acid, which are then moved between various wash buffers by magnetic forces, facilitating the removal of protein contaminants which may have an inhibitory effect on the downstream LAMP reaction. It is important that the extraction method is capable of purifying both undegraded genomic DNA, and RNA from the urine and swab samples that will be tested. The LAMP assays to be used may amplify RNA sequences, via the incorporation of reverse transcriptase, which can lead to increased assay sensitivities via the increased starting copy number often afforded by the use of an RNA target.

E.coli was used in place of *N. gonorrhoeae*, which was used throughout the extraction development process as *E. coli* can be grown to high cell densities, and is highly metabolically active. *E. coli* o/n culture contains more DNA, and RNA than an equivalent *N. gonorrhoeae* culture, enabling a better determination of nucleic acid quality via gel electrophoresis.

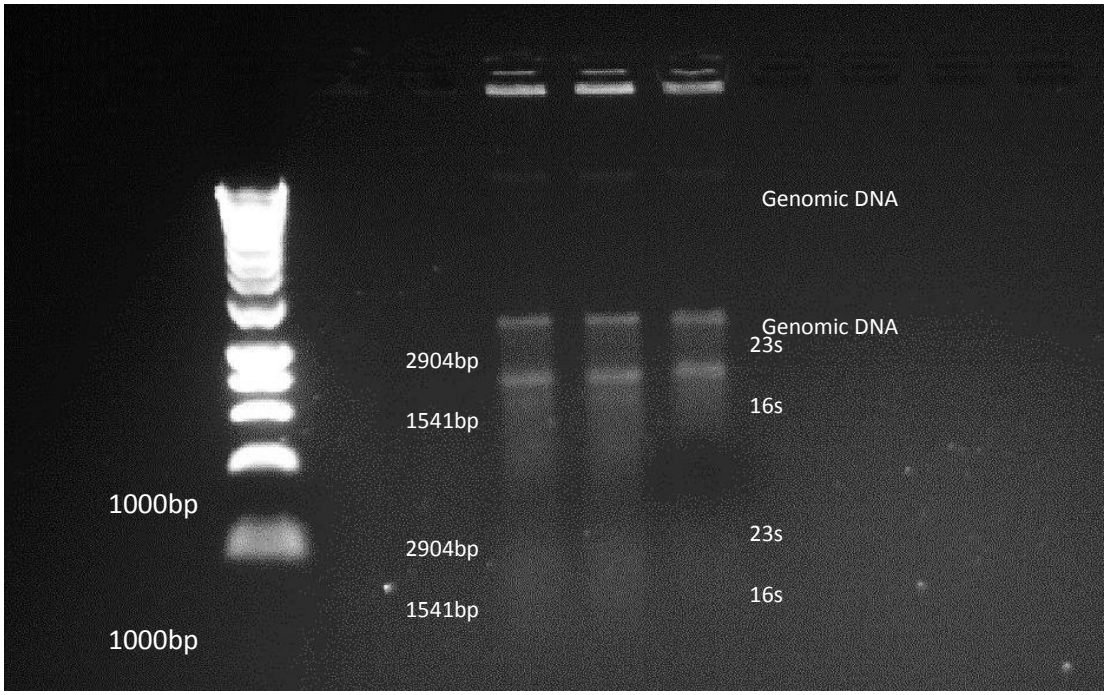

Fig.1.4. Agarose gel electrophoresis (1.5%) of total nucleic acid extractions from *E.coli* culture, using the magnetic silica based extraction method. Bands representing 23S rRNA, 16S rRNA and genomic DNA are indicated. Hyperladder II (Bioline) used as marker for fragment size.

The extracted genomic DNA could be seen as a distinct high molecular weight (>10kbp) band, indicating the extraction of unfragmented genomic DNA from the sample (Fig.1.4).

A bright band could also be seen inside the loading well on the gel. This is likely to be cellular proteins from the extraction, which are unable to pass through the agarose gel due to their large size. Positively charged proteins could bind to the negatively charged DNA, which would in turn bind to ethidium bromide molecules in the gel, producing a fluorescent band when exposed to the UV light source.

The two bright bands at 2904bp and 1541bp indicate the 23s and 16s ribosomal RNA subunits respectively. The 23s and 16s bands were sharp and unbroken, with equal intensity. The integrity and ratio between these two bands are important indicators of overall RNA integrity (Jahn *et al*., 2008).

A light smearing could be seen between the 1000bp and 100bp mark. This could be degraded portions of the 23s or 16s fragments, or indicative of the population of low weight undegraded mRNA.

1.3.2. A comparison of thermal and chemical lysis methods for total nucleic acid extraction from *E. coli* culture

In the magnetic silica based total nucleic acid extraction protocol, the bacterial cells were initially lysed in a lysis buffer containing guanidine isothiocyanate and Triton X-100, which act to break open the cell membranes and release intracellular nucleic acid. An alternative method would be thermal lysis, which uses rapid heating, typically to 95^{o}C, to break open any cells in the sample. Thermal lysis is a more rapid process than chemical lysis, however the heat can act to degrade released RNA, which would reduce the available LAMP template, if the assay is designed for an RNA target. The effect of a 30 second 95oC heat step, followed by a 30 second incubation, on the relative yield and purity of extracted nucleic acid was examined.

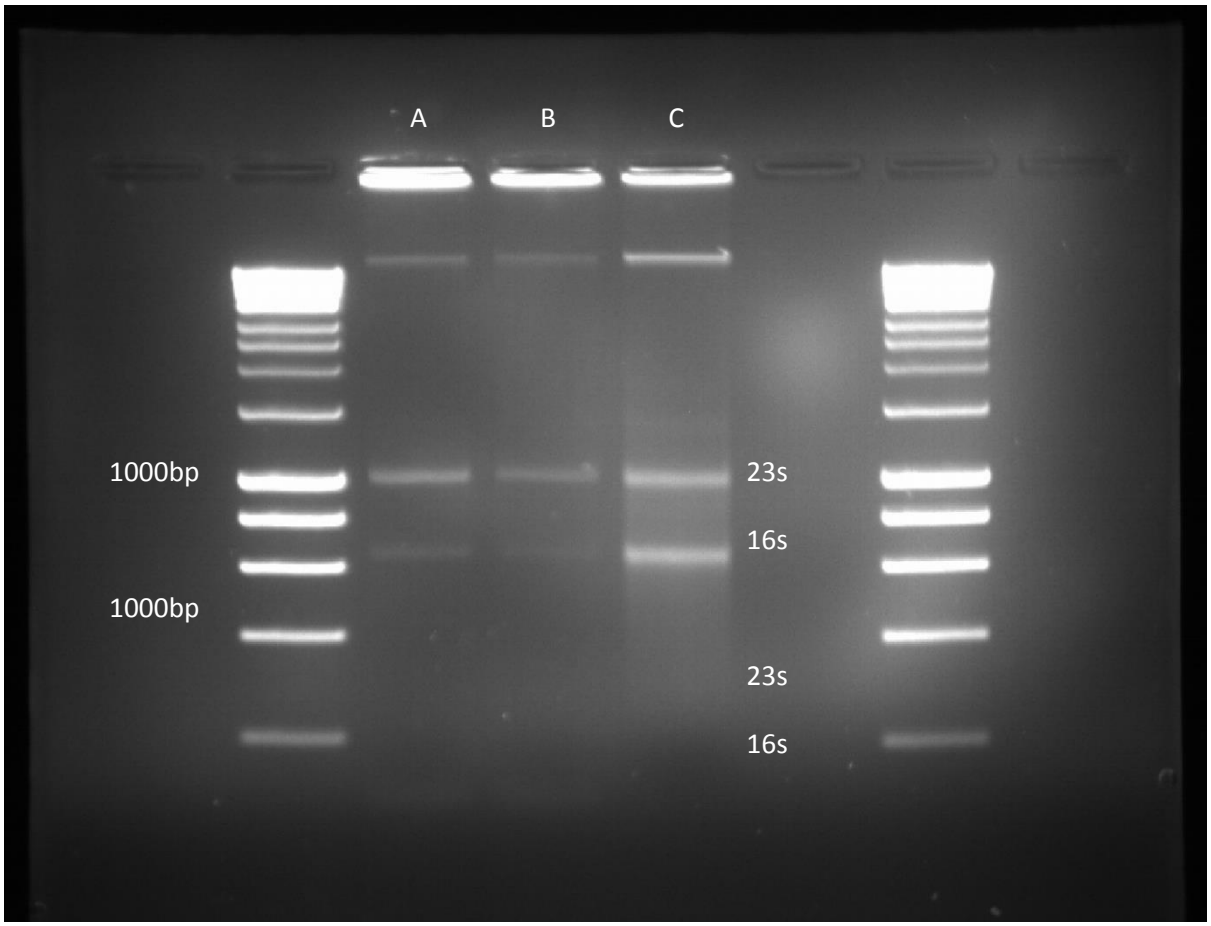

Fig.1.5. Agarose gel electrophoresis (1.5%) showing comparison of nucleic acid concentration and integrity obtained from *E. coli* using chemical lysis and thermal lysis during the nucleic acid extraction procedure. Well A was loaded with 30µl of extracted DNA from cells lysed via a 10 minute lysis buffer incubation. Well B was loaded with 30µl of extracted DNA from cells lysed via a 1 min lysis buffer incubation. Well C was loaded with 30µl of extracted DNA from cells lysed via a 30

second 95°C heat treatment, followed by a 30 second incubation in lysis buffer. Bands representing 23s rRNA and 16s rRNA are indicated. Hyperladder II (Bioline) used as marker for fragment size.

In all three samples the genomic DNA, 16s rRNA, and 23s rRNA bands appear as sharp bands, showing a low level of degradation during the extraction process (Fig.1.5). Sample C (nucleic acid extracted from cells lysed by a 30 second heat treatment of 95°C, followed by a 30 second incubation in lysis buffer) yielded a larger level of both DNA and RNA relative to samples A and B, evidenced by brighter fluorescence of the genomic DNA and rRNA bands.

The genomic DNA and rRNA bands of Sample A, nucleic acid extracted from cells lysed by a 10 minute buffer incubation, exhibited fluorescence similar to those of sample B, in which the cells were only exposed to lysis buffer for 1 minute.

1.3.3. Initial evaluation of the effect of chaotropic salt on lysis buffer performance

The use of heat during the lysis process produced a high yield of nucleic acid, whilst having no negative effects on RNA or DNA integrity (Fig.1.5). In the original lysis buffer, the lytic agent was guanidine isothiocyanate, a potent chaotropic salt which in addition to lysing cells in the sample also reduces proteins, preventing endonuclease activity. If complete cellular lysis could be achieved by heating the sample, then guanidine isothiocyanate could be replaced with guanidine hydrochloride, which is a less potent lytic agent, but would still provide protection from nucleic acid degradation. Guanidine hydrochloride is a safer compound than guanidine isothiocyante, which can produce hydrogen cyanide gas when mixed with bleach, and the inclusion of this chemical could lead to less stringent regulations on the use of the test.

A guanidine hydrochloride based lysis buffer, containing the same molarity of chaotropic salt as the previously used guanidine isothiocyanate buffer, was produced (Table 1.15). The performance of this buffer, with and without a 95°C heat step, was tested alongside the guanidine isothiocyanate lysis buffer.

Component	Weight/Volume of components	
	GuCN buffer	GuHCl buffer
Guanadine isothiocyante	10g	
Guanadine hydrochloride		8.12g
0.1M Tris/HCl pH 6.4	8.5ml	8.5ml
0.2M EDTA pH 8	1.8ml	1.8ml
Isopropanol (IPA)	10ml	10ml
Guanadine molarity (buffer)	3.86 M	3.86 M
Guanidine molarity (final)	3.05 M	3.05 M

Table.1.15. Composition of the guanidine isothiocyante and guanidine hydrochloride based lysis buffers

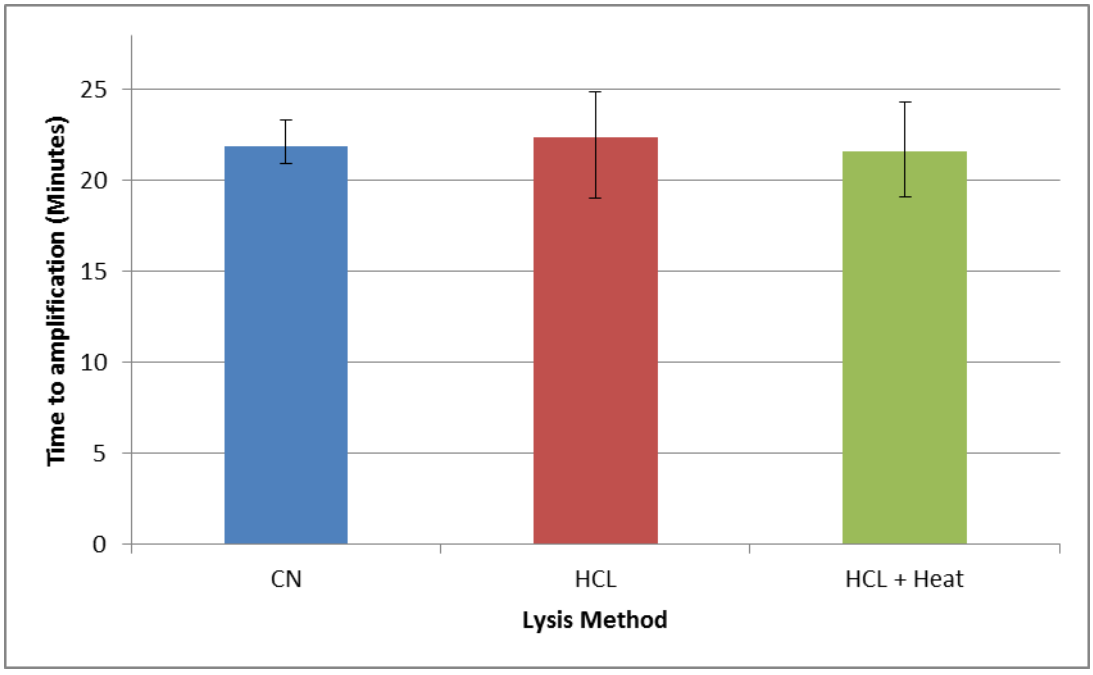

Fig.1.6. The average time to amplification in the GC *GroEL* LAMP assay from nucleic acid extracted from a suspension of *N. gonorrhoeae* (1 x 10^5 CFU/ml) in urine, using the various lysis methods. Error bars represent the range of 5 replicates.

The time to amplification in the LAMP assay from nucleic acid extracted using the different lysis methods remained constant, with an average time to amplification of 21.9, 22.4 and 21.6 minutes when nucleic acid extracted using the guanidine isothiocyanate buffer, guanidine hydrochloride buffer, and guanidine hydrochloride buffer with a pre-heating step, respectively (Fig.1.6). The range between the replicates was smaller for the reactions carried out on extractions using the guanidine

isothiocyante buffer (20.9 – 23.3 minutes), than those using the guanidine hydrochloride buffer (24.9 – 19 minutes) or the guanidine hydrochloride buffer with heating step (19.1 – 24.3 minutes).

1.3.4. Effect of guanidine hydrochloride lysis buffer and thermal lysis on a range of dilutions of *N. gonorrhoeae* cell suspensions in urine

One question regarding the use of thermal lysis that would need to be addressed is whether it is necessary to heat the sample in the presence of the lysis buffer, or whether it is optimal to heat the sample before the sample and lysis buffer are mixed.

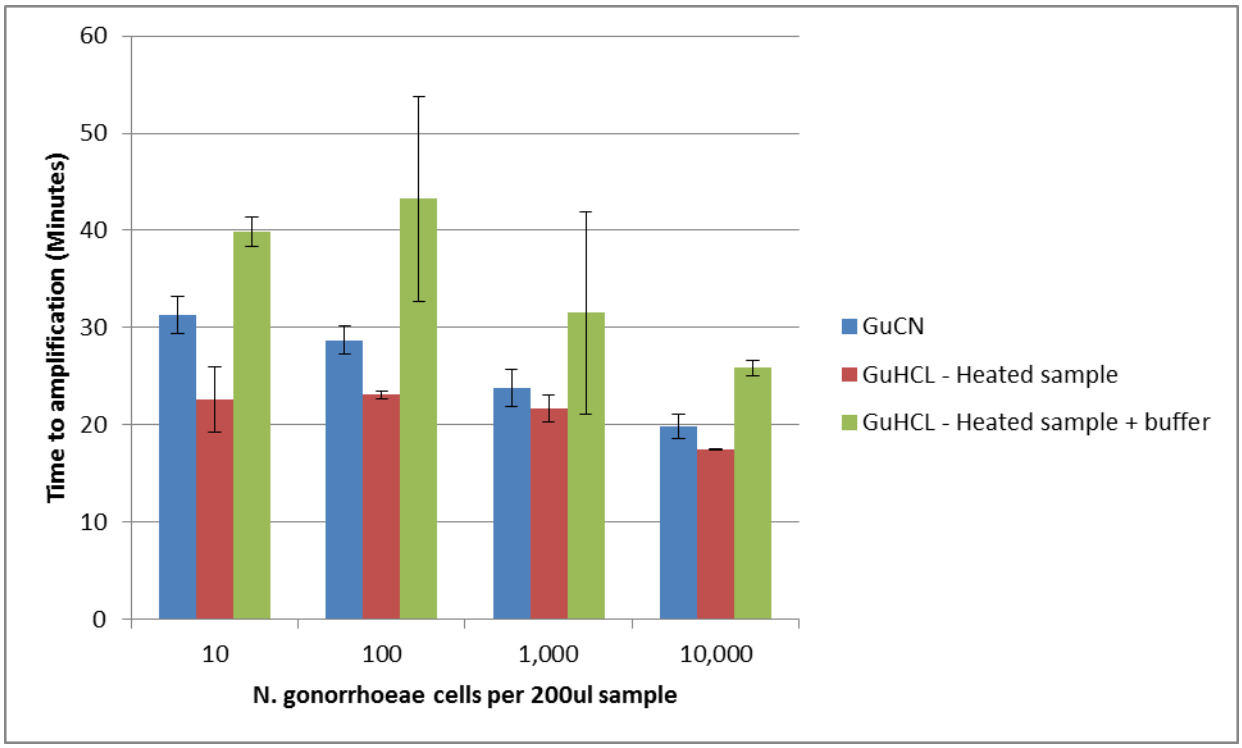

Fig.1.7. The effect of lysis method used during nucleic acid extraction from various concentrations of *N. gonorrhoeae* cell suspension on the subsequent time to amplification in the GC *GroEL* LAMP assay.

The shortest average time to amplification in the *GroEL* LAMP assay for each concentration of cell suspension was generated from nucleic acid extracted using the guanidine hydrochloride buffer and a pre mixing heat treatment (Fig.1.7). Compared to the use of guanidine isothiocyanate based buffer, the average times to amplification were 8.7, 5.6, 2.1 and 2.4 minutes shorter for the extractions containing 10, 1 x 10^2, 1 x 10^3, and 1 x 10^4 cells per reaction, respectively. The percentage

time difference between the use of the guanidine hydrochloride lysis buffer with pre heating, compared with the use of guanidine isothiocyanate lysis buffer and guanidine hydrochloride buffer with simultaneous heating are shown in Tables 1.15 and 1.16, respectively.

Organism load (per 200µl)	**Average time to amplification in *GroEL* LAMP assay (Minutes)**		**Time difference (%)**
	GuHCl - Heated sample	**GuCN**	
10,000	17.5	19.9	+13.7
1,000	21.7	23.8	+9.7
100	23.1	28.7	+24.2
10	22.6	31.3	+38.5

Table 1.16. Comparison of the average time to amplification in the *GroEL* LAMP assay using *N. gonorrhoeae* nucleic acid extracted using a guanidine hydrochloride lysis buffer and 95oC pre heating step, and nucleic acid extracted using a guanidine isothiocyante lysis buffer.

Organism load (per 200µl)	**Average time to amplification in *GroEL* LAMP assay (Minutes)**		**Time difference (%)**
	GuHCl - Heated sample	**GuHCl - heated sample + Buffer**	
10,000	17.5	25.9	+48
1,000	21.7	31.5	+45.2
100	23.1	43.3	+87.4
10	22.6	39.9	+76.5

Table 1.17. Comparison of the average time to amplification in the *GroEL* LAMP assay using *N. gonorrhoeae* nucleic acid extracted using a guanidine hydrochloride lysis buffer and 95oC pre heating step, and nucleic acid extracted using the same lysis buffer with a simultaneous heating step.

Compared with the use of the guanidine hydrochloride buffer and pre heating, amplification occurred 48.1% and 37.5% later when a simultaneous heating step or guanidine isothiocyanate lysis buffer were used, respectively (Table 1.16, Table 1.17). When extracting from the samples with the

lowest organism load tested, the difference in performance was more pronounced, with the pre heating method leading to an average amplification time of 22.6 minutes, compared with 39.85 minutes (a 76.32% increase in time to amplification) and 31.3 minutes (a 38.4% increase) when using a simultaneous heating step or guanidine isothiocyanate lysis buffer, respectively.

The extractions carried out utilising the heating of sample and guanidine hydrochloride buffer together generated the longest time to amplification during the LAMP assay, for all *N. gonorrhoeae* concentrations tested. This is indicative of either a reduced yield of nucleic acid from the extraction, or a reduction in the quality of nucleic acid obtained, caused by the degradation of the DNA or RNA. It is unlikely that the delay in time to amplification could be attributed to the insufficient removal of inhibitory compounds, as the efficiency of the wash steps is the critical parameter in the removal of impurities, rather than the lysis method.

1.3.5. Effect of the duration of the 95°C heating step during lysis on the yield of nucleic acid and LAMP amplification time

During the thermal lysis protocol, the sample is heated to 95°C before entering the lysis buffer. The duration of this heating step requires optimisation, to ensure that the sample is not heated for longer than is necessary. An increased duration of heating will increase the time needed to carry out the extraction, the power requirements of the test, and the likelihood that thermal degradation will occur to the DNA and RNA present in the sample.

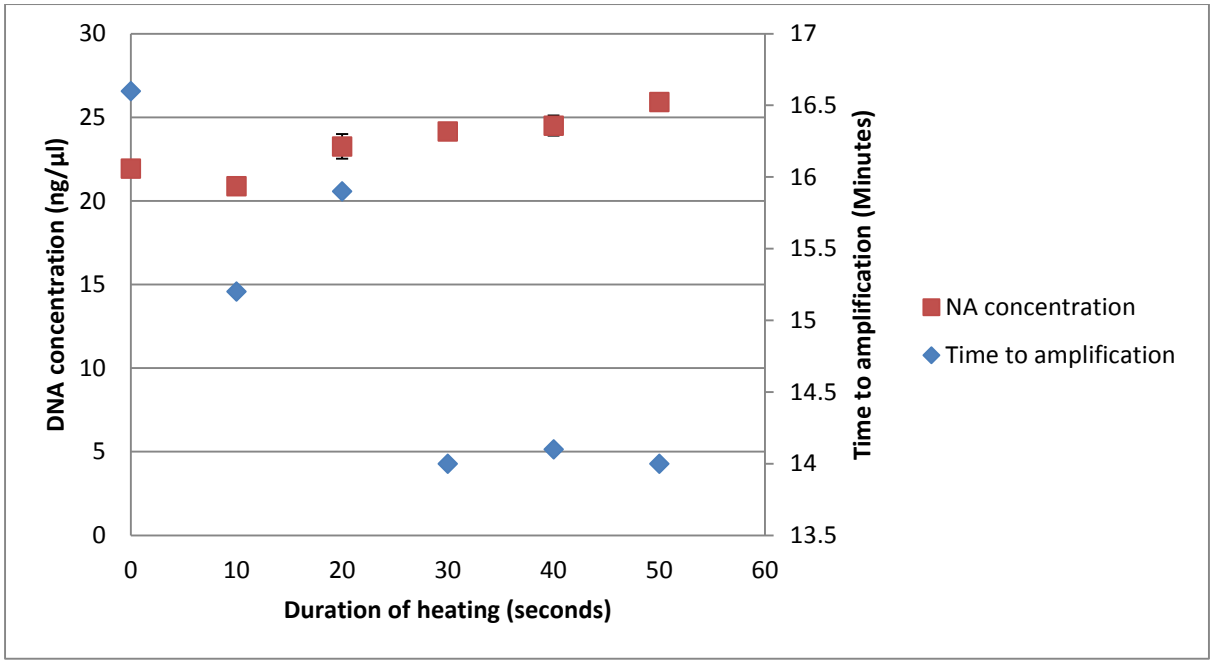

Fig.1.8. The effect of the duration of heat treatment during lysis on the concentration of nucleic acid extracted, and the time to amplification in the resulting GC *GroEL* LAMP assays.

The LAMP assay generated a detectable amplification from the nucleic acid extracted without a heat treatment after 16.6 minutes. A heat treatment of 10 and 20 seconds resulted in a decrease in this time to 15.2 and 15.9 seconds respectively (Fig.1.8). An increase of the heating duration to 30 seconds corresponded with a reduction in the time to amplification from 15.9 seconds to 14 seconds. Further heating after this point had no effect on the time to amplification.

The concentration of nucleic acid purified from the sample without heating was 21.9ng/µl, which increased as the heating duration increased, to 23.3ng/µl after 20 seconds and 25.9ng/µl after 50 seconds (Fig.1.8).

1.3.6. Effect of GuHCl concentration on concentration and quality of extracted DNA

A key element of the lysis buffer requiring optimisation is the concentration of guanidine hydrochloride. Guanidine hydrochloride concentration is likely to have a sizeable effect on adequate prevent of degradation by endogenous nuclease enzymes, and also the binding of DNA and RNA to the magnetic silica particles responsible for the capture and concentration of nucleic acids.

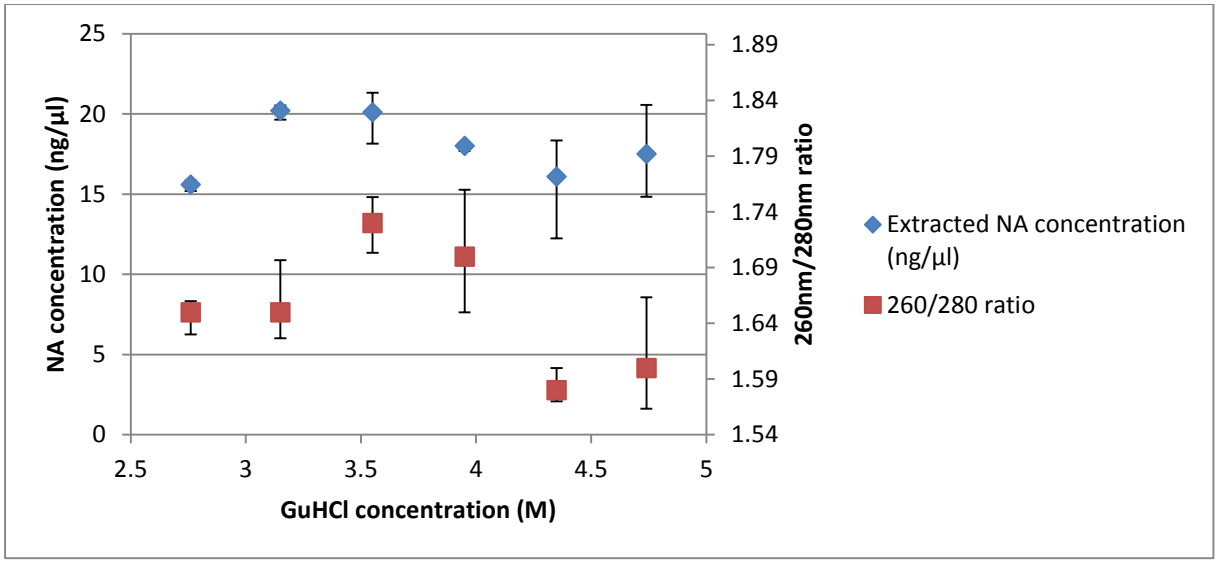

Fig.1.9. The effect of the final guanidine hydrochloride concentration in the lysis buffer and sample on the concentration and purity of eluted nucleic acid, extracted from a suspension of *N. gonorrhoeae* (1 x 10^6 CFU/ml). Error bars indicate the range of three replicates.

The guanidine hydrochloride concentration had an effect on both the yield and purity of the nucleic acid obtained (Fig.1.9). A final guanidine hydrochloride concentration of 3.15M to 3.55M, resulting from a buffer concentration of 4M to 4.5M produced the highest yield of nucleic acid, 20.2 and 20.1ng/µl respectively. The only lower concentration tested, a final concentration of 2.76M, yielded 15.6ng/µl. An increase in final concentration to levels above 3.55M corresponded with a reduction in yields, with final guanidine hydrochloride concentrations of 3.95, 4.35 and 4.74, resulting in yields of 18ng/µl, 16.1ng/µl, and 17.5ng/µl respectively. The A260/A280 ratio, used as an indicator of nucleic acid purity, was highest for the nucleic acid extracted using a final guanidine hydrochloride concentration of 3.55M, at 1.7. Increasing the final guanidine hydrochloride concentration to 4.35M and above resulted in a sizeable decrease in the A260/A280 ratio, which reduced to 1.59.

1.3.7. Buffer component concentrations

The lysis buffer contains a volume of both 0.2M EDTA, and 0.1M Tris/HCl, primarily in order to regulate the acidity and osmotic concentration of the lysed sample. A reduction in the concentration of these components would cause a minor decrease in the cost of production of the buffer, and would enable a reduction in the buffer volume required.

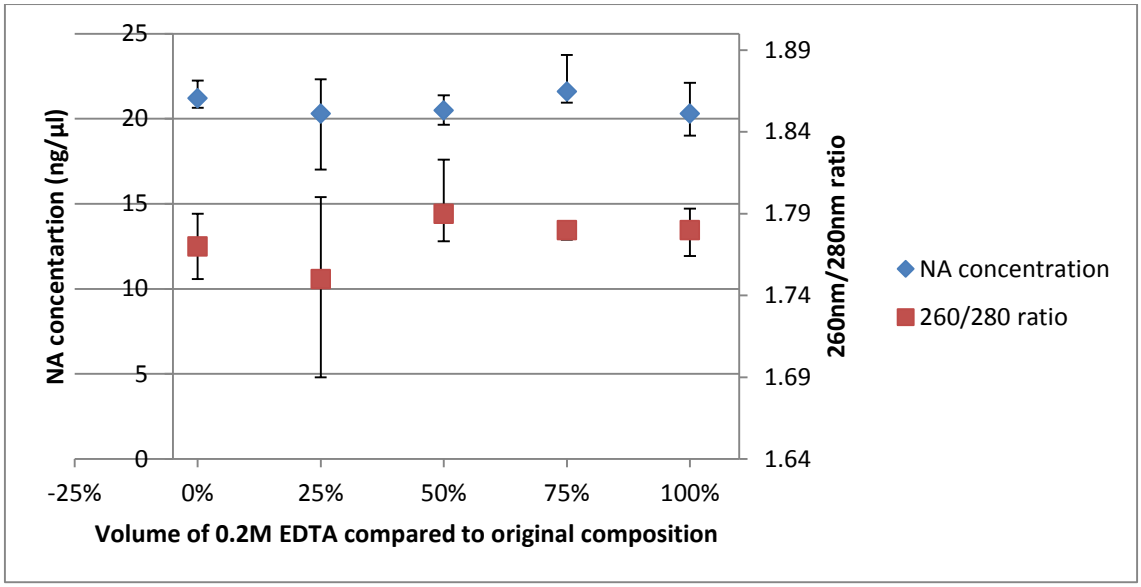

Fig. 1.10. The effect of the volume of 0.2M EDTA in the lysis buffer on the concentration and purity of eluted nucleic acid, extracted from a suspension of *N. gonorrhoeae* (1 x 10^6 CFU/ml). Error bars indicate the range of three replicates.

The concentration of extracted nucleic acid showed little variation when the volume of 0.2M EDTA was varied between 0% and 100% of the standard volume, with a concentration range of 20.3ng/µl to 21.6ng/µl obtained over all EDTA volumes tested (Fig.1.10). The 260/280 ratio also stayed relatively constant, ranging between 1.75 and 1.79 for all EDTA volumes tested. One replicate of the nucleic acid extracted with 25% of the standard EDTA volume contained a low concentration of nucleic acid (17.02ng/µl), and had a corresponding low 260/280 ratio of 1.69, however the remaining replicates at this concentration were markedly higher in concentration (22.33ng/µl, 21.85ng/µl) and 260nm/280nm ratio (1.8, 1.76).

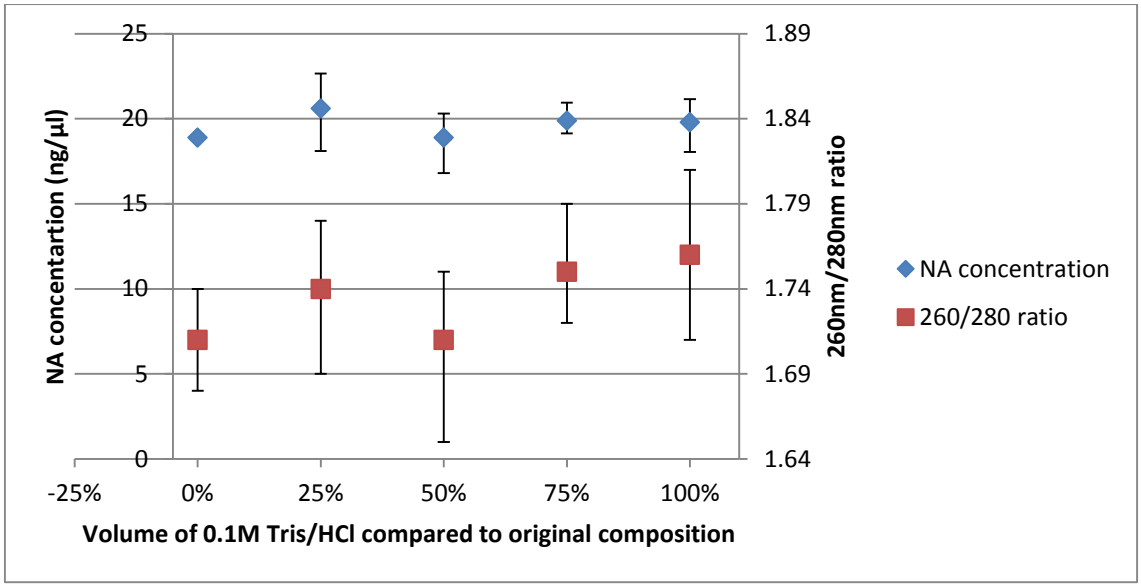

Fig.1.11. The effect of the volume of 0.1M Tris/HCl in the lysis buffer on the concentration and purity of eluted nucleic acid, extracted from a suspension of *N. gonorrhoeae* (1 x 10^6 CFU/ml). Error bars indicate the range of three replicates

The variation of the Tris/HCl volume in the lysis buffer from 100% to 0% of its original concentration had negligible effect on nucleic acid yield, with the concentration of extracted nucleic acid ranging from 20.6 to 18.9 across all Tris/HCl volumes tested (Fig.1.11). The average 260/280 ratio was seen to slightly decrease as the volume of Tris/HCl was decreased, with the 100% volume resulting in a 260nm/280nm ratio of 1.76, compared with the 260nm/280nm ratio of 1.71 for nucleic acid extracted with the buffer containing 0% Tris/HCl.

1.3.8. Comparison of 1x and 2x lysis buffer for total nucleic acid recovery from clinical *C. trachomatis* samples, using a silica based total nucleic extraction method.

Typically, with regards to sample management in a microfluidic device, a reduction in fluid volume results in a reduction in the time taken to process the sample (Kent *et al*., 2010). A smaller volume is able to be manoeuvred through channels and between compartments at a faster rate, and smaller volumes can also be heated and cooled more rapidly, speeding up these components of the extraction process. A 2x concentrated lysis buffer would enable a 50% reduction in lysis buffer volume, whilst retaining the concentration of active buffer components, and therefore buffer

activity. In order to examine any impact an increase in concentration would have on lysis buffer efficiency, extractions were carried out on three clinical samples using both the standard 1x buffer and a 2x concentrated buffer. Higher concentrations of buffer proved unfeasible, as the components would not solubilise in the reduced volumes.

Nucleic acid extractions were carried out on 4 clinical *C. trachomatis* samples supplied by Public Health England South West (2 positive urine samples, 1 positive endocervical swab sample, 1 negative urine sample), using the silica based total nucleic acid extraction method with both the standard 1x lysis buffer, and a 2x concentrated buffer. All extractions were tested for the presence of *C. trachomatis* nucleic acid with the CT-0332 LAMP assay.

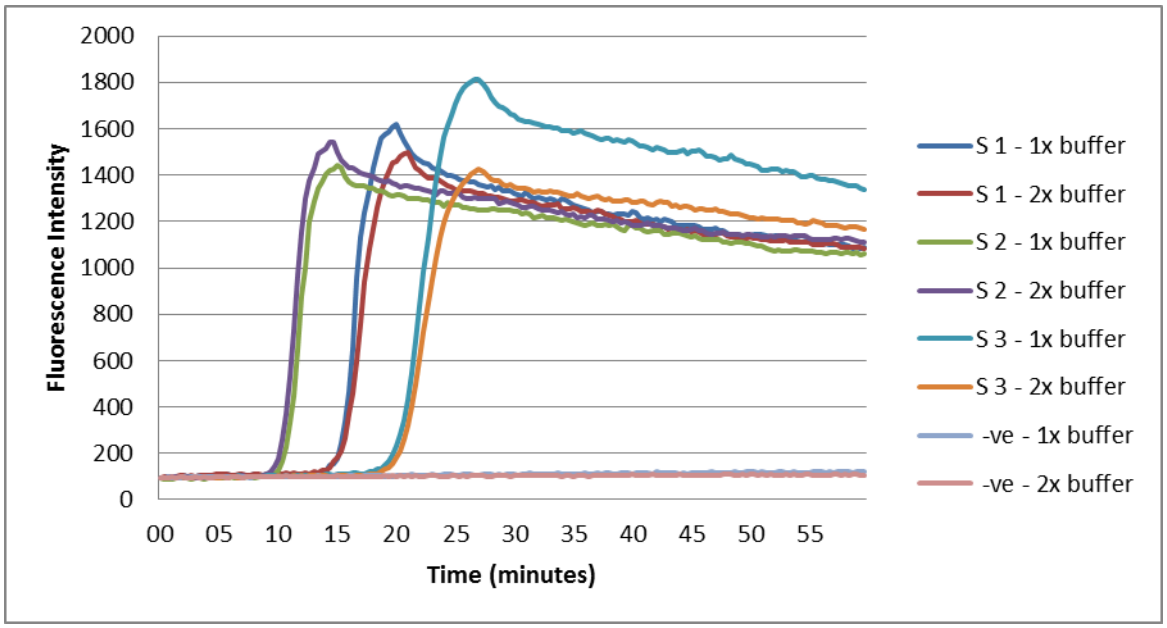

Fig.1.12. LAMP reaction amplification plot showing a comparison of the use of 1x and 2x concentrated lysis buffer during the nucleic acid extraction process. DNA extractions using both buffers were carried out with 2 clinical *C. trachomatis* positive urine samples (S 1 & S 2) and 1 endocervical swab sample (S 3). Extractions were then tested for the presence *of C. trachomatis* nucleic acid using the CT-0332 assay. A *C. trachomatis* negative clinical urine sample was used as a negative control.

For each clinical sample the use of both 1x and 2x lysis buffer during the extraction process yielded identical amplification times during the LAMP reaction (Fig.1.12). The fluorescence intensity and curve exhibited during the LAMP reaction of nucleic acid extracted from samples 1 and 2 were identical when either lysis buffer was used. For sample 3, an increase in fluorescence intensity was

detected during the LAMP reaction when the 1x buffer was used, although this is likely to have been caused by reasons other than nucleic acid concentration or purity, such as a pipetting error introducing an increased volume of reaction mix, and therefore fluorophore.

1.3.9. Robustness of nucleic acid extraction method in the presence of inhibitory compounds

Clinical samples contain a number of potential inhibitors of nucleic acid amplification, which need to be adequately removed during the extraction process in order to avoid any negative effects on the amplification reaction. Whilst LAMP is known to have a higher tolerance to a range of inhibitors than PCR, the exact tolerances are unknown. Also, the majority of studies have used end-point LAMP to investigate whether the LAMP reaction can generate a positive amplification in the presence of an inhibitor, and not determined any effect on amplification using real-time monitoring.

Nucleic acid extractions were carried out on 200µl aliquots of a suspension of *N. gonorrhoeae* cells in urine (1 x 10^5 CFU/ml), which were spiked with varying concentrations of whole blood (Table 1.18), *E. coli* culture (Table 1.19), or bovine serum albumin (BSA) (Table 1.20).

Clinical urine samples can contain whole blood, originating from bacterial infection of the genital tract. Additionally, high vaginal or endocervical swab samples can contain significant levels of blood during menstruation, which would enter in the nucleic acid extraction system and require removal before amplification. Lysed red blood cells contain a number of proteins that can interfere with PCR amplification, and therefore potentially LAMP amplification, principally haemoglobin and lactoferrin. Blood plasma also contains inhibitory proteins.

Extractions were carried out containing varying dilutions of whole defibrinated horse blood, and the red blood cells per ml were quantified. A range of between $5.5x10^7$ (representing a 50:50 dilution of whole blood and culture) and $5.5x10^3$ red blood cells per extraction were investigated.

Sample	RBC count	Result	Time to amplification (mins)
RBC 1	$5.5x10^7$	POSITIVE	12.6
RBC 2	$5.5x10^6$	POSITIVE	11.6
RBC 3	$5.5x10^5$	POSITIVE	11
RBC 4	$5.5x10^4$	POSITIVE	11.3
RBC 5	$5.5x10^3$	POSITIVE	11.6
Positive	0	POSITIVE	11.3
Negative	0	NEGATIVE	-

Table 1.18. Time to amplification in the GC *GroEL* LAMP assay from nucleic acid extracted from *N. gonorrhoeae* cell suspension (1 x 105 CFU/ml), in combination with varying concentrations of whole defibrinated horse blood.

The LAMP assay was able to detect the *N. gonorrhoeae* target from nucleic acid extracted from samples containing all of the concentrations of whole blood tested (a 50:50 sample:whole blood mix). This level is far higher than would be expected in any urine or genital swab sample. There was no effect on the LAMP amplification caused by the presence of $5.5x10^3$ to $5.5x10^6$ RBC's per ml, with all samples generating an amplification within +/- 0.3 minutes of the positive control extraction containing no blood component. The sample containing $5.5x10^7$ RBC's per ml, generated a signal 1 minute later than the positive control. (Table 1.18).

An additional consideration when evaluating the performance of the extraction method in the presence of various inhibitors, is the effect that excess non-target cells, and particularly non-target DNA will have on the yield and purity of target DNA acquired. Swab and urine samples are both likely to contain a proportion of human cells, commensal bacteria, and possibly very high concentrations of pathogenic bacteria, in the case that a urethral infection with these organisms has resulted in the test for a sexually transmitted infection. Excess non target nucleic acid will passively compete with target nucleic acid for binding space on the silica surface of the magnetic beads, and an excess of non target nucleic acid in the LAMP reaction could reduce the likelihood of the initial priming event necessary to instigate LAMP amplification.

Extractions were carried out on a 50:50 mixed sample of *N. gonorrhoeae* (1 x 10^5 CFU/ml), and *E. coli* culture of varying concentrations.

Sample	*E. coli* count (CFU)	Result	Time to amplification (mins)
E. coli 1	$1x10^8$	POSITIVE	13.3
E. coli 2	$1x10^7$	POSITIVE	11.3
E. coli 3	$1x10^6$	POSITIVE	12
E. coli 4	$1x10^5$	POSITIVE	13
Positive	0	POSITIVE	12.3
Negative	0	NEGATIVE	-

Table 1.19. Time to amplification in the GC *GroEL* LAMP assay from nucleic acid extracted from *N. gonorrhoeae* cell suspension (1 x 105 CFU/ml), in combination with varying concentrations of *E. coli*.

Amplification was generated in each positive LAMP reaction, irrespective of the accompanying concentration of *E. coli* (Table 1.19). A slight delay in amplification occurred in the reaction containing $1x10^8$ CFU of E. coli, which was generated at 13.3 minutes, 2 minutes later than the positive control reaction. It is worth noting that the reaction containing $1x10^7$ CFU generated amplification 1 minute sooner than the positive control, meaning that the small changes in amplification time could be due to variations in reaction speed rather than inhibition.

In healthy individuals, the protein content in urine should be minimal (<0.1 mg/ml). However, proteinuria, the excretion of excess serum proteins in the urine, can be caused by a number of pathological conditions, including diabetes, HIV infection and kidney disease. In extreme cases of proteinuria, concentrations as high as 2mg/ml of serum proteins, mostly made up of serum albumin, are encountered. It is unknown whether the presence of such concentrations in the pre-extraction sample will have an impact on the yield of nucleic acid obtained, or the amplification during the LAMP process.

Nucleic acid extractions were carried out from samples containing varying concentrations of BSA, ranging from 10mg/ml to 0.1mg/ml.

Sample	BSA concentration (mg/ml)	Result	Time to amplification (mins)
BSA 1	10	POSITIVE	23
BSA 2	2	POSITIVE	26.3
BSA 3	1	POSITIVE	25.3
BSA 4	0.5	POSITIVE	24.3
BSA 5	0.1	POSITIVE	24
Positive	0	POSITIVE	26.3
Negative	0	NEGATIVE	-

Table 1.20. Time to amplification in the GC *GroEL* LAMP assay from nucleic acid extracted from *N. gonorrhoeae* cell suspension (1 x 10^5 CFU/ml), in combination with varying concentrations of BSA.

The GC *GroEL* LAMP assay was able to amplify the *N. gonorrhoeae* target sequence from nucleic acid extracted in the presence of all of the concentrations of BSA tested. The range of time to amplification was 3.3 minutes for all concentrations tested (23 minutes – 26.3 minutes), and there was no correlation between BSA concentration and the amplification time (Table 1.20).

1.3.9. Effect of lysis buffer lyophilisation on nucleic acid extraction.

Lyophilisation, or freeze drying, is a dehydration process in which water is removed from a frozen substance by a process of sublimation. This versatile and well used process has been utilised for many applications, including food production and pharmaceutical manufacture. Lyophilising a substance significantly reduces its mass, and also allows it to be stored at ambient temperatures, reducing shipping and storage costs. The substance can then be rehydrated at the time of use, and will recover the chemical structure and properties of the substance prior to the lyophilisation process (Slater *et al*., 2003). The lyophilisation of the lysis buffer component in a microfluidic chip based device would reduce fluid volume in the chip itself, enable a faster processing of the clinical sample, which is a primary concern for a point of care microfluidic platform. It would also simplify the manufacture process, as a dried reagent is typically easier to integrate into chips during manufacture than liquid reagents. Chip shelf live could also be lengthened; an important consideration of any diagnostic test destined for clinical use.

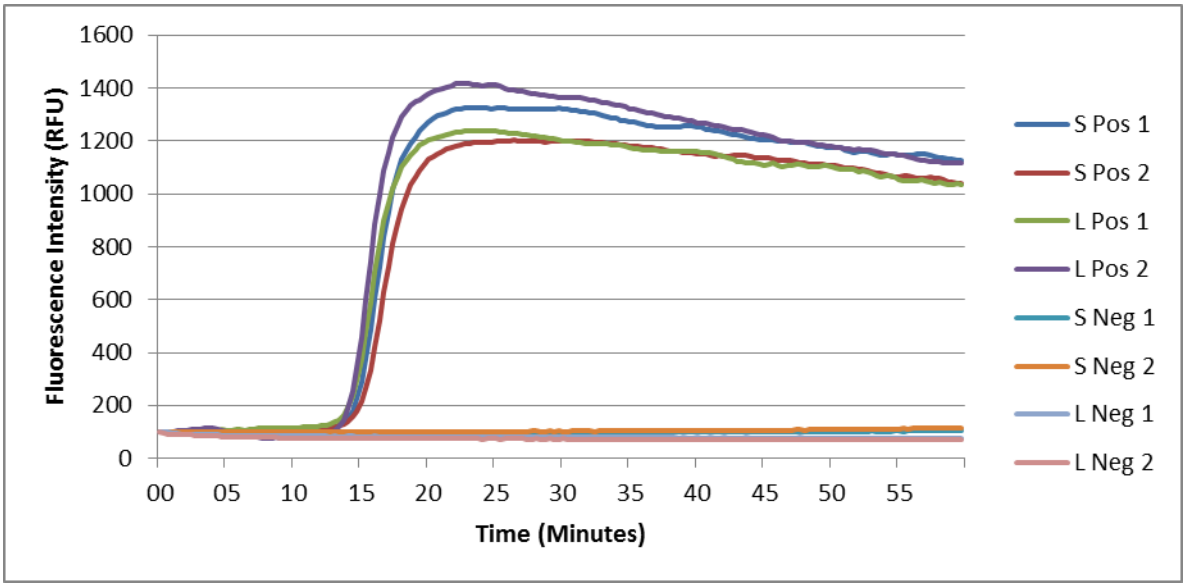

Fig.1.13. LAMP amplification plot showing effect of lysis buffer lyophilisation on nucleic acid extraction efficiency. Nucleic acid extractions were carried out using lyophilised (L) and standard buffers (S), from a *N. gonorrhoeae* cell suspension, in duplicate. Extractions were then tested for the presence of *N. gonorrhoeae* nucleic acid using the GC *GroEL* LAMP assay. Uninoculated culture media was used as a negative control.

Nucleic acid from both of the extractions carried out using lyophilised 2x lysis buffer was successfully amplified during the GC *GroEL* LAMP assay. There was no difference in the time to amplification (Ta) generated using DNA extracted with either the rehydrated lyophilised buffer, or the standard liquid buffer (Fig.1.13). This shows that the freeze drying process had no negative effect on the performance of the lysis buffer in this experiment.

1.3.10. Tablet Manufacture

During manufacture, the freeze dried buffer will need to be added into the correct partition of the microfluidic LabDisk, along with reagents for the LAMP reaction mix, and primer sets. In its freeze dried powdered form the lysis buffer would need to be weighed out individually for each disk, adding complication and time needed for the manufacturing process. Also, if the lysis buffer is supplied as free powder, there is the risk that it could disperse from the chamber during transfer, and find it sway into fluid channels on the LabDisk. In order to address this problem, the feasibility of the manufacture of tablets made from the lysis buffer was examined. The process involves

compressing the freeze dried lysis buffer powder into a cast, producing a solid tablet than can be more easily handled. As well as producing tablets containing 100% active ingredient, it is possible to improve the properties of the final tablet via the inclusion of excipients. These excipients include lubricating agents, which improve the efficiency of the tabletting process; disintegrants which assist in the break up of the tablet and release of the active ingredient; and bulking agents, which can improve the flow of the powder through the tabletting machine, and the solidity of the final product.

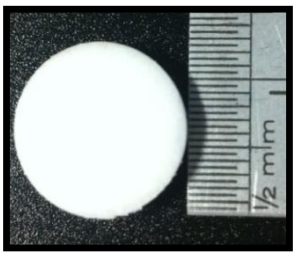

Fig. 1.14. Lysis tablet 1

Two tablets were produced; tablet 1 (Fig.1.14), containing 516mg of 100% active ingredient, and measuring 12mm in diameter; and tablet 2, containing 516mg of a 50:50 ratio of excipients and the active buffer components.

1.3.11. Initial test of tablet performance

Although it has been confirmed that freeze drying the lysis buffer does not lead to a degradation in performance (Fig.1.13), It is important that the lysis tablets produced also do not have any negative effect on the time to amplification of the LAMP reactions taking place using the extracted nucleic acid.

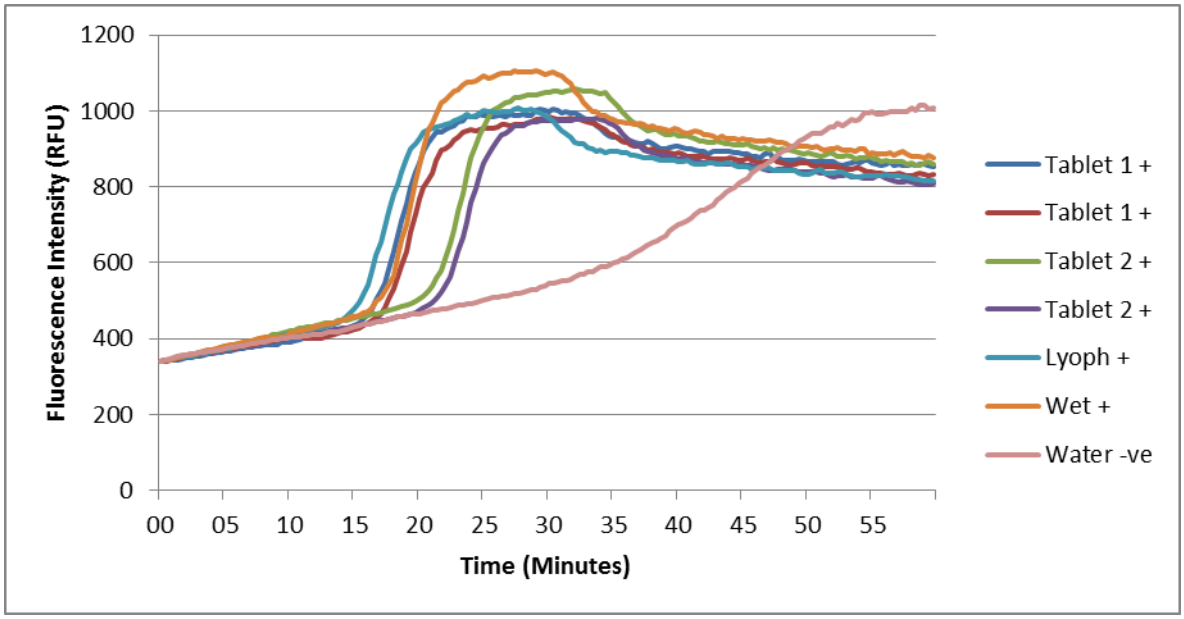

Fig.1.15. Amplification plots generated by the GC *GroEL* LAMP assay from nucleic acid extracted from a *N. gonorrhoeae* urine suspension (2 x 10^6 per ml), using lysis tablet 1, lysis tablet 2, lyophilised powdered lysis buffer and standard "wet" lysis buffer.

The two LAMP reactions containing DNA extracted using tablet 1 generated detectable amplification after 16.6 and 17.6 minutes. The extractions carried out using lyophilised lysis buffer and standard "wet" lysis buffer generated an amplification after 15.6 and 16.6 minutes respectively (Fig.1.15). These amplifications were fairly closely grouped within a 2 minute period. The use of tablet 2 during the extraction process caused a delay in the downstream LAMP assay of ~4.5 minutes, with a detectable amplification generated after 20 minutes and 21.3 minutes in these reactions.

The negative control reaction generated a late amplification of signal, with a curve more gradual than those generated in the true positive reactions. The signal generated equalled those of the true positives (1000 RFU), but appeared like an extension of the gradually increasing baseline fluorescence rather than a positive reaction. This experiment was the first carried out with a new formulation of reaction mix provided by Mast Group Ltd, and it was thought that this may be responsible. A total of eight negative reactions were carried out with a range of LAMP primer sets, and all exhibited this gradual late amplification. Due to the difference in amplification curve and time, and the occurrence of this phenomenon with a range of primer sets, it is assumed that there is no contamination of the reagents that would influence the positive reactions.

Tablet 2 also did not fully dissolve in the urine and IPA; the tablet took longer to fully break up, despite the included disintegrants, and was not fully soluble. Whilst tablet 2 was the same weight as tablet 1, it contained 50% of the active compounds, and two tablets were required per reaction, increasing the material present in the extraction. As the performance of tablet 2 was inferior during this test, would create problems due to the increased material, and were less cost effective to make, their use was discontinues in further experiments.

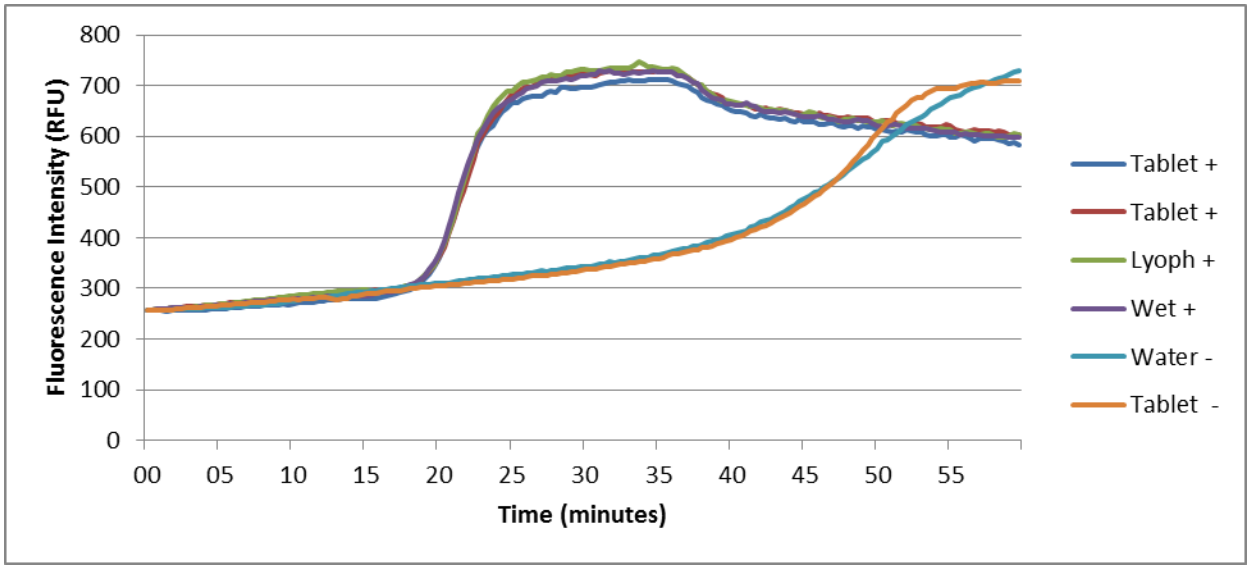

Fig.1.16. Amplification plots generated by the GC *GroEL* LAMP assay from nucleic acid extracted from a *N. gonorrhoeae* urine suspension (2 x 10^2 per ml), using lysis tablet 1, lyophilised powdered lysis buffer and standard "wet" lysis buffer.

The performance of the lysis tablet 1, lyophilised buffer, and standard "wet" lysis buffer during the extraction of nucleic acid from a low density *N. gonorrhoeae* urine suspension (2 x 10^2 per ml) was equivalent, with a Ta of 20.5 minutes achieved during LAMP amplification of nucleic acid extracted using each method (Fig.1.16).

1.3.12. Lysis buffer pellets

Due to difficulties encountered during the tabletting process, caused by the freeze dried lysis buffer damaging the steel tooling of the machine, the use of freeze dried lysis buffer pellets was examined.

Liquid lysis buffer was frozen overnight at -80°C in 790µl aliquots in plastic circular moulds (2.7cm diameter). These moulds were then placed in the cold chamber of a freeze drier, and subjected to lyophilisation for 24 hours.

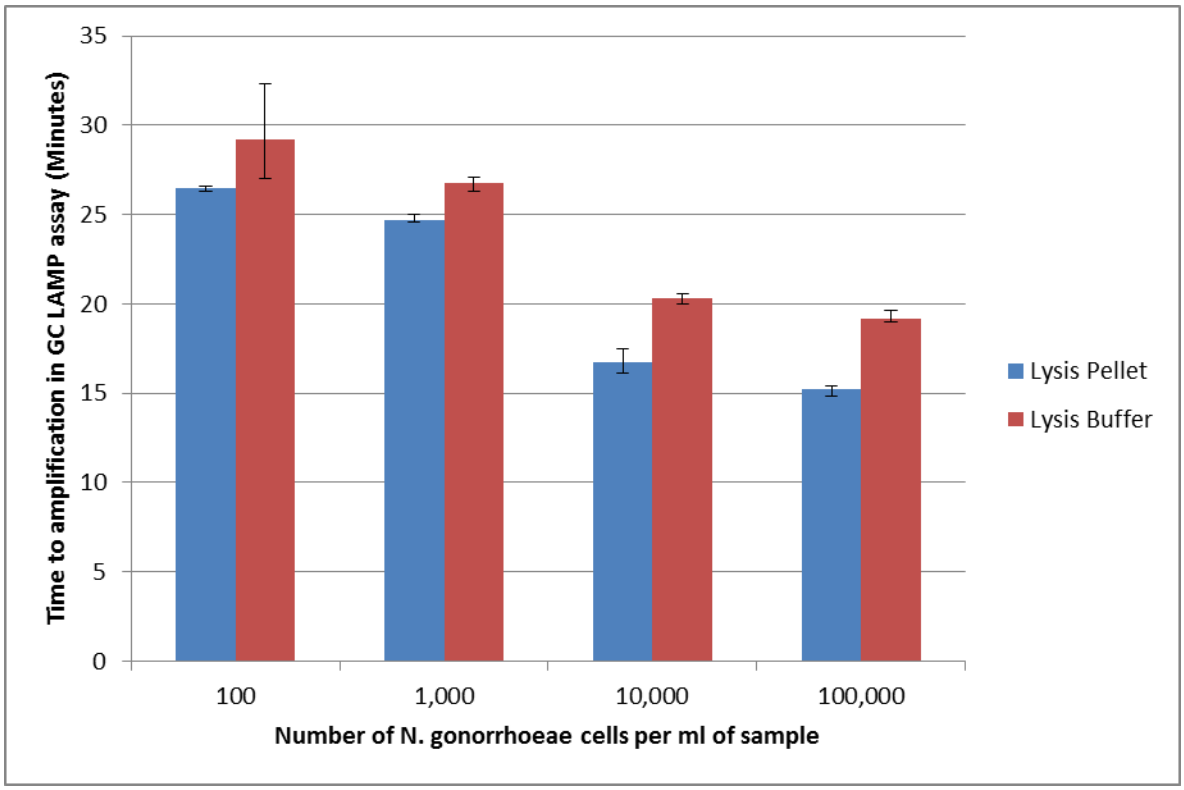

Fig.1.17. Time to amplification of the GC *GroEL* LAMP assay using nucleic acid extracted from various concentrations of *N. gonorrhoeae* cell suspension using the 790µl lysis pellet and liquid 2x lysis buffer

The effect of the use of the lysis pellets compared with the use of lysis buffer is shown in Fig.1.17. Overall, the use of the lysis pellets resulted in an average reduction in amplification time of 3.1 minutes for all cell concentrations tested. This ranged from a 2.7 minute reduction for the lowest cell concentration (1 x 10^2), to a 4 minute reduction for the lowest cell concentration (1x 10^5).

1.3.13. Lyoprotectants

In order to improve the consistency of the freeze dried lysis buffer pellets, the effect of the inclusion of a number of lyoprotectants was investigated. These lyoprotectants were added to the lysis buffer at a range of concentrations before the freeze drying process was initiated. Lyoprotectant compounds add stability to lyophilised products, and can improve consistency of the finished product, maintaining a more solid structure which can be more easily manipulated.

Final concentrations of polyvinylpyrrolidone (PVP) of 1%, 2%, 4%, 8% and 10% were tested, as PVP is a well known lyoprotectant at these concentrations. PVP is a water soluble polymer, used as an amorphous bulking agent during the lyophilisation of pharmaceuticals, in order to provide a more robust cake.

Two proprietary buffers from Mast were also included in the study; Lyo B and Lyo D. The exact formulation of these buffers was not disclosed, only that they include a concentration of poly(ethylene glycol) (PEG) 8000, and a variety of buffering compounds and ions. These buffers were designed to protect LAMP reaction mix, *bst* polymerase and reverse transcriptase enzymes, and primers, during the freeze drying process. These buffers also provide a solid freeze dried cake, which can be handled in one piece, which is an essential property of the lysis pellets. The concentrations found to work best for the reaction mix was 10%, so the concentrations of 5%, 10% and 15% were tested.

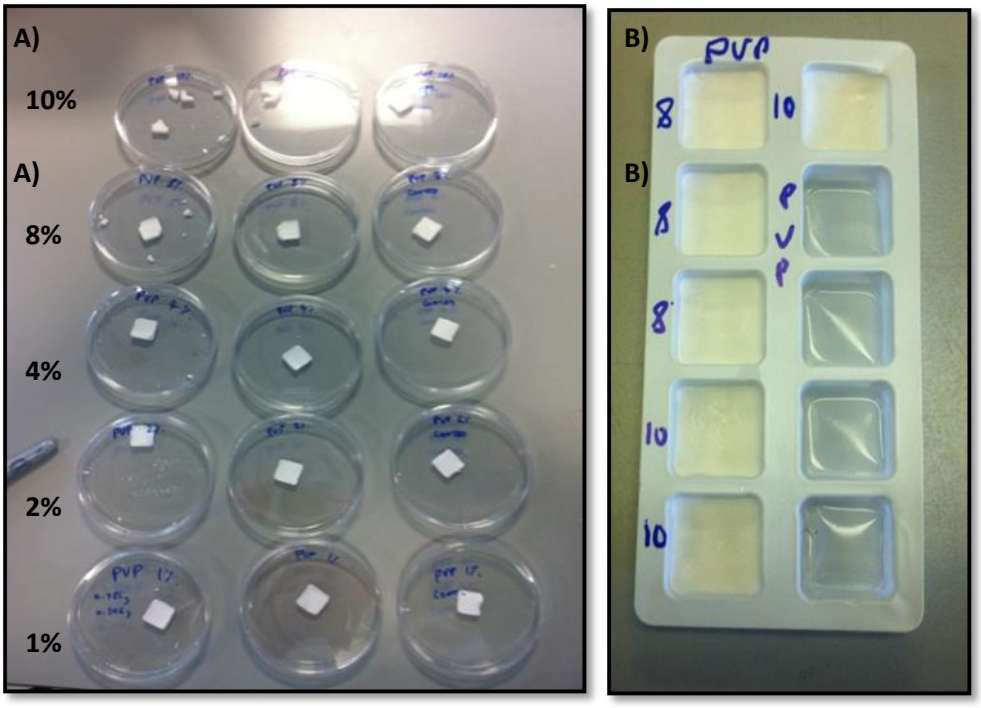

Fig. 1.18. A photograph showing the integrity of lysis pellets including varying concentrations of PVP (A), and a photograph demonstrating the appearance of the lysis tablets in the molds, after the freeze drying process (B).

A PVP concentration of 1% - 8% was found to increase the integrity of the pellet, in comparison to pellets formed without the inclusion of a lyoprotectants (Fig. 1.18). The inclusion of 10% PVP lead to the pellets becoming heavily stuck to the mold, and difficult to retrieve without breaking.

PVP concentration (%)	Time to amplification (Minutes)					
	Replicate 1	Replicate 2	Replicate 3	Average	Difference from 0%	Negative Control
0	18.0	18.4	18.7	18.4	0.0	NA
1	21.8	21.6	24.0	22.5	4.1	NA
2	22.2	22.2	25.2	23.2	5.0	NA
4	23.4	23.0	24.4	23.6	5.2	NA
8	25.6	25.2	26.0	25.6	7.2	NA
10	29.1	-	-	29.1	10.7	NA

Table.1.21. GC *GroEL* amplification times from nucleic acid extracted from aliquots of *N. gonorrhoea* culture (2 x 10^5 cells per ml), using lysis buffer pellets containing various concentration of PVP

The inclusion of PVP in the lysis pellet inhibited the LAMP reaction, with an increase in PVP concentration correlating with an increase in the Ta (Table 1.21). A PVP concentration of 1% resulted in an average delay of 4.1 minutes compared with the pellets prepared without PVP. This delay increased to 10.7 minutes when pellets containing 10% PVP were used. Amplification was not generated in the negative control reactions, irrespective of the absence or inclusion of PVP, at any concentration.

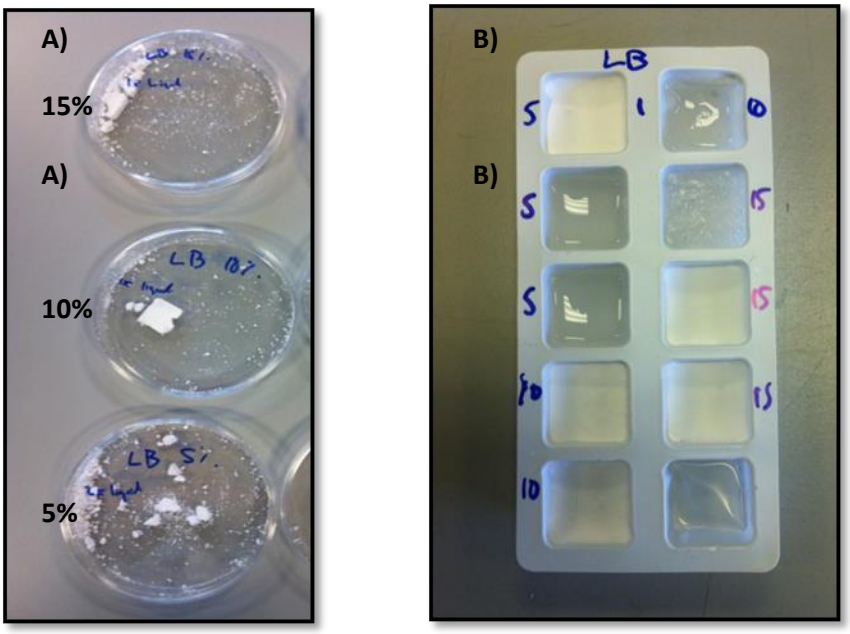

Fig. 1.19. A photograph showing the integrity of lysis pellets including varying concentrations of Lyo B (A), and a photograph demonstrating the appearance of the lysis tablets in the molds, after the freeze drying process (B).

15%

The inclusion of the Lyo B buffer at a 5% v/v concentration caused incomplete lyophilisation of the lysis buffer in two out of three replicates, whilst one of the three replicates failed to lyophilise when Lyo B was included at 5% or 15% concentrations (Fig.1.19 B). The pellets were difficult to remove from the molds, and stuck to the plastic surface, making retrieval problematic (Fig.1.19. A).

10%

5%

Lyo B concentration (%)	Time to amplification (Minutes)					
	Replicate 1	Replicate 2	Replicate 3	Average	Difference from 0%	Negative Control
0	18.0	18.4	18.7	18.4	0.0	NA
5	18.3	17.8	19.0	18.4	0.0	NA
10	23.1	25.0	26.1	24.7	6.3	NA
15	18.2	17.9	23.5	19.9	1.5	NA

Table.1.22. GC *GroEL* amplification times from nucleic acid extracted from aliquots of *N. gonorrhoea* culture (2 x 105 cells per ml), using lysis buffer pellets containing various concentration of Lyo B.

A Lyo B concentration of 5% had no inhibitory effect on subsequent LAMP assays, causing no change in the average amplification time (Table 1.22). However, the presence of 10% and 15% Lyo B caused an average delay in amplification of 6.3 minutes and 1.5 minutes respectively. No false-positive amplification was generated in any of the negative control reactions.

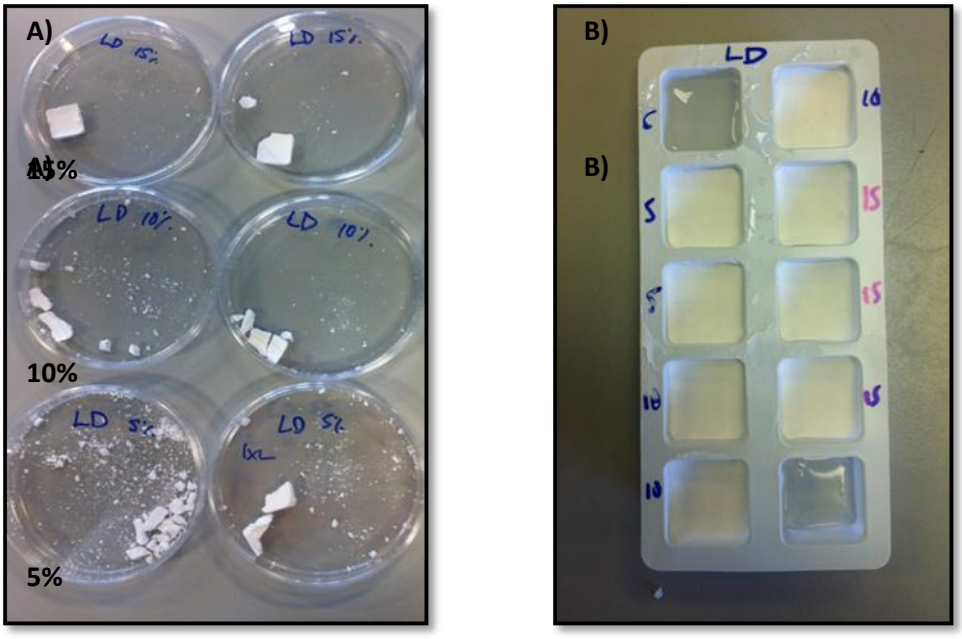

Fig. 1.20. A photograph showing the integrity of lysis pellets including varying concentrations of Lyo D (A), and a photograph demonstrating the appearance of the lysis tablets in the molds, after the freeze drying process (B).

The inclusion of the Lyo D buffer at a concentration of 15% resulted in the formation of solid pellets, that were easily removed from the molds intact (Fig.1.20 A). At concentrations of 10% and 5% the integrity of the pellets were affected, and the fragility was increased in comparison with the pellets without a lyoprotectants (Fig. 20 B).

Lyo D concentration (%)	Time to amplification (Minutes)					
	Replicate 1	Replicate 2	Replicate 3	Average	Difference from 0%	Negative Control
0	18.0	18.4	18.7	18.4	0.0	NA
5	17.5	19.0	18.8	18.4	0.0	NA
10	18.2	18.7	18.5	18.5	0.1	NA
15	18.3	18.1	18.1	18.2	-0.2	NA

Table.1.23. GC *GroEL* amplification times from nucleic acid extracted from identical aliquots of *N. gonorrhoea* culture (2 x 105 cells per ml), using lysis buffer pellets containing various concentration of Lyo D.

Lysis pellets containing 5%, 10%, or 15% Lyo D had no effect on the amplification time of the *GroEL* LAMP assay, compared to lysis pellets containing no lyoprotectants (Table 1.23). The negative control reactions did not generate amplification, in the presence or absence of Lyo D.

1.3.14. Lyoprotectants: PEG

The absence of any negative effect on the LAMP assay arising from the presence of Lyo D in the lysis pellet prompted further investigation of its constituents. As the exact formulation was undisclosed, the concentrations of the reagents were unknown. As the buffer was primarily designed to maintain the stability of the enzymes during lyophilisation of the reaction mix, a number of the reagents, particularly pH buffers and salts, are not applicable to improving the composition of the cake obtained by freeze drying the lysis buffer. Lyo D contains an unknown quantity of PEG 8000, which is responsible for producing a solid, consistent, product.

PEG 8000 concentration (%)	Time to amplification (Minutes)					
	Replicate 1	Replicate 2	Replicate 3	Average	Difference from 0%	Negative control
0.0	20.8	20.3	20.0	20.4	0.0	NA
1.0	20.4	20.4	20.8	20.5	0.1	NA
2.0	20.2	20.4	20.4	20.3	-0.1	NA
4.0	20.1	20.4	19.6	20.0	-0.4	NA
8.0	20.5	20.4	20.4	20.4	0.1	NA
10.0	21.3	20.5	20.4	20.7	0.3	NA

Table.1.24. GC *GroEL* amplification times from nucleic acid extracted from identical aliquots of *N. gonorrhoea* culture (2 x 10^5 cells per ml), using lysis buffer pellets containing various concentration of PEG.

The inclusion of PEG at any concentration between 1% and 10% had negligible effect on the time to amplification of the LAMP reactions carried out from the extracted nucleic acid (Table 1.24). The average amplification times using each concentration of PEG tested were within 0.5 minutes of the average amplification times when pellets not containing a lyoprotectants compound were used.

1.3.15. Scanning electron microscopy analysis of lysis pellets

Scanning electron microscopy (SEM) was used in order to image the surface of the lysis pellet cake, produced with and without 2% PEG, to examine for topological features characteristic of a properly formed cake. A key element of a well formed cake is the presence of micropores, which provide routes for water to vapour to escape the cake during the drying cycle. Insufficient frequency or complete absence of these pores prevents the escape of the water vapour, leading to a larger volume of water being retained in the final product. As guanidine hydrochloride is highly hygroscopic, any retained water is likely to draw the molecule back into solution, ruining the consistency of the pellet.

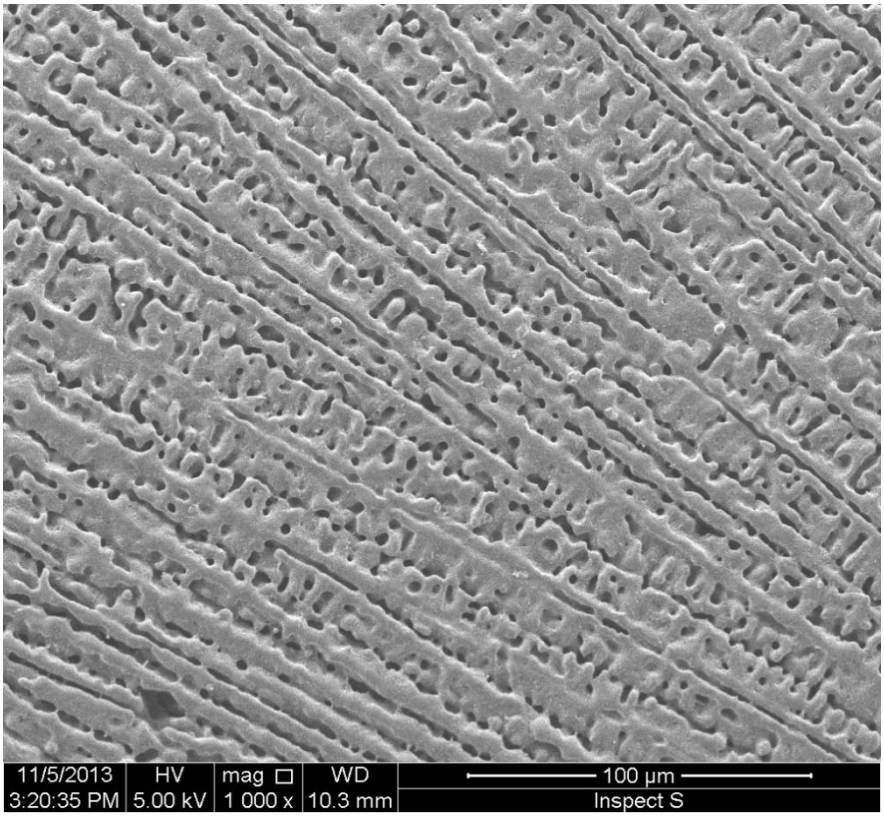

Fig. 1.21. SEM image of the surface of the lysis pellet (2% PEG) at 1000x mag.

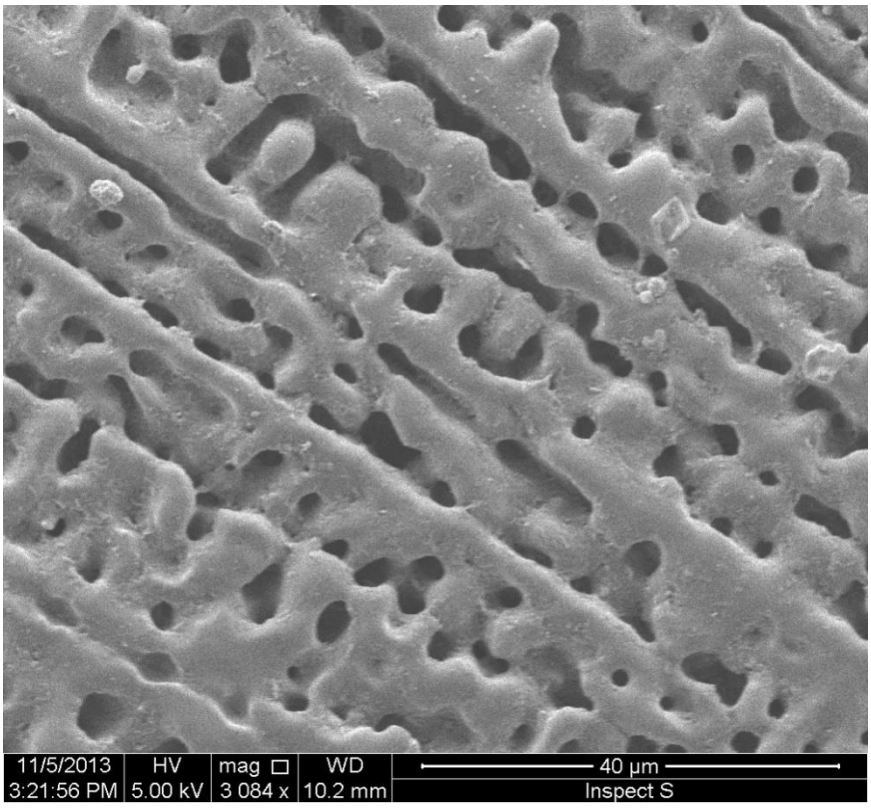

Fig.1.22. SEM image of the surface of the lysis pellet (2% PEG) at 3084x mag.

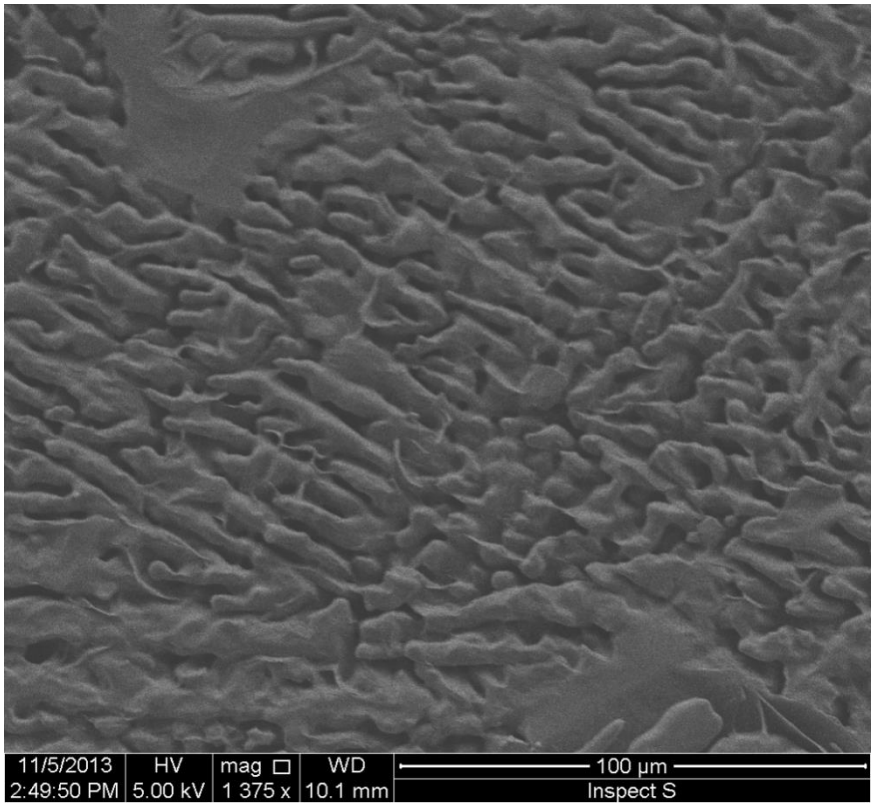

Fig.1.23. SEM image of the surface of the lysis pellet produced with buffer without PEG, at 1375x mag.

Micropore structures were clearly visible on the surface of the lysis pellets containing 2% PEG, indicating the presence of escape routes for water vapour sublimated during the freeze drying process (Fig. 1.21; 1.22). This is evidence that there is likely to be very low residual water content retained in the pellets, which is essential for their performance. The surface of the pellet formed from without the inclusion of 2% PEG did not have any observable micropore structures (Fig.1.23).

1.3.16. Swab performance

As the test will be required to be able to process swab samples, in addition to urine samples, it was necessary to determine the most suitable swab type for inclusion with the test. The recovery rate of the swab, which is the ability of the swab to retrieve cells, and then also subsequently elute the cells into a sterile media, will be an important factor in the overall sensitivity of the test.

Three swabs were tested; Copan flocked swabs, Sterilin cotton swabs, and Copan cotton swabs. The swabs were used to collect cells from 1ml aliquots of *N. gonorrhoeae* cell suspensions of varying concentrations, and then elute the cells into transport media. Total viable counts using the initial suspension, and inoculated transport media, were then carried out in order to work out the overall percentage recovery rate.

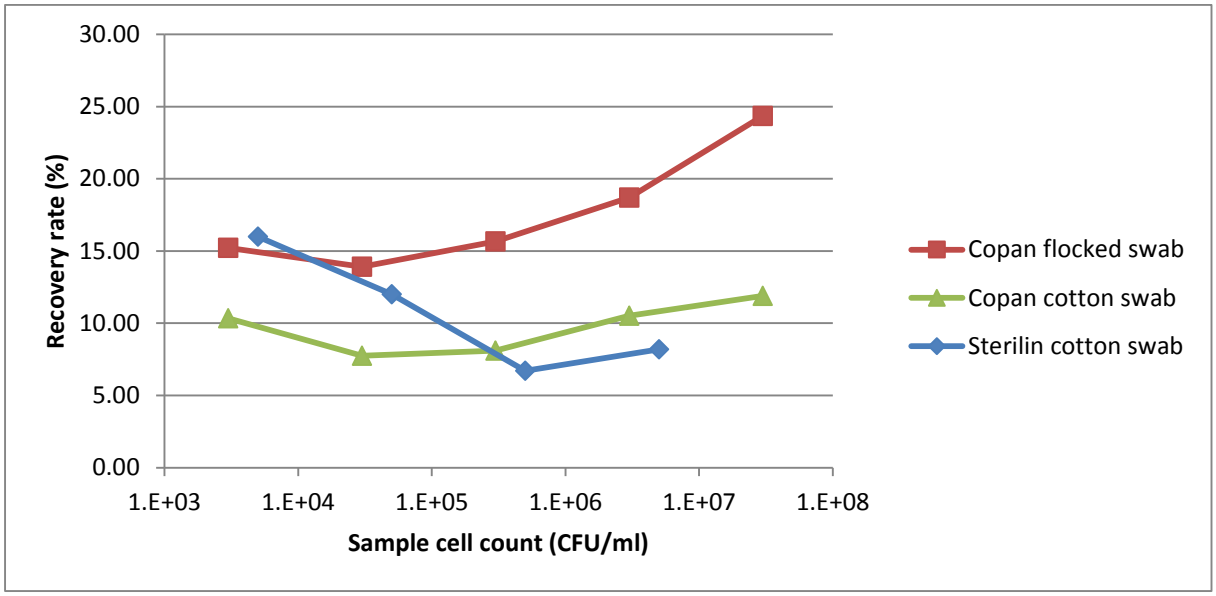

Fig.1.24. The recovery rate of bacteria from various concentrations of *N. gonorrhoeae* cell suspension, using Copan flocked swabs, Copan cotton swabs and Sterilin cotton swabs

The recovery rate using each swab for various cell suspension concentrations are shown in Fig.1.24. The highest recovery rate was seen using the Copan flocked swab, which enabled an average recovery of 17.6% over all concentrations tested. The Copan cotton swab and Sterilin cotton swab had overall recovery rates of 9.7% and 10.7% respectively, across all the concentrations of cell suspension. The Copan flocked swab performed best with more concentrated cell suspensions, recovery 24.4% of cells from the 3×10^7 dilution, compared with 15.2% from the 3×10^3 dilution.

1.3.17. Effect of Vortexing speed on swab recovery

Due to the low recovery rates achieved with all swab types in previous experiments, where no mechanical force was used to assist during the elution of cells, the effect of vortexing during this

process was examined. It was hoped that the force exerted during elution would improve the removal of cells from the swab into the transport media, by forcefully dislodging the cells from the swab.

A five second period of vortexing was carried out during the elution of the swab into transport media, at speeds of 0rpm, 500rpm, or 1000rpm.

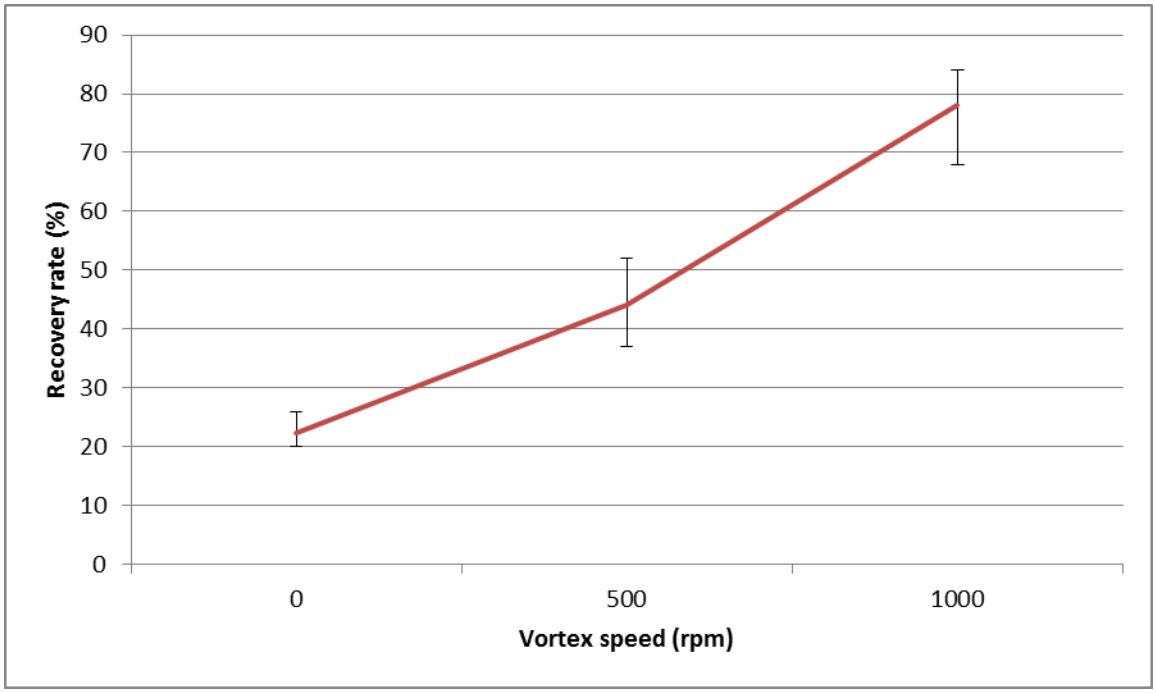

Fig.1.25. The effect of vortexing on the recovery rate of *N. gonorrhoeae* from Copan flocked swabs

Without vortexing, a recovery rate of ~24% was achieved, showing good agreement with the data shown in Fig. 1.24. The use of vortexing significantly increased recovery rate, with a speed of 500rpm increasing the recovery rate to ~45% (range 37% - 52%), and a speed of 1000rpm bringing it up to ~78% (range 68% - 84%) (Fig.1.25).

1.3.18. Swab buffer performance

In order to determine the optimum transport media for use with the Copan flocked swabs, an experiment was carried out to determine the effect of three transport media on the organism recovery rate from *N. gonorrhoea* suspensions of varying cell concentration. The three transport

media tested were Copan UTM, PBS and PBS Tween (0.02% Tween). The Copan UTM medium is frequently used in conjunction with Copan swabs for the collection of genital swab samples, with which it is frequently provided alongside. It is designed for the collection and transport of swabs containing C*hlamydiae*, *Mycoplasma* or *Ureaplasma*, but is also widely used for collection of *N. gonorrhoeae*. PBS and PBS Tween (0.02% Tween) are alternative commonly used transport media, which are simpler and less expensive than commercially available transport media such as the Copan UTM.

N. gonorrhoeae CFU/ml	Average PBS recovery (CFU/ml)	Average PBS recovery overall (%)	Average PBS Tween recovery (CFU/ml)	Average PBS Tween overall recovery (%)	Average Copan TM recovery (CFU/ml)	Average Copan TM overall recovery (%)
8,500,000	1,400,000	16	1,500,000	18	1,700,000	20
850,000	155,000	18	135,000	16	180,000	21
85,000	18,500	22	12,500	15	19,000	22
8,500	1,500	18	1,500	18	1,700	20

Table.1.25. Comparison of the effect of various transport media on the recovery rate of *N. gonorrhoeae* cells from Copan flocked swabs.

The percentage recovery of each buffer remained relatively stable for each starting concentration of cells in the suspension (PBS range 16 – 22%; PBS Tween range 15% - 18%; Copan TM range 20 – 22%). The average recovery across all concentrations of the PBS, PBS Tween and Copan UTM were 18.5%, 16.75%, and 20.75%, respectively (Table.1.25.).

1.3.19. Evaluation of the use of lysis buffer as swab transport media

In a clinical setting, after a swab is used to collect a sample from a patient it is then placed into a transport media, into which the cells are eluted. For NAAT testing, nucleic acid is then extracted from cells in this eluate. As previous experiments (Fig.1.25) have shown, the recovery rate from the swab is less than 100%, with only 24% recovered without vortexing, and a recovery rate of 78%

achieved with a 1000rpm vortex. Therefore, it is possible that a higher yield of nucleic acid could be achieved by lysing the cells at this point, whilst still on the swab, mitigating any loss from cells remaining on the swab. The replacement of the transport media with 1x GuHCl lysis buffer was tested in order to determine the subsequent effect on the time to amplification in the down-stream LAMP assays. This would provide an indication of the yield and quality of nucleic acid obtained from the sample.

	Time to amplification (Minutes)								
	1×10^6 Ng per ml			1×10^5 Ng per ml			1×10^4 Ng per ml		
	TM	LB	Difference	TM	LB	Difference	TM	LB	Difference
1	19.3	18.8	-0.5	21.3	19.8	-1.5	26.3	22.7	-3.6
2	17.7	17.8	0.1	22.4	18.9	-3.5	24.8	20.6	-4.2
3	20.1	20.6	0.5	22.5	21.8	-0.7	28.7	25.9	-2.8
Average	19.3	19.7	0.4	22.1	20.2	-1.9	26.6	23.1	-3.6

Table.1.26. Comparison of the effect of the use of Copan transport media and 1x GuHCl lysis buffer during the elution of N. gonorrhoeae from Copan flocked swabs on the time to amplification in subsequent GC *GroEL* LAMP assays. Assays that generated more rapid amplification when lysis buffer was used are highlighted green.

At the higher cell concentration, 1×10^6 CFU per ml, there was minimal difference in time to amplification, with the samples placed in transport media generating amplification 0.4 minutes quicker than the samples placed in lysis buffer (Table.1.26). However, at the lower starting concentration of 1×10^4 CFU/ml, the use of lysis buffer rather than transport media lead to an average decrease of 1.9 minutes in time to amplification. This difference was greater for the lowest concentration, 1×10^4 CFU/ml, where an average decrease of 3.5 minutes was acheived using the lysis buffer.

1.3.20. Comparison of the effect of swab incubation time in lysis buffer and Copan transport media

An important function of the transport buffer provided with the swab is the protection of the target nucleic acid in the sample. In the transport media this is accomplished by ensuring the cells remain intact, and do not release their nucleic acid into the extracellular environment, where it may be degraded by DNase or RNase enzymes present in the sample. The lysis buffer protects the released nucleic acid, after lysis has taken place, via the strong reducing action of the chaotropic salt, which deactivates these nucleases.

Swabs were incubated for 0, 10, 20, and 30 minutes in either lysis buffer or Copan transport media. Nucleic acid extractions were subsequently carried out, and the *GroEL* LAMP assay was used to test the extracted nucleic acid. Real time monitoring was carried out in order to determine whether there was a delay in signal due to lengthier incubation. The experiment was repeated using the lysis buffer, with the time period extended to 1 hour, as this was thought to be the maximum length of pre assay incubation for the POC system.

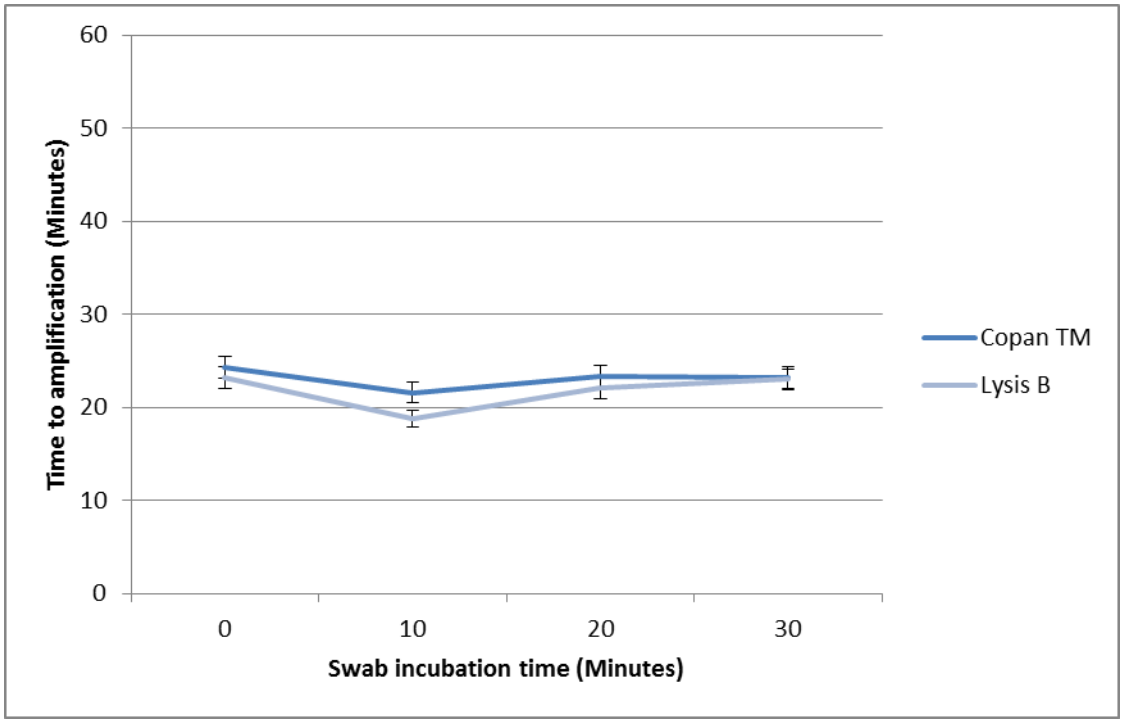

Fig.1.26. Comparison of the effect of the length of swab incubation in Copan TM and lysis buffer on the time to amplification in downstream GC *GroEL* LAMP assays. Error bars indicate a range of three replicates.

The incubation in lysis buffer had little effect on the time to amplification achieved in the subsequent LAMP assays, irrespective of time spent. The length of time that buffers were incubated in the Copan media prior to extraction also had little effect on the time to amplification of downstream LAMP assays. Similar time to amplification occurred when transport media or lysis buffer was used, which was also seen in previous experiments when using this cell concentration (Table.1.26)

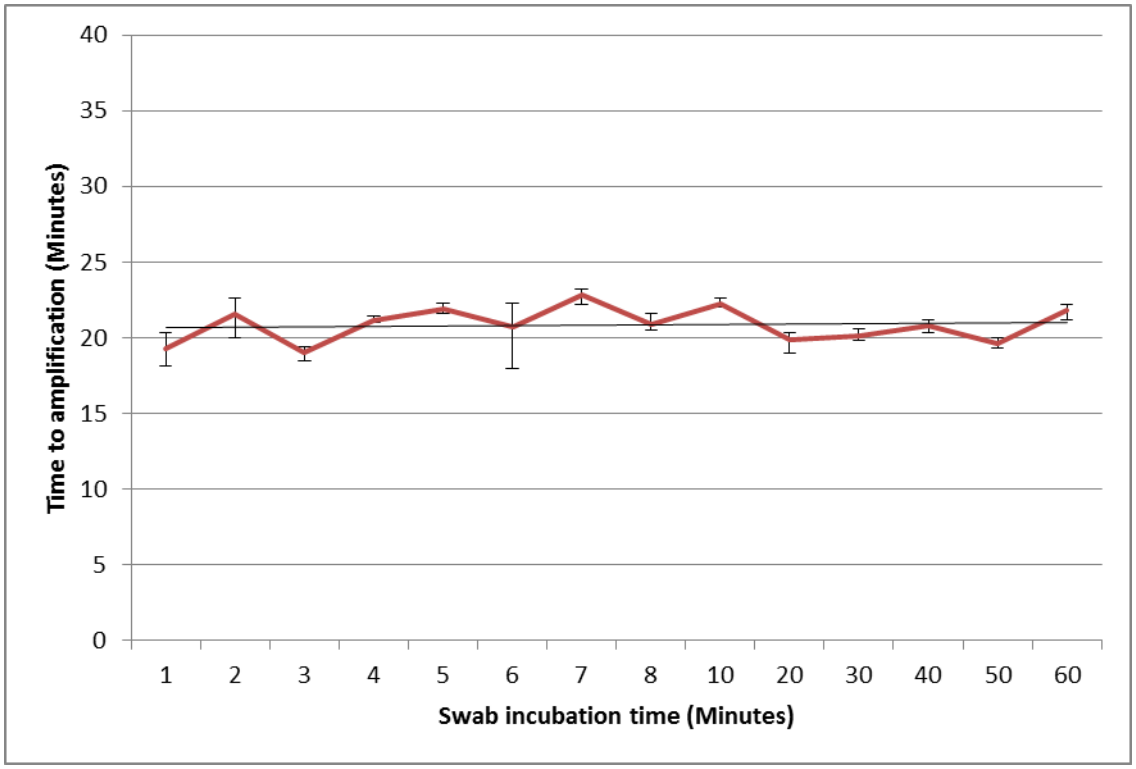

Fig.1.27. The effect of the duration of swab incubation in 1x GuHCl lysis buffer on the time to amplification in downstream *GroEL* LAMP assays. Error bars indicate a range of three replicates.

The experiment was repeated on another occasion solely using the lysis buffer, for an hour, in order to determine whether any degradation of the nucleic acid content of the sample occurred in the one hour duration with this buffer (Fig.1.27). There was no increase in the time to amplification seen as a result of an hourly incubation, indicating the stability of the released nucleic acid in the lysis buffer solution.

1.4. Discussion

1.4.1. Total nucleic acid extraction from *E. coli* culture using a magnetic silica based total nucleic acid extraction method

The image of the agarose gel electrophoresis of total nucleic acid extracted from *E. coli* culture in Fig.1.4 shows the various nucleic acid fractions successfully extracted using this method. The extracted genomic DNA is visible as a high weight band (>10kb) in all three samples, and shows no smearing. This indicates the extraction of unfragmented genomic DNA from the sample, showing that the DNA is preserved during the reaction process. This is important, as the extraction of unfragmented DNA from the sample will ensure that the DNA target sequence of the LAMP primers remains intact, and viable for the annealing of the primers and subsequent amplification.

The 23s rRNA (2904bp) and 16s rRNA (1541bp) bands are also clearly visible as sharp bands on the gel. There is a small degree of smearing, which could indicate minor degradation of the RNA, or be indicative of the varied mRNA population. The optimum intensity ration between the 23s rRNA band and 16s rRNA band should be around 2:1. This is because that although there is an identical number of 16s and 23s RNA transcripts in each cell, and each should degrade at the same rate, the fluorescence seen is a function of how many ethidium bromide molecules can bind to each nucleic acid strand. This depends on the number of bases available, so as the 23s rRNA contains roughly twice as many bases as the 16s rRNA, a 2:1 ratio should be seen. The ratio between the bands in Fig.1.4. is closer to 1:1, possibly indicating some degradation of the RNA during extraction.

A bright band was also present in each of the sample loading wells on the gel. This is likely to be fragments of cellular proteins released by the lysed cells during the extraction, which have survived the extraction process and been eluted into the final nucleic acid sample. These proteins are unable to pass through the agarose gel due to their large size. Positively charged proteins could bind to the negatively charged DNA, which would in turn bind to ethidium bromide molecules in the gel. As the extraction does not include a proteinase digestion step, typically found in most kit based extraction methods, it relies solely on the wash buffers to remove any contaminating proteins. This is an important step to remove any proteins inhibitory to PCR, such as haemoglobulin, and also ribonucleases, which could degrade RNA in the sample once free of the denaturing guanidine isothiocyanate present in the lysis buffer.

If LAMP amplification from samples processed by this extraction method is inhibited due to the carry-over of these proteins, it may be necessary to include a Proteinase K digestion during the process, or improve the efficiency of the wash procedure.

1.4.2. A comparison of thermal and chemical lysis methods for total nucleic acid extraction from *E. coli* culture

The use of a 30 second 95^{o}C heat treatment to lyse the *E. coli* cells during the nucleic acid extraction process resulted in a higher concentration of both DNA and RNA during elution compared to extraction carried out using a 10 minute lysis buffer incubation (Fig.1.5). This is possibly due to a decreased exposure of the nucleic acids to ribonucleases before the first wash step removes protein contaminants, the heat assisting in ribonuclease inactivation, or an increase in the percentage of cells fully lysed. Importantly, the heat treatment did not result in thermal degradation of the nucleic acid.

The sample lysed by a 1 minute incubation in lysis buffer also yielded a similar concentration of nucleic acid when compared to the sample lysed by a 10 minute incubation step, indicating that the 10 minute incubation is excessive and does not significantly increase nucleic acid yields. The performance of both the 1 minute buffer mediated lysis, and the 1 minute thermal lysis procedure, show that this part of the extraction process can easily be carried out within these time scales without any detrimental effect on the quality or quantity of nucleic acid retrieved.

1.4.3. Initial evaluation of the effect of chaotropic salt on lysis buffer performance

Although guanidine isothiocyanate (GuCN) is a more potent reducer of proteins than guanidine hydrochloride (GuHCl) (Rajeshwara and Prakash, 1994) and is also a more potent lytic agent, it carries safety risks associated with its use. Although both chemicals are classified in the same risk brackets in their respective MSDS (Health, 2; fire, 1; Reactivity, 0; personal protection, E), GuCN reacts with bleach to produce hydrogen cyanide gas, complicating the cleaning process in case of spillage. The inclusion of GuCN in the extraction method may lead to more stringent safety precautions surrounding the use of the test, and may lead to a reduced uptake of the platform once commercialised.

The initial test of the viability of a GuHCl based lysis buffer showed that there was negligible difference in the average LAMP amplification time between nucleic acid extracted using the GuCN buffer, or GuHCl buffer, with or without a heating step. A greater range of amplification time was seen across the five replicates for extractions carried out with the GuCHl buffer, suggesting a greater variability in extraction performance using this method. It was surprising that there was no difference in amplification time seen when the lysis process was aided by a heat step, as previous results have suggested that a greater yield of nucleic acid would be obtained in this manner. However, those experiments were carried out using *E. coli* as a model organism, and at higher concentrations than those used in this experiment, which may have affected the results.

1.4.4. Effect of guanidine hydrochloride lysis buffer and thermal lysis on a range of dilutions of *N. gonorrhoeae* cell suspension in urine

The tests that were carried out to examine the performance of the GuHCl lysis buffer, compared with the GuCN buffer, over a range of *N. gonorrhoeae* concentrations, showed that the GuHCl buffer, in conjunction with a prior 95^{o}C heating step of the sample, resulted in a faster LAMP reaction. The difference in amplification time was particularly pronounced for the extractions carried out on low density suspensions, which suggests that the use of this method may improve sensitivities of the assay, when dealing with samples containing at organism loads close to the limits of detection.

A critical parameter in the use of a thermal lysis procedure was the timing of the heating step; if the heating step was carried out after the mixing of the sample and buffer, a large increase in the time to amplification occurred. The reasons for this are unclear; by lysing the cells in the presence of GuHCl it was thought that a greater deal of protection would be from nucleases present in the urine, as when heating is carried out prior to buffer mixing, the released nucleic acid is at risk from this enzymatic degradation until it encounters the lysis buffer. One possible explanation is that by heating the lysis buffer along with the sample a proportion of the IPA was lost via evaporation. The IPA component is necessary for the efficient binding of the nucleic acid to the silica beads, and a reduction in concentration could possibly result in a reduction on the nucleic acid yield.

Due to this degradation, it is imperative that the application of heat to the sample in the LabDisk occurs prior to the mixing of the sample and buffer. This will actually simplify the process, as the volume at this stage will be at the lowest point throughout the process, and there will be no issue of

heating in the presence of IPA, which could cause evaporation, moving IPA vapour into other compartments of the system.

1.4.5. Effect of the duration of the 95°C heating step during lysis on the yield of nucleic acid and LAMP amplification time

The duration of the 95°C heating step during lysis effected the time to amplification of the LAMP reactions; an increased heating duration improved the speed of the LAMP reaction, up to a 30 seconds duration, at which time the amplification time reached a plateau and did not increase any further. This reason for this is likely that 30 seconds at 95°C is enough to ensure complete lysis of all cells present in the sample, while a reduced heating duration leads to incomplete lysis and a reduction in nucleic acid release. The concentration of the purified nucleic acid did increase as the duration of heating was extended, although the gain was minimal, increasing from 21.94ng/µl to 24.16ng/µl from 0 to 30 seconds of heating.

Whilst thermal lysis is capable of lysing cells in the sample at a fast rate, it also risks degrading the nucleic acid in the sample, which could reduce intact target sequence for the LAMP assay, reducing assay sensitivity. It is important that the duration of heating is enough to lyse the cells, but insufficient to cause damage to the released nucleic acid. It has been demonstrated that a 5 minute 100°C heat treatment during the extraction of DNA from *M. tuberculoisis* does not degrade DNA (Bemer-Melchior and Drugeon, 1999), although studies have shown that by increasing the incubation at this temperature to 30 minutes results in the significant shearing of the DNA (Zwadyk *et al.*, 1994). Heat shocks of 95°C for 30 seconds have been found to lyse over 90% of viable Gram-negative bacteria in a microfiuidic chip (Privorotskaya *et al.*, 2010). The use of thermal lysis in a microfluidic device has also been shown to produce DNA with the same integrity as that extracted using sonication, which is considered the safest lysis method for maintaining DNA integrity (Baek *et al.*, 2010). The extraction of RNA using thermal lysis has also been demonstrated, and been found to result in comparable yields and purity to RNA extracted using commercial kit lysis buffer s (Baek *et al.*, 2010).

As the use of a 95°C heat step decreased the Ta, and agarose gel electrophoresis of nucleic acid extracted in this manner did not reveal any evidence of degradation (Fig. 1.5), this lysis methodology seems well suited to use on the LabDisk.

1.4.6. Effect of GuHCl concentration on concentration and quality of extracted DNA

The concentration of GuHCl is an important parameter of the lysis buffer composition; the reversible absorption of nucleic acid to the surface of the silica microparticles is driven by the presence of this chaotropic salt, and will vary depending on its concentration (Vandeventer *et al.*, 2012). The chaotropic activity of GuHCL disrupts the lattice of water molecules in the sample solution, which combined with the high ionic strength of the solution, due to the presence of both GuHCl and NaCl from the lysis buffer, enable the formation of salt bridges between the negatively charged silica surface, and negatively charged oxygen atoms in the phosphate backbone of DNA or RNA molecules (Tan and Yiap, 2009). At high salt concentrations, as provided by the lysis buffer, the sodium ions break the bonds between the negatively charged oxygen ions in the silica matrix, and hydrogen atoms in water molecules in the solution, enabling the formation of the salt bridge (Tan and Yiap, 2009). GuHCl is also a potent reducer of proteins, and has been shown to denature RNase A at concentrations >3M (Ahmad, 1984). This protects the released nucleic acid during the lysis process from the action of endonucleases in the sample, maintaining the integrity of the nucleic acid prior to the amplification step. A final GuHCl concentration of between 2M (Kim and Morrison, 2009) and 5M (Shaw *et al.*, 2009) has been shown to be effective for SPE of nucleic acid using a silica matrix, although concentrations as high as 7M have been used in buffers lacking additional salts (Mathot *et al.*, 2013). Minimising the amount of GuHCL in the lysis buffer, to the lowest effective level, will be an important part of the buffer optimisation, as this will help to reduce costs during manufacture. It will also reduce difficulties in ensuring the GuHCl is fully dissolved in solution, as concentrations over ~5M require heating in order to be completely soluble in water.

The lysis buffer containing the lowest concentration of GuHCL tested, 3.5M, which resulted in a final concentration of 2.76M, resulted in the lowest yield of nucleic acid, 15.6ng/µl. This concentration is below the ~3M required to denature endonucleases (Ahmad, 1984), and it may be the absence of protection from these enzymes that has reduced the yield obtained using this buffer.

The highest concentration of extracted nucleic acid, and 260nm/280nm ratio was obtained using the buffer containing 4.5M GuHCl, which results in a final concentration of 3.55M. Buffers containing higher concentrations of GuHCl than this generated a reduction in both yield and 260nm/280nm ratio. This was slightly unexpected, as buffers containing these concentrations and above have been demonstrated in the literature as being effective for silica SPE (Mathot *et al.*, 2013). The difference in yield between the concentrations was fairly low, with a decrease from 20.1ng/µl to 17.5ng/µl recorded between the 4.5M and 6M buffers. Additionally, the values for the nucleic acid yield

obtained using the 5.5M and 6M had a comparatively large inter-replicate variance of 6.1ng/µl and 5.7ng/µl, respectively, so a lack of experimental reproducibility could factor in these results.

1.4.7. Buffer component concentrations

Another component of the lysis buffer that requires optimisation is EDTA. This was tested as percentage reductions of the initial 0.2M EDTA volume, which was 1.8ml per 20.3ml of buffer. EDTA is included in the lysis buffer as a chelating agent, with the purpose of binding 2+ ions, and preventing their involvement in any reaction processes. Certain DNase enzymes require Mg2+ for functionality, and the presence of EDTA will reduce their activity, protecting DNA from degradation (Guéroult *et al.*, 2010). It is important that the EDTA is sufficiently removed before the amplification process, as EDTA can inhibit polymerase enzymes, by sequestering the Mg2+ ions required for enzyme activity (Huggett *et al.*, 2008), although the concentration of EDTA required for inhibition of the LAMP reaction appears to be higher than those used in the lysis buffer (Francois *et al.*, 2011).

The difference in nucleic acid yield for buffers containing 100% to 0% of the volume of EDTA present in the standard lysis buffers was minimal. This was to be expected, as the main benefit of EDTA in this system is the reduction in nuclease activity, which is already effectively prevented by the presence of GuHCl. The volume of EDTA added is comparatively low, at around 9% of the total buffer volume, and EDTA is cheap and simple to make, so the presence at these volumes will not have a great impact on the price of manufacture. This experiment shows that the EDTA component could likely be removed, without any negative effect on assay performance.

The volume of 0.1M Tris/HCl pH 6.4 in the lysis buffer is greater than that of EDTA, comprising 42% of the total fluid volume. The purpose of the Tris/HCL component is to provide a level of control over the pH of the lysate and lysis buffer, to ensure optimal binding conditions. The formation of salt bridges between the phosphate backbone of the nucleic acid molecules and the silica beads will only form at <pH 7 (Tan and Yiap, 2009), and the Tris/HCL buffer will maintain these conditions.

The presence of Tris/HCl had little effect on the nucleic acid yield obtained in the experiment, with the 100% volume generating a yield of 19.8ng/µl, compared with a yield of 18.9ng/µl for the buffer not containing any Tris/HCl. A possible explanation for this is that as guanidine hydrochloride is acidic in solution, and although urine pH is highly variable, it is typically ~pH 6 (+/- 0.8) (Welch *et al.*, 2008), the combination of the lysis buffer and sample were unlikely to exceed the pH of 7 necessary for the binding of the nucleic acid to the silica beads. However, urine samples with a pH of up to 7.7

are encountered clinically, and the test needs to be able to process samples in this range (Proudfoot *et al.*, 2004).

1.4.8. Comparison of 1x and 2x lysis buffer for total nucleic acid recovery from clinical *C. trachomatis* samples, using a silica based total nucleic extraction method.

The use of a 2x concentrated lysis buffer during the extraction of nucleic acid from clinical samples had no effect on the amplification time of the CT-0332 LAMP assay, with nucleic acid extracted using the standard 1x GuHCl buffer generating an amplification at the same time-point. This result was expected, as the 2x concentrated buffer delivers an identical final concentration of GuHCl and IPA, the two components chiefly responsible for the yield and quality of the purified nucleic acid. The LAMP amplification curves generated by nucleic acid extracted using the two different buffers start at identical timepoints, showing a high level of reproducibility between extractions from the same sample.

1.4.9. Robustness of nucleic acid extraction method in the presence of inhibitory compounds

One of the primary objectives of the extraction system is the removal of compounds inhibitory to the LAMP assay from the clinical specimen. Inhibition of amplification can manifest as a failure of the reaction to produce any detectable product, a reduction in product produced, or an increase in the time to amplification in comparison to an uninhibited reaction (Huggett *et al.*, 2008). Diagnostically this leads to an increase in the likelihood of false-negative results and a reduction in assay sensitivity (Chernesky *et al.*, 1997). A large scale study investigating the presence of PCR inhibitors in STI samples using the COBAS AMPLICOR *C. trachomatis* test found that PCR inhibition occurred in 7% of cervical swabs, 45% of urethral swabs, and 1.1% of urine samples (Toye *et al.*, 1998). Urine samples contain a number of compounds known to interfere with PCR amplification, including urea, and protein components in cases of proteinuria. Significant concentrations of red blood cells can be rarely found in urine samples, especially samples from patients with urethral or kidney infections, which have symptoms that can overlap those of STD infection, leading to testing. Traces of blood are more commonly found in swab samples (Toye *et al.*, 1998), and are particularly prevalent in vaginal swab samples, with concentrations varying during the menstrual cycle. Blood contains haemoglobin and lactoferrin, which are both inhibitory to PCR (Al-Soud and Radstrom,

2001), and therefore may also be inhibitory to LAMP. The anticoagulant molecule heparin, a glycosaminoglycan present in blood plasma, can also inhibit PCR at physiological concentrations (García *et al.*, 2002).The robustness of the combination of the nucleic acid extraction system and LAMP assay was demonstrated by the lack of inhibition in the LAMP reaction, despite the presence of whole blood in the pre extraction sample in concentrations of up to $5.5x10^7$ RBC's per ml, equating to a 50:50 dilution of whole blood and *N. gonorrhoeae* cell suspension. This is a much higher level concentration of whole blood in the extraction system than would be encountered when sampling for sexually transmitted disease. Studies have defined haematuria as RBC counts over 1.1×10^3 (Hyodo *et al.*, 1997), and the assay system was able to process samples containing a greater than 1000-fold increase of this concentration. The LAMP reaction itself is known to be more tolerant than PCR to inhibitory compounds present in blood (Francois *et al.*, 2011), and the direct detection of pathogenic DNA sequences from un-processed whole blood has been demonstrated (Ebbinghaus *et al.*, 2012). Despite the fact that inhibition of the LAMP reaction itself by low concentrations inhibitory compounds carried through the extraction process is unlikely, it is important that the presence of blood does not interfere with the lysis of the sample, or the binding of nucleic acid to the silica particles during lysis.

There is a significant overlap between the symptoms of a number of STI's, and urinary tract infection (UTI) caused by bacteria that are not sexually transmissable. Additionally, co-infection between STI and UTI caused by organisms such as *E. coli* and *Klebsiella pneumoniae* can occur. The result of this is that both negative and positive samples submitted for STI testing can contain high densities of non-target bacterial species. This potential overabundance of nucleic acid could potentially compete for binding space on the silica bead surface with target nucleic acid, reducing the concentration available for amplification in the eluate. Lysed cells will also release their contents into the lysis buffer, increasing the concentration of RNAses leading to a greater likelihood of RNA degradation in the case of incomplete protection by the chaotropic salt. The absence of a reduction in amplification time in the LAMP reactions containing up to 1×10^7 *E. coli* cells shows that no inhibitory effect was found from the presence of bacteria at these levels. There was a small increase in the amplification time in the reaction containing 1×10^8 *E. coli* cells, 1.15 minutes more than the average of the reactions containing fewer *E. coli* cells. This could indicate that this is the threshold at which the concentration of non-target cells has an inhibitory effect on the extraction process, or it could be variance between reaction speed.

1.4.10. Effect of lysis buffer lyophilisation on nucleic acid extraction.

The use of a rehydrated lyophilised lysis buffer during the extraction of nucleic acid from a *N. gonorrhoeae* cell suspension had no effect on the time to amplification in the LAMP reaction, compared with the use of standard liquid lysis buffer. This shows that the freeze drying process has not impacted on the chemical properties of the guanidine hydrochloride; the identical amplification curves obtained show that the nucleic acid yield has not been reduced either by nuclease activity or reduced binding to the silica particles. This result is important as it shows that there is no loss of performance when freeze drying the buffer, and rehydrating it back to the original concentrations. This suggests that the storage of the lysis buffer in the microfluidic system will be able to occur as in a freeze dried form. This will have two major benefits; firstly the storage of liquid reagents is problematic, due to difficulties in applying them to the disk during manufacture, evaporation and leakage. Secondly, freeze drying the lysis buffer allows for the possibility of rehydrating using the sample itself, decreasing fluid volumes in the system and allowing for faster processing. This will also reduce the buffer concentration of the reagents necessary to generate the optimum final reagent concentration in the sample, reducing the cost of the buffer preparation. The absence of false positive amplification in the LAMP reactions using negative extractions carried out with the freeze dried lysis buffer shows that the use of this buffer does not cause non-specific amplification events, and will not impact on the sensitivity of the assay.

1.4.11. Tablet Manufacture

Tablet 1, produced using 100% freeze dried guanidine hydrochloride lysis buffer, formed solid tablets which were easily handled without breaking. They also dissolved completely in urine and IPA, with minimal agitation. The major benefit of these tablets is that just a single 516mg tablet is required per extraction, whereas the use of tablet 2 requires two tablets per extraction.

It was thought that tablet 2, containing 50% freeze dried lysis buffer, and a 50% mix of excipients, would break up more readily in solution, and have an improved consistency when compared with tablet 1. However, it was found that the consistency was actually less optimal, as the tablets easily crumbled and tended to disintegrate during contact. The tablets did not fully dissolve into solution; the rehydrated guanidine forms an almost saturated solution, leading to the lactose, which has a poor solubility in water and IPA, forming a suspension. The saturation of the solution could prevent

the active buffer components from dissolving into solution, and reduce the concentration in the lysis reaction down from the optimum level. Additionally, due to the 516mg tablets only containing 50% of the necessary active buffer component, two tablets are required per reaction, increasing the amount of materials required for the disk, and complicating the extraction process. Due to these reasons, tablet 2 was deemed unsuitable for use on the disk, unless the performance in the extraction was improved by using this tablet.

1.4.12. Initial test of tablet performance

The performance of tablet 1 during the lysis process was equal to the performance of the liquid lysis buffer, with the LAMP reactions from nucleic extracted from the high density (2×10^6 per ml) cell suspension of *N. gonorrhoeae* using these lysis buffers all generating an amplification between 16.6 and 17.6 minutes. The extractions carried out on the low density cell suspension (2×10^2 per ml) using tablet 1, lyophilised buffer, and liquid lysis buffer, all generated identical amplification curves after 20.5 minutes in the subsequent LAMP assay, demonstrating an equal yield and relative purity produced by the various buffers. Although the fact that freeze drying and subsequent rehydration does not diminish the performance of the lysis buffer during the extraction process has been demonstrated, this experiment shows that the same is true for the freeze dried buffer when compressed into tablets.

Extractions using tablet 2 were only carried out on the high density cell suspensions, due to the small volume of tablets produced, and their poor performance in this, and previous tests. The use of tablet 2 caused a delay of ~4.5 minutes compared with the use of tablet 1 or liquid lysis buffer. This could be due to carry over of the excipients present into the tablet into the eluted nucleic acid, inhibiting the LAMP reaction. Alternatively, the presence of the excipient compounds could interfere with the binding of the nucleic acid to the silica beads, reducing the starting copy number in the LAMP reaction. The increase in the time to amplification of the LAMP assay caused by the use of tablet 2, in combination with the need for two tablets to achieve the correct concentration, and the poor tablet consistency means that the tablet 2 formula is inappropriate for the extraction system.

Despite the encouraging performance of the lysis tablets, some problems with their manufacture arose during development. The buffer components had a strong oxidising effect on the stainless steel molding and punch, causing a rust build up and discolouration, which was then transferred to the tablets. This also appeared to cause the tablets to dissolve slightly, becoming wet and sticky.

Additionally, although tablet 1 performed well in the tests, due to the lack of a bulking agent and lubricant, the “flow” of the powder was poor. This is acceptable when manually producing the tablets, but would have caused major difficulties when the process was scaled up for automation. In light of this, it was decided to investigate freeze drying the buffer in individual molds.

1.4.13. Lysis buffer pellets

The use of lysis pellets resulted in an average reduction of the time to amplification in the LAMP assay, with an overall average reduction of 3.1minutes being recorded. This is likely to be due to the increased sample volume afforded by the use of tablets compared to the use of liquid lysis buffer. The extractions were carried out using the KingFisher ml extraction platform, in order to ensure reproducibility, and the fact that an unacceptable proportion of the beads are lost when attempting to manually carry out the liquid handling steps using a magnet. As the system can process only 1ml of sample in total, a 1ml portion of the mixture of tablet, 1ml of sample, and 0.5ml of IPA was used. This reduces the volume of sample processed to around 66% of the total that will be processed on the disk. This 660µl volume is larger than the 200µl volume of sample used during the standard extraction method with liquid reagents, and provides a higher concentration of target for the LAMP assay, reducing amplification times.

1.4.14. Lyoprotectants

The inclusion of PVP in the lysis pellets at concentrations of 1% to 8% produced a well formed cake, with good consistency. Unfortunately, even the lowest concentration of PVP tested, 1%, had a marked inhibitory effect on the subsequent LAMP amplification of extracted nucleic acid. The inhibitory effect was proportional to the PVP concentration; the higher the PVP concentration used, the greater the delay in amplification during the LAMP assay. The inclusion of 1% PVP resulted in an average delay of 4.1 minutes, and the inclusion of 2%, 4% and 8% PVP resulted in delays of 5 minutes, 5.2 minutes and 7.2 minutes, respectively. Target nucleic acid was only detectable in extractions carried out using pellets containing 10% PVP in one of the three triplicates, and the amplification was 10.7 minutes later than the average amplification using nucleic acid extracted using the 0% PVP pellets.

The reasons for this inhibitory effect are unclear; PVP is not a known inhibitor of PCR at low concentrations, and is used at concentrations of 0.5%-2% in PCR reactions to prevent inhibition arising from polyphenolic inhibitors, particularly from plant tissues (Koonjul *et al.*, 1999), although PCR inhibition has been noted at concentrations in excess of 2% (Koonjul *et al.*, 1999). Even so, LAMP is known to have a markedly higher tolerance than PCR to inhibitors, including those found in blood or stool (Francois *et al.*, 2011), and it would be surprising if the LAMP reaction was less tolerant to PVP than PCR. An inhibitory effect was noticed with a 1% concentration of PVP in the pellet, which would correlate to a 0.66% concentration in the lysis buffer. A possible explanation would be that the PVP is being co-purified in this system alongside the nucleic acid, by binding to the silica beads. This could either exert an inhibitory effect by interfering with the final LAMP reaction, or by competing for binding space on the silica beads, and reducing the yields of nucleic acid obtained.

The presence of polyvinylpolypyrrolidone (PVPP), a highly crossed linked version of PVP, has been shown to reduce the yield of DNA extracted using a sodium dodecyl sulphate (SDS) based extraction method (Zhou *et al.*, 1996), so there does exist evidence in the literature of PVP based compounds negatively impacting on DNA purification. The negative effect of PVP on the LAMP reaction has also been encountered by Mast Group Ltd during their development of the LAMP reaction buffer, so it is not a phenomenon peculiar to our laboratory.

1.4.15. Lyoprotectants: PEG 8000

Lyo B and Lyo D solutions were both developed by Mast as lyoprotectant solutions for the LAMP reaction buffer and enzyme during freeze drying. The full list of components was not disclosed, apart from the fact that the primary lyoprotectant compound was polyethylene glycol (PEG). The pellets produced containing the Lyo B solution were not suitable, due to the incomplete freeze drying of the product that occurred.

However, as the Lyo D buffer produced a solid stable cake at concentrations of 5%-15%, whilst having no negative effects on amplification time during LAMP amplification of the extracted nucleic acid (table 3), the use of the primary lyoprotectant in Lyo D, PEG 8000, was evaluated. Although the precise concentration of PEG 8000 in the Lyo D buffer was undisclosed, it corresponded to a final concentration of between 2% and 10%, so this range of concentrations was tested.

The optimal consistency of the pellet was achieved with a PEG 8000 concentration of 2%-8%. The pellet readily dissolved in urine and IPA within seconds, yet the cake was solid and easy to handle. The inclusion of PEG had a negligible effect on the amplification time in the LAMP assay at any concentration tested, with the largest average differences being a less than 30 seconds increase or decrease in amplification time.

The use of PEG 8000 as a lyoprotectants has been previously noted as creating a well formed cake (Amin *et al.*, 2004), in comparison with lower molecular weight PEG compounds. PEG 8000 has also been used in PCR reaction mix in microfluidic chips in order to prevent surface interactions, without compromising amplification efficiency (Panaro *et al.*, 2004), evidence that PEG 8000 carry over into the LAMP reaction is unlikely to have inhibitory effects. In fact, it has been demonstrated that the inclusion of PEG 8000 or PEG 20000 in the LAMP reaction mix can actually increase the speed of the reaction (Nose *et al.*, 2013).

1.4.16. Scanning electron microscopy analysis of lysis pellets

SEM analysis of the surface of the lyophilised lysis buffer pellets revealed the structural differences resulting from the inclusion of 2% PEG in the buffer prior to lyophilisation. The pellets containing PEG possessed clearly visible pore structures, 2µm - 5µm in size, that are indicative of a well formed product. These pores provide a route for water vapour to escape the dry later, ensuring a low residual water content, which is paramount when dealing with highly hygroscopic materials such as GuHCl, and also reducing drying time (Wang, 2000).

There was no evidence of any pore structure on the surface of the lysis pellets produced without a lyoprotectants. This could explain the poor consistency of these pellets, as it is possible that incomplete sublimation had resulted in a higher residual water content than in the pellets containing PEG.

1.4.17. Swab performance

An important factor determining the overall sensitivity of the testing process on the LabDisk will be the collection of cells during sampling. The design and composition of the swabs used during swab sampling can have a large bearing on the organism recovery rate. It is essential that the swab

collects as many pathogenic cells from the anatomical site as possible, and that as high a proportion as possible are eluted in the transport media in order to be applied for the disk, ultimately providing as high a starting copy number as possible in the LAMP assay.

The use of Copan flocked swabs enabled the highest recovery rate of *N. gonorrhoeae* from aliquots of culture media; an average recovery of 17.6% over all concentrations tested compared with overall recovery rates of 9.7% and 10.7% achieved by the Copan cotton swabs and Sterilin cotton swabs, respectively. This increase was expected; nylon flocked swabs have been shown as providing a greater recovery rate during bacterial sampling, providing an increase of up to 45% total recovery efficiency when compared with cotton swabs (Probst *et al.*, 2010). Additionally, previous studies have found that the elution of trapped cells occurs more readily from nylon flocked swabs, further enhancing the recovery rate, by up to 6-fold (Benschop *et al.*, 2010).

However, the recovery rates were much lower than those expected. Recovery rates achieved from bacterial cultures using flocked swabs have been determined to be between 56% and ~100% by various studies (Dube *et al.*, 2013; Harry and Madhusudhan, 2014; Tan *et al.*, 2014), considerably higher than the rates obtained by our tests. One difference in the methodologies employed by our test, compared with those detailed in the literature, is the absence of a vortexing step to aid in elution in our methods. This absence of a vortexing step was decided on by Mast, as there was a concern that a vortxing step prior to sample application would constitute a complication of the single-step sample handling that is stipulated by the terms of the TSB project. The lack of vortexing could be hindering the elution of cells from the swab, due to the absence of any mechanical agitation. Vortexing has been shown to increase swab recovery, with one study finding the use of vortexing increased the recovery rate from 17.7% to 43.7% (Rose *et al.*, 2004).

It is worth noting that the swabs were only tested using N. *gonorrhoeae*, rather than the full set of target organisms. These swabs have been tested extensively in the literature, and have been shown to provide improved capture rates for a large range of microbial species (Probst *et al.*, 2010; Benschop *et al.*, 2010) Once confirmed experimentally in our laboratory using *N. gonorrhoeae*, it was felt that this was sufficient evidence to support the data in the literature.

1.4.18. Effect of Vortexing speed on swab recovery

The use of a vortex period yielded a sizeable increase in recovery rate, with a 30 second 1000rpm vortex increasing the average recovery rate from 24% to 78%. This shows that lower than expected

recovery rates achieved using the Copan swabs in the previous study was due to incomplete elution of captured cells from the swabs, rather than the swab failing to retrieve the majority of the cells. The increase in recovery seen with vortexing was dependent on the strength of the agitation, illustrating that the greater the force applied, the less likely the cells are to remain caught in the fibres of the swab. The increased gained by using the vortex could be essential in ensuring as high a sensitivity as possible for the LabDisk test, especially for samples containing low cell numbers. For example, whilst the average organism load from a *C. trachomatis* positive high vaginal swab sample is 7728 cells per ml, the range includes samples with 10 – 100 cells per ml (Michel *et al.*, 2007). With organism loads at this low level, a high recovery rate becomes imperative in enabling the test to determine the sample as positive.

1.4.19. Swab buffer performance

There was minimal difference in the recovery rate from the Copan flocked swabs between the use of any of the buffers tested. The Copan universal transport media provided the highest average recovery rate (21%) across all concentrations tested, compared with the PBS buffer and PBS Tween buffer, which retrieved an average of 19% and 17% of the cells, respectively. These experiments were carried out without the use of a vortexing step, hence the low recovery rates encountered. A large scale study examining the effect of the elution buffer on organism recovery rate from wipes found that PBS and PBS-Tween (0.04%) enabled optimum recovery rates, which could be enhanced by a vortexing period (Downey *et al.*, 2012).

1.4.20. Evaluation of the use of lysis buffer as swab transport media

The replacement of a swab transport media with lysis buffer was able to increase the concentration of nucleic acid retrieved during the extraction process, particularly at low sample concentrations, evidenced by the decrease in the time to amplification of subsequent LAMP assays. Lysing the cells on the swab alleviates the low swab recovery rates caused by the incomplete elution of cells from the swab, by ensuring that all nucleic acid is released from cells that have been picked up during sampling. This would also remove the need for a vortexing step to be carried out prior to the application of the sample.

The practical application of the use of lysis buffer rather than a transport media is not without difficulty; the use of the lysis pellets requires a liquid sample in order to rehydrate the buffer. The pellets would not be soluble in a lysis buffer already containing a ~3M concentration of guanidine hydrochloride. If lysis was to take place off disk, and then the lysed sample applied and processed, the urine samples would also have to be lysed off disk. If a liquid buffer was to be used, this would dilute the sample, potentially impacting on the analytical sensitivity of the system. A possible solution to this problem would be to lyse the urine off disk with a lysis tablet, and apply the entire sample to the disk, whilst doing the same for swab samples with the liquid media.

1.4.21. Comparison of the effect of swab incubation time in lysis buffer and Copan transport media

The pre extraction incubation of the sample in either lysis buffer or Copan UTM transport media had no effect on the time to amplification in the LAMP assay, demonstrating that there was a negligible level of nucleic acid degradation over the hour tested. Whilst the lysis buffer causes the release of nucleic acid into the extracellular environment during lysis, the reducing activity of the guanidine salt protects the nucleic acid from degradation by denaturing nuclease enzymes. The Copan UTM works by maintaining cell viability and cell integrity, ensuring that the intracellular nucleic acid remains protected within the cell, and is not degraded. The initial test was only carried out for one hour, as it is unlikely that a sample will be left unprocessed for longer than this in a point of care setting.

1.4.22. Evaluation and recommendations

The design of a suitable nucleic acid extraction system, and sampling handling methodology, for the proposed POC assay required the optimisation of each step of the process. The utilisation of the LabDisk as the microfluidic processing unit for the assay, with liquid movements controlled via centrifugation, resulted in the initial testing of a magnetic silica based extraction system. A variant of this method has been successfully used on centrifugally operated disk platforms previously, it enables the rapid concentration of nucleic acids from clinical samples, concentrates both RNA and DNA, and can be modelled using the KingFisher Ml extraction robot, which was available in our laboratory.

The assay would need to be able to process both urine samples, and vaginal/endocervical swab samples. The urine samples will simply be collected as is standard practice at the point of operation. A 1ml volume of this sample will then be applied to the LabDisk using a fixed volume pipette. Swab samples would be collected, placed in a transport media, and then a 1ml volume of this transport media would be applied to the LabDisk using the same fixed volume pipette. For the swab samples it was necessary to determine the optimum swab type, and also transport media required. It was found that out of the swab types under consideration, the Copan flocked swabs outperformed the Copan and Sterilin cotton swabs (Fig.1.24), and provided the highest recovery rate. This swab type was therefore recommended for use.

The use of lysis buffer as the transport medium for swab samples, rather than a classic transport medium, resulted in an increase in assay speed, presumably due to an increase in the quality or quantity of nucleic acid being presented to the subsequent LAMP assays (Table 1.26). This was particularly evident when extracting from samples containing low concentrations (1×10^4 cells per ml). However, the use of lysis buffer in the transport media would require the use of two separate disks; one for urine samples with on-chip lysis, and another for swabs with in-tube lysis. This would complicate manufacture of the LabDisks, and could make the test less attractive to consumers. If lysis buffer was not used, it was found that Copan TM, which can be supplied alongside the Copan flocked swabs which have been chosen for use with this system, enabled a slight increase in the percentage of eluted cells when compared with other PBS based buffers tested (Table 1.25).

It was found that the absence of mechanical agitation during the elution of cells from the swabs resulted in poor recovery rates, which could be greatly increased by a vortexing step. A five second vortex at 1000rpm, of the swab in transport media, increased the recovery rate from ~24% to 78% (Fig.1.25). This is a highly significant increase in the recovery rate and its inclusion could lead to a

sizeable increase in clinical sensitivity. It has been suggested that a vortex is sold alongside the POC system, in order to be able to provide this agitation step.

Once the samples, either transport media from a swab, or a urine sample, are applied to the LabDisk they will undergo a lysis step. A 95^{o}C heat step at this stage was shown to increase the nucleic acid yield obtained (Fig.1.7), reducing the time to amplification of downstream LAMP assays (Fig.4.), and also reduce the time needed for complete sample lysis. It is imperative that this heat step occurs before the sample contacts the lysis pellet, silica beads, and IPA, in order to have this positive effect on amplification time (Fig.1.7). The optimum duration of the heat treatment was found to be 30 seconds, as this was the minimum time that generated the full benefits of the thermal lysis step (Fig.1.8).

After the thermal lysis step, the sample should be channelled into the extraction chamber, where it would be mixed with 0.5ml of IPA, and rehydrate the 15µl of dried MagnaSil beads (Thermo), and 790µl 2x lysis buffer pellet. Rehydrated freeze dried buffer was equally effective as liquid lysis buffer (Fig.1.13). The use of a freeze dried lysis buffer pellet was shown to decrease assay amplification time (Fig.1.17.), by enabling a larger volume of sample to be processed. It also removes the need for storage of liquid lysis buffer on the LabDisk, which reduces issues of evaporation and spillage, and the need for the expensive blister packs required for the IPA and wash buffers. The inclusion of PEG 8000 in the lysis buffer at a concentration of 2% assisted in the formation of a solid, easily handled cake, whilst having no negative effect on the extraction process or downstream LAMP assays (Table.1.24.). A 30 second incubation involving the beads, sample, and lysis reagents, was sufficient to enable binding of the nucleic acids to the silica beads (Fig.1.5).

After the lysis procedure is completed, the beads are captured via an external magnet, whilst the disk is moved around their position, bringing them through the three wash buffers; wash A, wash B, and wash C. A total of 30 seconds is spent in each buffer. The beads are then placed over a heat pad, in a procedure designed by IMTEK, to ensure residual 75% v/v ethanol is evaporated before the elution stage. The nucleic acid is then eluted in molecular grade water, and used to rehydrate the LAMP reaction mix. This mixture is then divided between the LAMP assay wells, where it rehydrates the freeze dried primers, and undergoes amplification and fluorescence detection.

An overview of the suggested sample handling and extraction process, based on the findings of the experimental work detailed in this chapter, is shown in Fig.1.28.

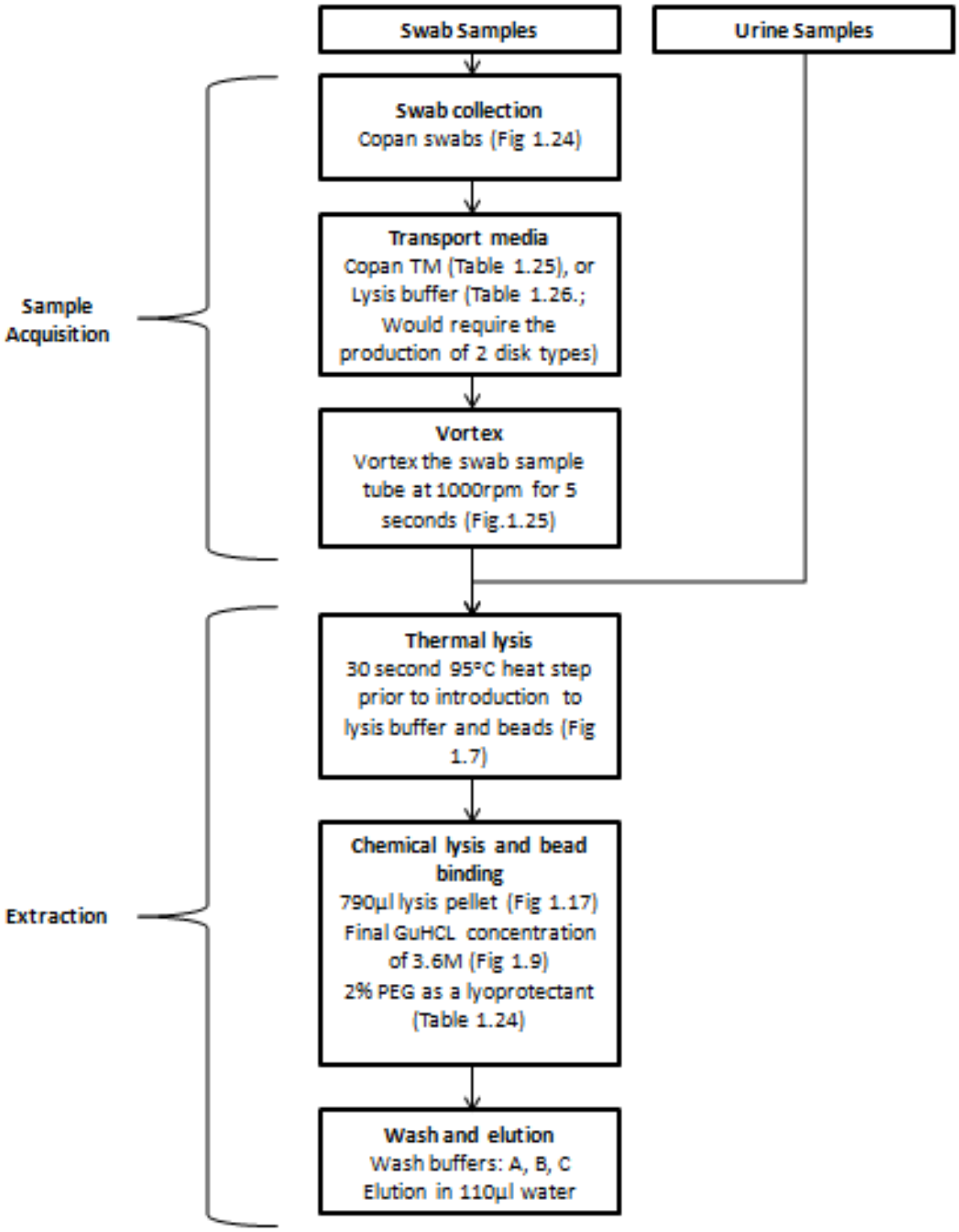

Fig.1.28. An overview of the recommended sample handling and extraction process, showing suggested processes. The figures referred to show supporting experimental data.

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Chapter 2

Neisseria gonorrhoeae

2.1. Introduction

Neisseria gonorrhoeae is a species of Gram-negative, aerobic, fastidious bacteria, and the causative agent of the disease gonorrhoea, the second most common human sexually transmitted infection in the developed world (Newman *et al.*, 2007). An estimated 106 million incidences of gonorrhoea infection are reported yearly worldwide (World Health Organisation, 2012), representing a significant global health burden. Gonorrhoea is transmitted by sexual contact; colonisation usually occurs as a localised infection in the genital or cervical mucosa. Infection of the pharynx, anorectum and disseminated infections are also encountered (Sherrard, 2005), including endocarditis (Butterly *et al.*, 2011) and arthritis (Dalla Vestra *et al.*, 2008).

90% to 95% of urethral infections in men are symptomatic, compared with only 20% of genital infections in women and 5% to 10% of pharyngeal infections (CDC, 2010). In men, symptoms include mucopurulent urethral discharge and pain upon urination. If left untreated infection can lead to epididymititis with associated testicular pain, swelling and fever (Little, 2006). In women, symptoms, if present, tend to be milder. They include pain upon urination, vaginal bleeding and discharge. Pelvic inflammatory disease (PID) also occurs in 10% to 20% of untreated women, which can lead to infertility and ectopic pregnancy, with symptoms including chronic abdominal pain, fever and cervical motion tenderness (Pearson *et al*., 2004).

2.1.1 Pathogenesis

Transmission of *N. gonorrhoeae* occurs via sexual contact with an infected partner, although transmission to a child during pregnancy can also occur (Brocklehurst, 2002). In heterosexual sex, transmission is thought to occur more readily from an infected man to an uninfected woman (50 – 70% probability per exposure), than vice-versa (20 – 35% per exposure) (Edwards and Apicella, 2004). Infection is initiated by adherence to host epithelia, which is mediated by a number of *N. gonorrhoeae* surface proteins and pili. *N. gonorrhoeae* possesses type IV pili, which enable twitching motility, providing a means for the *N. gonorrhoeae* cells to move through the mucosal epithelia during colonisation (Plant and Jonsson, 2006). Type IV pili also play a key role in the adherence of the gonococcus to the host epithelial surface, via the presence of a number of pili-associated adhesins. PilC, a pilus associated 110-kDa protein, is expressed in the tip of the pilus, and cell membrane, and is essential for adherence to epithelial cells (Wolfgang *et al.*, 1998). *N. gonorrhoeae* mutants lacking

PilC are unable to adhere to epithelia (Wolfgang *et al.*, 1998), and purified PilC conjugated to latex beads enables bead binding to epithelial cells (Rudel et al., 1995), demonstrating the function of PilC as an essential adhesion. Additionally, PilE, the major pilus subunit, has been determined as having binding affinity to epithelial cells itself, and antigenic variants of this protein modulate epithelial adherence (Winther-Larsen *et al.*, 2001; Plant and Jonsson, 2006). The fibre subunit PilV has also been demonstrated to have a key role in pili mediated adherence, by promoting the functional display of the PilC adhesin on the pilus fibre itself (Winther-Larsen *et al.*, 2001). Interaction between the pili and the host cell membrane has been shown to result in cyto-skeletal rearrangement in of the host cell, activating microvilli which promote the engulfment of the bacterial cells, enhancing invasion(Griffiss *et al.*, 1999). This pili-host membrane interaction also instigates the redistribution of membrane associated proteins such as actin, ezrin and CD46, a membrane cofactor receptor that has been demonstrated to act as a receptor for PilC in some cell lines (Merz and So, 1997; Edwards and Apicella, 2004).

After this initial pili-mediated attachment, the intimate attachment and subsequent invasion of *N. gonorrhoeae* is chiefly mediated by Opacity (Opa) proteins; a family of outer membrane proteins that modulate attachment, tissue tropism, and the avoidance of the immune response. A strain of *N. gonorrhoeae* may harbour up to 12 variants of the *Opa* gene, the expression of which is constantly varied. These proteins can be grouped into two classes dependent on their binding targets; Opa_{HS} proteins target host cell heparin sulphate proteoglycans (HSPG) (Grant *et al.*, 1999), whilst Opa_{CEA} proteins bind to members of the carcinoembryonic related cell adhesion molecule (CEACAM) family (Sadarangani *et al.*, 2011). Binding of Opa_{HS} proteins to surface HSPG is mediated by bridging molecules such as vitronectin or fibronectin, in a process resulting in host cell invasion caused by a cytoskeletal rearrangement that facilitates the endocytosis of the membrane associated bacterial cell (Gomez-Duarte *et al.*, 1997; van Putten *et al.*, 1998). Binding of Opa_{CEA} to CEACAM proteins, also results in endocytosis of the gonococcus following cytoskeletal activity, in a process resulting from the activation of host cell Src tyrosine kinases, and subsequent activation of Rac GTPase (Hauck *et al.*, 1998).

N. gonorrhoeae porins have also been implicated in having a number of roles in *N. gonorrhoeae* pathogenesis. Porins form hydrophilic ion and water channels in the gonoccocal membrane, where they are abundantly expressed. *N. gonorrhoeae* possesses two porins, PIA and PIB, expressed by the *PoR* and *PorB* genes, respectively (Olesky *et al.*, 2006). The porins of pathogenic *Neisseria* are unique in their ability to insert into the cell membranes of eukaryotic cells that they are in close contact with, to form gated ion channels (Ayala *et al.*, 2002), which has been shown to impede neutrophil

activity and macrophage maturation (Lorenzen *et al.*, 2000). Porin IB has been shown to activate NF-KappaB in human urethral epithelial cells *in vitro*, which results in the increased expression of a number of anti-apoptotic genes such as cox-2, bfl-1 and c-IAP-2 (Binnicker *et al.*, 2004). It is thought that the prevention of apoptosis allows the gonococcus to evade the host immune response, and multiply intracellularly, enhancing the likelihood of successful colonisation (Binnicker *et al.*, 2003).

A primary mechanism of inflammation encountered during the course of *N. gonorrhoeae* infection occurs via the release of peptidoglycan monomers, which instigate the release of host pro-inflammatory cytokines, damage ciliated epithelia, and activates the Nod signalling cascade, ultimately increasing production of Interleukin-8 (Cloud-Hansen *et al.*, 2008). One peptidoglycan monomer released by *N. gonorrhoeae* is tracheal cytotoxin (TCT), a 921 dalton glycopeptide, shown to be cytopathogenic to vaginal and fallopian ciliated epithelia (Swaminathan *et al.*, 2006; Melly *et al.*, 1984). The damage and subsequent sloughing of ciliated epithelia seen in pelvic inflammatory disease occurring from persistent infection may be a result of this peptidoglycan-host cell interaction (Cloud-Hansen *et al.*, 2008); purified peptidoglycan fragments released by *N. gonorrhoeae* cause sloughing of ciliated fallopian tube cells in the absence of *N. gonorrhoeae* cells (Melly *et al.*, 1984).

2.1.2. Epidemiology and clinical presentation

In 2008 there were an estimated 106.1 million new cases of *N. gonorrhoeae* infection worldwide, with an estimated 36.4 million people infected at any one time (WHO, 2012). This represents an estimated 21% increase in incidence compared to the 2005 WHO figures (WHO, 2008), illustrating the increasing spread of this disease. It is worth noting, however, that this sharp increase could be explained by the increased use of sensitive NAAT based diagnostics, especially in resource poor settings, increasing the likelihood of diagnosis, and inflating the number of cases reported. The incidence of gonoccocal infection is higher in developing countries, such as those found in South East Asia, the Western Pacific region, and Africa (Table 1), due in part to lower levels of safe-sex education, reduced condom use, and lack of access to testing facilities (Mayaud and Mabey, 2004).

WHO Region	Incidence (per 1000)		Prevalence (%)	
	Female	Male	Female	Male
Africa	49.7	60.3	2.3	2.0
The Americas	18.5	27.6	0.8	0.7
South East Asia	16.2	37.0	0.8	1.2
European	8.3	7.0	0.3	0.2
Eastern Mediterranean	8.1	11.6	0.3	0.3
Western Pacific	34.9	49.9	1.5	1.3

Table 2.1. Global incidence and prevalence of *N. gonorrhoeae* infection, according to 2008 WHO estimates. Data taken from WHO (2012).

In England the incidence of gonorrhoea is increasing; 25,525 cases were diagnosed in 2012, a 21.4% increase since 2011 (Health Protection Agency, 2012) and a 51.6% increase since 2010 (Health Protection Agency, 2011). As with the increase seen in the WHO figures, the increase may partially be down to an increase in test sensitivity driven by increased NAAT use, however it is thought that these figures do indicate a genuine increase in gonorrhoea incidence in the UK (Savage *et al.*, 2012). In the UK, around half of all diagnosed cases are found in men who have sex with men (MSM), and the incidence in this group rose 61% from 2010 to 2011, making this group the target of particular attention from public health bodies in the UK (Savage *et al.*, 2012). A large scale study in the US screened MSMs at an STD clinic (n=5539) and at a gay men's community health centre (n=895) and found an overall prevalence of 6%, 6.9% and 9.2% of detectable gonoccocal infection at urethral, rectal, and pharyngeal sites, respectively (Kent *et al.*, 2005). A total of 64% of the infections were at non-urethral sites, and it is imperative that extra-urethral testing is offered to MSM in order provide effective testing to this population.

Infections in males are frequently symptomatic, with urethral infections causing symptoms in 90 – 95% of cases. Symptoms occur 2-5 days post-exposure, with urethral discharge and dysuria present in >80% and >50% of infections, respectively (BASHH, 2011 guidelines). Pharyngeal infections are frequently asymptomatic, with symptoms occurring in 5% - 10% of cases, and include tonsillitis and pharyngitis. Anal *N. gonorrhoeae* infection, particularly prevalent in MSM, is also rarely symptomatic, with symptoms such as proctitis and discharge occurring in <15% of cases (Hamlyn and Taylor, 2006). *N. gonorrhoeae* infection in males has also been documented as causing epididymitis (Trojian *et al.*, 2009) and prostatitis (Zhou *et al.*, 2003).

N. gonorrhoeae infections in females can occur in multiple anatomical sites, including the endocervix, urethra, anus and pharynx. Endocervical infections are asymptomatic in 80% of cases (CDC, 2010); possible symptoms, if present, include increased or altered vaginal discharge, lower abdominal pain, and rarely, intermenstrual bleeding (BASHH, 2012). Untreated endocervical infection, which is a possibility due to its asymptomatic nature, is thought to progress to PID in 10%-20% of cases. This serious ascending infection leads to uterine and tubal scarring, which causes infertility, chronic pelvic pain, and ectopic pregnancy in 20%, 18% and 9% of PID sufferers, respectively (Walker, 2011). Rectal infection in women presents in a similar fashion to those found in males, and often occurs via transmucosal spread of genital secretions to the anus, rather than by anal intercourse (BASHH, 2012). Occurrences of gonococcal infection during pregnancy can lead to transmission to the neonate during childbirth, with the likelihood thought to be around 50% (Hammerschlag, 2011). Neonatal infection can occur as conjunctivitis, arthritis or sepsis (Walker, 2011). Disseminated gonoccocal infections can occur in either sex, with *N. gonorrhoeae* cells migrating from the primary site of infection via the circulatory system (Sherrard, 2005), which can lead to arthritis (Dalla Vestra *et al.*, 2008), endocarditis (Butterly *et al.*, 2011), or even sepsis (Walker, 2011).

2.1.3. Relationship with other pathogens

Co-infection of *N. gonorrhoeae* and *C. trachomatis* is common, with a large UK based study involving 1239 women and 1141 men with gonorrhoea, chlamydia, or both, finding the 24% of heterosexual men and 38.5% of heterosexual women who had gonorrhoea were simultaneously infected with *C. trachomatis* (Creighton *et al.*, 2003). Of patients with *C. trachomatis* infection, 18.8% of men and 13% of females were also concurrently infected with *N. gonorrhoeae*. A large study in the US youth detention system, involving over 100,000 samples found that of patients with *N. gonorrhoeae* infection, *C. trachomatis* co-infection was present in 51% of males and 54% of females (Kahn *et al.*, 2005). Co-infection with sexually transmitted pathogens is not uncommon, as the methods of transmission, and high-risk populations are the same. Also, the frequent asymptomatic nature of STIs increases the likelihood that an infection will go undetected, allowing the patient to become exposed to another pathogen prior to treatment. The presence of an STI can also damage the genital epithelia, increasing the likelihood of the transmission of other pathogens across this surface. The high prevalence of *C. trachomatis* co-infection in patients diagnosed as harbouring *N. gonorrhoeae*

infection has lead the CDC in the US, and PHE in the UK, to recommend that *C. trachomatis* is treated for concurrently with antibiotic therapy for *N. gonorrhoeae* (CDC, 2012; GRASP, 2012).

Infection with *N. gonorrhoeae* has been identified as a risk factor for increased HIV-1 transmission, with a study in Zaire finding women were 3.8 times as likely to become infected with HIV-1 whilst infected with *N. gonorrhoeae* (Laga *et al.*, 1993). One study examining 431 initially HIV negative heterosexual women, of which 9.8% seroconverted over the two year study period, found that HIV infection was 4.8 times more likely in women with gonoccocal infections (Laga *et al.*, 1993). Anal *N. gonorrhoeae* infection has been associated with increased risk of HIV acquisition in MSMs in Australia (Jin *et al.*, 2010) and America (Bernstein *et al.*, 2010).

N. gonorrhoeae releases extracellular heptose during a growth, which has been shown to induce HIV-1 expression in CD4+ lymphocytes in an NF-κB dependent manner, driving viral production (Malott *et al.*, 2013). This increases viral shedding, and therefore the infectivity of the patient, providing a direct link between *N. gonorrhoeae* infection and increased HIV transmission. Additionally, the human defensins 5 and 6 (HD5 and HD6), released as a response to *N. gonorrhoeae* infection, have been found to increase HIV transmission, by facilitating viral entry (Klotman *et al.*, 2008). These defensins, and human beta defensins (HBDs) which are also released during gonococcal infection, can also up regulate inflammation via a range of interleukins and pro-inflammatory cytokines, which act to recruit HIV target cells, providing a larger pool of potential host cells for initial HIV infection and replication(Jarvis and Chang, 2012). HD5 and HD6 have also been shown to prevent the anti-HIV activity of polyanionic microbicides, and HIV entry and fusion inhibitors in vitro (Jarvis and Chang, 2012). It has also been demonstrated that N*. gonorrhoeae* can enhance the susceptibility of primary CD4+ cells to HIV-1 infection, via the activation of toll-like receptor 2 (TLR2) (Ding *et al.*, 2010). These mechanisms contribute to the increased risk of HIV-1 acquisition in people who are already harbouring a *N. gonorrhoeae* infection. Control of *N. gonorrhoeae*, along with other sexually transmitted pathogens implicated in increased HIV transmission risk, is an important aspect of the control of HIV worldwide.

2.1.4. Diagnosis

Traditionally, diagnosis of gonorrhoea has been carried out via microscopy and culture of the organism on selective media. Microscopy of Gram stained genital specimens enables detection of the coffee bean shaped diplococcus, and offers acceptable sensitivities of 90-95% in symptomatic

men. The sensitivity of this method for the diagnosis of asymptomatic men or females is considerably lower; 50% - 75% and 20% - 50% respectively (BASHH, 2011). Microscopy from urethral exudates of symptomatic men is sensitive and specific enough to be utilised diagnostically, and has the added advantage of being fast enough to be carried out at the point of care (POC), enabling immediate treatment (Tapsall, 2001).

Culture from clinical specimens is carried out using a non-selective agar, typically chocolate agar, in conjunction with a selective media such as Thayer martin agar in order to remove commensal microorganisms frequently picked up by genital sampling (Ng and Martin, 2005). Plates are incubated at 35^{o}C to 37^{o}C, supplemented by 5% CO_2, for 24 hours and then inspected for growth. The recovery of Gram negative diplococcus from *N. gonorrhoeae* selective media, testing oxidase positive, is considered a positive diagnosis. Ideally, specimens should be inoculated onto culture media and incubated immediately after acquisition in order to maintain maximum viability of the organisms. Realistically this is only possible if there is a dedicated microbial diagnostics laboratory at the site of testing. If this is not the case, the sample is added to a transport media designed to maintain viability prior to culture, or the plates are inoculated and held in a 5% CO_2 enriched atmosphere in a candle jar or incubator during transit. The more sophisticated of these transport media's include mechanisms to provide a CO_2 enriched atmosphere, such as the InTray GC system (BioMed Diagnostics, USA), which relies on a CO_2 tablet to provide conditions conducive to growth of the gonococcus. Various studies have demonstrated the sensitivity of culture for *N. gonorrhoeae* to be 69.8%, 66.7%, 47.4% and 72.2% for endocervical swabs (Van Dyck *et al.*, 2001), rectal swabs (Bachmann *et al.*, 2010), pharyngeal swabs (Shafer *et al.*, 2002) and male urethral swabs (Buimer *et al.*, 1996), respectively.

Nucleic acid amplification testing (NAAT) for gonorrhoea is becoming more widespread, and in the developed world is commonly used, often in conjunction with culture, to provide a diagnosis in suspected cases of gonorrhoea. Commonly used NAATs include those based on the polymerase chain reaction (PCR), ligase chain reaction (LCR) and novel amplification assays such as APTIMA GC (Gen-Probe) assays, which utilise transcription mediated amplification technology, and the BD ProbeTec (PT) energy transfer amplified DNA assay (Chernesky *et al.*, 2005). NAATs offer unparalleled specificities and sensitivities, with PCR assays typically boasting sensitivities and specificities of >95% and 100% respectively, compared with sensitivities of ~68% obtained via culture (Luijt *et al.*, 2005). NAAT provides a more rapid diagnosis than culture, due to the removal of the need for lengthy incubation periods.

The first NAAT s to be described for *N. gonorrhoeae*, as with most organisms, were home-brew PCR assays, published in the literature. PCR assays have been described for amplification of a number of conserved gene targets, including *cppB* (Ho *et al.*, 1992) and the *glnA* gene (Chaudhry and Saluja, 2002). An abundance of commercial PCR, and real-time PCR assays have been released, to accord for the high levels of testing occurring for *N. gonorrhoeae* infection, owing to the high prevalence of infection, and overlap of symptoms with other prevalent infections such as those caused by *C. trachomatis* and *T. vaginalis*.

The Cobas CT/NG test, is a commercially available and FDA approved NAAT, processed on the c4800 platform (Roche Cobas, US), providing a fully automated testing system for the diagnosis *of C. trachomatis* and *N. gonorrhoeae* infection. An automated magnetic silica based nucleic acid extraction system purifies nucleic acid from the sample, which then undergoes real-time PCR amplification using two primer sets, specific to the highly conserved DR-9 repeat region (Van Der Pol *et al.*, 2012). The sensitivity of the assay has been determined to be between 95.6% and 100%, with a specificity of >99.8% (Van Der Pol *et al.*, 2012).

Another available FDA approved NAAT for the combined detection of *N. gonorrhoeae* and *C. trachomatis* is the CT/NG GeneXpert assay (Cepheid, US). This assay uses disposable single use assay cartridges, which enable automated sample processing and rapid real-time PCR based detection, which removes the need for liquid handling by the operator. The test takes 90 minutes to complete, from the application of the patient sample to the retrieval of the results, enabling same day testing. A comparison with the APTIMA Combo 2 assay (Hologic GenProbe, US) and the BD ProbeTec BDPT assay (Becton Dickinson, US) demonstrated that the GeneXpert assay had sensitivities of 100%, 100% and 95.6%, and 98% when processing endocervical, vaginal, female urine, and male urine samples, respectively (Gaydos *et al.*, 2013). Specificities were over 99.8% with all sample types.

Simple point of care (POC) tests have been developed for the detection of *N. gonorrhoeae*, but have not found widespread use, due to low analytical sensitivities. Leukocyte esterase (LE) dipstick tests, which detect the presence of leucocytes present due to inflammation caused by infection of the urthera, have been used to screen for gonoccocal infections. These tests are simple to carry out, and typically take no longer than two minutes to provide a result, enabling their use at the POC (Watchirs Smith *et al.*, 2013). A systematic review of evaluations of a number of LE tests for *N. gonorrhoeae* detection found an overall sensitivity of 71%, and a specificity of 70% (Watchirs Smith *et al.*, 2013). These tests only identify the presence of inflammation caused by infection, and are therefore non-specific for gonorrhoeae, but can indicate the need for antibiotic therapy, or for more specific testing. The application of these tests is limited by this un-specific nature, and their low sensitivities,

but they remain a viable option for screening of populations unlikely to return for follow up (Olshen and Shrier, 2005). Rapid immunochromatographic assays, for the detection of *N. gonorrhoeae* specific antigen, are also available, and well suited to POC testing. An evaluation of the immunochromatographic NOW Gonorrhoea test using a sample of 58 male patients (34 positive) found that the assay sensitivity and specificity to be 94.1% and 95.8%, respectively (Suzuki *et al.*, 2004). A comparative study of three POC immunographic assays found a median sensitivity and specifity of 70% and 96% (Watchirs Smith *et al.*, 2013). The authors did note that the sensitivity could have been overestimated, due to the predominance of samples from symptomatic patients used in the study, who will typically have a higher organism load, and therefore antigen concentration in the sample. These immunochromatographic POC tests are relatively expensive, and the lower analytical sensitivity of around 1 x 10^4 *N. gonorrhoeae* cells per ml, a 100 fold decrease in sensitivity compared with modern NAAT technologies such as the AC2 assay (Harkins and Munson, 2011), means that they are not regularly used clinically.

Rapid isothermal amplification technologies are beginning to be applied to the detection of this pathogen. A multiplex helicase-dependent amplification end-point assay has been described for the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* (Doseeva *et al.*, 2011). Also, an on-chip recombinase polymerase amplification assay capable of detecting 100 *N. gonorrhoeae* CFU has been developed (Kersting *et al.*, 2014). To the best of our knowledge, there has not yet been a loop mediated isothermal amplification assay designed for the detection of *N. gonorrhoeae*. A N. gonorrhoeae specific LAMP assay would enable rapid molecular testing, without the need for expensive real-time PCR technology, and with a greater tolerance to amplification inhibition, which may allow for the direct detection from urine samples.

2.1.5. Treatment and Resistance

Treatment for gonorrhoea infection consists of appropriate antimicrobial therapy, with azithromycin (1g orally) in conjunction with ceftriaxone (500mg intramuscularly) currently administered in the UK and US (GRASP report, 2011; CDC MMWR, 2010). The primary anti-gonoccocal agent in this dual therapy is the cephalosporin ceftriaxone, which has excellent efficacy against *N. gonorrhoeae*. The supplementation of ceftriaxone with azithromycin was instigated as a response to growing resistance rates in *N. gonorrhoeae* to third generation cephalosporins, and the high incidence of co-infcetion with *C. trachomatis* (CDC, 2010). Doxycycline has also been administered for this purpose,

in place of azithromycin, but is regarded as less preferable, as isolates possessing resistance to third generation cephalosporins frequently also display decreased tetracycline susceptibility (MMWR Morb Mortal Wkly Rep, 2010).

Antimicrobial resistance in *N. gonorrhoeae* isolates is extremely widespread, with resistance to most classes of antibiotic, including penicillins, macrolides, tetracyclins, quinolones (Whiley *et al.*, 2012) and most recently cephalosporins (Ross and Lewis, 2012), documented in clinical isolates. Wide spread resistance to previously extensively used therapies, such as penicillins, tetracyclins,and macrolides, has rendered their use ineffective, leaving expensive third generation cephalosporins as the only remaining drugs with high efficacy available. The recent emergence of isolates with decreased susceptibility to extended spectrum cephalosprins (ESC), such as cefixime and ceftriaxone (Ohnishi *et al.*, 2011), which are considered to be the "last line" treatment options, indicate the potential future difficulties facing the treatment of *N. gonorrhoeae* infection, and raise the possibility of untreatable gonoccocal infection (Bolan *et al.*, 2012). The loss of effective treatment for this disease would significantly raise the morbidity and mortality associated with gonoccocal infection, and has lead the World Health Organisation to produce a global action plan to control the spread and impact of antimicrobial resistance in Neisseria gonorrhoeae (WHO, 2012). Treatment failures with 500mg ceftriaxone in infections caused by the multi drug resistant NG-MAST genotype 1407 clone have been described in Sweden (Golparian *et al.*, 2014a). This strain has also been recorded in Japan as causing a treatment failure with 2g azithromcyin (Morita-Ishihara et al., 2014), and has been responsible for multiple cefixime treatment failures since 2010; in the UK (Ison *et al.*, 2011), Austria (Unemo *et al.*, 2011) Norway (Unemo *et al.*, 2010), and the US (Hess *et al.*, 2012) which have lead to cefixime being removed from recommended treatment lists by leading public health bodies worldwide (CDC, 2010; WHO, 2012; BASHH, 2012).

Since the beginning of the antibiotic era, *N. gonorrhoeae* has demonstrated the ability to rapidly generate and maintain antibiotic resistance, via the acquisition of chromosomal and plasmid-mediated resistance genes. *N. gonorrhoeae* is known to be highly transformable, and has the unique ability to take up double stranded DNA sequence harbouring specific 10bp sequence elements, termed DNA uptake sequences (DUS), present in the *N. gonorrhoeae* genome and those of related species, in a highly efficient manner (Elkins *et al.*, 1991). This process has been shown to rely on the expression of genes required for the production of type IV pili, in combination with three associated proteins; ComP, PilT, and ComE (Aas *et al.*, 2002). *N. gonorrhoeae* is also capable of taking up double stranded DNA lacking this particular recognition sequence, bus does so with much less efficiency. Absorbed DNA is then incorporated into the homologous area of the receiving cells genome in a

process of recombination, providing the gonococcus with a mechanism for genetic diversification, and potential route for the acquisition of resistance genes from closely related species or other *N. gonorrhoeae* cells (Aas *et al.*, 2002). This mechanism is thought to be responsible for the transfer of antimicrobial resistance genes from commensal *Neisseria* species, which are likely to be exposed to antimicrobials more frequently than the transient pathogenic gonococcus (Unemo and Shafer, 2011). *N. gonorrhoeae* also displays a high frequency of internal recombination and mutation (O'Rourke and Stevens, 1993), increasing the likelihood of mutations leading to antimicrobial resistance arising as a response to exposure to antimicrobial drugs. A feature of *N. gonorrhoeae* antimicrobial resistance is the ability of *N. gonorrhoeae* to maintain its resistant phenotype, even after extended periods without the selection pressure of the drugs in question. For example, a high proportion of *N. gonorrhoeae* isolates in areas where the application of tetracyclins, quinolones and penicillins for the treatment of gonorrhoea has long been discontinued still exhibit high level plasmid-mediated resistance to these drugs (WHO, 2012).

A primary route to ESC resistance is mutation of the *penA* gene, which encodes the protein target of ESCs, the PBP2 transpeptidase(Unemo and Nicholas, 2012). Mosaic alleles of *penA*, and non-mosaic alleles containing a base substitution at A501, have been shown to lead to a reduced ESC susceptibility phenotype (Golparian *et al.*, 2010; Ohnishi *et al.*, 2010; Huang *et al.*, 2010). Mosaic PBP2 mediated resistance tends to lead to decreased susceptibility to cefixime in comparison with ceftriaxone (Zhao *et al.*, 2009; Unemo and Nicholas, 2012), and this may be one of the reasons that decreased susceptibility to cefixime ha been detected more readily than to ceftriaxone in clinical isolates. Resistance arising from single base substitutions at A501 tend to equate to increased ceftriaxone resistance, and is less commonly encountered. This particular mutation has not been discovered in any species other than *N. gonorrhoeae*, and is therefore thought to have arisen via mutation in these species rather than via the acquisition of genetic elements from alternate species of *Neisseria* (Unemo and Nicholas, 2012).

One mechanism of ESC resistance that has been demonstrated in ESC resistant clinical isolates is the overexpression of the MtrC-MtrD-MtrE efflux pump, which increases the rate of the removal of ESC drugs from the intracellular environment (Zhao *et al.*, 2009; Folster *et al.*, 2009). Overexpression of the two other pump systems known to remove antimicrobial compounds in this organism, MacA-MacB and NorM, is also thought to contribute to ESC resistance, but to a lesser degree (Golparian *et al.*, 2014b). The demonstration that the inactivation of these efflux pumps absolves the resistance phenotype from ESC resistant isolates, reducing MICs to clinically susceptible breakpoints, has lead

to the suggestion that efflux pump inhibitors could have a part to play in the future treatment of resistant *N. gonorrhoeae* (Golparian *et al.*, 2014b).

One difficulty facing the monitoring of global resistance profiles in *N. gonorrhoeae* is the increased reliance on molecular detection methods for gonorrhoea diagnosis. NAATs are typically carried out from nucleic acid extracted directly from samples, precluding the need for prior culturing (WHO, 2012). Compounding this, transport buffers for samples destined for NAAT are designed to protect nucleic acid integrity rather than organism viability, making the retrieval of viable organisms difficult. The development of methods for detecting antimicrobial resistance in *N. gonorrhoeae* directly from nucleic acid faces difficulties due to the genetic complexity behind the resistant phenotypes, with multiple variations in large numbers of genes all potentially involved in any isolate(Golparian *et al.*, 2014b). Therefore, culture of the organism from clinical isolates and subsequent determination of MIC's of relevant antimicrobial compounds is an essential process for the monitoring of global AMR in *N. gonorrhoeae*.

Aims

- To maintain the culture of *N. gonorrhoeae*, in order to provide a source of *N. gonorrhoeae* DNA to ourselves and also Public Health England
- To provide confirmation of the suitability of the magnetic silica based extraction system for the purification of nucleic acids from *N. gonorrhoeae*.
- To determine the limits of detection of the *GroEL* LAMP assay designed by PHE.
- To design both a LAMP assay and PCR assay for a suitable *N. gonorrhoeae* target sequence, and compare the sensitivity of both methods.
- To compare the performance of the designed LAMP assay with PHE designed LAMP assays for alternative targets.
- To determine the tolerance of the *N. gonorrhoeae* LAMP assay to the presence of amplification inhibitors in urine, such as urea and blood.
- To explore the use of the LAMP assay for the direct detection of *N. gonorrhoeae* from urine samples, using a visual detection method.

2.2. Materials and Methods

2.2.1. Culture of *N. gonorrhoeae*

N. gonorrhoeae (ATCC 19424) was cultured on chocolate agar (blood agar base, Sigma Aldrich, UK; defibrinated horse blood, TCS, UK) at 37°C at 5% CO_2. Broth cultures were maintained under the same conditions, in brain heart infusion broth (Oxoid, UK).

2.2.2. Culture of *Escherichia coli*

E. coli K-12 (lab strain) was cultured on nutrient agar (Oxoid, UK) incubated at 37°C. Broth cultures were maintained under the same conditions in nutrient broth (Oxoid, UK).

2.2.3. Use of human urine

For each experiment were human urine was used, the urine was a mix of urine taken from one individual over a 12 hour period. Urine was stored at 6°C and used no later than 24 hours after excretion.

2.2.4. PCR primer design

PCR primers were designed for ORF1 of the glutamine synthetase (*glnA*) gene of *N. gonorrhoeae*. The target sequence was first downloaded from the NCBI GenBank database (Accession number M84113), and primers were designed using Primer3 (http://frodo.wi.mit.edu/). ORF1 PCR primer sequences are shown in Table 1. Primer specificities were checked using BLAST (http://blast.ncbi.nlm.nih.gov/).

Primer	Sequence	Length (bp)	Product size (bp)	Annealing Temp (^{o}C)
N. gonorrhoeae ORF1 FWD	GACGTATTTCATATCTTGGG	20	139	47
N. gonorrhoeae ORF1 RVS	GGTGAACATTTTGGAAG	17		

Table 2.2. ORF1 PCR primer sequences

2.2.5. LAMP primer design

In this study, LAMP primers were designed for ORF1 of the *glnA* gene of *N. gonorrhoeae*. The target sequence was first downloaded from the NCBI GenBank database (Accession number M84113). LAMP primers were designed using PrimerExplorer Version 4 (http://primerexplorer.jp/e/). The additional optional Loop primers were not included. ORF1 LAMP primer sequences are shown in Table 2.3. Primer specificities were checked using BLAST (http://blast.ncbi.nlm.nih.gov/). Both PCR and LAMP primers were synthesised commercially (Eurofins, Germany).

LAMP Primer	Sequence	Length (bp)
N. gonorrhoeae ORF1 S1 F3	GGGAAACGCGCTTCGATG	18
N. gonorrhoeae ORF1 S1 B3	ACCGCGCTCTACTACATCG	19
N. gonorrhoeae ORF1 S1 FiP	GGCCTATTCCGCCAAAAACCGTGCCTTGCTGCTGTTCACA	40
N. gonorrhoeae ORF1 S1 BiP	ACGAGGCGTTTGTAGGAGTTGGCAAACACGCCAAAGCCCT	40
N. gonorrhoeae ORF1 S2 F3	TGGTCGGTGCTTCAAAGTG	19
N. gonorrhoeae ORF1 S2 B3	GCACGTCCACCAATCCATT	19
N. gonorrhoeae ORF1 S2 FiP	CAAACACGCCAAAGCCCTGAACGCACGAGGCGTTTGTAGG	40
N. gonorrhoeae ORF1 S2 Bip	TGTAGTAGAGCGCGGTATCGGACGGTCAAAACCTGTTCGCA	41

Table 2.3. Sequences of the *N. gonorrhoeae* ORF1 S1 and ORF1 S2 LAMP primer sets.

2.2.6. Public Health England (PHE) *N. gonorrhoeae* LAMP primer sets

Sequences were provided by PHE for *N. gonorrhoeae* LAMP primer sets, specific to the *GroEL* and *PorA* genes. The primer sequences are shown in table 2.4 and 2.5.

LAMP Primer	Sequence	Length (bp)
N. gonorrhoeae GroEL F3	ACTTTATCAACGACGCGGAA	18
N. gonorrhoeae GroEL B3	ACGGTTTTCAGGATGCCG	19
N. gonorrhoeae GroEL FiP	AGGTCGCGGATGTTGCTGATTTAAATCGCCGGTCTGGACA	40
N. gonorrhoeae GroEL BiP	TGGAACAAGTGGCGAAAGCCAGACCAAAGTCGCCAAGGC	40
N. gonorrhoeae GroEL Loop F3	GTCGAACAGCAAAACAAACGG	21
N. gonorrhoeae GroEL Loop B2	CCCGCTGTTGATTATCGCTGAAG	23

Table 2.4. Sequences of the *N. gonorrhoeae GroEL* LAMP primer sets, designed by PHE.

LAMP Primer	Sequence	Length (bp)
N. gonorrhoeae PorA F3	ACCAAAAACAGTACGACCGA	19
N. gonorrhoeae PorA B3	AAGTGCGCTTGGAAAAATCG	19
N. gonorrhoeae PorA FiP	ATGGGCATAGCTGATGCGCGAATTGCCGCCACTGCTTC	40
N. gonorrhoeae PorA Bip	TCGACTTTGTCGAACGCAGTCAAATCGACACCGGCGATGA	41
N. gonorrhoeae PorA Loop F3	GCGAACATACCAGCTATGATCAA	23

Table 2.5. Sequences of the *N. gonorrhoeae PorA* LAMP primer sets, designed by PHE.

2.2.7. DNA extraction

DNA extractions were carried out using a QIAmp DNA mini kit (QIAGEN Inc., USA), following the protocol for bacterial cells. RNase A (New England Biolabs, UK) was used during the RNA digestion step. DNA was eluted in a single 60µl volume of molecular grade water. DNA quantification was carried out using a Spectrostar plate reader in conjunction with an LVIS plate (BMG Labtech, UK). The corresponding genome copy number was calculated from the weight of the *N. gonorrhoeae* genome, which is 2.45fg (2.2×10^6 bp × 665 Da/bp × 1.67×10^{-24} g/Da) (Geraats-Peters *et al.*, 2005a). Where indicated, total nucleic acid extractions were also carried out using the KingFisher ML automated extraction system (See chapter 1.2.7.).

2.2.8. ORF1 PCR Reactions

PCR reactions were set up as follows; 25µl RedTaq reaction mix (Sigma Aldrich, UK), 0.5pmol forward primer, 0.5pmol reverse primer, 5µl DNA sample. Reactions were then made up to 50µl with water. Reaction conditions were as follows; 94°C for 5 minutes initial denaturation; 35 cycles of 94°C for 30 seconds, 47°C for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes final extension. Reactions were controlled using an ABI 2720 thermocycler (Applied Biosystems, UK).

2.2.9. LAMP Reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), as follows; 5µl five times LAMP reaction buffer, 12µl molecular grade water, 1µl intercalating dye (propriety dye, emission in FAM channel), 1µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 1µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers. Loop primers, if present, were included at a 25pmol/µl concentration in the primer mix. Finally 5µl of DNA sample or water was added to the reaction. Reactions were carried out in a ESEQuant Tube Scanner (Qiagen Inc., CA), at 63°C for 60 minutes. Reactions were terminated by heating to 80°C for 1 minute in order to denature the *Bst* polymerase.

2.2.10. Reaction Product Detection

Detection of reaction products from the PCR and LAMP reactions was carried out primarily by electrophoresis, using 1.5% w/v agarose gels with transillumination and photography provided by a BioRad Gel/Chem Doc system (Bio-Rad Inc, UK).

LAMP reactions were also monitored in real-time using the ESEQuant Tube Scanner (Qiagen Inc, UK), through the FAM channel. The "Tube Scanner Studio" software package was used to analyse the real time data and produce a manually set threshold line for the purposes of determining time to amplification.

End point visual detection was also carried out on the reactions assaying raw urine samples. In these reactions the 1µl intercalating dye was replaced with 1µl of calcein (fluorescent detection reagent; Eiken Chemical Co., Japan). A colour change from orange to green was seen in the positive reactions, easily visible in natural light. The negative reactions remained orange. Visual detection of fluorescence in the positive reactions was also possible, by placing reactions on a 365nm UV light source.

2.2.11. Limits of detection of the *N. gonorrhoeae GroEL* LAMP assay.

A 1ml aliquot of overnight culture of *N. gonorrhoeae* was taken, and cells were concentrated by centrifugation at 5000 x g for 1 minute. The cells were then resuspended in urine, and a series of 1:10 dilutions were carried out in urine. Total viable counts were then carried out, with 50µl of each dilution being spread over chocolate agar plates, in triplicate. The colonies on each plate were counted after 48 hours of incubation at 37^{o}C and 5% CO_2, to quantify the cells in the suspension. Nucleic acid was then extracted from a 200µl aliquot of each suspension using the KingFisher ML automated extraction system. LAMP reactions using the *GroEL* primer set were then carried out, and monitored in real time using the Qiagen ESEquant Tubescanner. Nuceic acid extracted from a 200µl aliquot of *Escherichia coli* culture was used as a negative control.

2.2.12. Initial testing of the ORF1 primer sets

LAMP reactions containing 0.1µg of *N. gonorrhoeae* DNA were carried out using the ORF1 S1, ORF1 S2, *GroEL* and *PorA* primer sets. The DNA was extracted using a Qiagen genomic DNA kit (Qiagen), and quantified using a Spectrostar plate reader (BMG), with LVIS attachment. Negative control reactions were carried out simultaneously for each primer set, with the DNA replaced by molecular grade water. The reactions were carried out in duplicate, and monitored in real time. The products of the positive reactions from both duplicates, and a single negative control, were analysed by agarose gel electrophoresis (1.5%).

2.2.13. Relative sensitivity of the *GroEL* and ORF1 S2 primer sets

Nucleic acid extractions were carried out on *N. gonorrhoeae* cell suspensions in urine containing 5000, 500, 50 and 5 cells per 200µl aliquot. The extracted DNA was then tested using the GroEL and ORF1 S2 LAMP assays, and the reactions were monitored in real-time using the Qiagen ESEquant Tubescanner.

2.2.14. Specificities of ORF1 PCR and LAMP assays

DNA was extracted from the following organisms; *N. gonorrhoeae* (ATCC 19424), *C. trachomatis* (lab strain, serovar L2), *Trichomonas vaginalis* (ATCC 30001) and *Pseudomonas aeruginosa* (ATCC 15692). The DNA was diluted to 20ng/µl and 5µl of each DNA sample (0.1µg) was used as a template in both ORF1 PCR and ORF1 LAMP reaction.

2.2.15. Sensitivities of ORF1 PCR and LAMP assays

DNA was extracted from a 200µl sample of an overnight culture of *N. gonorrhoeae* and quantified after RNA digestion. Copy number was determined, and the DNA sample was diluted to a concentration of 4×10^4 copies/µl using molecular grade water. 1:10 serial dilutions of the DNA

stock were carried out, down to a concentration of 0.4 copies/µl. ORF1 PCR and ORF1 LAMP reactions were carried out using 5µl of each dilution of DNA.

2.2.16. Urea tolerance of ORF1 PCR and LAMP assays

ORF1 PCR and ORF1 LAMP reactions, including 0.1µg of *N .gonorrhoeae* DNA, were spiked to include the following concentrations of urea; 1mM, 10mM, 100mM, 1M, 2M. Additional duplicate ORF1 LAMP reactions were spiked to include the following urea concentrations; 1M, 1.2M, 1.4M, 1.6M, 1.8M, 2M.

2.2.17. Direct detection from urine samples

ORF1 LAMP reactions were carried out on 5µl samples of both molecular grade water and human urine spiked with $2x10^3$ copies, $2x10^2$ copies, 20 copies, and 0 copies of the *N. gonorrhoeae* genome. The reactions contained 1µl of calcein to enable visual detection. Real-time detection of the intercalating dye was not possible due to the interference exerted by urine on the signal.

2.2.18. Tolerance of the ORF1 LAMP assay to the presence of blood

ORF1 LAMP reactions containing 0.1µg of *N. gonorrhoeae* DNA were spiked to include the following concentrations of defibrinated whole horse blood (TCS); 5%, 10%, 20%, 30%, 40%, 50%. The volume of blood used replaced the same volume of molecular grade water in the reaction. A reaction containing *N. gonorrhoeae* DNA, without blood was used as the positive control, whilst negative controls contained molecular grade water, or 10% blood, without *N. gonorrhoeae* DNA.

2.3. Results

2.3.1 Limits of detection of the *N. gonorrhoeae GroEL* LAMP assay

The limit of detection of the *GroEL* LAMP assay for the detection of *N. gonorrhoeae* was determined by carrying out *GroEL* LAMP assays on total nucleic acid extracted from varying concentrations of *N. gonorrhoeae* cells suspended in urine, using the silica based extraction method

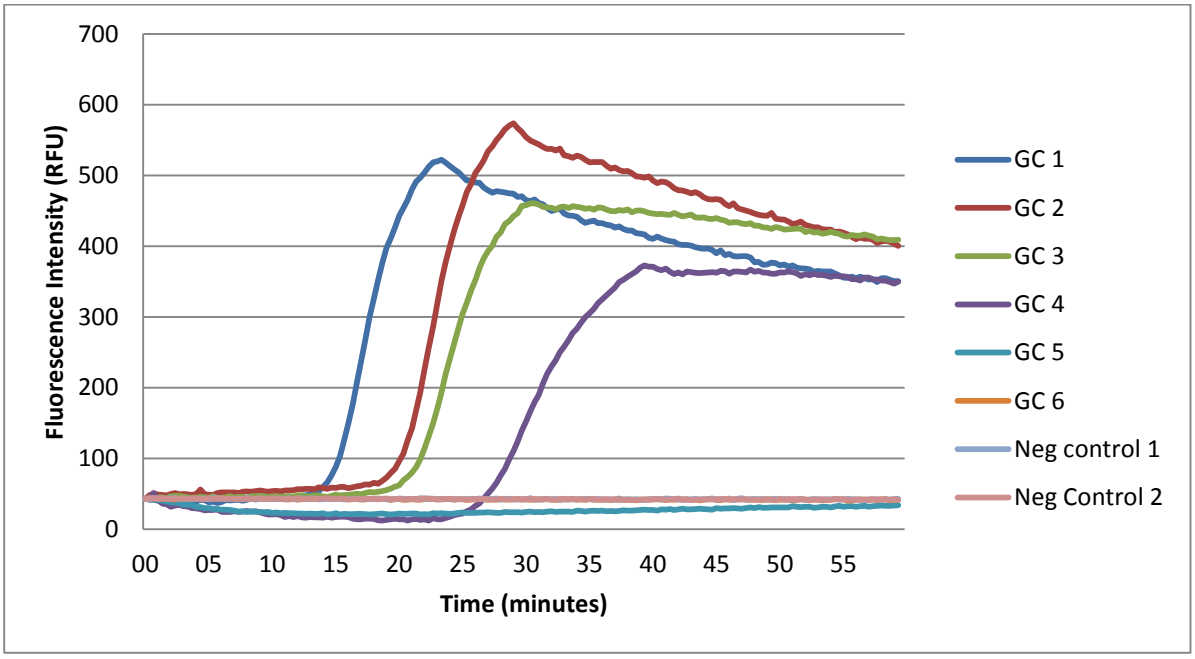


Fig.2.1. LAMP amplification plot showing the limit of detection of the *GroEL* LAMP assay. Nucleic acid extractions were carried out on 1/10 serial dilutions of a quantified *N. gonorrhoeae* suspension. The cell count of each sample is shown in Table 2.6. Extractions were then tested for the presence of *N. gonorrhoeae* nucleic acid using the *GroEL* D2 assay. Molecular grade water (Neg control 1) and 0.1µg of *E. coli* DNA (Neg control 2) was used as a negative control.

The *GroEL* LAMP assay, in conjunction with the silica based extraction method, was able to detect the presence of *N. gonorrhoeae* nucleic acid from a 200µl aliquot containing a minimum of 100 *N. gonorrhoea* cells, equating to 6.7 cells worth of nucleic acid per reaction (Table 2.6.). The assay was able to generate a positive result from all cell suspensions containing a greater number of cells.

There was no detectable signal generated in the reactions containing 0.67 or 0.067 *N. gonorrhoeae* cells, or in the negative controls containing non-target DNA or molecular grade water (Fig.2.1)

The time to amplification was dependent on the concentration of *N. gonorrhoeae* cells in the sample, with the reactions containing DNA equivalent to that from 6,700, 670, 67 and 6.7 cells generating an amplification after 15.3, 20.3, 22 and 28.6 minutes respectively (Fig.2.1).

Sample	GC cell count (per ml)	Volume used in extraction	Cells used in extraction	Elution Volume	Volume amplified	Equivalent number of cells	Results
1	500,000	200µl	100,000	75µl	5µl	6700	POS
2	50,000	200µl	10,000	75µl	5µl	670	POS
3	5,000	200µl	1,000	75µl	5µl	67	POS
4	500	200µl	100	75µl	5µl	6.7	POS
5	50	200µl	10	75µl	5µl	0.67	NEG
6	5	200µl	0.2	75µl	5µl	0	NEG
Negative	0	200µl	0	75µl	5µl	0	NEG
Negative	0	200µl	0	75µl	5µl	0	NEG

Table 2.6. The concentration of *N. gonorrhoeae* cells in samples used for the determination of the detection limits of the *GroEL* LAMP assay, and equivalent number of cells entering the LAMP reactions.

2.3.2. Initial testing of the ORF1 primer sets

The two primer sets specific to ORF1 of the *glnA* gene, ORF1 S1 and ORF1 S2, required initial testing in order to determine the best primer pair, and their performance with previously designed and optimised assays to the *GroEL* and *PorA* genes was also compared. Real-time monitoring of the reactions gave some evidence as to the superior primer set from a reaction speed perspective, which can also inform as to the likely comparative sensitivity. The comparison of the ORF1 sets, and their *glnA* target, with the *GroEL* and *PorA* LAMP assays compared these primer sets in terms of reaction speed with the primer sets designed by Public Health England Southwest, to determine whether it would be beneficial to fully test them clinically, potentially with a view to adopting this set for the LabDisk.

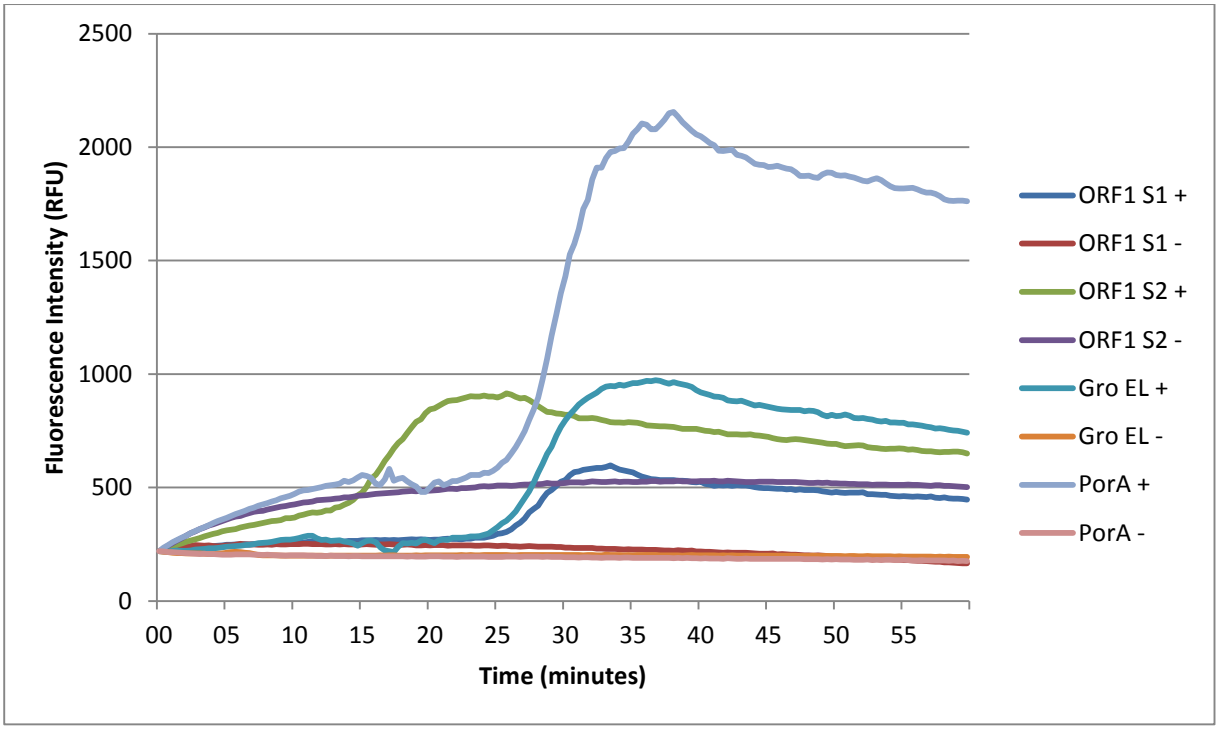


Fig.2.2. Amplification curves produced by real-time monitoring of the ORF1 S1, ORF1 S2, *GroEL* and *PorA* LAMP reactions. 0.1µg of *N. gonorrhoeae* DNA was used as the positive control reactions. Molecular grade water was used in place of the DNA in the negative reactions.

The shortest time to amplification occurred using the ORF1 S2 primer set, which generated a positive amplification after 15.3 minutes (Fig.2.2). This was ~10 minutes sooner than the ORF1 S2, *GroEL* and Por A primer sets, which produced amplification after 26.6, 26 and 26 minutes, respectively. The ORF1 S2 and *PorA* positive reactions both generated a gradual increase in fluorescence starting from the initiation of the reaction, through to the point of amplification. When the negative control reactions using these primer sets were analysed using gel electrophoresis, there was no visible product, barring the presence of a low weight (<100bp) band representing the primers (Fig.2.4). It could be that an element of primer dimerisation was occurring with these primer sets, resulting in a gradual increase in fluorescence but falling short of (and not preventing) a true exponential amplification. This gradual increase in signal was also encountered in the ORF1 S2 negative, but not the *PorA* negative reaction.

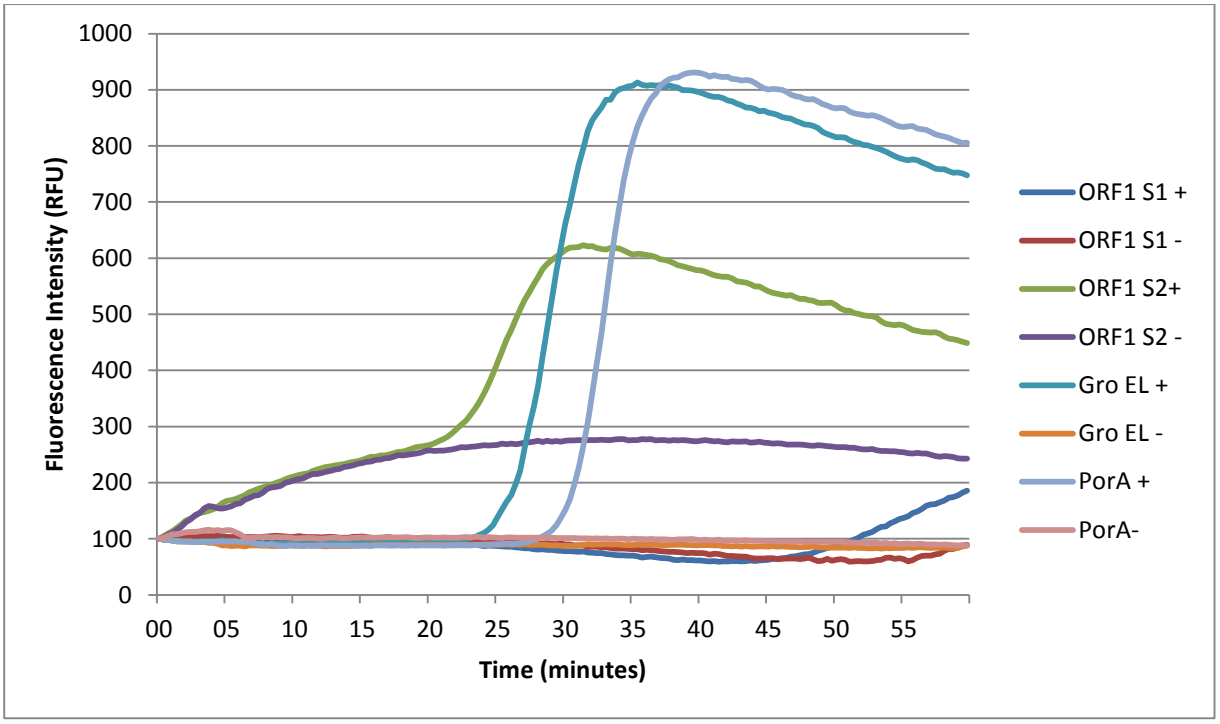


Fig.2.3. Amplification curves produced by real-time monitoring of the ORF1 S1, ORF1 S2, *GroEL* and *PorA* LAMP reactions; a repeat of the experiment detailed in Fig.2.2. 0.1µg of *N. gonorrhoeae* DNA was used as the positive control reactions. Molecular grade water was used in place of the DNA in the negative reactions.

A repeat of the experiment again found the ORF1 S2 primer set to result in the fastest reaction, generating an amplified signal at 22.3 minutes, compared with 25.3 minutes in the *GroEL* assay, and 30 minutes in the *PorA* assay (Fig.2.3). The gradual increase in fluorescence was again encountered in the ORF1 S2 positive and negative reactions, but not the *PorA* positive, as in the previous experiment (Fig.2.2). The ORF1 S1 positive reaction failed to generate a detectable amplification curve, although an increase in signal did occur between 52 and 60 minutes.

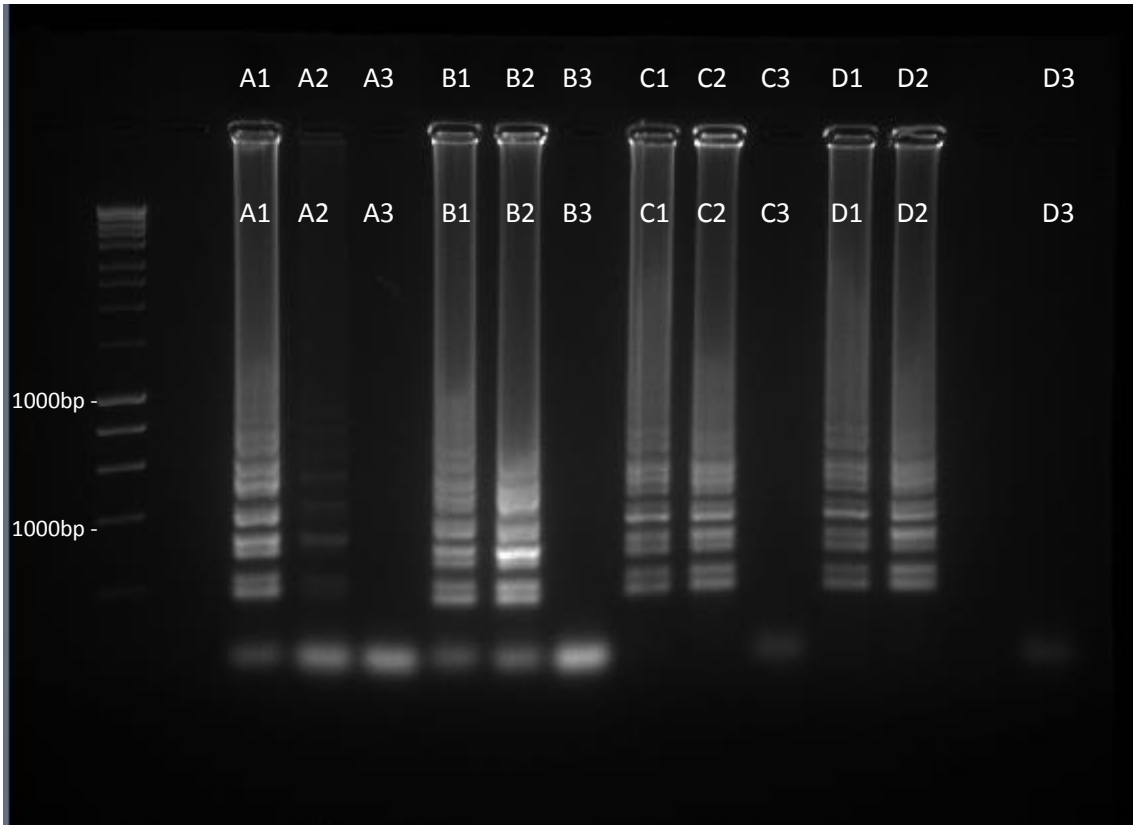


Fig.2.4. Comparison of the reaction products of LAMP reactions using ORF1 primer sets 1 (A) and 2 (B), with the *GroEL* (C) and *PorA* (D) primer sets. The positive control reactions were carried out in duplicate (1, 2), and a reaction containing molecular grade water was used as the negative control (3).

Agarose gel electrophoresis of the reaction products from Fig.2.2, Fig. 2.3. (Fig.2.4) showed the characteristic profile of LAMP reaction product, in each of the positive reaction duplicates for each primer set. The positive reaction from the second ORF1 S1 duplicate was markedly fainter on the gel, which was to be expected after the late amplification that was recorded in that reaction (Fig.2.3). There was no evidence of any amplified product in any of the negative control lanes.

2.3.3. Relative sensitivity of the *GroEL* and ORF1 S2 primer sets

The ORF1 S2 primer set was found to enable a faster reaction speed than the ORF1 S1 primer set in both of the initial tests of performance (Fig.2.2, Fig.2.3). For this reason the ORF1 S2 set was chosen for all further investigation, and will be referred to as the ORF1 primer set from here on.

As the ORF1 primer set enabled a more rapid reaction than the *GroEL* and *PorA* assays, when tested with a quantity of 0.1µg of *N. gonorrhoeae* genomic DNA, it is possible that this increase in speed would also be seen at lower concentrations of DNA. A faster LAMP reaction can be indicative of an increase in starting copy number, which would result in an improved sensitivity, or it can be down to the compatibility and sequence identity of the primer sets themselves, leading to a faster reaction but not an improvement in sensitivity.

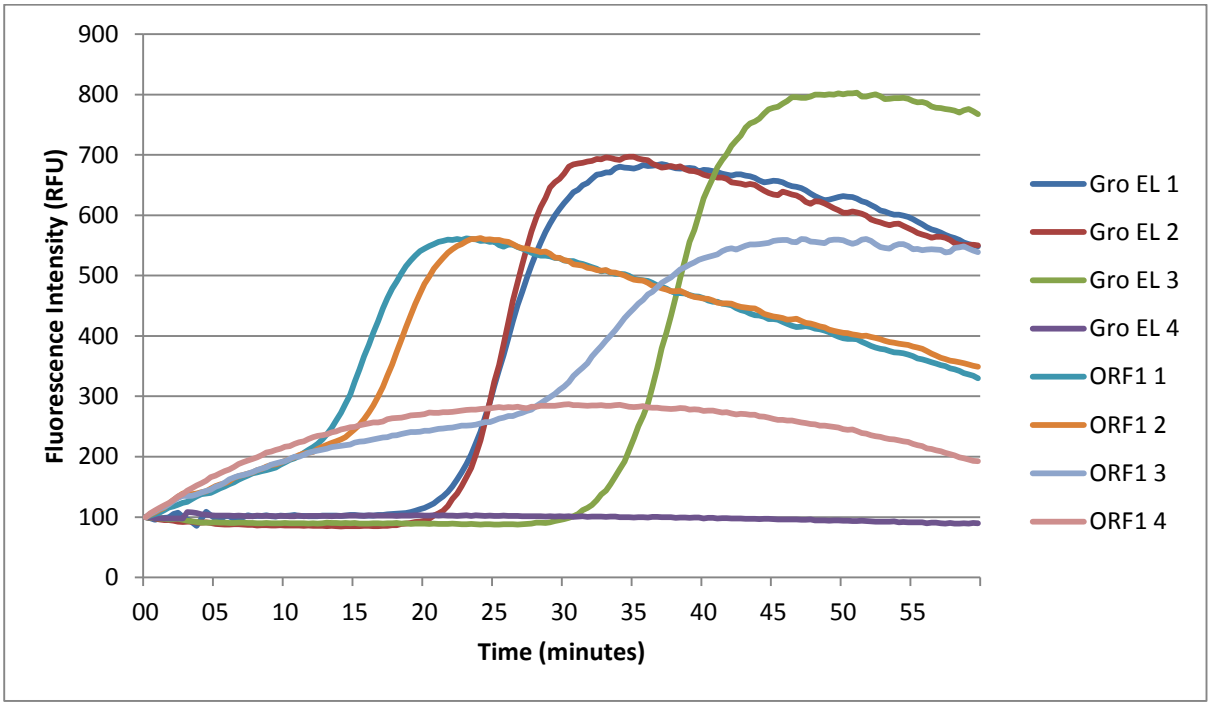
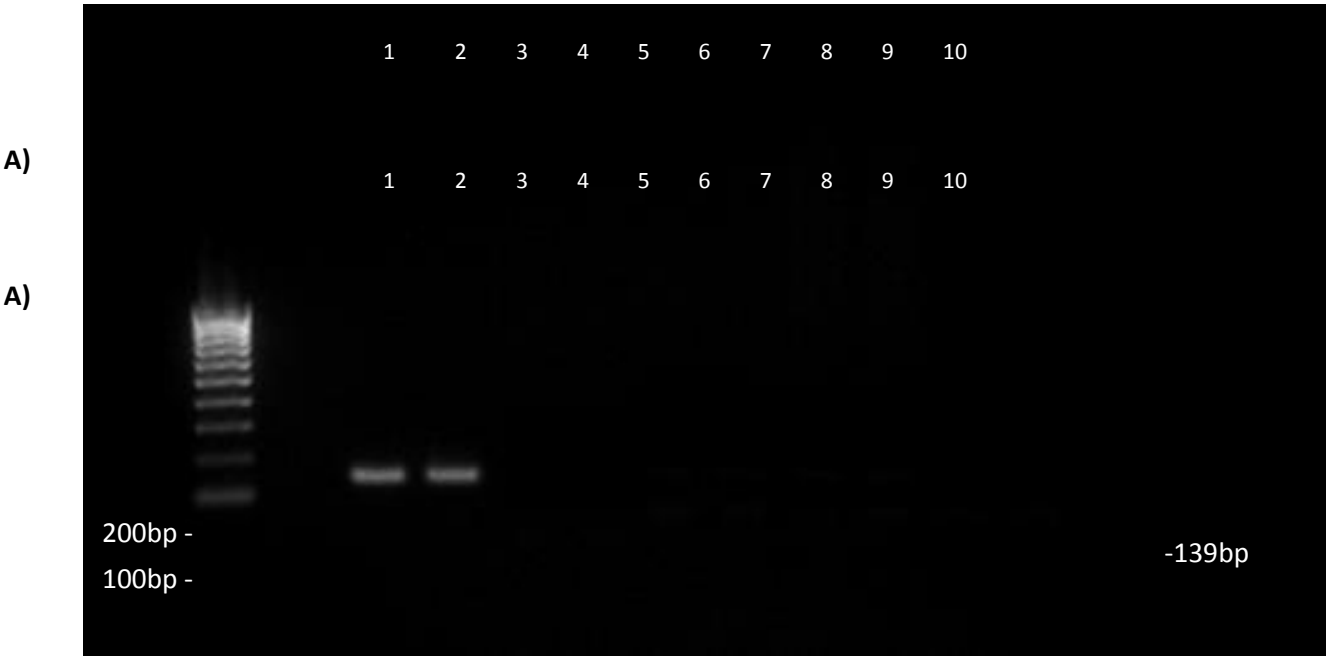


Fig.2.5. Comparative sensitivity and reaction speed of the *GroEL* and ORF1 LAMP assays. *GroEL* and ORF1 LAMP reactions containing nucleic acid extracted from 200µl aliquots containing 5000 (1), 500 (2), 50 (3) and 5 (4) *N. gonorrhoeae* cells were monitored in real-time.

The ORF1 LAMP assay generated faster amplification than the *GroEL* assay for each dilution tested, with the reactions containing 5000, 500 and 50 *N. gonorrhoeae* cells amplifying at 14.6, 17, 29.3 minutes, respectively (Fig.2.5). The corresponding reactions utilising the *GroEL* primer sets generated detectable amplification at 22, 22.6 and 33.3 minutes.

2.3.4. Specificities of ORF1 PCR and LAMP assays

The ORF1 LAMP reaction will be required to detect *N. gonorrhoeae* nucleic acid from samples presented for STI testing. As such, it is essential that the assay can accurately distinguish the *N. gonorrhoeae* DNA from that of other non-target organisms that may be encountered in the samples. The small scale specificity test included the organisms *T. vaginalis* and *C. trachomatis*; both sexually transmitted pathogens of the human genital tract that are sampled for in the same manner as *N. gonorrhoeae*, and cause overlapping symptoms. The commensal organism, and opportunistic pathogen, *Pseudomonas aeruginosa* was also included as it is frequently encountered in urine samples.



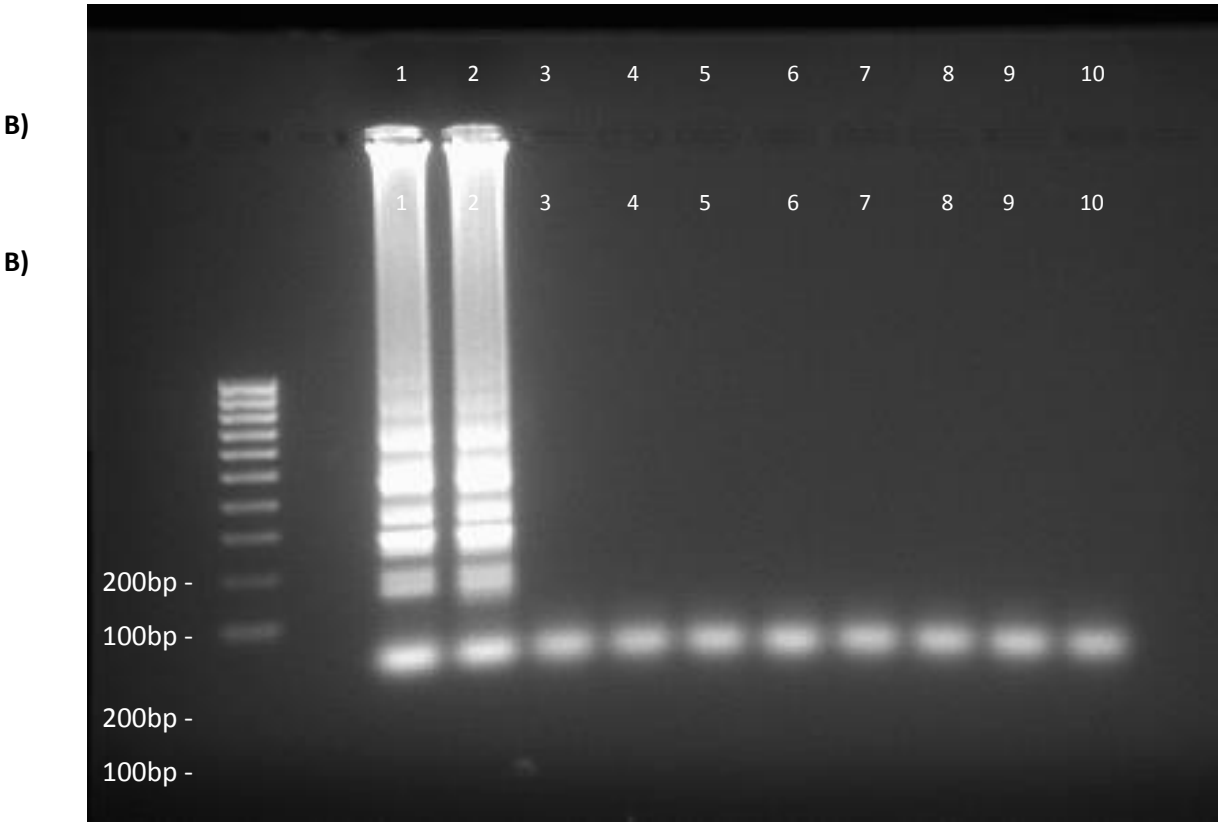


Fig.2.6. Specificity of *N. gonorrhoeae* ORF1 PCR and LAMP assay; agarose gel electrophoresis of (A) PCR and (B) LAMP reaction products from reactions containing 0.1µg of *N. gonorrhoeae* (lanes 1, 2), *C. trachomatis* (lanes 3, 4), *T. vaginalis* (lanes 5, 6), *P. aeruginosa* (lanes 7, 8) DNA. Water was used as a negative control (lanes 9, 10).

The ORF1 PCR assay produced a 139bp fragment from *N. gonorrhoeae* DNA, as expected. No non-specific amplification or primer dimerization could be detected from the gel (Fig.2.6.A). The ORF1 LAMP assay generated a successful amplification from *N. gonorrhoeae* DNA, evidenced by the presence of various sized DNA fragments in a characteristic ladder pattern seen on the gel (Fig.2.6.B). No amplification was seen for any non-target organism tested when using either the PCR or LAMP assay.

2.3.5. Sensitivities of ORF1 PCR and LAMP assays

The analytical sensitivities of LAMP assays are typically greater than, or at least equal to, standard PCR reactions for the same target. In order to determine whether this LAMP primer set was as sensitive as a PCR for the same target gene, the limits of detection of each assay were determined using *N. gonorrhoeae* genomic DNA

The ORF1 PCR assay was able to detect a minimum level of 20 copies of the *N. gonorrhoeae* genome per reaction (Fig.2.7). However, even after 35 cycles the amount of product generated was very low, demonstrated by the faintness of the band seen on the gel. A stronger signal was seen for reactions containing >200 copies.

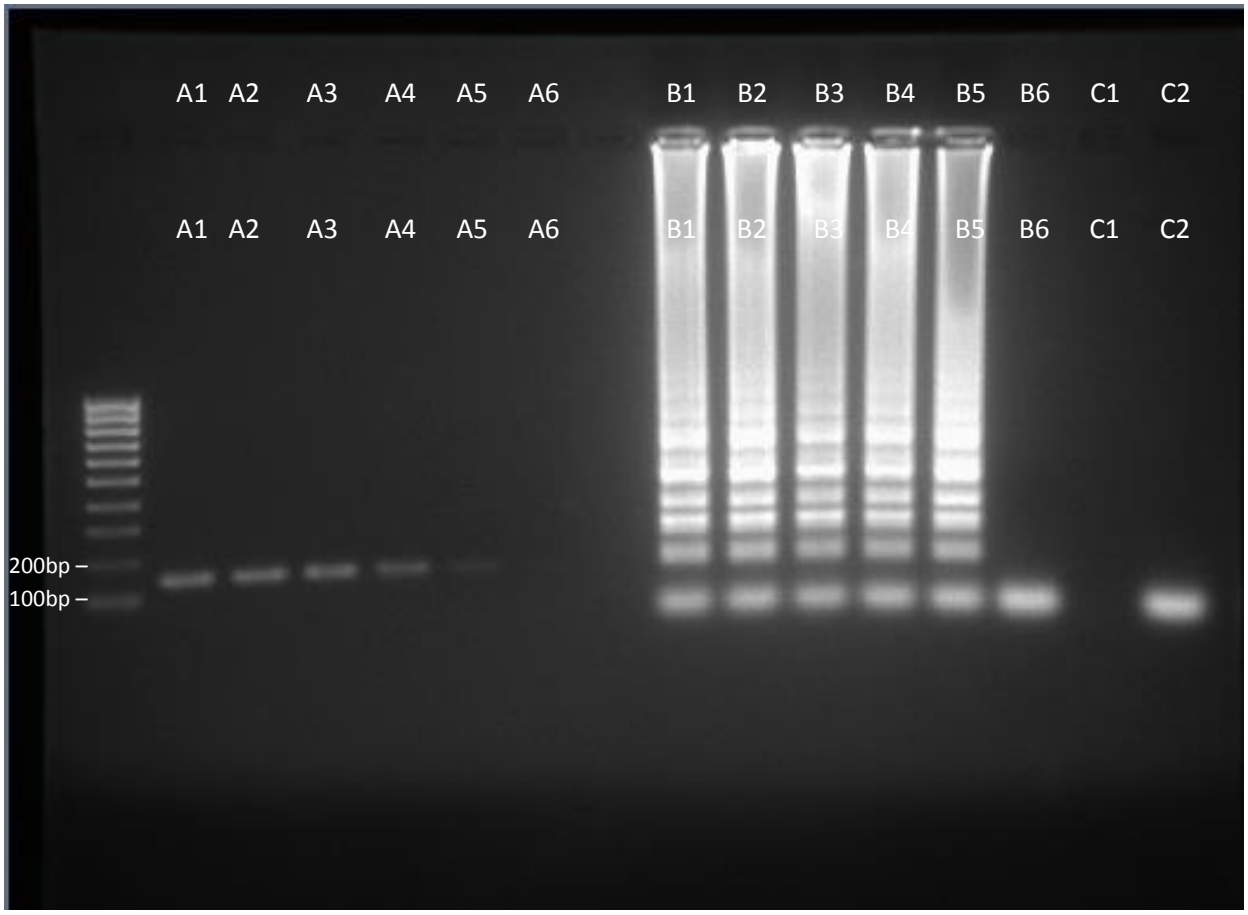


Fig.2.7. Sensitivity of ORF1 PCR and LAMP assays; agarose gel electrophoresis of ORF1 PCR (lanes A1-A6) and ORF1 LAMP reactions (lanes B1-B6) containing $2x10^5$ (lanes A1, B1), $2x10^4$(lanes A2, B2), $2x10^3$(lanes A3, B3), $2x10^2$(lanes A4, B4), 20 (lanes A5, B5)and 2 copies (lanes A6, B6)of the *N. gonorrhoeae* genome. PCR (lane C1) and LAMP (C2) reactions containing 5µl of water instead of DNA were used as a negative control.

The ORF1 LAMP reaction was able to detect a minimum of 20 copies of the *N. gonorrhoeae* genome (Fig.2.7). Real-time analysis of the amplifications using the ESEQuant Tube Scanner (QIAGEN, CA.) showed that the time to amplification was shorter with an increased starting concentration of template, with the reaction containing $2x10^5$ copies showing the earliest amplification, crossing the manually set threshold line at 17 minutes (Table 2.7). The latest time to amplification, 27.3 minutes, was seen in the reaction containing 20 copies.

***N. gonorrhoeae* genome copy number**	**Time to amplification (minutes)**
$2x10^5$	17
$2x10^4$	18.6
$2x10^3$	20.6
$2x10^2$	25
20	27.3
2	No amplification
0	No amplification

Table.2.7. Sensitivity of ORF1 LAMP assay; Average time to amplification of ORF1 LAMP reactions containing $2x10^5$, $2x10^4$, $2x10^3$, $2x10^2$, 20 and 2 copies of the *N. gonorrhoeae* genome. Time to amplification was taken as the time point that the fluorescent signal exceeded a manually set threshold line on the Tube Scanner Studio software package (Qiagen). A LAMP reaction containing 5µl of water instead of DNA was used as a negative control.

2.3.6. Urea Tolerance

In order to examine the suitability of the ORF1 LAMP assay for the direct detection of *N. gonorrhoeae* from urine, the tolerance of the LAMP assay to urea, the primary amplification inhibitor in urine, was determined. This was compared with PCR, as the most commonly used amplification technology, and one ill suited for use with urine samples, without a prior nucleic acid purification step.

A)

A)

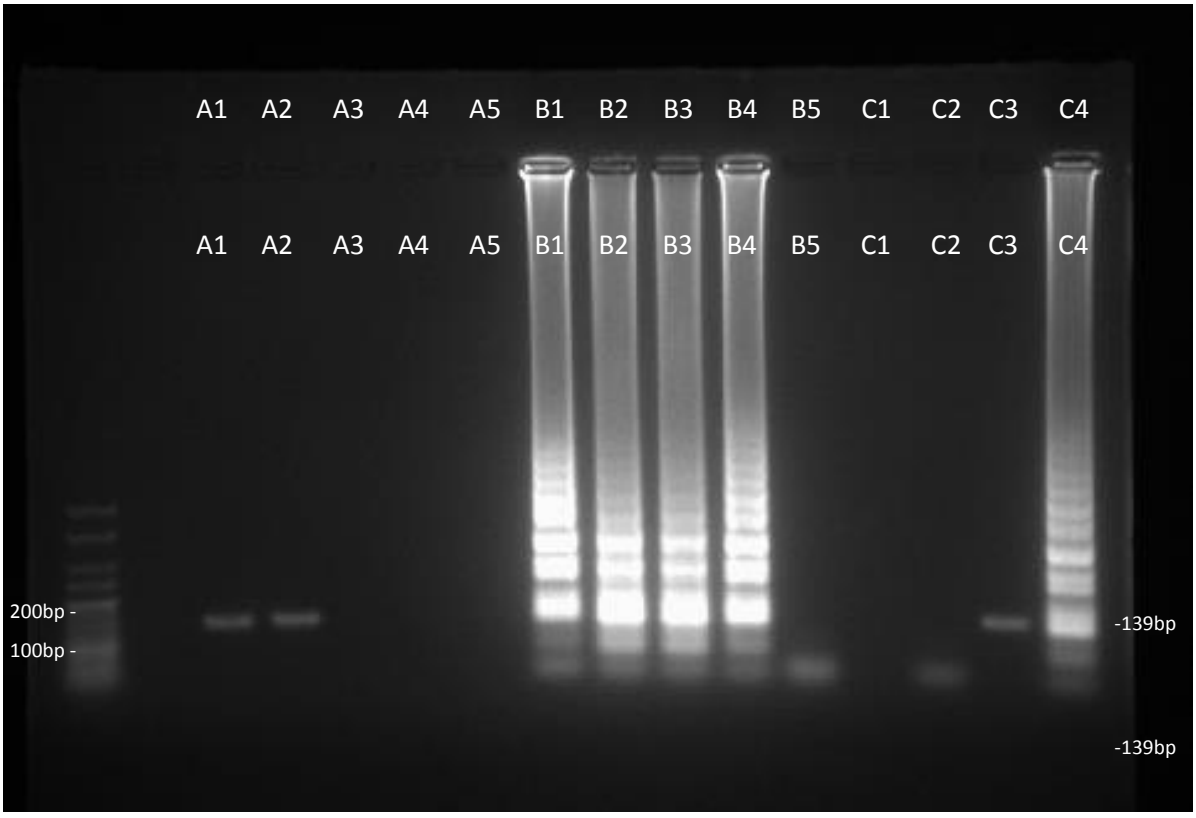


Fig.2.8.A. Tolerance of the ORF1 PCR assay and ORF1 LAMP assay to urea; agarose gel electrophoresis of ORF1 PCR (lanes A1-A5) and LAMP (lanes B1 – B5) reaction products from reactions spiked with urea concentrations of 1mM (lanes A1, B1), 10mM (lanes A2, B2), 100Mm (lanes A3, B3), 1M (lanes A4, B4), 2M (A5, B5). Positive reactions contained 0.1µg of *N. gonorrhoeae* DNA. PCR (lane C3) and LAMP (lane C4) reactions containing no urea were carried out as positive controls. PCR (lane C1) and LAMP (lane C2) reactions containing 5µl of water instead of DNA was used as a negative control.

B)

B)

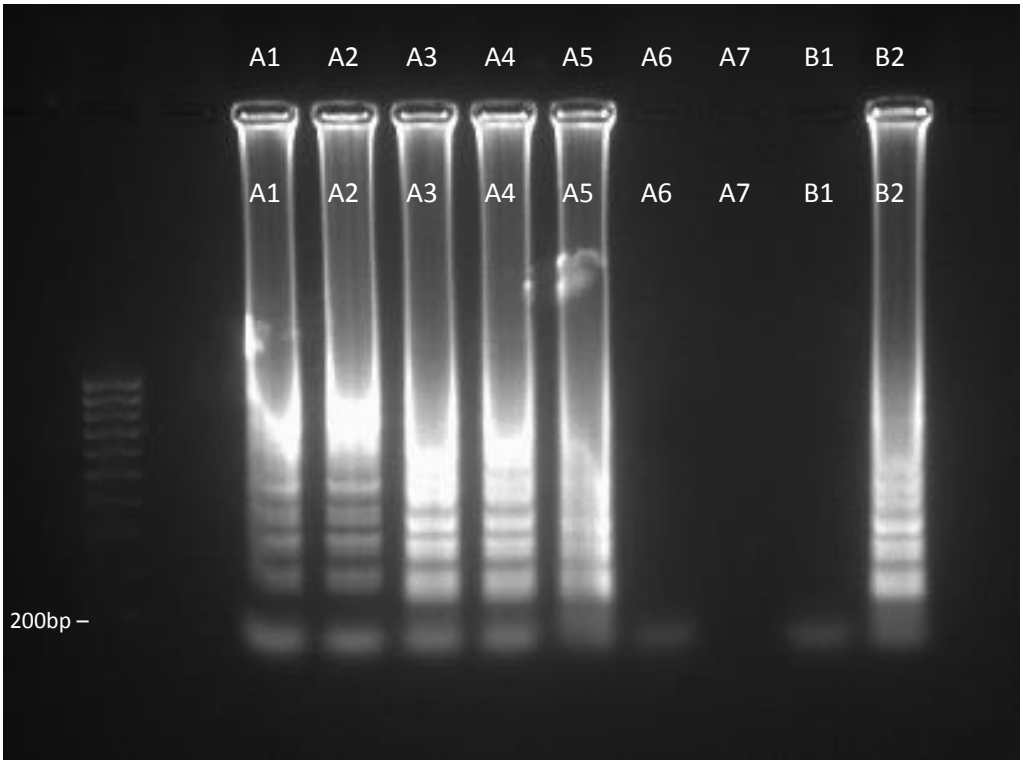


Fig.2.8.B. Agarose gel electrophoresis of reaction products from LAMP reactions spiked with urea concentrations of 1M (lane A1), 1.2M (lane A2), 1.4M (lane A3), 1.6M (lane A4), 1.8M (lane A5), 2M (lane A6). Positive reactions contained 0.1µg of *N. gonorrhoeae* DNA. A LAMP reaction (B1) containing 5µl of water instead of DNA was used as a negative control. A LAMP reaction containing no urea was carried out as a positive control.

The ORF1 PCR reaction was able to tolerate a urea concentration of 10mM, whereas a concentration of 100mM proved inhibitory (Fig.2.8.A). The LAMP reaction was totally inhibited by a urea concentration of 2M. At concentrations of 1.8M and below, no inhibitory effects, such as a reduction in product or increase in time to amplification, were seen (2.8.B).

2.3.7. Detection of *N. gonorrhoeae* directly from urine samples

The simplest option available for positive reaction determination using the LAMP assay is direct amplification detection, using an intercalating dye or indicator dye to provide a visually detectable colour change. One of the detection dyes most frequently used in conjunction with the LAMP assay is calcein, a fluorescent dye with excitation and emission wavelengths of 495nm/515nm. During LAMP amplification, pyrophosphate is released, which removes manganese ions bound to calcein, which are replaced with magnesium ions, resulting in fluorescence.

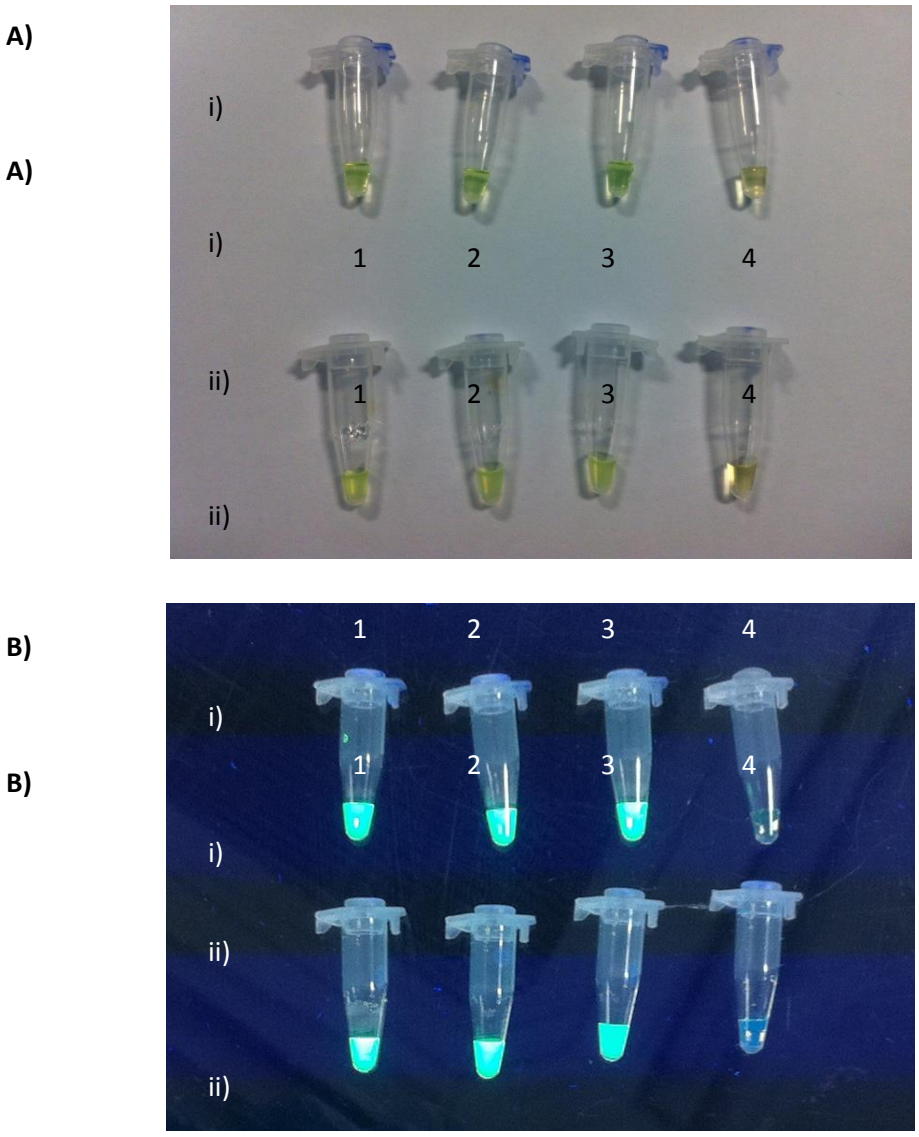


Fig. 2.9. Comparison of calcein-mediated end point detection of positive *N. gonorrhoeae* ORF1 LAMP reactions, using either urine (i) or water (ii) spiked with *N. gonorrhoeae* DNA. Detection was carried out by observing the amplification dependent color change in daylight (A) or with excitation of the fluorophore via a UV transilluminator (B). The LAMP reactions contained $2x10^3$ copies (1), $2x10^2$ copies (2), 20 copies (3), and 0 copies (4) of the *N. gonorrhoeae* genome.

The inclusion of calcein in the LAMP reaction, in place of the intercalating dye, resulted in an easily distinguishable colour difference between the positive and negative reactions (Fig.2.9.). Under ambient light the positive reactions appear green, whilst the negative reactions appear yellow (A). The presence of urine in the reaction (ii) did not affect this. Under UV light (365nm) the positive reactions emitted bright green fluorescence irrespective of the presence of urine (B). The water-

containing negative emitted a faint green fluorescence, whilst the urine-containing negative emitted a slightly brighter blue/green fluorescence.

2.3.8. Tolerance of the ORF1 LAMP assay to the presence of blood

The tolerance of the ORF1 LAMP assay to the presence of blood, another potential source of amplification inhibition that could be found in a clinical sample taken for STI testing, was characterised.

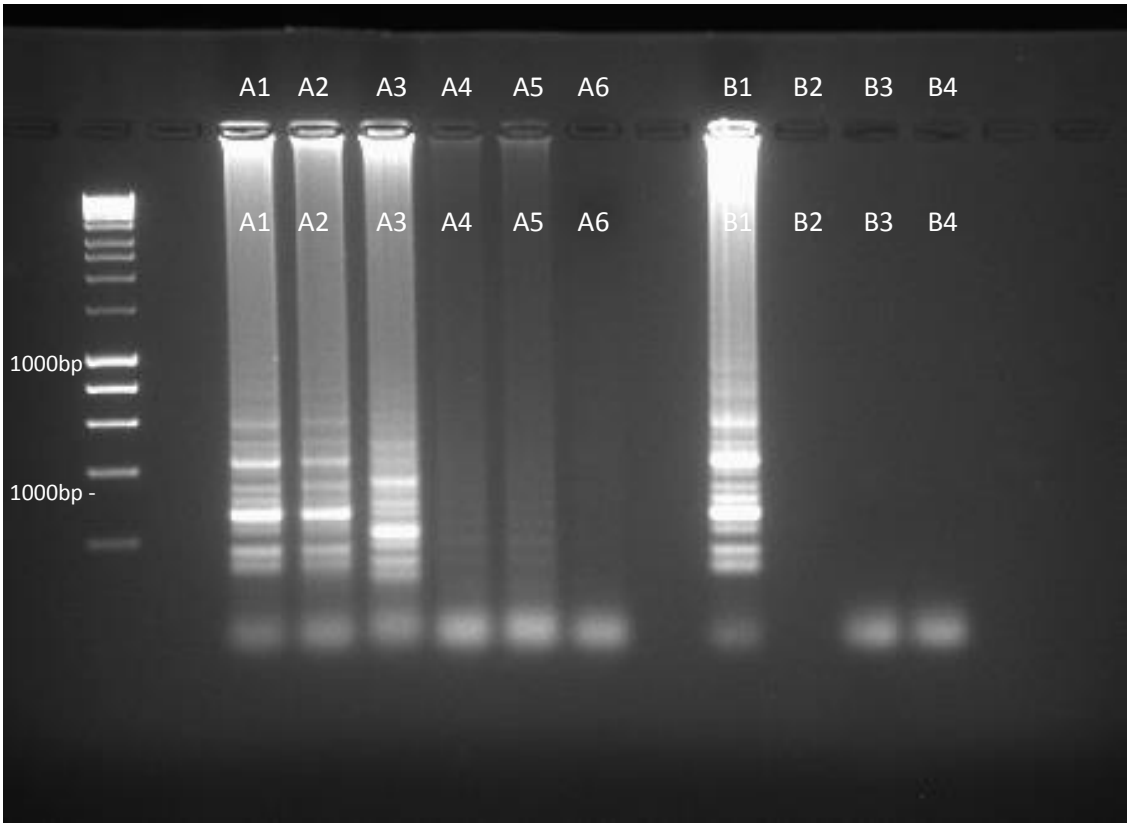


Fig.2.10. Agarose gel electrophoresis of reaction products of ORF1 LAMP reactions containing 0.1µg of *N. gonorrhoeae* DNA and the following concentrations of whole blood; 5% (A1), 10% (A2), 20% (A3), 30% (A4), 40% (A5), 50% (A6). The positive control reaction contained containing 0.1µg of *N. gonorrhoeae* DNA, without blood (B1). Negative reactions contained no *N. gonorrhoeae* DNA, with water (B3) or blood (B4). Well B2 was empty.

The ORF1 LAMP assay was able to tolerate up to a 20% volume of whole blood present in the reaction, equating to 5µl of the 25µl reaction, the standard sample volume (Fig.2.10). This shows that whole blood would be an acceptable sample type for the ORF1 LAMP assay, and would not cause inhibition in an end-point LAMP assay. At concentrations of 30% and 40%, and to a lesser extent 50%, there was a low level of the typical ladder-like pattern of LAMP reaction fragments present on the gel, indicating that the reaction was not completely inhibited, even at these high concentrations of whole blood.

The negative control, containing 5% whole blood without template, did not generate any product, showing that the presence of blood at this concentration does not cause non-specific amplification.

2.4. Discussion

2.4.1 Limits of detection of the *N. gonorrhoeae GroEL* LAMP assay

The *GroEL* LAMP assay, in conjunction with the magnetic silica based extraction method, was capable of detecting a minimum of 100 *N. gonorrhoeae* cells per extraction. This was the equivalent to 6.7 cells worth of nucleic acid per LAMP reaction. As the extraction was carried out using a 200µl sample, this equates to 500 *N. gonorrhoeae* cells per ml. The range of *N. gonorrhoeae* cells present in clinical samples depends greatly on the sample type, and stage of infection of the patient. One study found that the organism load in male first void urine (FVU) during symptomatic *N. gonorrhoeae* infection ranged from 3.7×10^4 to 2.6×10^7, with an average of 6.1×10^6 (Isbey *et al.*, 1997). FVU Samples taken during early stage infection (21 – 48 hours post inoculation) yielded an average of 1.8×10^3 CFU/ml (Isbey *et al.*, 1997). The organism load in vaginal or cervical swab samples is likely to be higher than these levels, as evidenced by the improved assay sensitivity obtained from these sample types (Fang *et al.*, 2008). The limit of detection of the *GroEL* LAMP assay, as determined in this set of experiments, lies below the range of organism loads given in this study, and is also lower than the ID50 (Infectious dose 50; the number of cells required to generate an infection in 50% of exposures) of 1.8×10^3 to 1×10^5, depending on the strain, as determined by human inoculation experiments (Hobbs *et al.*, 2011). The ability of the assay to detect the organism at levels below that typically encountered in clinical samples is a good predictor that the test will have a high clinical sensitivity; the assay will be unlikely to generate false negative results due the presence of an organism load lower than the limits of detection of the assay. However, care must be taken when equating this information to clinical samples; clinical samples are likely to contain a range of inhibitory compounds absent from a cell suspension in urine, such as blood and mucous. The LAMP assay, in combination with the magnetic silica based extraction process, has been shown to be tolerant of inhibitory compounds at supra-physiological levels (Fig. 2.8.B), so the impact of these inhibitors should be limited. A larger problem will be the decay of nucleic acid during the sampling process; it will be imperative that the degradation of nucleic acid during the period between sampling and the initiation of the assay is minimised. In order to accurately determine the performance of this assay, a clinical trial will be required, in order to assess the sensitivity and specificity of the assay when dealing with clinical samples. This work will be carried out by Public health England Southwest, at a later date.

2.4.2. Initial testing of the ORF1 primer sets

The initial test of the performance of the ORF1 primer sets showed that the ORF1 S2 primer set enabled a faster time to detection than the ORF1 S1, *GroEL* and *PorA* primer sets, generating a positive signal 10.7 minutes earlier than the next fastest set. Whilst in the duplicate run of this experiment the difference was smaller, the ORF1 primer set generated a detectable amplification 3 minutes sooner than the other primer sets.

The fact that the ORF1 S2 set generated an amplification 11.3 minutes earlier than the ORF1 S1 set was surprising, as the primers target the same gene, with the 5' F3 positions only lying 93bp apart on the *N. gonorrhoeae* genome, and will have the same starting copy number. This suggests that the increase in reaction speed is more a symptom of the actual primer sequences involved, and their compatibility with each other. The late amplification generated by the ORF1 S1 primer set in the duplicate experiment suggests that this primer set is sub-optimal, and this may have resulted in the slower reaction speed also seen in the first experiment.

The steady gradual increase in fluorescence that occurred in the ORF1 S2 negative reaction could be due to primer dimerisation or self-priming events. The gradual increase was maintained for ~15 minutes in the first experiment, and ~20 minutes in the second experiment, before reaching a plateau and maintaining this level. This could be at the point that all of the copies of the primer had formed dimers or self-primed. The examination of the products of the negative reaction revealed an absence of any LAMP reaction products, but the presence of a bright band around 50bp in size. This was approximately the same size as the primer sequences, but was brighter than in the negative reactions carried out with the alternate primer sets, indicating the generation of a greater quantity of DNA. The formation of primer dimers can have a deleterious effect on the amplification reaction, as the amplification of the dimer by the polymerase enzyme can lead to premature exhaustion of the PCR reagents, such as dNTP's and primer. Agarose gel electrophoresis of the LAMP products showed that the ORF1 S2 primer set generated an increased concentration of product compared to the other primer sets, indicating that the reaction was not limited by a shortage of components. The LAMP reaction mix contains a greater concentration of both primers and dNTP's than the recommended concentrations for PCR, and this could act to alleviate the impact of primer dimer formation. In these initial tests, the ORF1 S2 primer set provided the fastest reaction, and highest amount of product, whilst generating no false positive amplification. Therefore it was selected for further testing, in order to further characterise its performance and sensitivity.

2.4.3. Relative sensitivity of the *GroEL* and ORF1 S2 primer sets

The ORF1 primer set generated a faster amplification than the *GroEL* primer set from each dilution tested. Despite this there was no difference in the limit of detection of the two assays. The faster assay speed afforded by the ORF1 primer set would enable a more rapid diagnosis to be made, and allow an earlier cut-off point to be used for the assay without affecting the sensitivity. Although the *N. gonorrhoeae glnA* gene include a ~130bp hypervariable region, the ORF1 region of *glnA* is highly conserved, and has little variation (Vidovic *et al.*, 2011). This is in contrast to the *PorA* gene, as *PorA* mutants are now more frequently encountered, and have previously been determined as the cause of a range of sequence related false-negative results in clinical samples (Whiley *et al.*, 2011). Mutations in other genes commonly used as the targets of in-house PCR assays for this organism such as *cppB* and *opa* have also been identified as the cause of false negative results in a diagnostic setting (Geraats-Peters *et al.*, 2005b; Bruisten *et al.*, 2004).

2.4.4. Specificities of ORF1 PCR and LAMP assays

The ORF1 PCR assay produced a 139bp fragment from *N. gonorrhoeae* DNA, as expected. No non-specific amplification or primer dimerization could be detected from the gel (Fig.2.6.A).

The ORF1 LAMP assay was able to detect *N. gonorrhoeae* DNA, with a positive reaction demonstrated separating the amplified products using agarose gel electrophoresis (Fig.2.6.B), in order to visualise the characteristic ladder effect caused by the various sized concatemers of double stranded DNA formed during the LAMP reaction (Notomi *et al.*, 2000). No amplification was seen for any non-target organism tested when using either the PCR or LAMP assay. This experimentally confirmed the high levels of primer specificity previously determined using BLAST. The non-target organisms tested were all common urinary pathogens, and like *N. gonorrhoeae*, potential causes of urethritis, so it is essential that these organisms are not incorrectly identified by a diagnostic test. The correct identification of the pathogen in question when diagnosing sexually transmitted disease is important, due to the recommendation of different antimicrobial treatment programmes for each of these infections (Health Protection Agency, 2011).

LAMP assay specificities are typically higher than PCR assays targeted to the same sequence, due to the use of 4 primers specific to 6 regions of target DNA, rather than the use of 2 primers specific to two targets as used in PCR (Notomi *et al.*, 2000). The high specificity of LAMP assays has been

demonstrated by the development of species-specific malaria LAMP assays (Iseki *et al.*, 2010), and LAMP assays capable of identifying specific subtypes of virus (Zhou *et al.*, 2011).

2.4.5. Sensitivities of ORF1 PCR and LAMP assays

The ORF1 LAMP assay was able to detect a minimal level of 20 copies of the *N. gonorrhoeae* genome. The more sensitive diagnostic LAMP assays presented in the literature have typically been shown to have limits of detection ranging from 20 to 5 copies per reaction (Shan *et al.*, 2012; Kim *et al.*, 2011; Nakao *et al.*, 2010). However, limits of detection ranging from 100 copies (Iseki *et al.*, 2010; Uemura *et al.*, 2008), to single copy detection (Zhou *et al.*, 2011) have been reported. As the ORF1 LAMP assay was chosen to examine the inhibitory effects of urine on LAMP assays in general, it was important that the ORF1 assay was representative of a sensitive LAMP assay. Monitoring the reactions in real time using the ESEQuant TS showed that amplification occurred after 17 minutes for the reactions containing the highest copy number (2 $x10^5$ per reaction) and 27.3 minutes for the lowest (20 per reaction). The time to amplification was similar (within 2 minutes) during all 3 replications of the experiment. The assay ran for 60 minutes, but the cut off time could be reduced by up to 30 minutes without affecting the results.

2.4.6. Urea Tolerance

Urine is a complex, and highly variable sample. Composition varies between individuals and depends on the diet, hydration and disease state of the patient. Urine consists primarily of water, and soluble excreted compounds, including sodium chloride, urea, and ionic potassium, calcium and sulphate (Lind, 2001). The primary compound found in urine responsible for PCR inhibition is urea (Khan *et al.*, 1991), present in urine as an excreted by-product of the metabolism of nitrogen containing compounds, primarily proteins (Brosnan and Brosnan, 2010).

The inhibition of PCR via compounds present in urine has been shown to have a detrimental effect on assays for the detection of bacterial (George JA, 2003) and viral pathogens (A. Behzadbehbahani, 1997). A study on the evaluation of the performance of an internal positive DNA control for a PCR assay for the detection of *C. trachomatis* found that 23% of 100 clinical urine samples tested were inhibitory to PCR unless a pre-assay DNA extraction was carried out (Betsou *et al.*, 2003). If a

potential false negative result due to inhibition of the reaction has been identified through the use of an internal control, the dilution of the sample 1:10 has been reported as a likely solution (Mahony *et al.*, 1998). This dilutes the concentration of inhibitory compounds sufficiently to allow amplification to take place. However, this practice will reduce overall assay sensitivity by reducing the target nucleic acid concentration in the sample by 90%, which will prevent the positive identification of samples with a starting organism load close to the assay detection limits. A recent evaluation of a direct urine PCR test for *N. gonorrhoeae,* which relied on a pre-assay dilution step, reported a sensitivity of 83% when compared with a commercial PCR test (Rahimi *et al.*, 2013).

The urea tolerance of the ORF1 PCR reaction was shown to be <100mM, which is in agreement with previous research, which has variously determined the minimum inhibitory concentration to be >50mM (Khan *et al.*, 1991) and >20mM (A. Behzadbehbahani, 1997). The average concentration of urea in adult urine is ~300mM, above the determined minimum inhibitory threshold for PCR. This means that simple DNA extraction procedures, such as chelex based methods, or the use of a lysate are not suitable for PCR when dealing with urine samples.

The LAMP assay was able to detect *N. gonorrhoeae* DNA at urea concentrations of 1.8M and below and urea concentration had no effect on time to amplification (data not shown), or a noticeable effect on the amount of product generated when visualised on an agarose gel. This minimum inhibitory urea concentration of 2M represents a 20 fold increase of the minimum concentration inhibitory to the ORF1 PCR, demonstrating the increased robustness of the LAMP assay to urea mediated inhibition, when compared to PCR. A urea concentration of >1.8M is higher than the maximum urea concentrations found in samples of human urine, and this high tolerance for urea makes the LAMP assay more suitable for use with unextracted samples than PCR assays.

PCR inhibition by urea is mediated by interference in non-covalent bonding between the polymerase enzyme and template, and also the annealing primer and template (Hedman and Radstrom, 2013) . The reasons for the lack of inhibition of the LAMP assay to urea is currently undetermined, but could involve the stability of the *Bst* enzyme itself, or the isothermal nature of the reaction maintaining constant favourable conditions for annealing of the primers and template.

The ability of the LAMP reaction to withstand higher levels of urea than those found in human urine, and therefore enable the detection of target nucleic acid from urine samples that have either not undergone a nucleic extraction process, or undergone a very simple process such as a heat treatment, has implications for the use of LAMP assays to diagnose sexually transmitted infections in a resource poor setting. The removal of the need for a nucleic acid purification/extraction

procedure, coupled with the lack of specialist equipment needed to process the reactions, and detect the outcomes, mean that LAMP technology is well suited for use in laboratories without the equipment or staff necessary to perform conventional PCR based testing.

2.4.7. Detection of *N. gonorrhoeae* directly from urine samples

In order to demonstrate the suitability of the LAMP assay for use with unprocessed urine samples, ORF1 LAMP reactions were carried out on both urine and water samples spiked with $2x10^4$ copies, $2x10^3$ copies, $2x10^2$ copies, and 0 copies of the *N. gonorrhoeae* genome. Real-time detection using V13-01184 dye monitored by the ESEQuant fluorometer proved difficult, as the presence of urine increased the baseline fluorescence via an unknown mechanism, making it difficult to differentiate between the positive and negative reactions. This was not seen for the LAMP reactions containing any concentration of urea, so must occur due to another component of the urine. The urine would be expected to contain a component of endogenous DNA, but the amount was too low to measure, and would not be sufficient to cause the size of increase in fluorescent intensity that occurred.

As an alternative, and in order to demonstrate a more rapid and cost effective method of detection, end point detection using the fluorescent dye calcein was carried out. The inclusion of an indicator or dye, such as calcein, enables visual end point detection and removes the need for real-time analyzers, which although provide the fastest result, are costly. It also enables detection without any further post-reaction steps, such as separating reaction products on an agarose gel, which necessitate opening the reaction tubes and introducing high levels of contaminating amplicon into the laboratory environment. Several dyes and fluorophores have been used in conjunction with LAMP for this purpose, including SYBR green (Tao *et al.*, 2011), hydroxy napthol blue (Goto *et al.*, 2009), and calcein (Tomita *et al.*, 2008).

Calcein is added to the reaction, along with manganous ions (Mn^{2+}), which bind to the calcein molecule and act as a quencher, preventing fluorescence. The rapid synthesis of large quantities of double stranded DNA during the LAMP reaction generates high concentrations of pyrophosphate as a by-product, which displaces manganous ions from the calcein molecules, resulting in a colour change from orange to green, and fluorescence when exposed to UV light (Tomita *et al.*, 2008). Additionally, magnesium ions (Mg^{2+}) are then able to bind to calcein, which enhances the intensity of fluorescence (LaBarre *et al.*, 2011).

The end point detection of positive LAMP reactions was equally accurate for both the spiked urine and water samples, with positive reactions undergoing an orange to green colour change. This

colour change was obvious in daylight. The negative reactions remained orange, whether urine or water was used. A minor colour difference was seen between the urine negative and water negative under UV, with the urine negative exhibiting a low blue fluorescence. This assisted in distinguishing between the positive and negative results when un-processed urine was used. However, the colour chance was unambiguous in ambient light, meaning that the only equipment needed once the reactions had been set up was a method of heating to a constant temperature of 63^{o}C, such as a water bath.

2.4.8. Tolerance of the ORF1 LAMP assay to the presence of blood

The ability of the ORF1 LAMP assay to successfully generate an amplified product with up to 20% of the reaction volume taken up by whole blood demonstrates the greatly increased robustness of LAMP when compared with PCR. The 20% volume is also significant; this equates to 5µl of the 25µl reaction, which is the suggested sample volume for the LAMP reaction. This shows that whole blood could be used as the sample for the LAMP reaction, without a purification or extraction step. This would enable sensitive NAAT testing of blood borne disease, such as malaria or hepatitis B, without the need for complicated and expensive extraction methodologies. LAMP assays for the direct detection of malaria (Ebbinghaus *et al.*, 2012; Poon *et al.*, 2006) Methecilin-resistant Staphylococcus aureus (Hanaki *et al.*, 2011) and BK virus (Bista *et al.*, 2007) from blood samples demonstrate this.

PCR is inhibited by a number of compounds in urine, including immunoglobulins, haemoglobin, and lactoferrin (Al-Soud and Radstrom, 2001), and PCR is not capable of the detection of pathogenic DNA directly from blood samples without prior purification. However, the use of mutant variations of taq polymerase, in combination with a PCR enhancer cocktail including heparin and other anticoagulants, has been shown to enable PCR amplification in the presence of up to 25% whole blood (Zhang *et al.*, 2010).

The ability of the ORF1 LAMP reaction to detect *N. gonorrhoeae* DNA from whole blood, containing higher levels of blood-borne amplification inhibitors than will be found in urine or swab samples, show that inhibition by these compounds would not be a problem when using this assay to test for *N. gonorrhoeae*.

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Chapter 3

Mycoplasma genitalium

3.1. Introduction

Mycoplasma genitalium is a sexually transmissible, pathogenic bacterium, belonging to the class *Molicutes*, and genus *Mycoplasma* (Sethi *et al.*, 2012). *M. genitalium* carries one of the smallest genomes of any self-replicating prokaryote, consisting of a ~580kb genome containing ~480 protein coding regions (Suthers *et al.*, 2009), and is considered to have close to the minimal gene complement of a living cell that can be grown in pure culture (Glass *et al.*, 2006). *M. genitalium* was first isolated from two patients presenting with non-gonococcal urethritis (NGU) at the sexually transmitted disease clinic of St. Marys's Hospital, London, UK in 1980. In the previous decade it had been noticed that tetracycline therapy was shown to have success in some instances of NGU, despite the absence of bacteria in the urethra, and it was hypothesised that an as yet unknown bacterium was responsible for the infections (Tully *et al.*, 1981). Samples were taken from a number of patients and sent to the National Institute of Health, MD, USA, where two different strains of a mycoplasma were isolated from the samples, that differed serologically from all previously encountered mycoplasma. These two strains, G-37 and M-30, were then classified as a new organism, *M. genitalium* (Taylor-Robinson and Jensen, 2011).

3.1.1 Pathogenesis

Morphologically, *M. genitalium* is a 0.2µm – 0.3µm, flask shaped cell, with a defined tip known as the adhesion organelle, which is used for adhesion and invasion of host cells (Cazanave *et al.*, 2012). Adhesion has been demonstrated on a number of cell types, including a variety of genital epithelial cells, spermatozoa, erythroyctes, and also non biological surfaces such as glass and plastic (Taylor-Robinson and Jensen, 2011). In cell culture, adhesion to vaginal and cervical epithelial cells has been shown to occur within two hours of infection, with invasion occurring at 3 hours post infection (McGowin *et al.*, 2009). It is thought that only subsets of *M. genitalium* cells actually internalise during an infection (Ueno *et al.*, 2008), and also that not all host cells are susceptible to this internalisation (Taylor-Robinson and Jensen, 2011). During the infection of Vero cells in tissue culture, only around 10% of cells will harbour internalised mycoplasma (Jensen, 1994). *M .genitalium* is considered a facultative intracellular pathogen; unlike obligate intracellular pathogens such as *Chlamydia trachomatis*, it is able to remain viable and reproduce either intracellularly or extracellularly (Jensen, 2006). During colonisation of lung fibroblasts in vitro, *M. genitalium* invades the cells, and migrates through the intracellular environment to the nucleus. Intranuclear replication

then occurs, followed by lysis of the host cell and the release of infectious mycoplasma into the extracellular environment, which can then further infect surrounding cells(Mernaugh *et al.*, 1993). This process results in the lysis of the cells in 96 hours. Contrastingly, a study on the colonistaion of HEp-2 epithelial cells found that the internalisation of *M. genitalium* resulted in the long term colonisation and persistence of intracellular infection, without host cell lysis (Dallo and Baseman, 2000). *M. genitalium* replication was found to be slower intracellularly than extracellularly, and this gives rise to the possibility that cell invasion is used as a survival strategy, to maintain a state of low growth maintenance whilst evading the host immune system.

Adhesion to the host cell is mediated by the localisation of a number of surface exposed adhesion proteins, linked to the internal cytoskeleton apparatus of the adhesion organelle (Burgos *et al.*, 2006). The primary adhesion proteins are MgPa, also referred to as P140 or MG191, and P110, also known as MG192. These adhesins, in combination with a number of cytoskeletal accessory proteins, are required for adhesion to the host cell, and therefore colonisation (Pich *et al.*, 2008). The binding of *M. genitalium* to host cells can be blocked by either removing the surface exposed binding proteins with trypsin, or by binding MgPa to a monoclonal antibody in order to prevent any cell surface interactions (Baczynska *et al.*, 2007), illustrating their importance to this process. Gene knockouts of MgPa or P110 result in reduced haemadsorption, reduced expression of associated cytoskeletal proteins such as MG386 and Dnak, and also the incomplete formation of the adhesion organelle itself (Burgos *et al.*, 2006).

3.1.2. Epidemiology and clinical presentation

Epidemiological studies have estimated the overall prevalence of *M. genitalium* infection to be 2% and 7.3% for low and high risk populations, respectively (McGowin and Anderson-Smits, 2011). A large UK study of 2378 sexually active female students found the prevalence of *M. genitalium* infection in this population to be 3.3% (Oakeshott *et al.*, 2010). Similar, smaller scale studies on female populations in Denmark (Andersen *et al.*, 2007) and Japan (Hamasuna *et al.*, 2008) found comparable levels of infection. A large scale study in the US tested 1714 female and 1218 male urine samples from adolescents and young adults for a range of sexually transmitted infections, and found a prevalence of 1.0%, 0.4%, 4.2%, and 2.3% for *M. genitalium*, *Neisseria gonorrhoeae*, *C. trachomatis* and *Trichomonas vaginalis* respectively. Some European studies have found the prevalence of *M.*

genitalium infection to be greater than that of *C. trachomatis* infection (Anagrius *et al.*, 2013), whilst other similar studies have found the prevalence to be less (Oakeshott *et al.*, 2010).

M. genitalium is now known to be a prominent cause of urethritis in males, and is thought to be responsible for approximately 20% - 35% of the NGU cases in men that are not caused by *C. trachomatis* infection (McGowin and Anderson-Smits, 2011). *M. genitalium* infection of the male urethra is more frequently symptomatic than asymptomatic (Falk *et al.*, 2004) with symptoms present in between 61% and 73% of patients (Anagrius *et al.*, 2005; Falk *et al.*, 2004), and the likelihood of discharge is higher than with NGU caused by other infectious agents (Wetmore *et al.*, 2011). *M. genitalium* infection is also strongly correlated with chronic NGU, often resulting from doxycycline and azithromycin treatment failures (Taylor-Robinson *et al.*, 2004). *M. genitalium* is not thought to be a significant cause of prostatitis (Taylor-Robinson, 2002), however studies have found *M. genitalium* in both prostate biopsy specimens (Krieger *et al.*, 1996), and in semen of men with chronic prostatitis (Mandar *et al.*, 2005). *In vitro* studies have found that chronic exposure of human prostate cells to *M. genitalium* can lead to a malignant phenotype (Namiki *et al.*, 2009), although further work is required to establish whether *M. genitalium* infection is a risk factor in human prostate cancer. Urethral to rectal transmission of this organism has been demonstrated in men who have sex with men (MSM) (Edlund *et al.*, 2012) and a screening of 438 MSM in the UK found an overall prevalence of rectal positivity of 4.4% (Soni *et al.*, 2010).

M. genitalium infection in female patients is associated with cervicitis (Mobley *et al.*, 2012), pelvic inflammatory disease (Haggerty *et al.*, 2006) and endometriosis (Cohen *et al.*, 2002). The vaginal inoculation of mice results in the ascendance of the organism through the urethral tract, to the cervix, uterus, fallopian tubes and ovaries (McGowin *et al.*, 2010). In humans, *M. genitalium* has been isolated in tissues of the cervix, uterus and fallopian tubes, demonstrating this model of ascending infection (McGowin *et al.*, 2010). There has been no causal link found between *M. genitalium* infection and miscarriage or ectopic pregnancy (Manhart *et al.*, 2011; Oakeshott *et al.*, 2004), although the available studies are of small populations, and are difficult to draw conclusive observations from. Infection in women is often asymptomatic (Falk *et al.*, 2005), which increases the likelihood of the infection lying undetected and causing more serious sequelae. The instigation of a routine M*. genitalium* screening programme, as is carried out for *C. trachomatis* in the UK and US, has been suggested, partially because of this reason (Ross *et al.*, 2009).

3.1.3. Relationship with other pathogens

Co-infection of *M. genitalium* with a number of other sexually transmitted bacterial, viral and protozoan pathogens has been noted by a number of screening studies, as both the mode of transmission, and behavioural risk factors are the same for these infections (Andersen *et al.*, 2007), a level of co-infection is to be expected. A study on a population of 390 *N. gonorrhoeae* culture positive men in Japan found the rate of *M. genitalium* co-infection to be 4.1% (Yokoi *et al.*, 2007). A large study of 1182 symptomatic men in Australia found an overall *M. genitalium* prevalence of 8.1%, with 14.6% also infected with *C. trachomatis* and 3.1% with *N. gonorrhoeae* (Mezzini *et al.*, 2013).

M. genitalium infection has been associated with an increased risk of HIV infection (Mavedzenge *et al.*, 2012), with some studies recording a greater than two-fold increase in risk of HIV acquisition (Mavedzenge *et al.*, 2012). A study on a high risk population in Kenya found that HIV positive patients were more likely to also be coinfected with *M. genitalium* than HIV negative patients (Cohen *et al.*, 2007), which is likely to reflect high risk behaviour, or HIV mediated immunosupression, facilitating *M. genitalium* colonisation (Manhart *et al.*, 2008). Infection by *M. genitalium* in HIV positive patients has been linked to an increase in viral shedding from the cervix, but only in patients with a high *M. genitalium* organism load (Manhart *et al.*, 2008). A high organism load is typically indicative of a recent infection, with lower organism loads occurring once specific immunity is generated, and the infection cleared to some degree (Manhart *et al.*, 2008). This increase in viral shedding makes transmission more likely (Anderson and Cu-Uvin, 2008), and is another mechanism by which the prevalence of *M. genitalium* in a population can affect the prevalence of HIV. Experiments in mice have shown that for effective antibiotic treatment of *M. genitalium* a functioning immune system is also required (Taylor-Robinson and Furr, 2000), although it is yet to be established whether immunocompromised human patients have the same difficulties in clearing mycoplasma infections during antimicrobial therapy (Taylor-Robinson, 2008).

3.1.4. Treatment

Currently, the primary antibiotic used in treatment of *M. genitalium* infections, is azithromycin (Skov Jensen, 2009). Azithromycin is a macrolide antibiotic, which exerts its antimicrobial effects by competing for [14C]erythromycin ribosome binding sites, and preventing the translation of mRNA

needed for protein synthesis (Retsema *et al.*, 1987). A single 1g dose of azithromycin has been shown to eradicate ~87% of *M. genitalium* infections (Mena *et al.*, 2009), whilst an extended dosage of 500mg on day one, followed by a 250mg dose for a subsequent 4 days has been shown to improve outcomes, clearing >95% of infections (Björnelius *et al.*, 2008). At least one study however, has found no difference in cure rates between these two programmes of azithromycin therapy (Jernberg *et al.*, 2008). Cases of NGU in which the aetiological agent has not been identified are often treated with either azithromycin, or doxycycline. Doxycycline has been shown to be effective in treating around 17% to 37% of *M. genitalium* infections (Björnelius *et al.*, 2008), and is therefore not recommended once *M. genitalium* has been identified as the cause of the symptoms. It is partly for this reason that recent guidelines for NGU recommend an azithromycin regimen (Workowski and Berman, 2010). Treatment failures after single dose azithromycin therapy have been associated with the generation of resistance, with follow up extended azithromycin treatment failing in up to 66% of patients (Jernberg *et al.*, 2008). This resistant phenotype has been linked to point mutations at positions 2058 and 2059 in region V of the 23S rRNA gene (Jensen *et al.*, 2008), which have previously been linked to macrolide resistance in other organisms including S*taphylococcus aureus* and *Helicobacter pylori* (Vester and Douthwaite, 2001). A recent study in the UK (Pond *et al.*, 2014) diagnosed *M. genitalium* in 16.7% of 217 men with asymptomatic urethritis, and found of this group that 41% (95% confidence interval: 20%-62%) were infected with strains possessing genotypic macrolide resistance.

In cases of treatment failure, patients are treated with moxifloxacin, which is given at 400mg daily for seven to ten days, and is not known to have been unsuccessful in clearing *M. genitalium* infection (Manhart *et al.*, 2011). Moxifloxacin, like all quinolones, exert their antimicrobial effect by inhibiting the activity of type II topoisomerases, DNA gyrase, and topoisomerase IV (Hooper, 1999). Despite the lack of clinical resistance encountered, point mutations in the quinolone resistance determining region (QRDR) of the *parC* gene, encoding a subunit of topoisomerase IV, have been discovered in clinical isolates, which could indicate the emergence of quinolone resistance (Shimada *et al.*, 2010). Studies have found the prevalence of these mutations to currently be between occurring in 10% to 15% of isolates encountered clinically (Tagg *et al.*, 2013; Shimada *et al.*, 2010). QRDRs also exist in the *M. genitalium gryA*, *gyrB*, and *parE* genes, and mutations in these genes causing a fluoroquinolone resistant phenotype have been detected in clinical isolates (Hamasuna *et al.*, 2011; Shimada *et al.*, 2010). *In vitro* data suggests alternative quinolone therapies utilised for the treatment of both NGU and cervicitis, including levofloxacin, ofloxacin and ciprofloxacin, have limited effectiveness against *M. genitalium* (Renaudin *et al.*, 1992).

A considerable obstacle in the susceptibility testing of *M. genitalium* is the extreme difficulty in obtaining a pure culture from a clinical specimen. Conventional broth dilution, or agar dilution susceptibility testing can be carried out once an axenic culture is obtained from the clinical specimen (Taylor-Robinson and Bebear, 1997). However, some strains are incapable of adaptation to axenic culture, and can therefore not be tested in this manner (Hamasuna *et al.*, 2005). A method based on real-time PCR has been developed, which uses a primer set specific to the single copy *mgpB* gene, to detect *M. genitalium* DNA levels in co-culture with Vero cells (Hamasuna *et al.*, 2005). This removes the need for the generation of an axenic culture, and allows for much earlier susceptibility testing. In one evaluation, the MICs obtained for macrolides using this method were slightly higher than those obtained using the broth diffusion method (Hamasuna *et al.*, 2008), whilst the MICs for flouroquinolones and tetracyclines showed good agreement. Due to the extremely high macrolide MICs seen for macrolide resistant strains, this difference would not be clinically relevant.

3.1.5. Diagnosis

The laboratory diagnosis of *M. genitalium* infection is almost exclusively carried out using nucleic acid amplification tests (NAATS), usually in the form of either commercially available or "in house" PCR assays (Cazanave *et al.*, 2012; Shipitsyna *et al.*, 2009). The culture of *M. genitalium* is difficult and extremely time consuming, requiring incubation periods in excess of a month to provide a maximally sensitive culture protocol (Hamasuna *et al.*, 2005). Obtaining an axenic culture from a clinical specimen can take over 6 months using standard culture methods, and around 5 weeks using the Vero cell method (Hamasuna *et al.*, 2005), making traditional culture based antibiotic susceptibility very difficult. Culture by the Vero cell method involves the propagation of the mycoplasma in Vero cells, before broth cultures are attempted, and then finally the growth of the organism on solid media (Jensen *et al.*, 1996; Jensen, 2004). The high failure rate and lengthy incubation times mean that culture of *M. genitalium* is very rarely attempted using clinical samples, especially now alternative molecular diagnostics methods are available (Waites *et al.*, 2012). The development of serological diagnostic assays has also proven difficult, due to the cross reactivity between *M. genitalium* and the closely related, and antigenically similar, *M. pneumoniae*(Lind *et al.*, 1984). This cross reactivity has been overcome with some success by targeting lipid associated membrane proteins (LAMPs) as antigens, due to the lack of response that antibodies to *M. genitalium* LAMPs show towards the corresponding proteins of *M. pneumoniae* (Wang *et al.*, 1997). In one of the few published evaluations of serological tests for *M. genitalium*, an enzyme linked

immunosorbent (ELISA) assay developed using recombinant fragments of the MgPa adhesin protein (rMgPa) was shown to have a sensitivity and specificity of 59% and 81% respectively, when compared with a polymerase chain reaction (PCR) assay specific to the *mgpB* locus (Svenstrup *et al.*, 2006). The lack of availability of commercial ELISA tests for *M. genitalium* in the UK or USA (Waites *et al.*, 2012), the difficulty of their design, and their poor performance when compared to NAAT tests such as PCR, means that serology based diagnostics are infrequently used in the clinical diagnosis of this organism.

M. genitalium specific PCR and rtPCR assays have been designed for a number of targets, including the 16S rRNA region (Jensen *et al.*, 2003), and genes in the MgPa operon (Jensen *et al.*, 2004). The 16S rRNA gene of *M. genitalium* shares 98% sequence homology with its counterpart in *M. pneumoniae*, complicating the selection of suitable primer binding sites (Jensen *et al.*, 2003a). The use of probe based real-time assays has helped in this regard, due to the added specificity provided by the internal probe sequence (Yoshida *et al.*, 2002). Despite the high level of sequence homology, several published 16S rRNA PCR assays have been demonstrated to have no cross reactivity with *M. pneumoniae* DNA (Eastick *et al.*, 2003), and assay sensitivities of ~10 gene copies per reaction have been reported (Yoshida *et al.*, 2002).

The MgPa operon includes the *mgpA*, *mgpB* and *mgpC* genes, which encode the highly expressed MgPa adhesin protein. Over 4.7% of the entire *M. genitalium* genome is composed of the MgPa operon, and repeated elements known as MgPars, which show significant homology to MgPa, and are interspersed at nine chromosomal locations (Ma *et al.*, 2012). These regions are attractive targets for NAATs, as the repeated sequences afford a high starting copy number per cell, enabling improved assay sensitivities. However, extensive variance has been shown between strains in the MgPar regions, demonstrated to be caused by recombination between the *mgpB* and *mgpC* genes and MgPar sequences (Iverson-Cabral *et al.*, 2007). This recombination results in MgPa antigenic variance of the mycoplasma during the course of infection, assisting the organism in evading the host immune response. Samples taken from a patient persistently infected with a single strain of *M. genitalium* yielded 17 *mgpB* variants when sequenced, illustrating this phenomenon (Iverson-Cabral *et al.*, 2007). The reported variability of these sequences has led to concern over their reliability as targets for NAAT assays, and has encouraged researchers to turn to more highly conserved, single copy targets, despite the reduction in sensitivity. Examples of these single copy targets include the *gap* housekeeping gene, which encodes a glyceraldehydes-3-phosphate dehydrogenase (Svenstrup *et al.*, 2005); the *pdhD* gene, encoding a dihydrolipoamide dehydrogenase (Muller *et al.*, 2012); and the 115-kDa gene (Dupin *et al.*, 2003).

In addition to “in house” assays detailed in the literature, a range of commercial rt-PCR assays are available for diagnostic use in the UK. These include singleplex assays such as the EuroClone (Pero, Italy) Duplica RealTime *Mycoplasma Genitalium* kit, which uses the Mgp-r2 repetitive element as a target, and Tib MolBiol (Berlin, Germany) LightMix Kits, for use on the Roche Lightcycler, which detect the *M. genitalium gap* gene. Multiplex assays capable of detecting a panel of sexually transmitted infections including *M. genitalium* are also on the market. Examples include the Bio-Rad (Hercules, US) CT/NG/MG assay for simultaneous detection of *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* in urogenital samples; The Amplex Biosytems (Giessen, Germany) Hyplex STD Mycoplasma kit for the detection of pathogenic mycoplasma species including *Mycoplasma hominis, M. genitalium, Ureaplasma urealyticum* and *Ureaplasma parvum;* and the Seegene (Seoul, South Korea) Seeplex^{TM} STD detection kit, which enables the user to choose from a range of 12 sexually transmitted pathogens including *M. genitalium*, and simultaneously detect between 4 and 6 different infections per test.

The application of novel isothermal amplification methods to *M. genitalium* detection has been limited. Gen-Probe (San Diego, US) has developed a transcription mediated amplification (TMA) assay for *M. genitalium* detection, for research use. TMA is an isothermal method of amplifying a target DNA or RNA sequence using a pair of target specific primers, and driven by reverse transcriptase and RNA polymerase enzymes (Wroblewski *et al.*, 2006). The reverse transcriptase catalyses the formation of a double stranded DNA template from single stranded RNA target sequences, and the RNA polymerase make copies of the single stranded complementary RNA sequence, providing more template for the reaction. In this exponential manner, TMA is capable of producing over a billion copies of amplicon in 30 minutes, per starting RNA or DNA target copy. The generated amplicon is detected by DNA probes, and fluorescently monitored. A comparison of the assay with a multi target rt-PCR assay, involving the screening of 607 clinical samples (101 positive), found the TMA assay to have both a sensitivity and specificity of 98.1%. The multi target rt-PCR assay had a sensitivity and specificity of 91.8% and 99.5% respectively (Hardick *et al.*, 2006).

To our knowledge, no loop mediated isothermal amplification (LAMP) assay has been designed for *M. genitalium*. Recent reviews of the diagnostic options available for *M. genitalium* detection do not identify any LAMP assays, or indeed alternative isothermal assays, bar the Gen-Probe TMA assay previously mentioned (Waites *et al.*, 2012). A LAMP assay has been published for the detection of *M. pneumoniae* (Saito *et al.*, 2005), which is closely related to *M. genitalium*. An *M. genitalium* LAMP assay would provide another assay to the limited number of diagnostic options for *M. genitalium* diagnosis, the vast majority of which are based on standard PCR. LAMP has several advantages over

PCR, including the potential for increased specificity (Notomi *et al*., 2000), higher tolerance to inhibitors (Francois *et al*., 2011), and speed. LAMP reactions also offer a number of different detection strategies, including the real time monitoring of the reaction via the incorporation of a fluorescent intercalating dye, or the end point detection of amplification using calcein (Tomita *et al*., 2008). The use of calcein mediated end point detection requires no additional equipment, and enables the use of sensitive NAAT diagnostics in a resource poor environment. This would enable rapid, sensitve testing for *M. genitalium* infection in laboratories without either expensive real-time PCR platforms, or thermocycling equipment.

Aims

- To maintain the culture of *M. genitalium*, in order to provide a source of *M. genitalium* DNA to ourselves and also Public Health England.
- To provide confirmation of the suitability of the magnetic silica based extraction system for the purification of nucleic acids from *M. genitalium*.
- To design a PCR assay for the detection of *M. genitalium*, in order to provide a tool for the determination of the successful culture of this organism.
- To determine the analytical sensitivity of PHE designed 16S rRNA LAMP primer sets for *M. genitalium*.
- To design a LAMP assay for a suitable gene target to enable the sensitive detection of *M. genitalium*.
- To determine the limit of detection and analytical specificity of the assay comparing this with gold standard PCR assays for the detection of this organism.

3.2. Materials and Methods

3.2.1. PCR primer design

The sequences of both the *M. genitalium* G37 strain 16S rRNA gene (NCBI Reference Sequence: NC_000908.2, Gene ID: 875400), and *M. genitalium* G37 strain *MgPa* gene (NCBI Reference Sequence: NC_000908.2, Gene ID: 875543) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The design of target specific primers was carried out using Primer-BLAST (http://www.blast.ncbi.nlm.nih.gov/) (Ye *et al*., 2012). Two sets were designed for the *mgpB* gene, designated *M. genitalium mgpB* 1, and *M. genitalium mgpB* 2. One set was designed for the 16S rRNA sequence. The primer sequences and predicted product sizes are shown in Table 3.1.

Primer	Sequence	Length (bp)	Product size (bp)	Annealing Temp (°C)
M. genitalium mgpB 1 F	CTGCACTTACCCTTGGGGTT	20	666	57
M. genitalium mgpB 1 R	ACCTTTGGGTGGGGTTGAAG	20		
M. genitalium mgpB 2 F	AGCAGGGTATCGCGTTCAAA	20	243	58
M. genitalium mgpB 2 R	AGCAACAGTTGATTGCGCTG	20		
M. genitalium 16S rRNA 1 F	TGCCCCTTATGTCTAGGGCT	20	270	58
M. genitalium 16S rRNA 1 R	ATCCTTGACATGCGCTTCCA	20		

Table 3.1. *M. genitalium* PCR primer sequences

3.2.2. LAMP primer Design

LAMP primer sets were designed for the *pdhD* gene of *M. genitalium*. Available *pdhD* gene sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), from the following *M.genitalium* strains; G37, M2288, M2300, M2321, M2341. Sequences were aligned using ClustalX (Chenna *et al*., 2003). LAMP primers were designed using PrimerExplorer Version 4 (http://www.primerexplorer.jp/e/), with the primer binding sites compared to the alignment data to ensure compatibility. The additional optional loop primers were not designed. Primer specificities were checked using BLAST (http://www.blast.ncbi.nlm.nih.gov/). LAMP primers were synthesised commercially (Eurofins, Germany). LAMP primer sequences are shown in Table 3.2.

Primer	Sequence	Length (bp)
M. genitalium pdhD 1 LAMP F3	ACTACAAACATCAACTACAAACA	23
M. genitalium pdhD 1 LAMP B3	ACAAAAGCAACTTCAGGATT	20
M. genitalium pdhD 1 LAMP FiP	TGCCCTGTTGGTAAGCGTAGCTATCTAATAGGTGATGTTAACACG	45
M. genitalium pdhD 1 LAMP BiP	TGCTGTTGATCAAATTTTGAACCATGTAAATACAAGCAGGACACTT	46
M. genitalium pdhD 2 LAMP F3	TCTGAACTGATAAGTAAAACCTT	23
M. genitalium pdhD 2 LAMP B3	TGTTTGTAGTTGATGTTTGTAGT	23
M. genitalium pdhD 2 LAMP FiP	GACTGTTCAACTCCATTAACTGTGTGAGTTCAGATTATTACCAATGCT	48
M. genitalium pdhD 2 LAMP BiP	TAGGAAGAATTGCTAACACAGAGTGTTTTGTTGTTATGGTCACG	45
M. genitalium pdhD 3 LAMP F3	ACAACACAACTTACACCACTA	21
M. genitalium pdhD 3 LAMP B3	TGGTCACTTCACTCCCTAA	19
M. genitalium pdhD 3 LAMP FiP	GTGCTTTTTCAAACCCTGGTAAAGTACAACATTATTGTTGCAACCG	46
M. genitalium pdhD 3 LAMP Bip	CATTGACTCAACCCAAGCTTTGGACTCAACCCCAATCACAC	41

Table 3.2. M. genitalium *pdhD* LAMP primer sequences.

3.2.3. Public Health England *M. genitalium* 16S rRNA LAMP primer sets

Sequences were provided by Public Health England for their *M. genitalium* 16S rRNA LAMP primer set. The primer sequences are shown in Table 3.3.

Primer	Sequence	Length (bp)
M. genitalium 16S F3	CGTGAACGATGAAGGTCTT	20
M. genitalium 16S B3	ACCACACTCTAGACTGATAGTT	22
M. genitalium 16S FiP	GCGACTGCTGGCACATAGTAATGACTCTAGCAGGCAATG	39
M. genitalium 16S BiP	ACATAGGTCGCAAGCGTTATCCCTGCCTTTAACACCAGACTT	42
M. genitalium 16S LoopF	TGGTACAGTCAAACTCCAGC	20
M. genitalium 16S LoopB	GGATTTATTGGGCGTAAAGCAA	22

Table 3.3. Sequences of the Public Health England *M. genitalium* 16S rRNA LAMP primer sets

3.2.4. Culture

M.genitalium (NCTC 10195) was cultured on Mycoplasma Agar Base (Oxoid, UK), containing 20% Mycoplasma Supplement G (Oxoid, UK). Plates were inoculated aerobically in a moist environment at 37^{o}C. Colonies were typically detectable using a dissecting microscope at a 60x magnification after a period of 2 to 3 weeks.

Broth cultures were also used, from the initial freeze dried culture. 100µl of *rehydrated M. genitalium* cell suspension was added to 100ml of mycoplasma broth base, containing 20% mycoplasma supplement G (Oxoid, UK). Cultures were incubated aerobically in a moist environment at 37^{o}C. DNA was extracted from 200µl of the culture, and tested using *M. genitalium* mgpB and 16S rRNA PCR assays for the presence of *M. genitalium*.

3.2.5. DNA extraction

M. genitalium cells were harvested from solid media plates after a 3 week period post inoculation, when colonies were visible with the aid of a dissecting microscope. As much material was removed from the plates as possible, using a cotton swab, and transferred to a 1ml aliquot of molecular grade water. Alternatively, if broth cultures were used, a 100µl volume of culture was removed from the vessel. The cells were then concentrated by centrifugation at 30,000 x g for 15 minutes at 4^{o}C. DNA extractions were carried out using a QIAmp DNA mini kit (QIAGEN Inc., USA), following the protocol for bacterial cells. RNase A (New England Biolabs, UK) was used during the RNA digestion step. Alternatively, in section 3.2.11, extractions were carried out on the centrifuged cells using the total nucleic acid extraction methodology (1.2.7). DNA was quantified spectrophotometrically using Spectrostar plate reader in conjunction with an LVIS plate (BMG Labtech, UK). Genome copy number was worked out from the size of the *M. genitalium* genome (580kb), and the following equation; 5.8 $\times 10^{5}$ bp $\times$ 665 Da/bp $\times$ 1.67 $\times 10^{-24}$ g/Da).

3.2.6. LAMP Reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), as follows; 5µl five times LAMP reaction buffer, 1µl intercalating dye (propriety dye, emission in FAM channel), 1µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 1µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers, 12µl molecular grade water. Finally 5µl of DNA sample or water was added to the reaction. Reactions were carried out in an ESEQuant Tube Scanner (Qiagen Inc., CA), at 63^{o}C for 60 minutes. Reactions were terminated by heating to 80^{o}C for 1 minute in order to denature the *Bst* polymerase. The ESEQuant Tube Scanner allows for real-time monitoring of the LAMP reactions, via optical detection of the fluorescent intercalating dye through the FAM channel. The “Tube Scanner Studio” software package was used to analyse the real time data and produce a manually set threshold line for the purposes of determining time to amplification (Ta).

3.2.7. PCR Reactions

PCR reactions were set up as follows; 25µl RedTaq reaction mix (Sigma Aldrich, UK), 0.5pmol Forward primer, 0.5pmol reverse primer. Reactions were then made up to 45µl with water, and 5µl DNA (or water as a no template control) was added. Reaction conditions were as follows; 94^{o}C for 5 minutes initial denaturation; 35 cycles of 94^{o}C for 30 seconds, the correct primer annealing temperature (specific to each primer set) for 30 seconds and 72^{o}C for 30 seconds; 72^{o}C for 5 minutes final extension. Reactions were heated using an ABI 2720 thermocycler (Applied Biosystems, UK). PCR reaction products were separated on 2% % w/v agarose gels, with transillumination and photography provided by a BioRad Gel/Chem Doc system (Bio-Rad Inc, UK).

3.2.8. Detection of *M. genitalium* in broth culture by PCR

A 100µl aliquot of the rehydrated freeze dried *M. genitalium* cell suspension purchased from NTCC had been reserved in order to provide a positive control, and a source of DNA in case culture proved impossible. DNA was extracted from both the original inoculum, and also from 100µl of the first attempt at a broth culture, that had been incubating for a period of 1 week. PCR reactions were then carried out with both extracted DNA samples, using the *M. genitalium* specific primer sets mgpB 1, mgpB 2 and 16S rRNA 1. For each primer set, a reaction was carried out with molecular grade water in place of the DNA sample, as a negative control. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised.

3.2.9. Detection of *M. genitalium* from culture on solid media by PCR

Cells were harvested, and DNA extracted, from an agar plate of *M.genitalium* culture as described above. The plates were harvested at 3 weeks post inoculation. PCR reactions were then carried out with the extracted DNA sample, using the *M. genitalium* specific primer sets mgpB 1, mgpB 2 and 16S rRNA 1. For each primer set, a reaction was carried out with molecular grade water in place of the DNA sample, as a negative control, and a reaction with DNA extracted from the initial freeze

dried cell suspension, as a positive control. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised.

3.2.10. Sensitivities of the *M. genitalium* 16S rRNA PCR and *mgpB* 2 PCR assays

DNA was extracted from *M. genitalium* cultured on solid media. The DNA was quantified spectrophotometrically, and the sample was diluted to 2ng/µl. As 5µl of DNA is used in each assay, this equates to 0.1µg per individual reaction. This stock DNA was then sequentially diluted 1:10 in molecular grade water to a level of 0.2fg/µl. PCR reactions were then carried out using the *M. genitalium* 16S rRNA and *mgpB* 2 PCR assays for each dilution of DNA from 20pg/µl to 0.2fg/µl. Molecular grade water was used as a negative control for each assay. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised.

3.2.11. Sensitivity of the 16S rRNA LAMP assay

DNA was extracted from *M. genitalium* cultured on solid media, using the total nucleic acid extraction protocol. The DNA concentration was measured spectrophotometrically, and the sample was diluted to 2ng/µl. As 5µl of DNA is used in each assay, this equates to 0.1µg per individual reaction. This stock DNA was then sequentially diluted 1:10 in molecular grade water to a level of 0.2fg/µl. PCR reactions were then carried out using the *M. genitalium* 16S rRNA PCR assay for each DNA dilution from 20pg/µl to 0.2fg/µl. LAMP reactions using the 16s rRNA primer set were then also carried out for each DNA dilution. Molecular grade water was used in lace of DNA as a negative control for both assays. Reaction products PCR reactions were then separated using agarose gel electrophoresis (2% w/v gel), and visualised. LAMP reactions were monitored in real-time using the ESEQuant tube scanner.

3.2.12. Initial testing of *pdhD* LAMP primer sets

DNA was extracted from *M. genitalium* colonies as described in section 2.1.4. The DNA concentration was measured, and the sample was diluted to 2ng/µl. A single positive reaction, containing the extracted DNA, and a single negative reaction, containing molecular grade water was carried out for each of the 3 *pdhD* primer sets (sequences shown in Table.2.).

3.2.13. Comparison of *pdhD* LAMP reactions with *pdhD* PCR, and notable M. *genitalium* PCR assays from the literature

DNA was extracted from *M. genitalium* colonies as described in section 2.1.4. The DNA concentration was measured, and the sample was diluted to 2ng/µl. As 5µl of DNA extract is used in each assay, this equates to 0.1µg per individual reaction. This stock DNA was then sequentially diluted 1:10 in molecular grade water to a level of 0.2fg/µl. Each dilution from 20pg/µl to 0.2fg/µl was then used as the template for a LAMP reaction using the *pdhD* 1 and *pdhD* 3 LAMP sets. PCR reactions were also carried out on the same DNA dilutions using the F3 and B3 primers from the *pdhD* 1 and *pdhD* 3 LAMP sets, and the annealing temperature and predicted product size is shown in Table 3.4.

Primer	Sequence	Length (bp)	Product size (bp)	Annealing Temp (°C)
M. genitalium pdhD 1 LAMP F3	ACTACAAACATCAACTACAAACA	23	183	52
M. genitalium pdhD 1 LAMP B3	ACAAAAGCAACTTCAGGATT	20		
M. genitalium pdhD 3 LAMP F3	ACAACACAACTTACACCACTA	21	225	51
M. genitalium pdhD 3 LAMP B3	TGGTCACTTCACTCCCTAA	19		

Table 3.4. Primer sequences and properties of the *pdhD* 1 and *pdhD* 2 F3 and B3 PCR reactions.

The same dilutions of extracted *M. genitalium* DNA were tested using two prominent PCR assays from the literature, a 16S rRNA PCR assay (Jensen *et al*., 2003), and a *MgPa* assay (Jensen *et al*., 2004). The primer sequences, annealing temperatures and expected product sizes are detailed in Table 3.5.

Primer	Sequence	Length (bp)	Product size (bp)	Annealing Temp (°C)
MgPa-355F	GAGAAATACCTTGATGGTCAG CAA	24	78	62
MgPa-432R	GTTAATATCATATAAAGCTCT ACCGTTGTTATC	33		
MG16-45F	TACATGCAAGTCGATCGGAA GTAGC	25	425	60
MG16-447R	AAACTCCAGCCATTGCCTGCT AG	23		

Table 3.5. Primer sequences and properties of the 16s rRNA PCR assay from Jensen *et al*. (2003), and *MgPa* PCR assay detailed in Jensen *et al*. (2004).

The reaction conditions for the 16S rRNA assay were as described in Jensen *et al* (2003), with the exception that Redtaq reaction mix (Sigma, UK) was used in place of the separate reaction components and enzyme previously detailed. The reaction contained 0.4µM of each primer. A touchdown PCR methodology is used in order to promote higher specificity. After an initial 94°C denaturing step for 2 minutes, 10 cycles of the following steps were carried out; denaturation at 94°C for 15 seconds; annealing at 72°C to 62°C for 30 seconds, with a 1 degree decrease per cycle; extension at 72°C for 15 seconds. A further 30 cycles were performed with the following steps; denaturation at 92°C for 15 seconds; annealing at 62°C for 30 seconds; extension at 72°C for 30 seconds.

The reaction conditions for the *MgPa* PCR assay were as described in Jensen *et al*. (2004), with a number of modifications. As the primer set was being used for conventional PCR, rather than real time PCR, for which the assay was designed, the inclusion of a an intercalating dye was not necessary. Also, the reactions could be performed in a standard thermocycler (ABI 2720), rather than the Smartcycler (Cepheid) used in the original study. Redtaq reaction mix (Sigma) was used in

place of the separate components and Hot Gold Star *taq* polymerase used in the original study, which necessitated an increase in reaction volume to 50µl. The reaction contained a 1µM concentration of each primer. A touchdown PCR methodology is used in order to promote higher specificity. After an initial 94°C denaturing step for 2 minutes, 1 cycle of the following steps were carried out; denaturation at 94°C for 15 seconds; annealing at 64°C for 30 seconds; extension at 72°C for 30 seconds. The next cycle was performed with the following steps; denaturation at 94°C for 15 seconds; annealing at 64°C for 30 seconds; extension at 72°C for 30 seconds. A further 48 cycles were performed with the following steps; denaturation at 95°C for 15 seconds; annealing at 60°C for 30 seconds; extension at 72°C for 30 seconds.

3.2.13. Specificity of the *pdhD* 1 LAMP assay

DNA was extracted from the following organisms using a QIAmp DNA mini kit (QIAGEN Inc., USA), following the protocol for bacterial cells; *N. gonorrhoeae* (ATCC 19424), *C. trachomatis* (lab strain, serovar L2), *T. vaginalis* (ATCC 30001) *Pseudomonas aeruginosa* (ATCC 15692), and *Escherichia coli* (lab strain). *M. pneumoniae* (NCTC 010119) genomic DNA was purchased commercially from Minerva Biolabs Gmbh (Germany).

DNA extractions from two clinical cervical swab samples which had been confirmed positive for *Ureaplasma urealyticum* and *Ureaplasma parvum* DNA using the PCR assays detailed in Cultrela *et al*. (2006), and negative for *M. genitalium* DNA using the 16S rRNA PCR assay (12) were used as a source of *U. urealyticum* and *U. parvum* DNA. A clinical cervical swab sample which had tested positive for *M. genitalium* DNA by 16S rRNA PCR was used as a clinical *M. genitalium* specimen. All clinical samples were negative for *C. trachomatis* and *N. gonorrhoeae* DNA, as determined by the APTIMA COMBI 2 assay (GenProbe, US).

Each DNA sample was diluted to 20ng/µl and 5µl of each DNA sample (0.1µg) was used as a template in the *pdhD* LAMP reaction, to test the specificity of the primers when in contact with non-target DNA, from organisms commonly found in samples from patients with urethritis.

3.3. Results

3.3.1. Detection of *M. genitalium* in broth culture by PCR

Due to the difficulty in visually detecting *M. genitalium* growth using liquid or solid media, order to determine the success of the initial culture of *M. genitalium* from the purchased cell stock, it was necessary to design a PCR assay capable of amplifying *M. genitalium* genomic DNA. Although standard endpoint PCR assays were developed, by comparing the brightness of the amplified product on an agarose electrophoresis gel, a comparison of relative product abundance, and hence starting copy number, can be made. PCR reactions were carried out on DNA extracted from both the rehydrated freeze dried inoculum (+) and first attempted broth culture, 1 week post inoculation (1). PCR reactions containing the *mgpB* 1, *mgpB* 2 and 16S rRNA primer sets were used. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised (Fig.3.1).

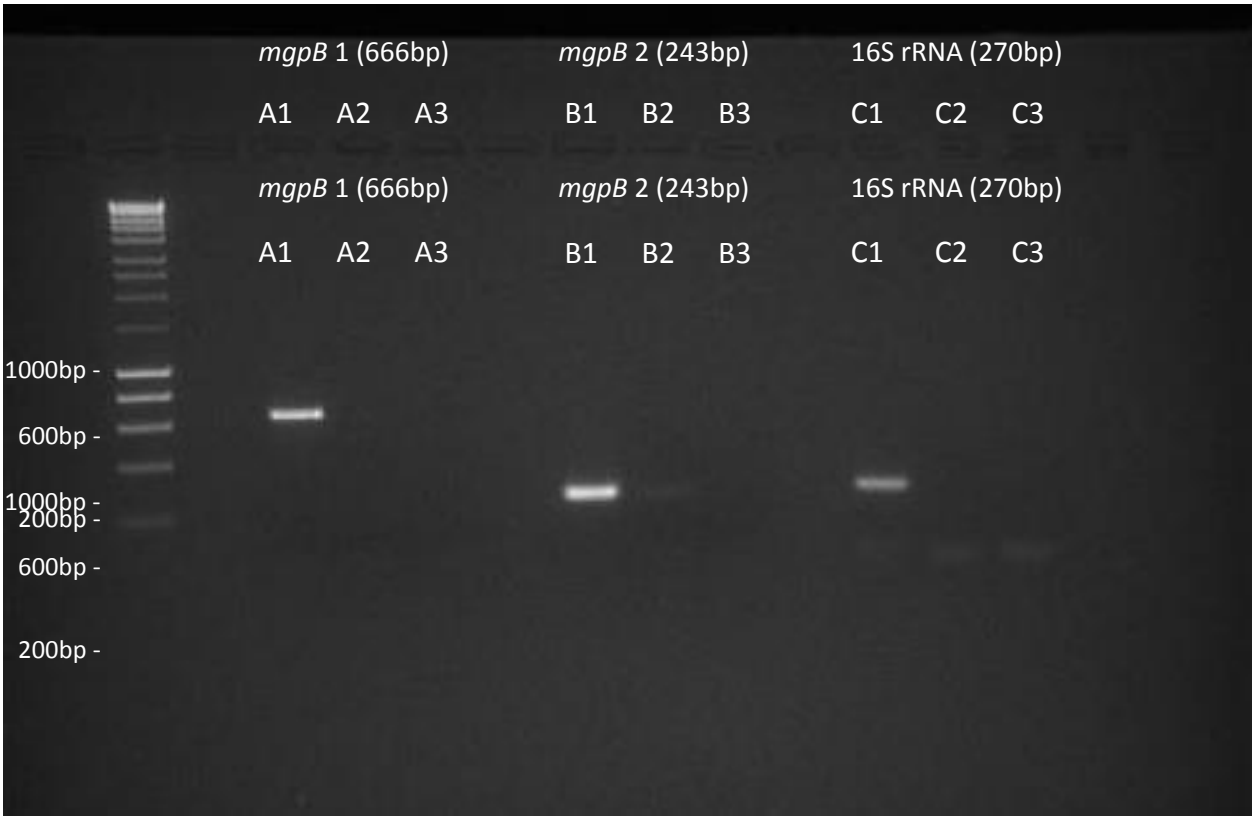

Fig.3.1. Agarose gel electrophoresis (2%) of reaction products from *mgpB* 1 (A), *mgpB* 2 (B) and 16S rRNA (C) PCR reactions, from DNA extracted from the rehydrated freeze dried inoculums (1), 1 week post inoculation broth culture (2), and molecular grade water (3).

The mgpB *1*, mgpB *2*, and 16S rRNA PCR assays all generated a product of the expected size from the positive control DNA; 666bp, 243bp, and 270bp, respectively (Fig.3.1). Additionally, no amplification was generated by any assay from the no template control, indicating a lack of baseline non-specific amplification. The *mgpB* 1 and 16S rRNA assay both failed to generate any product from the DNA extracted from the broth culture, indicating a failure of the culture to generate any biomass. The *mgpB* 2 assay did manage to generate a low level of product, faintly visible on the agarose gel at 243bp. This is likely to have been amplified from DNA present in the initial inoculum, indicating that the *mgpB* 2 assay is potentially the most sensitive PCR assay of the three tested.

3.3.2 Detection of *M. genitalium* from culture on solid media by PCR

The PCR assays used to test liquid culture (section 3.3.1) were also used to investigate whether the culture of *M. genitalium* on solid media had been successful.

PCR reactions were carried out on DNA extracted from both the rehydrated freeze dried inoculum (+) and *M. genitalium* cultured on solid media, 3 weeks post inoculation (1). PCR reactions containing the *mgpB* 1, *mgpB* 2 and 16S rRNA primer sets were used. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised (Fig.3.2).

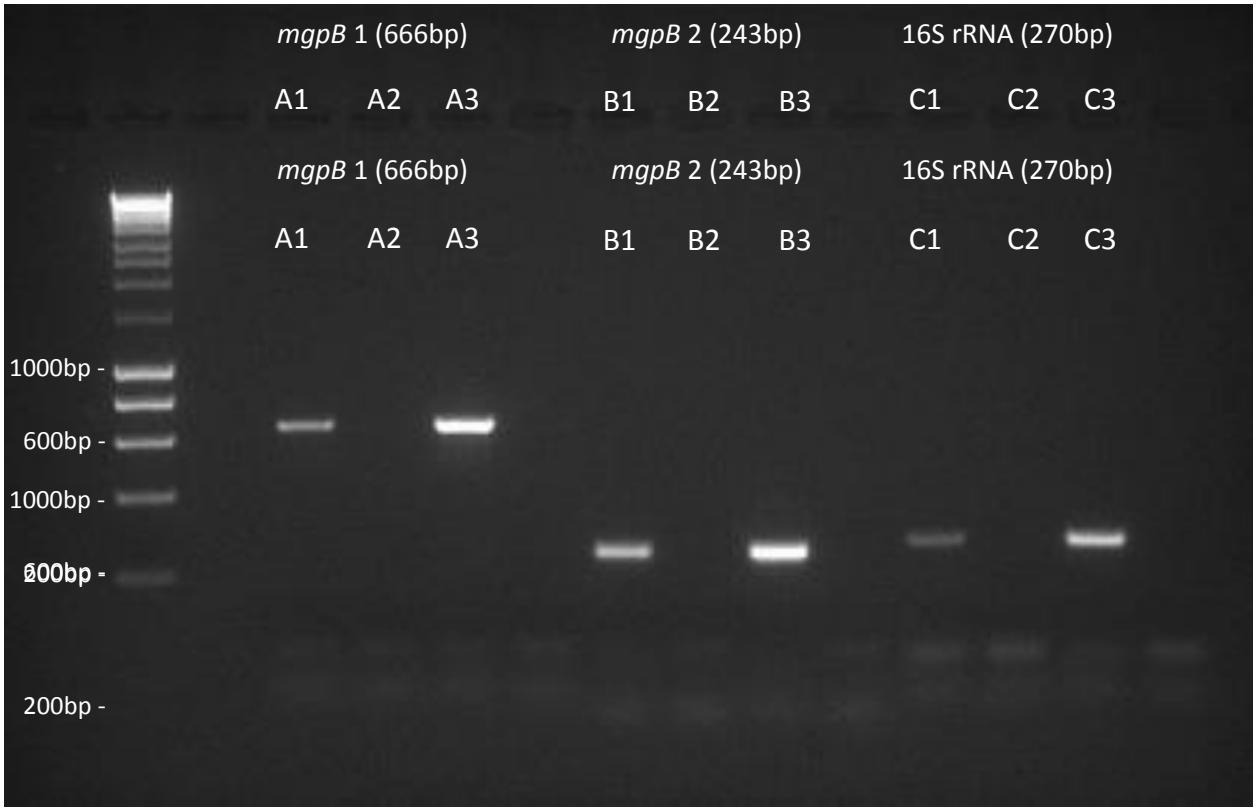

Fig.3.2. Agarose gel electrophoresis (2%) of reaction products from *mgpB* 1 (A), *mgpB* 2 (B) and 16S rRNA (C) PCR reactions, from DNA extracted from the rehydrated freeze dried inoculums (1), *M. genitalium* cultured on solid media (3), and molecular grade water (2).

As in Fig.3.1, all three PCR assays generated an amplified product from the positive control DNA, whilst failing to generate a product from the no template control (Fig.3.2). The *mgpB* 1, *mgpB* 2, and 16S rRNA assay all generated a product of the expected size from the DNA extracted from harvested cells from solid media, indicating the successful growth of *M. genitalium* cells by this method. The increased brightness and size of the amplified products from the cultured cells compared to the initial cell stock, when visualised using agarose gel electrophoresis, shows that the copy number, and hence cell mass, has increased.

3.3.3. Sensitivities of the *M. genitalium* 16S rRNA PCR and *mgpB* 2 PCR assays

In order to provide a comparison with the *M. genitalium* LAMP assays in development, and also be able to use the PCR assays to give an indication of copy number, the limits of detection of the 16S rRNA PCR and *mgpB* 2 PCR assays were determined. The *mgpB* 1 assay was not included in the study

as it appeared less sensitive than the *mgpB* 2 assay (Fig.3.1), and the excessive product size (666bp) is likely to strain the PCR reaction and prove disadvantageous.

PCR reactions were carried out on a dilution series of *M. genitalium* DNA, using the *mgpB* 2 and 16S rRNA primer sets. Final amounts of DNA per reaction of 0.1ng, 10pg, 1pg, 0.1pg, 10fg, 1fg. Molecular grade water was used as a negative control. PCR products were then separated using agarose gel electrophoresis (2% w/v gel), and visualised (Fig.3.3).

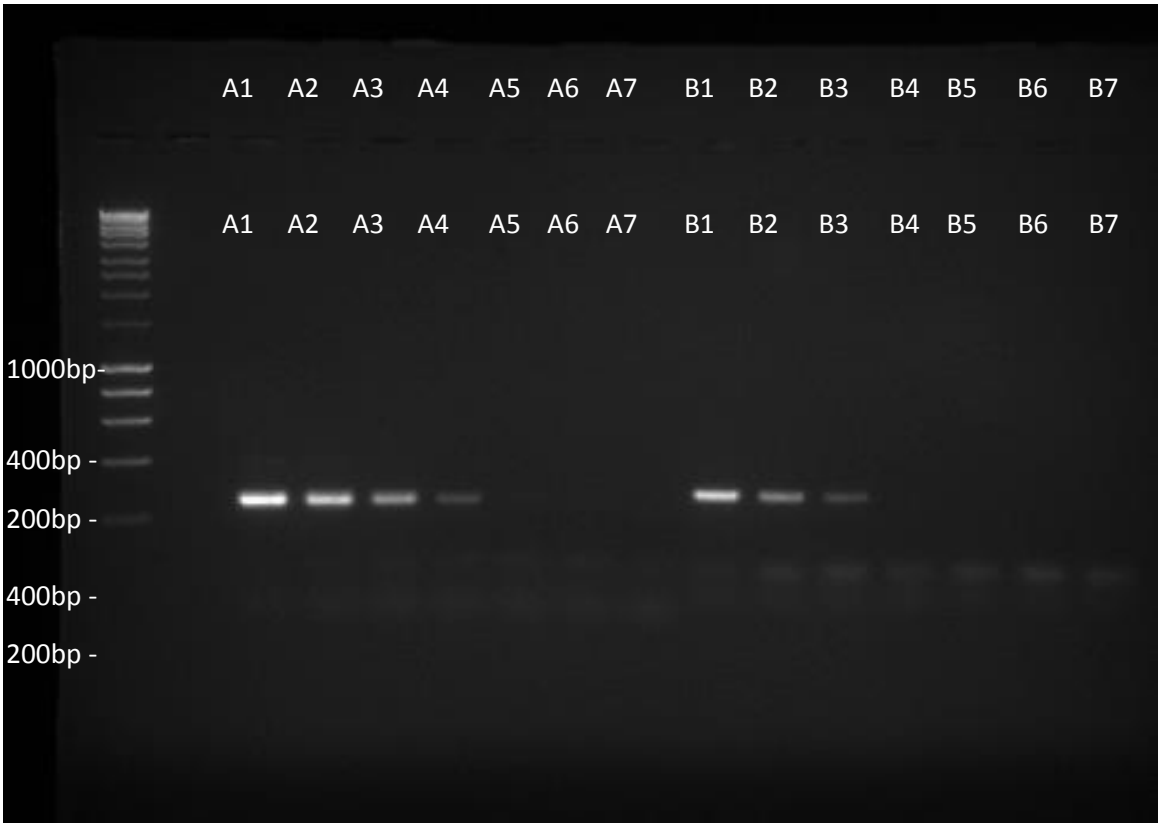

Fig.3.3. Agarose gel electrophoresis of reaction products of PCR reactions using primer sets *mgpB* 2 (A) and 16S rRNA (B). Final amounts of *M. genitalium* DNA per reaction were 0.1ng (A1, B1), 10pg (A2, B2), 1pg (A3, B3), 0.1pg (A4, B4), 10fg (A5, B5), 1fg (A6, B6). Molecular grade water was used in place of DNA as a negative control (A7, B7).

The 16S rRNA PCR reaction was able to detect a minimum of 1pg of purified *M. genitalium* DNA. The *mgpb* 2 assay was able to detect 0.1pg of *M. genitalium* DNA and above; a 10-fold increase in sensitivity when compared to the 16S rRNA assay. When visualised using agarose gel

electrophoresis, the size and brightness of the 243bp (*mgpB* 2) and 270bp (16S rRNA) products proportionally increased with an increase in starting DNA concentration.

3.3.4. Sensitivity of the 16S rRNA LAMP assay

In order to determine the sensitivity of the *M. genitalium* 16S rRNA LAMP assay it was necessary to identify the minimal concentration of *M. genitalium* genomic DNA detectable by the assay. As *M. genitalium* cells are too small to perform cell counts in broth culture, and the colonies are too small to perform plate counts, starting copy number was worked out from the concentration of genomic DNA in order to quantify the number of *M. genitalium* genomes present. DNA was extracted using the total nucleic acid extraction methodology, to ensure the capatability of this process with M. genitalium cells.

PCR reactions were carried out on a dilution series of *M. genitalium* DNA, using the 16S rRNA primer set. Final amounts of DNA per reaction were of 0.1ng, 10pg, 1pg, 0.1pg, 10fg, 1fg. LAMP reactions were carried out with the same diluted DNA samples, using the 16S rRNA LAMP primers provided by Public Health England. The reaction products from both the LAMP and PCR assays were separated using agarose gel electrophoresis (2% w/v gel), and visualised (Fig.3.4). The LAMP reactions were monitored in real-time using the ESEQuant tube scanner (Fig.3.5).

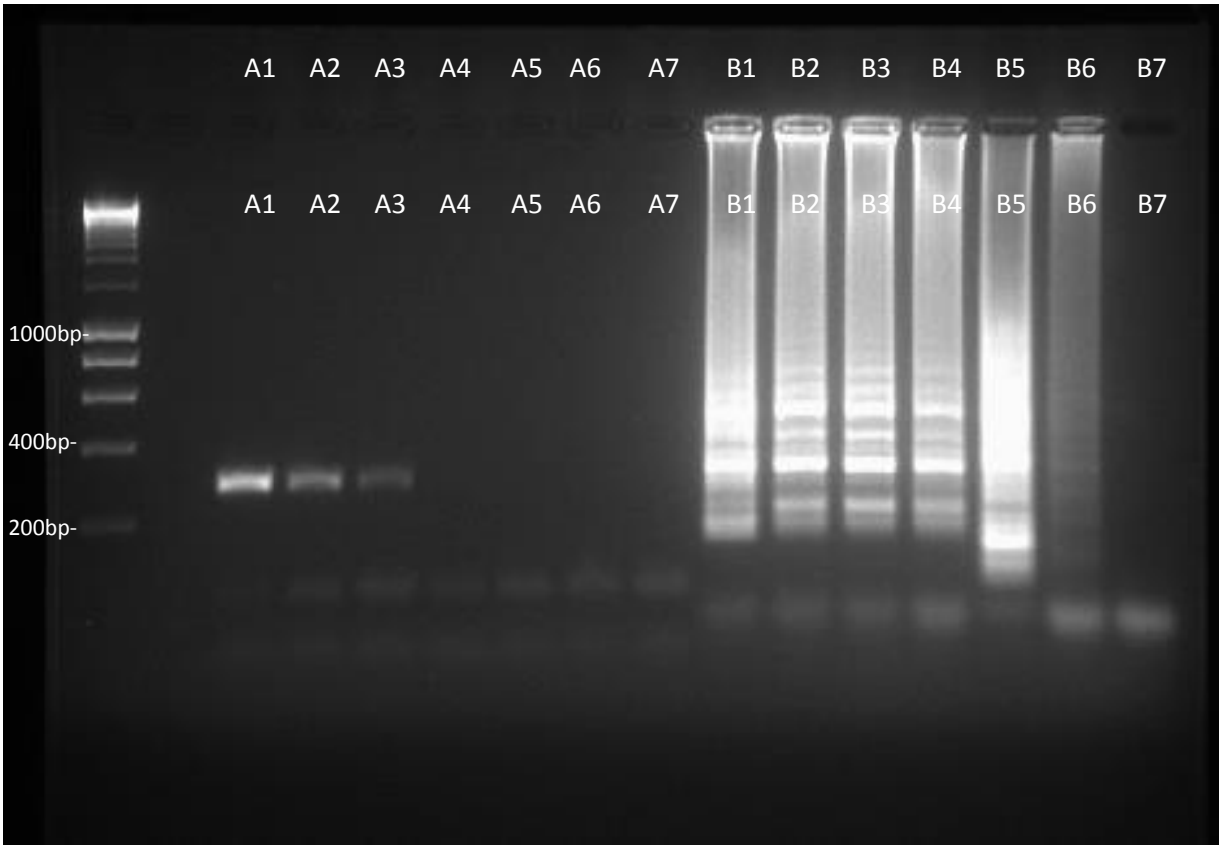

Fig.3.4. Sensitivity of the 16S rRNA LAMP reaction compared with the 16S rRNA PCR assay. Reaction products of the 16S rRNA PCR (A) and 16S rRNA LAMP (B) reactions were separated by agarose gel electrophoresis. Final amounts of *M. genitalium* DNA per reaction were 0.1ng (A1, B1), 10pg (A2, B2), 1pg (A3, B3), 0.1pg (A4, B4), 10fg (A5, B5), 1fg (A6, B6). Molecular grade water was used in place of DNA as a negative control (A7, B7).

The 16S rRNA PCR assay was able to detect a minimum concentration of 1pg of *M. genitalium* genomic DNA, evidenced by the presence of a visible 270bp product after agarose gel electrophoresis of the reaction products (Fig.3.4). The 16S rRNA LAMP assay was able to generate an amplified product from 1fg of *M. genitalium* DNA. Agarose gel electrophoresis of the reaction products enabled visualisation of the characteristic ladder like LAMP reaction products, formed by a range of DNA concatamers of varying size. The amount of product produced in the reaction containing 1fg was noticeably lower than in the other reactions, indicating the low starting copy number.

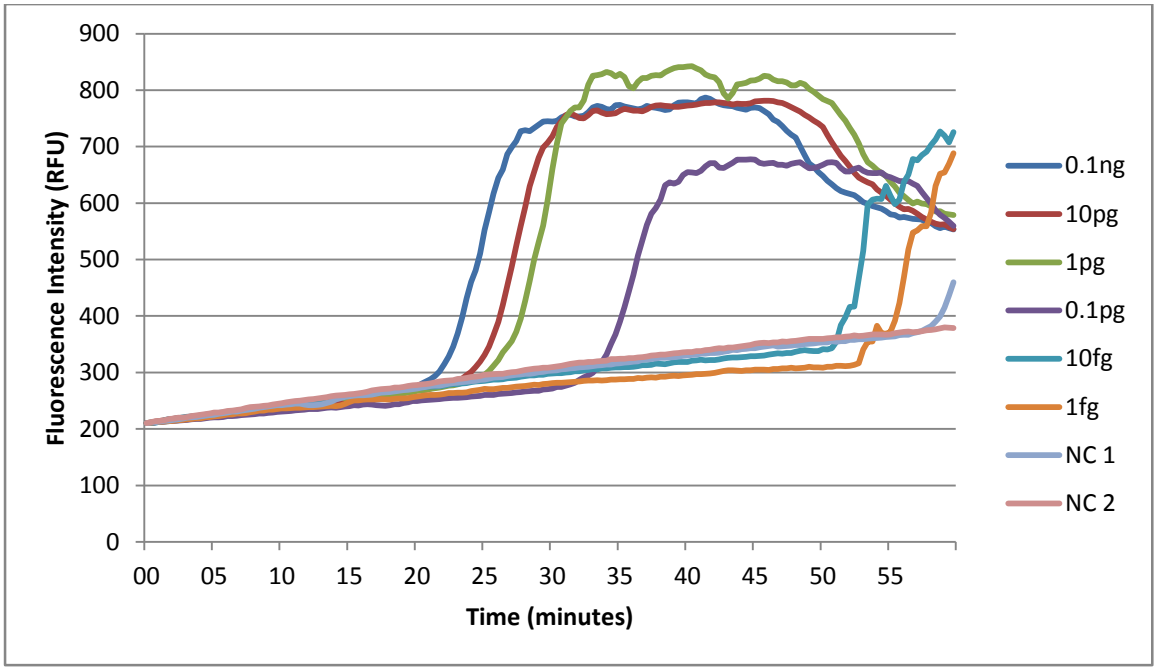

Fig.3.5. Real-time amplification curves of *M. genitalium* 16S rRNA LAMP reactions, showing assay sensitivity. LAMP reactions were carried out on a dilution series of DNA, with final amounts of *M. genitalium* DNA per reaction of 0.1ng, 10pg, 1pg, 0.1pg, 10fg, 1fg. The separation of these reaction products by agarose gel electrophoresis is shown in Fig.3.4.

Real time monitoring of the 16S rRNA LAMP reactions showed that the fastest time to amplification (ta) was seen in the reaction containing the largest amount of DNA, 0.1ng (Fig.3.5; Table 3.6), which generated a detectable amplification at 23 minutes. The reactions containing 10pg, 1pg and 0.1pg generated detectable amplification at 25.9, 27.3 and 35.1 minutes respectively. There was then a delay of 20.5 minutes before amplification was generated in the reactions containing 10fg and 1fg, at 55.8 and 55.6 minutes respectively. In this test, both the positive and negative reactions showed a gradual baseline increase. This was not due to amplification of the target sequence, as it occurred in the negative reaction, and agarose electrophoresis of the reaction showed a lack of product. Also, an amplification would produce a typical amplification curve. It is possible that the caps of the tubes in this run were not tight, enabling evaporation of the liquid, which would act to concentrate the fluorophore in the reaction, increasing the baseline fluorescence.

Amount of DNA per reaction	Time to amplification (Minutes)
0.1ng	23
10pg	25.9
1pg	27.3
0.1pg	35.1
10fg	55.8
1fg	55.6
Negative 1	58
Negative 2	n/a

Table.3.6. Time to amplification values of *M. genitalium* 16S rRNA LAMP assay calculated from amplification curves shown in Fig.3.5, using the Tube Scanner Studio software package.

3.3.5. Initial testing of *pdhD* primer sets

Three LAMP assays specific to the *pdhD* gene of *M. genitalium* were designed, and an initial evaluation was carried out in order to determine whether the primer sets would be able to amplify a product from *M. genitalium* DNA, and also whether they generated non-specific amplification in the absence of a template. LAMP assays for the same target can vary considerably in their speed and specificity, due to variations in primer sequences and the position of the loop sequences. Real time monitoring would provide a comparison of the reaction speed of all of the primer sets.

A single positive reaction, containing 0.1µg of *M. genitalium* DNA, and a single negative reaction containing molecular grade water was carried out using the *pdhD* 1, *pdhD* 2 and *pdhD* 3 LAMP primer sets. The reactions were monitored in real time to compare the reaction efficiency of each primer set.

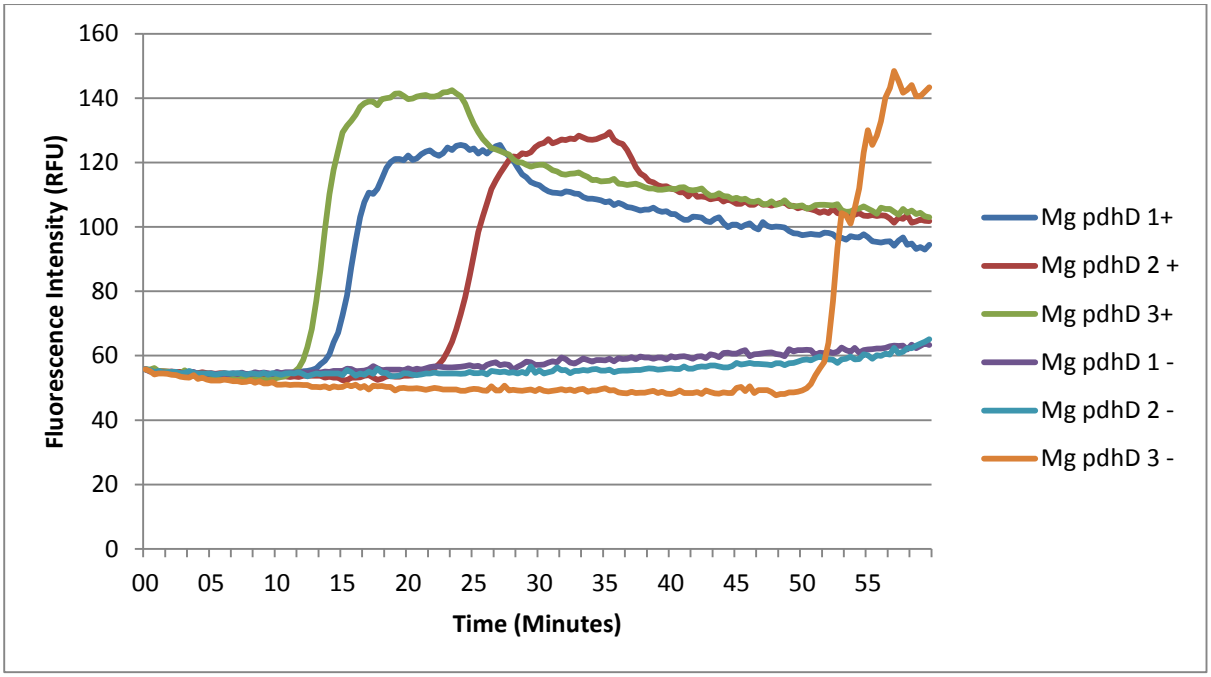

Fig.3.6. The initial screening of pdhD LAMP primer sets 1, 2 and 3. A single positive and negative reaction were carried out using each primer set and monitored in real time.

Real time monitoring of the LAMP reactions containing various *pdhD* primer sets showed that the *pdhD* 3 primer set had the shortest ta, generating a detectable signal at 12.3 minutes (Fig.3.6). The *pdhD* 3 set also generated an amplification in the negative control reaction, which contained no DNA template. This amplification occurred at 51 minutes, and so was likely to be a non-specific amplification, rather than the result of any contamination of DNA in the reaction. The *pdhD* 1 and *pdhD* 2 sets had a Ta of 13.8 and 22.9 minutes, respectively, and did not generate an amplification in the negative control. The sizeable delay in amplification when using the *pdhD* 2 set is indicative of a less efficient LAMP primer set.

3.3.6. Comparison of *pdhD* LAMP reactions with *pdhD* PCR, and notable *M. genitalium* PCR assays from the literature

In order to determine the sensitivity of the *pdhD* LAMP reactions, the minimum concentration of *M. genitalium* genomic DNA detectable by the *pdhD* 1 and *pdhD* 3 LAMP assays was determined. The *pdhD* 2 LAMP assay was not included in the tests due to its poor performance in previous tests (Fig.3.6). Using the same dilutions of DNA, the detection limits of the *pdhD* PCR assay and two prominent PCR assays from the literature, one targeting the 16S rRNA sequence (Jensen *et al*.,

2003), and one targeting the *mgpB* gene (Jensen *et al*., 2004). These assays use multi copy targets, in order to maximise sensitivity, and are the two most common non-commercial PCR tests. This was done in order to provide a relative comparison of analytical sensitivity.

The limit of detection of the following assays were determined; the *pdhD* 1 and *pdhD* 3 LAMP assays, PCR assays using the F3 and B3 primers of the *pdhD* 1 and *pdhD* 3 LAMP sets, the 16S rRNA PCR assay (Jensen *et al*., 2003), and the *mgpB* PCR assay (Jensen *et al*., 2004). Reactions were carried out using each assay, on a dilution series of *M. genitalium* DNA. Final amounts of DNA per reaction were 0.1ng, 10pg, 1pg, 0.1pg, 10fg, 1fg. The experiments were all carried out on the same DNA samples, over a period of 2 days. DNA samples were stored at -80^{o}C when not in use.

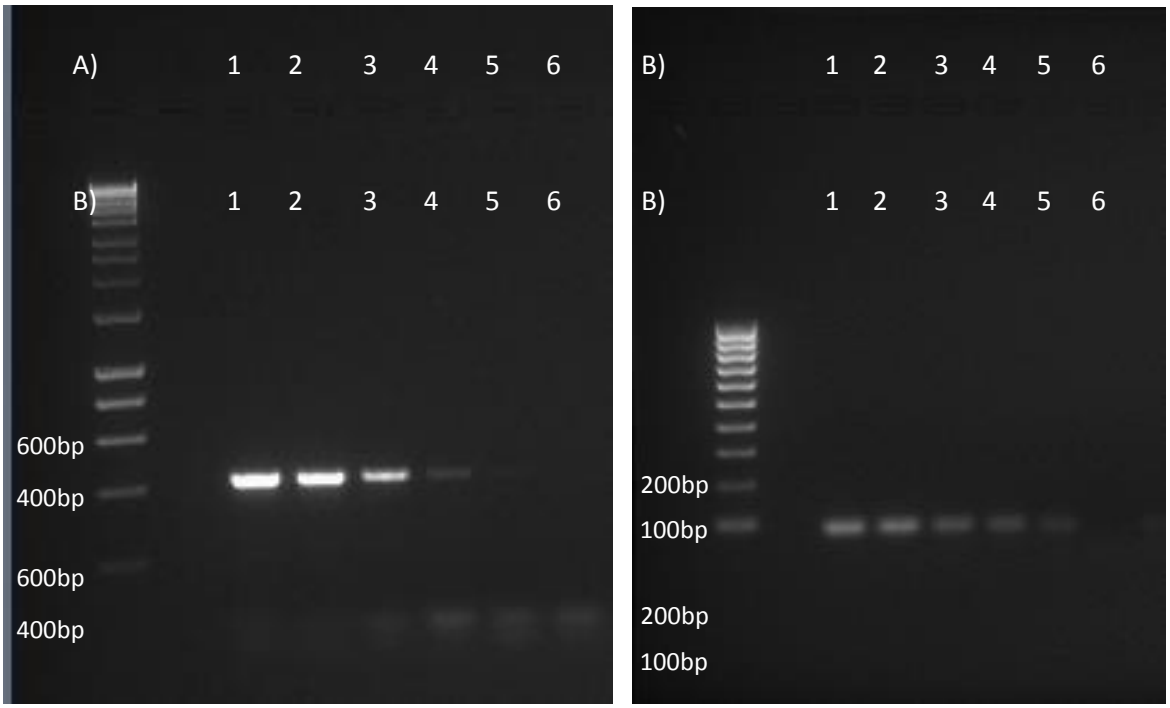

Fig.3.7. Agarose gel electrophoresis of PCR products showing the limits of detection of the 16S rRNA PCR assay from Jensen *et al*. (2003) (A), and the *MgPa* PCR assay detailed in Jensen *et al*. (2004) (B). PCR reactions with each primer set, containing the following concentrations of DNA were carried out; 0.1ng (1), 10pg (2), 1pg (3), 0.1pg (4), 10fg (5), 1fg (6). The expected fragment sizes were 425bp for the 16S rRNA assay (A), and 78bp for the *MgPa* assay (B).

The 16S rRNA PCR assay (Jensen *et al*., 2003) was able to generate a 425bp product from a minimum of 0.1pg of *M. genitalium* DNA (Fig.3.7. A). The *MgPa* PCR assay (Jensen *et al*., 2004) was able to

detect 10fg of DNA, a further 1:10 dilution. When visualised via agarose gel electrophoresis, the product from the *MgPa* assay was less bright than that of the 16S rRNA assay, due to the small product size of 78bp reducing the number of ethidium bromide molecules able to bind, and therefore produce fluorescence.

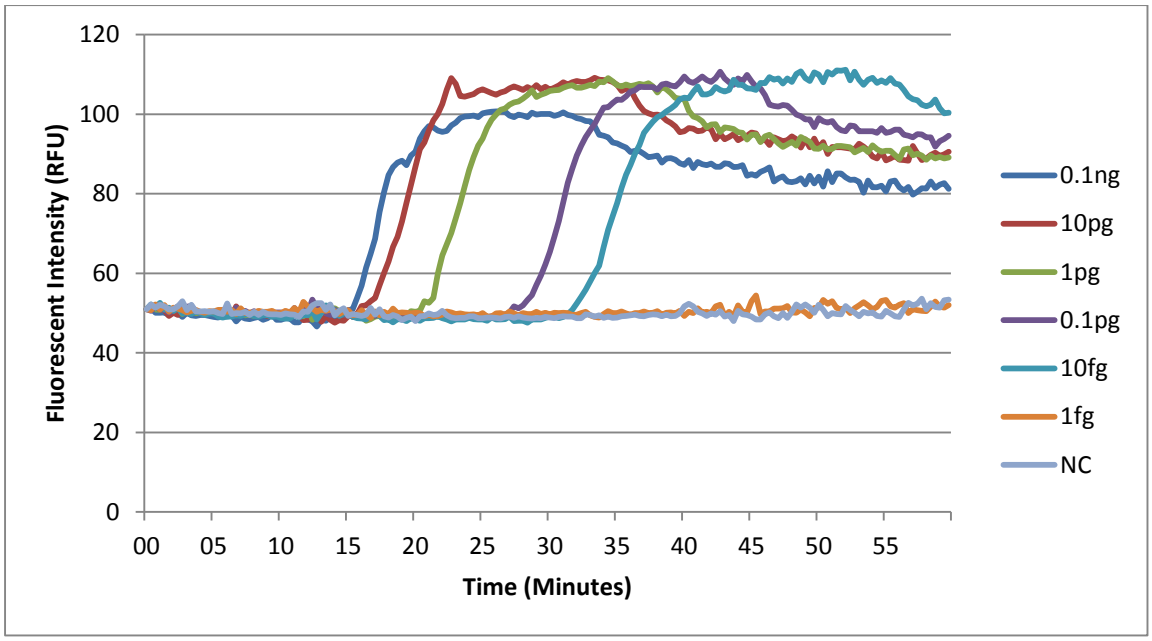

Fig.3.8. Amplification curves showing sensitivity of the *pdhD* 1 LAMP assay. LAMP reactions were carried out on a dilution series of DNA, with final amounts of *M. genitalium* DNA per reaction of 0.1ng, 10pg, 1pg, 0.1pg, 10fg, 1fg, 0.1fg. Molecular grade water was used as a negative control (NC).

The *pdhD* 1 LAMP assay was able to detect a minimum amount of 10fg of *M. genitalium* genomic DNA (Fig.3.8). Real time monitoring of the reactions showed that the amplification time for the reactions containing 0.1ng, 10pg, 1pg, 0.1pg, 10fg was 16.3, 17.5, 21.6, 28.8, and 32.7 minutes, respectively (Table 2). There was no amplification generated in the negative control reaction, or the reaction containing 1fg of *M. genitalium* DNA.

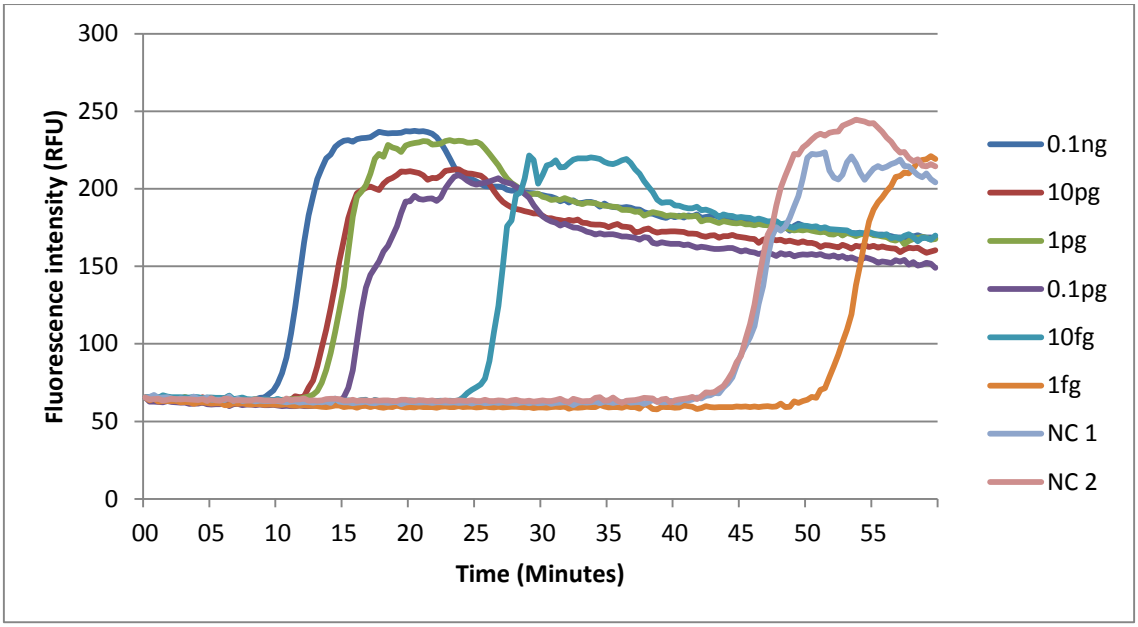

Fig.3.9. Amplification curves showing sensitivity of the *pdhD* 3 LAMP assay. LAMP reactions were carried out on a dilution series of DNA, with final amounts of *M. genitalium* DNA per reaction of 0.1ng, 1pg, 0.1pg, 10fg, 1fg, 0.1fg. Molecular grade water was used as a negative control (NC).

The *pdhD* 3 LAMP assay was able to detect a minimum amount of 10fg of *M. genitalium* genomic DNA (Fig.3.9), within the first 30 minutes of the reaction. Real time monitoring of the reactions showed that the amplification time for the reactions containing 0.1ng, 10pg, 1pg, 0.1pg, 10fg was 10.3, 12.8, 13.7, 15.6, and 25.9 minutes, respectively (Table 3.7). The Ta for these amplifications were lower than those of the *pdhD* 1 assay containing the same amount of DNA, by an average of 7.7 minutes. However, the reactions containing 1fg of M. *genitalium* DNA, and also both of the negative control reactions, generated a positive amplification between 44.3 and 52.6 minutes. Amplification in the negative control was also seen during the initial test of the primer set (Fig.3.6.). This is likely to be caused by complementary regions in the primer sequence leading to non-specific amplification events, making this primer set unsuitable for use diagnostically.

Assay	DNA per reaction						Water Negative
	0.1ng	10pg	1pg	0.1pg	10fg	1fg	
***pdhD* 1 LAMP**	+ 16.3 m	+ 17.5 m	+ 21.6 m	+ 28.8 m	+ 32.7 m	-	-
***pdhD* 3 LAMP**	+ 10.3 m	+ 12.8 m	+ 13.7 m	+ 15.6 m	+ 25.9 m	+ 52.6 m	+ 44.3/44.8 m
***pdhD* 1 PCR**	+	+	-	-	-	-	-
***pdhD* 3 PCR**	+	+	-	-	-	-	-
16S rRNA PCR (Jensen, 2003)	+	+	+	+	+	-	-
***mgpB* PCR (Jensen, 2004)**	+	+	+	+	+	-	-

Table 3.7. A Comparison of assay sensitivity. A "+" is indicative of a positive result, a "-" indicates a negative. Time to amplification is given for each LAMP assay in minutes (m), and was calculated using a manually set threshold value in the Tube Scanner Studio software.

3.3.7. Specificity of the *pdhD* 1 LAMP assay

In order to determine the analytical specificity of the *pdhD* LAMP assay it was necessary to challenge the assay with genomic DNA from a range of non-target organisms. The organisms chosen were all potential causes of urethritis, or could be encountered whilst sampling for *M. genitalium*; *U. parvum*, *U. urealyticum*, *C. trachomatis, N. gonorrhoeae, T. vaginalis, P. aeruginosa, E. coli.* Additionally, the ability of the assay to function in the presence of *M. pneumonia* DNA was tested, due to the genetic similarity between the organisms.

Species	Source	*pdhD* LAMP result
M. genitalium	NCTC 10195	+
M. genitalium	Clinical sample	+
M. pneumoniae	NCTC 010119	-
U. parvum	Clinical sample	-
U. urealyticum	Clinical sample	-
C. trachomatis	Lab strain (serovar L2)	-
N. gonorrhoeae	ATCC 19424	-
T. vaginalis	ATCC 30001	-
P. aeruginosa	ATCC 15692	-
E. coli	Lab strain	-

Table 3.8. Specificity of the *pdhD* LAMP assay. (NCTC, National Collection of Type Cultures, Public Health England, UK; ATCC, American Type Culture Collection, MD, USA; Clinical samples were collected from an STI clinic environment and provided by the Public Health Laboratory Bristol, Public Health England).

The *pdhD* 1 LAMP assay correctly identified both the *M. genitalium* type strain, and also the *M. genitalium* clinical isolate. The presence of genomic DNA of any of the non-target organisms did not result in a detectable amplification (Table 3.8).

3.4. Discussion

3.4.1. Detection of *M. genitalium* in broth culture by PCR

PCR reactions containing all three primer sets successfully amplified the predicted sized product from the control DNA (Fig.3.1). The faintest band, indicating a comparative reduction in DNA produced, and therefore starting copy number, was seen from the reaction containing the 16S rRNA primer set. This was expected, as the *mgpB* sequence used as a target for the *mgpB* 1 and *mgpB* 2 primer sets is also present in the MgPar repeat sequences, which together with the *MgPa* operon make up ~4.7% of the *M. genitalium* genome (Ma *et al.*, 2010). The *mgpB* 2 primer set yielded a slightly larger band on the gel, which could have been due to an increase in product formed. This could possibly have been due to the smaller product size (243bp compared with 666bp), which would place less stress on the PCR reaction, and would be less likely to exhaust surplus DNTPs (Cha and Thilly, 1993). The reactions containing molecular grade water instead of the positive control DNA contained no identifiable product when separated on the gel, indicating there was no non-specific amplification in the reactions.

The *mgpB* 1 and 16S rRNA PCR reactions containing DNA extracted from the *M. genitalium* broth cultures showed no amplified product. This suggests that the culture of the organism has failed, as after 1 week there should be a level of biomass detectable by PCR. A very faint band could be seen for the *mgpB* 2 PCR reaction containing this DNA sample. This primer set appeared to produce the largest yield of product from the positive control, and therefore could be assumed to be the most sensitive. The broth itself would contain *M. genitalium* DNA from the initial inoculum, whether there had been any growth or not, and it could be this low concentration of *M. genitalium* cells that the reaction has detected.

3.4.2. Detection of *M. genitalium* from culture on solid media by PCR

As in (Fig.3.1), the positive control DNA was amplified successfully by each primer set, whilst the negative control yielded no product (Fig.3.2). The DNA extracted from the *M. genitalium* cultured on solid media was successfully amplified by each PCR primer set. The bands, in each case, were brighter than those from the positive control DNA, indicating an increase in DNA concentration, and

therefore the starting copy number. This indicated the successful growth of the organism on this media type. All subsequent *M. genitalium* culture was carried out using this solid media (mycoplasma agar, 20% supplement G).

3.4.3. Sensitivities of the *M. genitalium* 16S rRNA PCR and *mgpB* 2 PCR assays

The 16S rRNA primer set was able to detect *M. genitalium* DNA at a concentration of 1pg per reaction, and above. The *mgpB* 2 primer set was able to detect a further 1:10 dilution of template DNA, at a level of ≥0.1pg per reaction. The increased sensitivity was expected, due to the brighter bands seen when using this primer set in previous PCR assays (Fig.3.3, Fig.3.2). The increased sensitivity is due to the increased copy number of the *mgpB* sequence, due to its replication in the MgPar repeats found in nine chromosomal locations throughout the *M. genitalium* genome (Ma *et al.*, 2012). Although this assay has performed with a higher sensitivity than the 16S rRNA assay with the exact *M. genitalium* G37 strain from which the primers were designed, the assay would be potentially less suitable for use as a diagnostic assay with modern clinical strains. Analysis of the primer set using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) showed two predicted products from the *M. genitalium* G37 strain genome, a 243bp fragment of the *mgpB* gene (Accession number EF458038) , and also a 240bp fragment of the MgPar 9 region (Accession number DQ248104). Analysis of products from alternate *M. genitalium* strains (M2288, M6320, M6282), showed no exact matches, with a minimum of five base mismatches per primer set present for any predicted product. Analysis of the 16S rRNA primer set however, shows that it would produce a 270bp fragment from all the *M. genitalium* strains with sequences available on GenBank (G37, M2288, M6282, M6320). The drawback of using the 16S rRNA target in a diagnostic setting is that the *M. genitalium* 16S rRNA sequence shares 98% homology with the equivalent gene in *M. pneumoniae* (Jensen *et al*., 2003). This is apparent in analysis of the 16S rRNA primer set, as there is only a single base mismatch in the reverse primer preventing 100% sequence complementarity for a 268bp fragment in the *M. pneumonia* 16S rRNA sequence. In order to adequately determine the sensitivity of this primer set they would need to be screened against *M. pneumonia* DNA to test for non-specific amplification, which with only a single 3’ mismatch on the reverse primer, is a possibility.

3.4.4. Sensitivity of the 16S rRNA LAMP assay

The 16S rRNA LAMP assay, designed by Public Health England South West Virology Lab, was able to successfully amplify *M. genitalium* DNA at concentrations of 1fg per reaction, and above. This was a 1000-fold greater sensitivity than the 16S rRNA PCR reaction, and 100-fold greater than the *mgpB* 2 PCR assay (Fig.3.4). The number of *M. genitalium* genome copies equating to 1fg of DNA is around 1.6, indicating the high level of sensitivity of the assay. Real-time monitoring of the amplification (Fig.3.5) found the Ta for the most highly concentrated sample to be 23 minutes, followed by 25.9 minutes and then 27.3 minutes for subsequent DNA dilutions (Table.3.6). There was a delay until the onset of detectable amplification in the reaction containing 0.1pg, which occurred at 35.1 minutes. The reactions containing 10fg and 1fg of *M. genitalium* DNA demonstrated detectable amplification after a period of 55.6 minutes and 55.8 minutes respectively. Amplification at this point is extremely late, and would not be detected on the final LabDisk platform, as the reaction will be proceeding for a period of 50 minutes. The sizeable delay between the amplification in the reaction containing 0.1pg and the reactions at lower concentrations suggest that the signal detected in these reactions may have resulted from non-specific amplification. The fact that a similar late amplification was seen in negative control 1 (NC 1, Fig.3.5), suggests that this may be the case, and that the actual detection limit could be considered to lie between 0.1pg and 10fg. This would equate to a detection limit of between 160 and 16 genome copies.

3.4.5. Initial testing of *pdhD* primer sets

The *pdhD* 3 primer set generated the earliest amplification from the positive reactions, with the reaction crossing the set threshold at 13.1 minutes (Fig.3.6). This was a faster amplification than seen when using the *M. genitalium* 16S rRNA set with the same concentration of DNA (Fig.3.5). This increase in time could be explained by an increase in copy number, as the *pdhD* gene is present as a single copy per *M. genitalium* genome, and the same concentration of *M. genitalium* genomic DNA was used in both reactions. LAMP primer sequences can affect the reaction efficiency in a number of ways, including influencing the likelihood of the initial binding event of any of the four primers to the target sequence, and the formation of the stem loop from the dumbbell structure at the beginning of the LAMP cycle (Fakruddin *et al.*, 2013). The *pdhD* 3 negative reaction, containing molecular grade water instead of a DNA template, generated detectable amplification at 52.3 minutes. This late false

positive result would be missed by the LabDisk assay, which runs for 50 minutes. However, it does indicate the possibility that this primer set has the ability to generate non-specific amplification, possibly due to complementary regions between the primers themselves.

The *pdhD* 1 primer set generated detectable amplification after 15.1 minutes, a 1.7 minute delay compared to the *pdhD* 3 positive reaction. The *pdhD* 1 negative reaction did not amplify during the 60 minute monitoring period, meaning that despite the slight delay in amplification in the positive reaction, this set may be more suitable than *pdhD* 3 set, due to the lack of non-specific amplification seen.

The *pdhD* 2 primer set generated detectable amplification after a period of 24.1 minutes in the positive reaction, a delay of 10.2 minutes when compared to the *pdhD* set 3 positive. This was a sizeable increase in the time to amplification (Ta), and represents a significant reduction in reaction efficiency when compared to the two other sets. It was on the basis of this difference in Ta that this primer set was disregarded for all subsequent experiments.

3.4.6. Comparison of *pdhD* LAMP reactions with notable M. *genitalium* PCR assays from the literature

The *pdhD* 1 and *pdhD 3* LAMP assays were able to detect a minimal level of 1fg of *M. genitalium* DNA per reaction. This is equivalent to approximately 16 genome copies of *M. genitalium* DNA (Fig.3.8, Fig.3.9).

The *pdhD* S1 primer set showed no non-specific amplification in the water negative reactions, whilst the *pdhD* S3 primer set generated a false positive result in both of the water negative results. This was possibly due to complementary areas within the primer sequences resulting in self-priming, and subsequent amplification. BLAST analysis of the specificity of the F3 and B3 primers revealed no alternative templates. Also, PCR reactions using the F3 and B3 primers resulted in successful amplification at concentrations of 1pg and above, and no amplification in the negative control. The generation of amplification in the negative control, leading to a false positive result makes this primer set unusable in an *M. genitalium* LAMP assay. The extremely rapid amplification seen using the *pdhD* 3 set could be due to the comparative ease with which these primer sequences bind, which is also leading to self-priming, or non-specific primer binding, and causing non-specific amplification.

The *MgPa* PCR assay, described in (Jensen *et al.*, 2004), is one of the most frequently cited *M. genitalium* PCR assays in the literature (Twin *et al.*, 2011). The primers, incorporated into a Taqman real-time PCR assay, have a limit of detection of 5 copies of the *M. genitalium* genome (Jensen *et al*., 2004). A clinical study found the real time *MgPa* assay using this primer set to be more sensitive than a real time PCR assay based on the single copy *gap* gene, and the conventional 16S rRNA PCR assay from Jensen et al. (2003) (Svenstrup *et al*., 2005). This result was replicated in a separate study (Edberg *et al.*, 2008). In the current study the assay was able to detect 1fg of *M. genitalium* DNA, but not 0.1fg (Fig.3.7). This equates to ~16 genome copies, and is in agreement with the limit of 5 copies presented in the literature. The fact a conventional PCR assay was used instead of a real time assay should not affect the sensitivity, with previous studies demonstrating a higher sensitivity for conventional PCR compared to real-time PCR, when using the same *M. genitalium* 16S rRNA primer sets (Jurstrand *et al.*, 2005).

The 16S rRNA PCR assay was also able to detect 10fg of *M. genitalium* DNA (16 genome copies) (Fig.3.7), however the band seen on the gel was very faint. A brighter band was seen for reactions containing 0.1pg of *M. genitalium* DNA. 16S rRNA PCR assays in the literature have been shown to have a detection limit of 10 copies per reaction (Yoshida *et al.*, 2002). Multiple studies have shown the *MgPa* PCR assays to be more sensitive than PCR assays relying on the 16S rRNA sequence as target (Edberg *et al.*, 2008), however the 16S rRNA gene is more highly conserved than the *MgPa* sequence, which may be the site of random recombination events (Iverson-Cabral *et al.*, 2007), and so is considered by some to be the more desirable target for NAATs.

The *pdhD* LAMP assays, and *MgPa* PCR assay, and 16S rRNA assay were able to detect a minimum level of 10fg *M. genitalium* DNA per reaction. The lower dilution tested was 1fg, or 1.6 genome copies, which none of the assays were able to detect. There would possibly be some difference between the assays at detecting DNA levels between these two values. The *pdhD* 1 LAMP assay was equally sensitive as the two widely used PCR assays, whilst enabling more rapid detection of target DNA. Monitoring of the reactions in real-time enabled detection of *M. genitalium* DNA in between 16.3 (0.1ng) and 32.7 (10fg) minutes. As well as offering a more rapid diagnosis than PCR, this assay also has the intrinsic benefits of a LAMP reaction, including higher tolerance to contaminating inhibitors (Francois *et al.*, 2011), the lack of need for thermocycling (Notomi *et al.*, 2000), and improved reagent stability (Thekisoe *et al.*, 2009).

3.4.7. Specificity of the *pdhD* 1 LAMP assay

Sampling for *M. genitalium* takes place using genital swabbing (urethral, cervical or vulvovaginal), or urine samples, all of which will potentially contain a range of commensal bacteria from the skin or genital mucosa, and any non-target pathogenic species present. In light of this it is essential that any diagnostic assay using these samples is only able to generate a positive result from its target organism. The *pdhD* gene, the target of the *pdhD* LAMP assay, is highly conserved in *M. genitalium*, and has a unique sequence in this organism. BLAST analysis of the primer sequences and target amplicon show that there are no identical, or highly similar sequences, in any alternative organism, including the genetically similar *M. pneumonia*. However, it important to confirm this predicted high level of specificity experimentally. The *pdhD* 1 LAMP assay did not generate a positive result when challenged with DNA from any of the non-target organisms tested, which included a number of sexually transmitted pathogens, and causes of urethritis.

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Chapter 4

Chlamydia trachomatis

4.1. Introduction

Chlamydia trachomatis is an obligate intracellular bacterium and a prevalent and highly clinically significant pathogen in humans, responsible for ocular and respiratory tract infections, in addition to being the most widespread sexually transmitted disease in developed countries (Bavoil *et al.*, 2000) Over 18 serovars of *C. trachomatis* have been identified, based on variations in the antigenically diverse major outer membrane protein (MOMP) (Gomes *et al.*, 2005). Serovars A-C are the causative agents of trachoma; infection of the cunjuctival follicles or lymphoid germinal centres of the eye. Serovars D-K are responsible for uritogenital tract infections, and serovars L1-L3 cause lymphogranuloma venereum (Borges *et al.*, 2010), and are found to a lesser extent in ano-rectal infections (Geisler *et al.*, 2002). Progression of the infection from the lower to upper genital tract is associated with an increase in the severity of the disease, frequently leading to epididymitis in men and pelvic inflammatory disease (PID) in women (Ostaszewska *et al.*, 2000; Paavonen and Eggert-Kruse, 1999). The inflammation seen in these conditions, and scarring seen in PID, can have a serious impact on the reproductive health of an individual and cause infertility or ectopic pregnancy.

4.1.1. Pathogenesis

C. trachomatis has a biphasic developmental cycle, which comprises of two stages. In its extracellular stage, *C. trachomatis* exists as a metabolically inactive elementary body (EB), a small coccus, around 0.3µm in diameter (Fig.4.1). This is the infectious stage of *C. trachomatis*, which can be passed between hosts via sexual contact. This occurs when EBs present in the penile urethra or vagina of the infected party, having been shed via infected epithelial cells, are passed to their sexual partner. The EBs are then internalised into the epithelial cells of the new host via receptor mediated endocytosis, of which only a small percentage are lost via fusion with host cell lysosomes. At this point the EBs inside the vacuoles differentiate into the next stage of the developmental cycle, the non-infectious and metabolically active reticulate body (RB) (Moulder, 1991), of around 1µm in diameter in size.

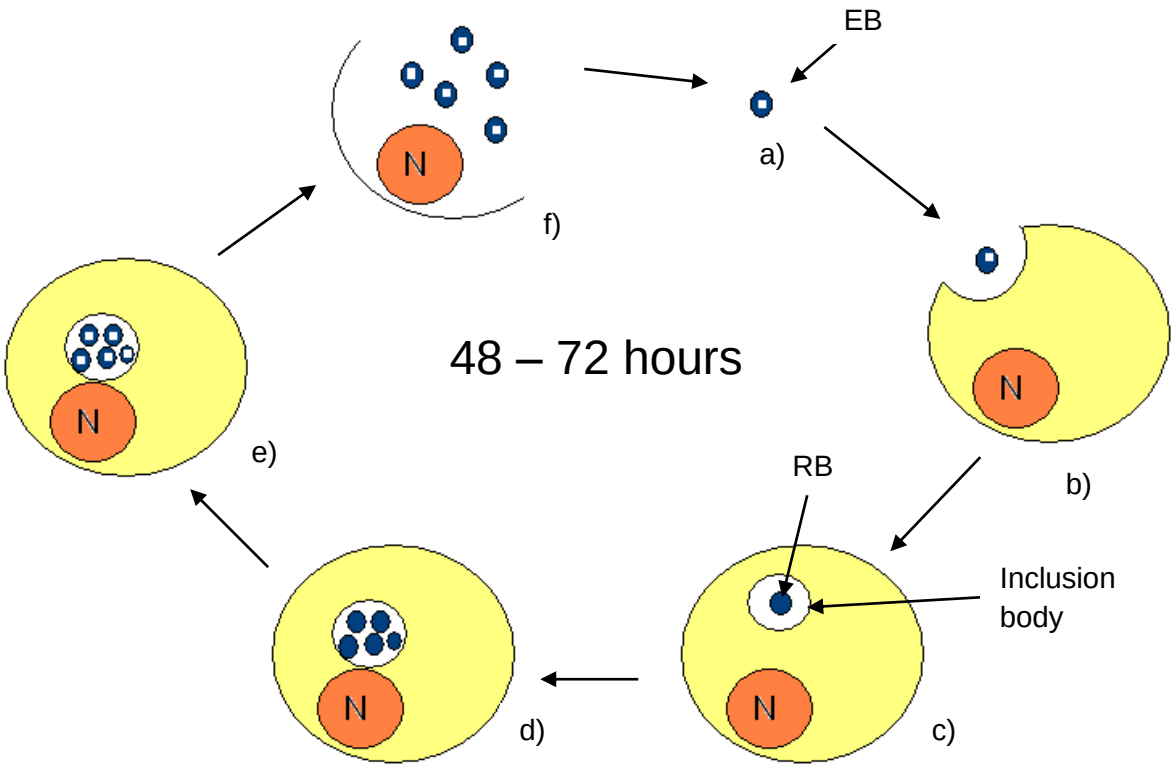

Fig.4.1. Diagram of the life cycle of *C. trachomatis,* showing: a) Extracellular EB, b) Entry of EB into host cell via phagocytosis, c) Primary differentiation of EB into RB, d) Rbs multiply by binary fission inside inclusion body, e) Secondary differentiation of RBs into EBs, f) Host cell lysis, release of EBs.

The initial stage of infection with *C. trachomatis* is the binding event between the infectious elementary body, and the cells of the host genital epithelia. This initial adhesion is a complex process, mediated by a number of *C. trachomatis* surface proteins and their corresponding ligands on the epithelial surface. The major outer membrane protein (MOMP) of *C. trachomatis* is known to act as a cytoadhesin, binding to heparin sulphate proteoglycans on the outer membrane of the epithelial cell (Su *et al.*, 1996). Additionally, the OmcB outer membrane peptide has been shown to have a major involvement in the binding of the EBs to epithelial cells, with the retardation of these proteins resulting in a 70% reduction in infectivity (Fadel and Eley, 2007). OmcB binds to glycosaminoglycan (GAG) like receptors on the host cell surface, in a similar fashion to the MOMP (Fadel and Eley, 2007). Bacterial membrane associated GAG proteins also contribute during the binding process, with a range of host cell receptors, including heparan sulfate, mannose receptor and estrogen receptor, facilitating these interactions (Cocchiaro and Valdivia, 2009). The presence of functional protein disulphide isomerase (PDI), a multifunctional peptide located on the surface of epithelial cells, is necessary for the binding and internalisation of EBs (Conant and Stephens, 2007).

This protein is not thought to act as a specific receptor for EB attachment, but is necessary for the correct structural arrangement of such receptors. After the initial binding between the EB and host cell, the enzymatic activity of PDI is a requirement of EB internalisation (Abromaitis and Stephens, 2009). Cell lines with defective PDI expression are inherently resistant to *C. trachomatis* infection, underlining the importance of this protein in pathogenesis (Conant and Stephens, 2007).

C. trachomatis also takes advantage of the host fibroblast growth factor 2 (FGF2) pathway to enhance its infectivity, by binding to FGF2, and interacting with the fibroblast growth factor receptor (FGFR) on the epithelial surface. This interaction results in FGFR activation at the location of the FGF2 bound-chlamydial EBs, resulting in the increased uptake of EBs into the cell (Kim *et al.*, 2011). The production of FGF2 is also upregulated in epithelial cells infected with *C. trachomatis*, and the extracellular release of this molecule during the lysis of the cell acts to promote further infection by extracellular EBs (Kim *et al.*, 2011).

The uptake of surface-bound Chlamydia EBs into the intracellular environment is facilitated by the reorganisation of the actin cytoskeleton to form microvillus like structures, which engulf the bacterial cells (Cocchiaro and Valdivia, 2009). This process is instigated by the translocation of an actin recruiting protein (translocated actin recruiting phosphoprotein; TARP) into the host cell membrane (Engel, 2004). This protein is activated by phosphorylation via the host membrane associate Src and Ab1 kinases, which results in the recruitment of guanine exchange factors Sos1 and Vav2, and subsequent activation of the Rac1 GTPase. Rac1 activates the Arp2/3 complex, which instigates actin reorganisation, via the activation of WAVE2 and Abi-1 (Carabeo *et al.*, 2007). These relationships have been visualised by imaging studies showing Rac1, WAVE2 and a number of actin-associated cytoskeletal proteins migrating to the invasion point, and co-localising with EBs during invasion (Carabeo *et al.*, 2004). This modulation of the actin cytoskeleton, and internalisation of *C. trachomatis* EBs is a fairly rapid process, taking ~90 seconds to occur, illustrating the rapid activation and suppression of the signalling molecules involved (Dautry-Varsat *et al.*, 2005). RNA sequencing of the host transcriptome at these early stages of infection (1 hour post infection) reveals the differential expression of a wide variety of extracellular matrix associated proteins, including collagens, mucins and metalloproteinases, showing the significant effect that the infection has on the organisation and composition of the host cell structure (Humphrys *et al.*, 2013).

Once the EB has been internalised, it is held in a membrane-bound compartment, containing a high concentration of cholesterol and glycosphingolipid molecules, termed an inclusion (Dautry-Varsat *et al.*, 2005). The involvement of clathrin mediated endocytosis in the uptake of *C. trachomatis* EBs is controversial; RNA interference and site-directed mutagenesis studies have given conflicting results,

with evidence presented that *C. trachomatis* internalisation is not inhibited by the production of non-functional clathrin (Dautry-Varsat *et al.*, 2005), and also that clathrin and its accessory factors are required for *C. trachomatis* internalisation (Hybiske and Stephens, 2007). This vacuole is able to avoid becoming sequestered to the host cell endosome-lysosome pathway, preventing the lysosome-mediated degradation of its bacterial contents (Scidmore *et al.*, 2003). The inclusion is then moved towards the endoplasmic reticulum (ER)/Golgi activity centre, via trafficking on cytoskeleton intermediate filaments, where gene expression dramatically shifts, and transcriptional and metabolic activity is upregulated, signalling the shift from the EB to RB form (Wyrick, 2010). During the growth and replication of the *C. trachomatis* RBs, components of the ER membrane are sequestered to the inclusion, enabling the inclusion to grow in size and accommodate the increasing biomass (Dumoux *et al.*, 2012). Materials are obtained from the rough ER (rER) membrane in a process requiring direct contact between the inclusion and rER membrane, and also an alignment with bacterial type 3 signalling systems (T3SS) present on RBs in the inclusion. It is thought that this alignment acts as a portal for the transport of materials from the rER to the RBs (Dumoux *et al.*, 2012). *C. trachomatis* infection induces fragmentation of the Golgi apparatus via proteolytic cleavage of goglin-84, a key protein component of the Golgi matrix, enabling to inclusion to obtain vital lipids from the remnants of the organelle (Heuer *et al.*, 2009). Vesicles are also intercepted from the host secretory pathway, and lipid droplets from the cytosol, providing the inclusion with a source of cholesterol and sphingolipids (Heuer *et al.*, 2009; Cocchiaro and Valdivia, 2009).

During infection, *C. trachomatis* prevents the recognition and killing of the infected cells by CD8+ cytotoxic T-lymphocytes by degrading intracellular transcription factors needed for the expression of the antigenic major histocompatability complex (Zhong *et al.*, 2001). This is carried out by the release of chlamydial protease-lie activity factor (CPAF) into the cytosol from the bacterial cells within the inclusion. CPAF additionally cleaves a number of other intracellular substrates, including the pro-apoptotic B3-only proteins, preventing the infected cell from undergoing apoptosis. The generation of CPAF in uninfected epithelial cells has shown that this protease causes large scale morphology changes, and eventual cell death via non-apoptotic mechanisms, and is a major factor in *C. trachomatis* pathogenesis (Paschen *et al.*, 2008).

Infected epithelial cells are responsible for instigating the immune response associated with chlamydial infection, and release a range chemokines and pro-inflammatory cytokines, including IL-8, GROα, GMCSF and IL-6, leading to inflammation of the infected epithelia (Rasmussen *et al.*, 1997). The release of proteases, cytokines and tissue growth factors from infected epithelial cells, and also immune cells, leads to cellular damage in the location of the infection site (Darville and Hiltke, 2010).

Upon lysis of the infected cell, after the maturation of the inclusion, high concentrations of IL-1α are released into the extracellular environment, which aggravates the inflammatory response by stimulating enhanced cytokine production in neighbouring uninfected epithelial cells (Rasmussen *et al.*, 1997). *C. trachomatis* has been shown to down regulate nectin-1, a cell adhesion immunoglobulin implicated in the maintenance of claudin-based tight junction complexes in epithelial monolayers (Sun *et al.*, 2008). Infection also results in the breakdown of the N-cadherin/β-catenin complex in epithelial cells, and subsequent sequestering of β-catenin within the *C. trachomatis* inclusion bodies (Prozialeck *et al.*, 2002). These processes are thought to accelerate the release of infected epithelial cells from the monolayer, increasing infectivity, whilst increasing damage to the epithelial monolayer which is a key component of chlamydial pathogenesis. It is through these mechanisms of cytotoxicity, and the instigation of a robust inflammatory response, that the epithelial damage and scarring that occurs during long term Chlamydia infection occurs.

C. trachomatis is a Gram negative bacterium and the EBs are found to entirely lack a peptideglycan layer, despite the existence of the genes required for peptidoglycan synthesis. However, antibiotics that act via disruption of peptidoglycan synthesis such a beta-lactams are found to be active against *C. trachomatis* and other members of the *Chlamydiae* genus. Treatment of *C. trachomatis* with these antibiotics results in morphologically aberrant RBs, preventing RB division and the differentiation into EBs (McCoy and Maurelli, 2006). This paradox is known as 'the Chlamydia anomaly'. The most widely held opinion is that a cell wall is present, in the RBs but not the EBs of *Chlamydiae*, which do not require the protection against low external osmolarity provided by a cell wall due to the abundance of cysteine rich envelope proteins, linked by disulphide bridges, which offer the same level of protection (Everett and Hatch, 1995). These envelope protein, on the surface of the EBs act to strengthen the cell wall considerably by forming a disulphide cross-linked supramolecular complex with the major outer membrane protein (MOMP). This cysteine rich protein layer gives the EB substantial structural integrity, explaining the resistance seen to detergent solubilisation of the outer membrane complex under non-reducing conditions (Everett and Hatch, 1995). The reliance of *C. trachomatis* on these disulphide bonds for structural stability can be demonstrated by the onset of cellular lysis upon the introduction of reducing agents such as dithiothreitol (Hackstadt *et al.*, 1985). Transcriptional analysis of expression of the genes involved in peptidoglycan synthesis in *C. trachomatis*, *murA, murB* and *pbp2,* revealed the peak expression to lie between 2 and 6 hours post infection, which would indicate their involvement in the early stages of the transition from EBs to RBs (McCoy *et al.*, 2003). Another study which carried out microarray analysis of the *C. trachomatis* genome found the highest peak in the expression of the same 3 genes to occur between 16 and 18 hours post infection, indicating a role for the genes in RB cell division (Belland *et al.*, 2003).

4.1.2. Epidemiology and clinical presentation

Worldwide prevalence and incidence data for 2008, published in 2012, estimate a worldwide incidence of 105.7 cases of *C. trachomatis*, with 100.4 million adults infected at any one time (World Health Organisation, 2012). Unusually amongst STI's, the prevalence is higher in developed countries, such as those in Europe, compared with developing countries in Africa and Asia (Table 4.1).

WHO Region	Incidence (per 1000)		Prevalence (%)	
	Female	Male	Female	Male
Africa	22.3	20.9	2.6	2.1
The Americas	72.6	38.2	7.6	2.9
South East Asia	9.2	6.2	1.1	0.6
European	37.1	54.2	3.9	3.8
Eastern Mediterranean	9.8	10.9	1.1	0.9
Western Pacific	38.4	42.5	4.3	3.4

Table 4.1. Global incidence and prevalence of *C. trachomatis* infection, according to 2008 WHO estimates. Data taken from (World Health Organisation, 2012).

The majority of *C. trachomatis* infections in women are asymptomatic, with symptoms occurring in 5%-15% of cases (Detels *et al.*, 2011). Due to its asymptomatic nature, *C. trachomatis* infections are liable to lie undetected, and therefore untreated, in patients unaware of the infection, although the instigation of screening programmes has helped to identify more of these infections. Untreated Chlamydia infection in women can lead to an ascending infection in the pelvis, causing PID in ~20% of cases (Paavonen and Eggert-Kruse, 1999). PID can lead to fallopian scaring, and lead to infertility and ectopic pregnancy. Chlamydia-mediated PID is the most prevalent preventable cause of infertility and adverse pregnancy outcome, making control of Chlamydia infection extremely important for public health bodies (Paavonen and Eggert-Kruse, 1999).

Urethral infections in males cause symptoms in ~40% of cases, with the most common symptom being urethritis (Falk *et al.*, 2005; Dixon *et al.*, 2002). The infection can progress to epididymitis,

although this tends to be a less severe form compared with epididymitis arising from other means (Ostaszewska *et al.*, 2000). It is thought prolonged untreated chlamdial infection can lead to chronic prostatitis (Shperling *et al.*, 2011; Weidner *et al.*, 2002), although unlike prostatitis caused by a number of other STIs, no association has yet been found with increased prostate cancer risk (Huang *et al.*, 2013).

4.1.3. Diagnosis

Current detection methods in the UK are based around both molecular detection methods and culture, with samples being taken from different biological sites. Molecular methods include LCR, PCR, Gen-Probe and also enzyme immune-assay (EIA) (Watson *et al.*, 2002). These methods tend to be time consuming, typically taking between 4 to 8 hours to obtain results from the raw sample. They also require skilled laboratory personnel and complex equipment to be carried out. Automated assays, featuring integrated sample preparation and nucleic acid amplification and detection are available, and widely used in the UK, but expensive. Many countries in the developed world, including the US and the UK, have instigated Chlamydia screening programmes, in order to identify the relatively large number of asymptomatic infections in the population. As a disease, chlamydia infection has many aspects of a disease that will typically benefit from screening as a mechanism of disease control; it is highly prevalent, frequently asymptomatic, associated with significant morbidity, and is easily diagnosed and treated (Paavonen and Eggert-Kruse, 1999).

Direct cytological inspection of urethral exudates, in conjunction with Giemsa staining, has been used in the past for diagnosing chlamydia infection, but is insensitive, and rarely used clinically (Chernesky, 2005). Culture of chlamydia from clinical samples is more sensitive, and has the added advantage of being the only diagnostic method that will detect live organisms. The clinical sample can be centrifuged over an animal cell monolayer, possibly after dilution to reduce toxicity(Yong and Paul, 1986). Cell lines commonly used for *C. trachomatis* propogation include McCoy cells, Hep-2, HeLa and Buffalo green monkey (BGM) cells (Krech *et al.*, 1989; Chernesky, 2005), which are often treated with cycloheximide in order to prevent cell division and provide a stable monolayer during infection. Chlamydia cells are cultured for 48 – 72 hours, after which the monolayer is examined for the presence of inclusion bodies indicative of infection, using microscopy aided by either Geimsa staining, or fluorescent microscopy aided by fluorescently conjugated *C. trachomatis* specific

antibodies. The culture of the organism is time consuming, requires animal cell culture capabilities, and skilled technical staff, and is difficult to carry out with a high throughput.

Enzyme immunoassays (EIA) for *C. trachomatis* are commercially available, and are used as a diagnostic, despite suffering from poor sensitivities compared with molecular tests. EIAs rely on the detection of *C. trachomatis* LPS using an enzyme labelled monoclonal or polyclonal antibody (CDC, 2002). Upon binding of the LPS, the enzyme then catalyses a colorimetric reaction, which can be read visually, or by using a spectrophotometer. One study found the sensitivity of a commonly used EIA test, the MicroTrak (Trinity Biotech, Ireland) to be 42% compared with that of LCR (Van Dyck *et al.*, 2001). Other studies have found the sensitivity to range between 65% and 75% (Chernesky, 2005). The specificity of these assays is also sub-optimal, due to the tendency of these assays to generate false positive results in the presence of LPS from non-target organisms (CDC, 2002). To alleviate this issue, confirmatory testing of positive samples is recommended, which increases specificity from ~97% to ~99.5% (Moncada *et al.*, 1990). Enzyme linked immunosorbant assays (ELISA) are also commercially available for detection of Chlamydia, and are used more frequently than EIAs due to the improved sensitivity, and decreased hands on time afforded by this technique (Witkin *et al.*, 1997).

Direct fluorescent antibody (DFA) tests have been applied to detecting *C. trachomatis* EBs directly from specimen material applied to a slide. Fluorescent antibodies specific to either *C. trachomatis* LPS or MOMP are then used to stain the slide, which is then examined using fluorescent microscopy (Phillips *et al.*, 1987). The sensitivity of this technique is ~70% compared with culture, and lower still when compared with NAATs (Phillips *et al.*, 1987; Chernesky, 2005). However, it is fast to carry out, and can be performed on-site to provide same day results.

The use of NAAT technologies for the detection of *C. trachomatis* is highly widespread. The first NAAT tests to find use in the laboratory diagnosis of Chlamydia were in-house PCR assays, which enabled a marked increase in assay sensitivity compared with culture based methods. Commercial PCR kits were also quickly made available, providing molecular diagnostics to laboratories without the need for lengthy assay development (Smith *et al.*, 1993). Common targets for *C. trachomatis* specific PCR assays are MOMP (Naidu *et al.*, 1997), and the multi copy 16S rRNA gene (Claas *et al.*, 1990) and cryptic plasmid (Jaton *et al.*, 2006), which are all highly conserved and offer high specificity.

One of the most widely used early PCR assays was the AMPLICOR PCR test (Roche, US), which one large scale evaluation found provided sensitivities of 88% from male urines, and 86% from

endocervical swab samples, compared with sensitivities of 50.7% and 67.3% when culture was used from the same sample types (Quinn *et al.*, 1996). Early PCR assays required post assay analysis, either via the separation of reaction products using agarose gel electrophoresis, or in the case of the AMPLICOR PCR test, DNA hybridisation (Quinn *et al.*, 1996). This increases the time taken to process each sample and achieve a diagnosis, and also increases the likelihood of amplicon contamination in the laboratory (Eickhoff *et al.*, 2003). The development of real-time PCR assays removed the need for post assay analysis, as detection is carried out in real-time during the amplification process, via the optical detection of fluorescence. Real-time PCR assays based on Taqman probe technology have been developed, with one assay specific to the *C. trachomatis* cryptic plasmid providing sensitivity and specificity of 95.7% and 100%, respectively (Jaton et al., 2006).

The instigation of *C. trachomatis* screening programmes in developed countries has lead to an increased volume of *C. trachomatis* testing, which has necessitated the development of high throughput, automated molecular diagnostics platforms (Van der pol et al., 2000). The COBAS AMPLICOR analyzer (Roche, US), is an automated platform for processing AMPLICOR PCR and subsequent DNA hybridisation steps, reducing hands-on time, and enabling same-day diagnosis (Jaton *et al.*, 2006). The automation of this assay has been shown to provide a slightly increased sensitivity in comparison to the manual assay (Livengood and Wrenn, 2001). More recent diagnostics platforms have incorporated the automation of not only the amplification and detection processes, but also the sample preparation and nucleic acid extraction steps, leading to a sample in-answer out system.

One such test is the Aptima Combo 2 test (Gen-Probe), a transcription mediated amplification (TMA) based test that utilizes target capture for the qualitative detection of *C. trachomatis* (via the 23S rRNA sequence) and *N. gonorrhoeae* from clinical swab or urine samples, which is run on the Gen-Probe Tigris or Panther platform. Package insert specificity for *C. trachomatis* detection is given as 98.9%, which has been replicated experimentally (Levett *et al.*, 2008). The limit of detection of the Aptima assay has been recorded as low as 0.008 EB's per assay, making it the most sensitive commercial diagnostic test for *C.trachomatis* (Lowe *et al.*, 2006).

A comparison between three of the most frequently used, FDA approved, automated molecular platforms for *C. trachomatis* detection over a large volume of female and male samples (n=3,832) at diverse geographical sites, found the sensitivity of the Abbot Realtime CT/NG assay (Abbot, US), the Aptima Combo 2 assay (Hologic Genprobe, US), and the ProbeTec ET assay (Becton Dickinson, US), to be 92.4%, 94.5%, and 90.3%, respectively (Gaydos *et al.*, 2010). The specificity of the Abbot realtime was 99.2%, the Aptima combo 2 was 99%, and the ProbeTec ET was 99.5%.

Point of care testing for Chlamydia infection is currently available, in the form of rapid immunoassay test kits. Whilst these kits are very simple to use, and provide a rapid time to result (as low as 15 minutes), they typically have much lower sensitivities than nucleic acid amplification assays (Mahilum-Tapay *et al.*, 2007). The most widely used rapid immunoassay in the UK, the Clearview Chlamydia MF test (Clearview, Inverness Medical, Bedford, UK), has been shown to have a sensitivity as low as 32.8% with vaginal swab samples, and 49.7% with endocervical swabs (Yin *et al.*, 2006), although previous studies had shown higher sensitivities, of 60.4% from male samples and 62% from female samples (Kluytmans *et al.*, 1993). A comparison of three POC tests widely available for home use, and available on the internet or in pharmacies, found assay sensitivities to be extremely low in comparison with NAATs. The study involved 772 female samples (11% positive), and the sensitivity of the Biorapid Chlamydia Ag test (Biokit, Spain), Quickvue chlamydia test (Quidel corporation, US), and Handi-lab C test (Zonda, US) were 17%, 27% and 12%, respectively (van Dommelen *et al.*, 2010). This low level of sensitivity seriously undermines the effectiveness of these assays, and despite their speed and ease of use, this group of tests is unlikely be able to provide the solution to the lack of sensitive POC Chlamydia test available to clinicians.

The current goal of POC technology is to provide simple, easy to use, rapid testing at the point of care, with equal sensitivity to laboratory based NAAT systems. Currently there is an FDA approved assay for simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* available on the GeneXpert platform (Cepheid, US), which enables POC NAAT. This platform utilises a fully automated nucleic acid extraction process, and real-time PCR based target amplification and detection, taking place on a disposable cartridge. The test provides results within 90 minutes, which enables same day diagnosis and treatment, and can be used as a POC diagnostic, provided that the patient can remain for that period of time on site. When compared with modern NAAT tests such as the Aptima Combo 2 assay (Genprobe, US), the GeneXpert CT/GC test has been shown to have a sensitivity of 97.4%, 98.7%, 97.6% and 97.5%, when dealing with endocervival swabs, vaginal swabs, female urines and male urines, respectively (Gaydos *et al.*, 2013). Specificity values for all sample types were over 99.4%.

A cost/benefit analysis of the implementation of POC NAAT in UK GUM clinics found that POC testing could reduce spending, and improve patient experience, mostly due to the prevention of presumptive treatment resulting in patients receiving antibiotics unnecessarily (Turner *et al.*, 2014). A similar study undertaken in the US also found that POC testing was likely to be more cost effective, and also identify more infections before they progress to PID (Huang *et al.*, 2013).

4.1.4. Treatment

The current recommendation for treatment of genital chlamydial infection from the UK PHE, and US CDC is 1g oral azithromycin, or a one week course of 100mg doxycyline twice a day, in case of contra-indications (CDC, X; PHE, X). Azithromycin is considered preferable, in part due to the improved compliance encountered when dealing with a single dose therapeutic (Taylor and Haggerty, 2011).

As an obligate intracellular pathogen, the MIC of an antibiotic is determined as the minimum concentration required to prevent intracellular proliferation. *C. trachomatis* also displays heterotypic resistance, in which only a sub-population of the *C. trachomatis* cells will be phenotypically resistant (Somani *et al.*, 2000), complicating MIC determination. Strains displaying homotypic resistance have not been encountered (Wang *et al.*, 2005). Due to the relative complexity in determining MIC values for this organism, MIC testing is not routinely carried out; in general only isolates that have caused clinical treatment failures will be tested.

Although *C. trachomatis* is able to evolve antimrocrobial resistance *in vitro*, reports of isolates with antimicrobial resistance are rare, and these isolates have not maintained their resistance once propagated in vitro (Sandoz and Rockey, 2010). In vitro studies have shown that *C. trachomatis* cells can enter a persistent state when challenged with sub-lethal concentrations of antibiotics, in which cell division is halted, but protein synthesis and DNA replication continue, and the instigation of this state of persistence can result in treatment failures (Hogan *et al.*, 2004). It is unknown whether this is significant in vivo, but it is possible that a certain proportion of treatment failures, or refractory infections, are occurring due to persistent *C. trachomatis* cells, which are inherently more resilient in the face of an antimicrobial challenge.

A small number of multi-drug resistant isolates of *C. trachomatis* have been previously described, although the infections have responded to an increased antibiotic dosage. isolates displaying resistance to azithromycin and ofloxacin have been reported as causing treatment failures, although the infections either resolved spontaneously or after the administration of an increased dosage, and the resistant isolates did not maintain their resistance *in vitro* (Somani *et al.*, 2000).

Macrolide antibiotics such as azithromycin exert their effects by binding to the 50S subunit of the bacterial ribosomes, inhibiting protein synthesis, leading to the death of the cell (Retsema *et al.*, 1987). Mutations in the 23S rRNA gene have also been shown to confer macrolide resistance in a number of other clinically significant bacterial species, such as *Pseudomonas aeruginosa* (Marvig *et*

al., 2012) and *N. gonorrhoeae* (Chisholm *et al.*, 2010), and mutations in the 23S rRNA gene of *C. trachomatis* are associated with a macrolide resistant phenotype, particularly mutations in the peptidyl transferase region (Misyurina *et al.*, 2004). Antimicrobial resistance surveillance of genital *C. trachomatis* isolates after mass azithromycin distributions in Ethiopia due to endemic trachoma detected no change in susceptibility, despite the obvious selection pressure (Hong *et al.*, 2009), demonstrating the relative slow generation of resistance in this organism. Indeed, it is thought that the majority of treatment failures are due to lack of treatment compliance, or re-infection from an infected partner (Lanjouw *et al.*, 2010).

Aims

- To maintain the culture of *C. trachomatis*, in order to provide a source of *C. trachomatis* DNA to ourselves and also Public Health England.
- To provide confirmation of the suitability of the magnetic silica based extraction system for the purification of nucleic acids from *N. gonorrhoeae*.
- To determine the limits of detection of the *C. trachomatis* 16S rRNA LAMP assay designed by PHE.

4.2. Methods

4.2.1. Growth and maintenance of McCoy cells

Monolayers of McCoy cells were cultured in 75cm^3 flasks in Eagles MEM (Invitrogen Life Technologies), supplemented with 10% foetal calf serum (PAA Laboratories), 1% streptomycin/vancomycin (Sigma) and 1% glutamine (Invitrogen Life Technologies). Monolayers were initially seeded with 12ml of cell suspension at a concentration of 3.5 x 10^5 cells per ml, and incubated at 35^{o}C with 5% CO_2. Once the monolayers were observed to have grown to 90% confluence they could be infected with *C. trachomatis*, or passaged. During passage, cells were washed twice with PBS, trypsinised with 0.25% trypsin solution for 5 minutes at 35^{o}C, before resuspending in 10ml of growth medium. Cells were then counted using an improved Neubauer counting chamber, and an appropriate volume was then added to a sterile 75cm^3 culture flask for each fresh culture required, along with fresh growth media, ensuring a final concentration of 3.5 x 10^5 cells per ml. After 72 hours the cells could then be passaged again if >90% confluence was reached, or the media was removed, and fresh growth media was added.

4.2.2. Chlamydia trachomatis culture

Monolayers of McCoy cells produced as described. Once the monolayers were observed to have grown to 90% confluence, the media was replaced, and 1ml inoculums of harvested *C.trachomatis* elementary bodies were added. The culture flasks were then centrifuged at 1,000 x *g* for 30 minutes, to enhance the rate of inclusion formation. The infected cultures were then incubated at 35^{o}C and 5% CO_2 for 72 hours, to insure inclusions predominantly contained elementary bodies. The supernatant was removed and stored. The monolayer was then trypsinised with 0.25% trypsin solution for 5 minutes at 35^{o}C. The trypsin was deactivated by the addition of 6ml of growth media, and the cell suspension was removed and added to the previously collected supernatant. The recovered cells and supernatant where mixed, sonicated for 30 seconds at 30kHz, and separated into 1ml aliquots. These aliquots were then stored at -80^{o}C, in order to provide infectious material to initiate future cultures or provide a positive sample for assays.

4.2.3. Confirmation of successful *C. trachomatis* infection of McCoy cells using fluorescence microscopy

Infected McCoy cell monolayers were harvested at 48 hours post infection, when inclusions should have formed, but cell lysis not yet occurred. Cells were trypsinised with 0.25% trypsin solution for 5 minutes at 35^{o}C, before resuspending in 10ml of growth medium. A 2.5µl aliquot of the cell suspension was taken and incubated with 2.5µl of bacterial LIVE/DEAD baclight stain (Invitrogen), formulated according to the manufacturer's instructions, in the dark for 10 minutes, on a glass slide. After the incubation, a coverslip was placed over the slide and the slide was visualised using an Olympus (Model) fluorescence microscope, using the x60 lens. An excitation wavelength of 480nm was applied, and emissions were collected via the green (500nm) and red (630nm) wavelengths.

4.2.4. Purification of *C. trachomatis* Elementary Bodies via gradient centrifugation

Chlamydia cells were harvested from an infected McCoy cell monolayer at 70 hours post infection, as described in Appendix 2. Any intact McCoy cells were then lysed via sonication for 1 30 second burst at 30kHz. The suspension was then centrifuged at 250 *g* for 5 minutes, in order to remove mammalian cell debris. The osmolality of the suspension was then increased by the addition of an equal volume of PBS containing 0.4M sucrose. A density barrier solution was made containing 180ml OptiPrep solution, 210ml H2O, and 210ml of solution A (0.13 M NaCl, 3 mM KCL, 0.3mM $CaNa_2EDTA$, 5 mM Tris-HCL, pH 7.2) (modified from Everson *et al*., 2002). 15ml of the *C. trachomatis* cell suspension was layered over 10ml of the barrier solution in 30ml centrifuge tubes. The tubes were then centrifuged at 30,000g for 1 hour in a swinging bucket rotor. A sterile syringe was then used to harvest the visible grey fraction at the interface, typically yielding a ~1.5ml suspension of purified EB's. The presence of *C.trachomatis* was confirmed using a PCR reaction, with primers specific to the C.trachomatis 16s rRNA region, as described below. Purified *C.trachomatis* elementary bodies (EB's) were then quantified using a GeneSig *C. trachomatis* absolute quantification qPCR kit (PrimerDesign Ltd., UK).

4.2.5. *C. trachomatis* PCR reaction

PCR reactions were set up as follows; 25µl RedTaq reaction mix (Sigma Aldrich, UK), 0.5pmol Forward primer, 0.5pmol reverse primer. Reactions were then made up to 45µl with water, and 5µl DNA (or water as a no template control) was added. Reaction conditions were as follows; 94°C for 5 minutes initial denaturation; 35 cycles of 94°C for 30 seconds, the correct primer annealing temperature (specific to each primer set) for 30 seconds and 72°C for 30 seconds; 72°C for 5 minutes final extension. Reactions were heated using an ABI 2720 thermocycler (Applied Biosystems, UK). PCR reaction products were separated on 2% % w/v agarose gels, with transillumination and photography provided by a BioRad Gel/Chem Doc system (Bio-Rad Inc, UK).

4.2.6. PCR primer design

Primers were designed to the *C. trachomatis* 16S rRNA gene (NCBI reference sequence: NC_020967.1) using Primer-BLAST (http://www.blast.ncbi.nlm.nih.gov/) (Ye *et al*., 2012). The primer sequences are shown in Table 4.2.

Primer	Sequence	Length (bp)	Product size (bp)	Annealing temperature (°C)
C. trachomatis 16S rRNA Forward	CGGTTGGAAACGGCCGCTAAT	21	449	63
C. trachomatis 16S rRNA Reverse	CCTACACGCCCTTTACGCCC	20		

Table.4.2. *C. trachomatis* 16S rRNA PCR primer sequences

4.2.7. LAMP Reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), as follows; 5µl five times LAMP reaction buffer, 1µl intercalating dye (propriety dye, emission in FAM channel), 1µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 1µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers, 12µl molecular grade

water. Finally 5µl of DNA sample or water was added to the reaction. Reactions were carried out in an ESEQuant Tube Scanner (Qiagen Inc., CA), at 63°C for 60 minutes. Reactions were terminated by heating to 80°C for 1 minute in order to denature the *Bst* polymerase. The ESEQuant Tube Scanner allows for real-time monitoring of the LAMP reactions, via optical detection of the fluorescent intercalating dye through the FAM channel. The "Tube Scanner Studio" software package was used to analyse the real time data and produce a manually set threshold line for the purposes of determining time to amplification (Ta). The CT-0332 primer set, designed by Public Health England, South West Laboratory, was used in all *C. trachomatis* LAMP reactions.

4.2.8. Public Health England *C. trachomatis* 0332 LAMP primer set

Sequences were provided by Public Health England for their *C. trachomatis* 0332 primer set. The primer sequences are shown in Table 4.3.

Primer	Sequence	Length (bp)
C. trachomatis 0332 F3	AAATGGAAAGACGGGGAG	18
C. trachomatis 0332 B3	AAAAAAAACGCAAGGCTCT	19
C. trachomatis 0332 FiP	CATGCCTAACCGCTCAGTGACTCAAAGACCGCCCAGTA	38
C. trachomatis 0332 BiP	CTGCTTGGGGCTACAGAGATCCAAAAAATAGGTTAGACGGAA	42

Table.4.3. Sequences of the Public Health England *C. trachomatis* 0332 primer sets

4.2.9. Detection of *C. trachomatis* nucleic acid from *C. trachomatis* infected McCoy cell culture using the CT-0332 LAMP assay.

Cells were harvested from *C. trachomatis* infected McCoy cell culture, as described in 2.2. Nucleic acid was extracted from the mix of McCoy cells and elementary bodies using the total nucleic acid

extraction method. Extractions were also carried out on uninfected McCoy cells as a negative control. Four LAMP reactions were carried out using the nucleic acid from the uninfected and infected McCoy cells.

4.2.10. Detection of *C.trachomatis* in clinical samples using the 0332 LAMP assay in conjunction with the magnetic silica based extraction method.

Nucleic acid extractions were carried out on a *C. trachomatis* positive urine and endocervical swab sample (from different patients) in duplicate, using the magnetic silica based total nucleic acid extraction method. Extractions were carried out on a *C. trachomatis* negative urine swab in order to provide a negative control. The samples provided were un-extracted excess transport media, from Aptima CT/GC assay tubes (Hologic Genprobe, US), provided by PHE South West. The positivity of each sample had been previously been determined by the Aptima assay, the current gold standard for the detection of this organism. Eluted nucleic acid from all extractions was then tested for the presence of *C. trachomatis* nucleic acid using the CT-0332 LAMP assay.

4.2.11. The determination of the limits of detection (LOD) of the 0332 LAMP assay, 16S rRNA taqman qPCR assay, and Genprobe Aptima Combi 2 assay for the detection of *C. trachomatis*

Purified *C.trachomatis* elementary bodies (EB's) were quantified using a GeneSig *C. trachomatis* absolute quantification qPCR kit (PrimerDesign Ltd., UK). The stock was diluted to 2 x 10^5 EB per ml. 2.9ml of purified EB stock was added to 2.9ml of transport media in an Aptima assay tube (Genprobe, US). After adequate mixing, 800µl of media was removed, leaving a 5ml volume. A total of 6 tenfold serial dilutions were then carried out into additional Aptima assay tubes containing transport media. The final EB concentration of each tube is shown in Table 4.4.

Sample Tube	EB concentration (per ml)
1	100,000
2	10,000
3	1,000
4	100
5	10
6	1
7	0

Table.4.4. Concentration of *C. trachomatis* elementary bodies in each Aptima sample tube.

Total nucleic acid was then extracted from 200µl of each sample using the silica based total nucleic acid extraction method (chapter 1.2.7). The sample tubes then underwent the Aptima Combi 2 CT/GC assay (Hologic Genprobe, US), carried out using a Tigris (Hologic, Genprobe US) automated NAAT system. The nucleic acid extractions were then tested for the presence of *C.trachomatis* nucleic acid via the 16SrRNA qPCR assay (an in-house diagnostic assay at the Public Health England South West Laboratory), carried out on an ABI 7500, and also the CT 0332 LAMP assay, carried out using the Qiagen ESEquant.

4.3. Results

4.3.1. Confirmation of successful *C. trachomatis* infection of McCoy cells using fluorescence microscopy

LIVE/DEAD staining of McCoy cells infected with *C. trachomatis* was used in order to visually identify, via fluorescence microscopy, whether the infection was successful.

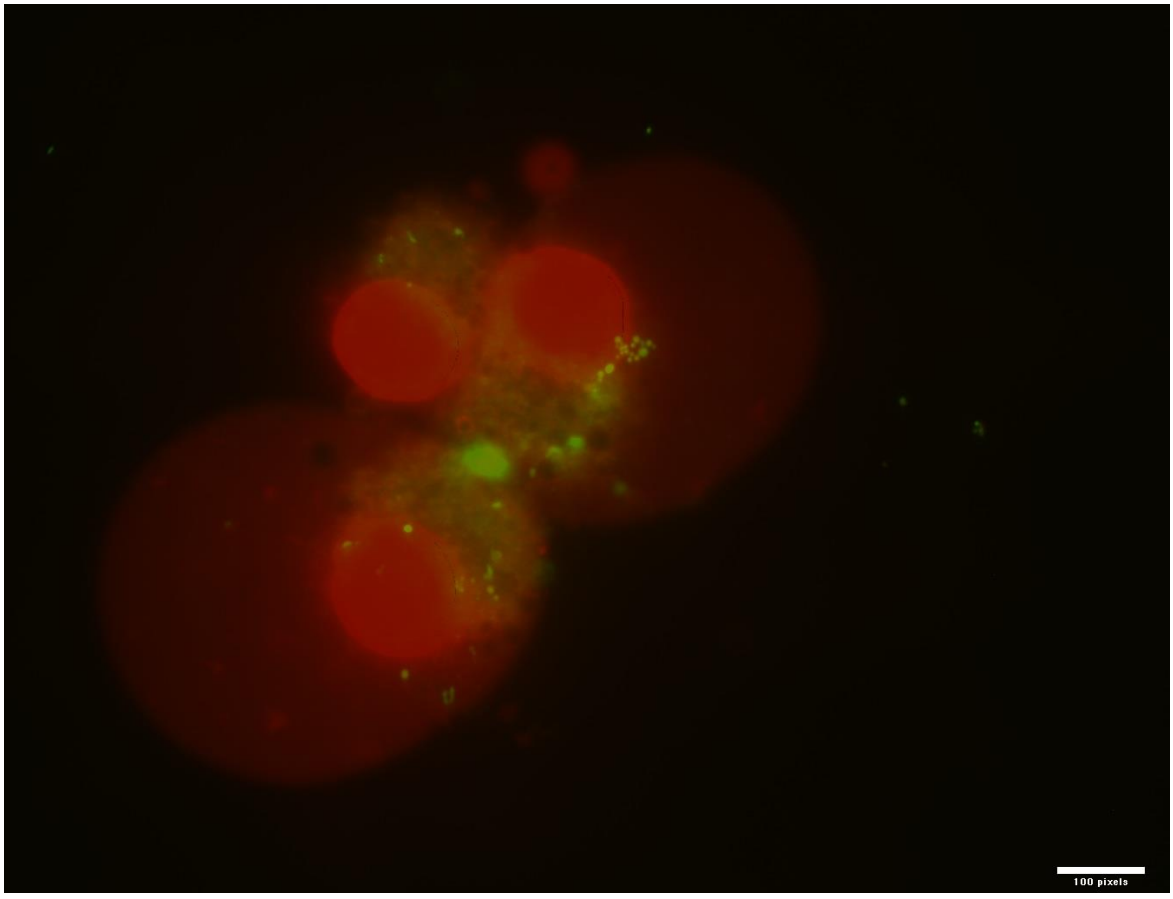

Fig.4.2. Image of McCoy cells (stained red; nuclei bright red) infected with *C. trachomatis* (stained green), acquired using fluorescence microscopy. Staining carried out using LIVE/DEAD backlight cell viability kit (Invitrogen, US).

The McCoy cells are visible (Fig.4.2.), the cytoplasm stained dark red, whilst the nuclei are a brigher red, having taken up a larger concentration of propidium iodide. The *C. trachomatis* inclusion can be seen as a green area surrounding the nuclei of the cells.

4.3.2. Detection of *C. trachomatis* nucleic acid from *C. trachomatis* infected McCoy cell culture using the CT-0332 LAMP assay.

An experiment was carried out In order to determine whether the CT-0332 LAMP assay, in conjunction with the total nucleic acid extraction method, was capable of detecting the presence of *C. trachomatis* in infected McCoy cell culture. This was carried out to demonstrate primer specificity and confirm the particular *C. trachomatis* strain that we are working with is detectable with the primer sets, and also show the ability of the extraction method to extract nucleic acid from this organism.

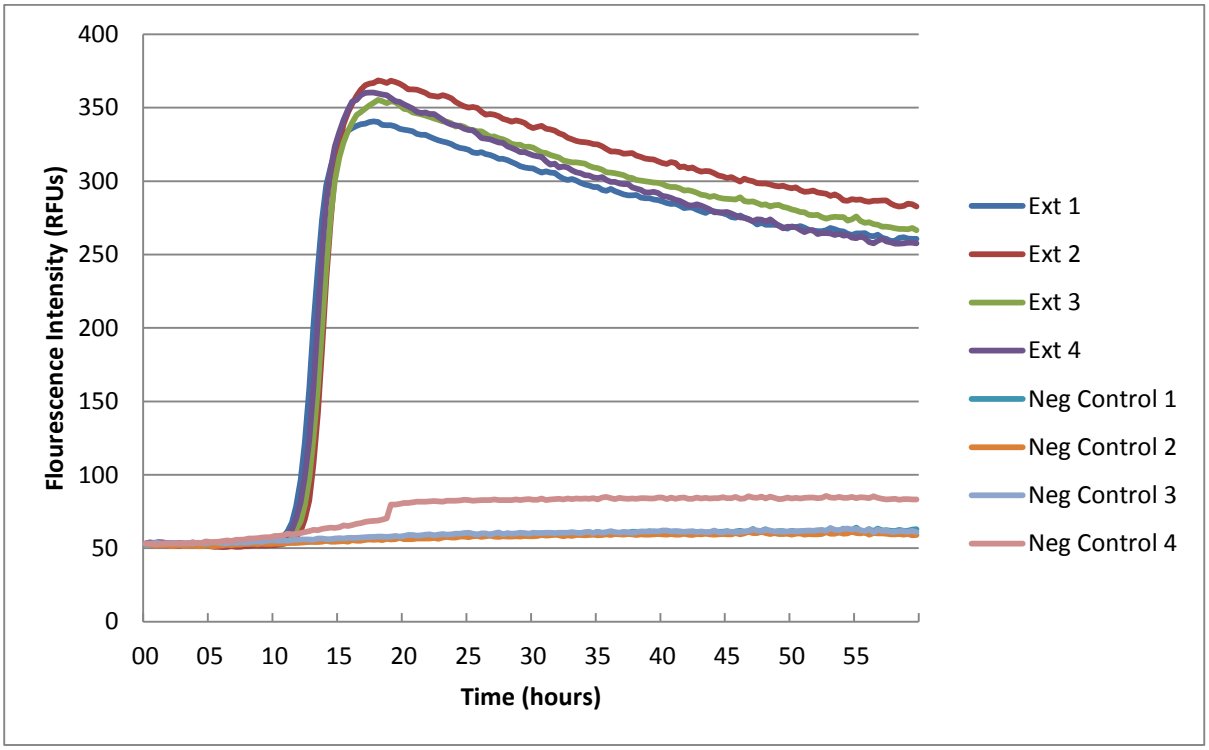

Fig.4.3. LAMP reaction amplification plot of nucleic acid extracted from McCoy cells infected with *C. trachomatis* (Ext 1-4). Nucleic acid extracted from uninfected McCoy cells was used as a negative control.

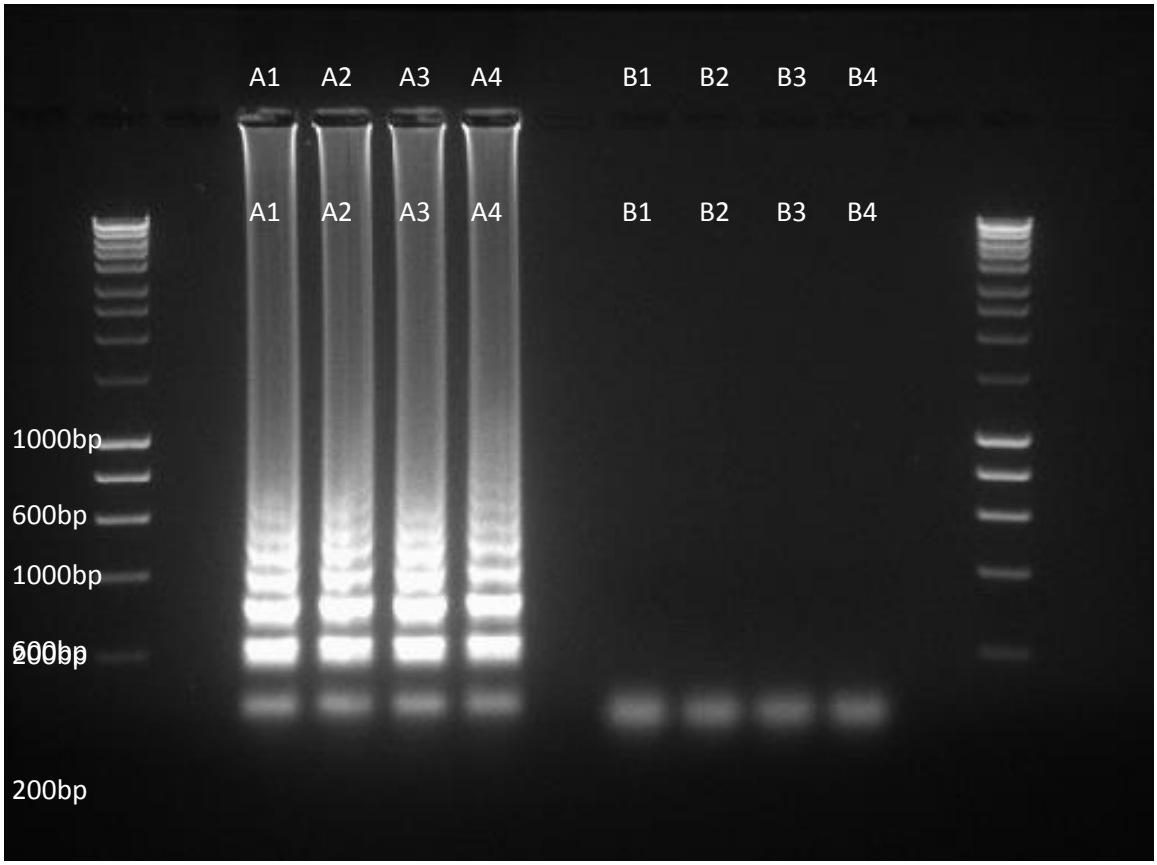

Fig.4.4. 1.5% agarose gel electrophoresis of LAMP reaction products from fig.3.1. Extractions 1-4 in well A1-A4, negative control 1-4 in wells B1-B4

Amplification occurred in all of the LAMP reactions containing nucleic acid extracted from *C. trachomatis* elementary bodies, as expected (Fig.4.3). No amplification was seen in the negative control reactions. Amplification in all 4 of the positive reactions was detectable after 11-12 minutes, with uniform grouping of the amplification curves. The level of fluorescence intensity generated was similar between all positive reactions (340 – 370 RFUs).

The agarose gel electrophoresis of the end point reaction products (Fig 4.4) shows the characteristic ladder formation of LAMP reaction products (Notomi *et al*., 2000), formed by a high concentration of nucleic acid concatamers of certain undefined lengths, between 600bp and ~100bp. The negative reactions showed no amplified nucleic acid, only low molecular weight bands representing the 4 primers used in the reaction.

4.3.3. Detection of *C. trachomatis* in clinical samples using the 0332 LAMP assay in conjunction with the magnetic silica based extraction method.

An experiment was carried out in order to determine whether the CT- 0332 LAMP assay, in conjunction with the magnetic silica based extraction method, was capable of detecting *C. trachomatis* DNA from clinical endocervical swab and urine samples.

Nucleic acid extractions were carried out on a *C. trachomatis* positive urine and endocervical swab sample in duplicate, using the magnetic silica based total nucleic acid extraction method. Extractions were carried out on a *C. trachomatis* negative urine swab in order to provide a negative control. Eluted nucleic acid from all extractions was then tested for the presence of *C. trachomatis* nucleic acid using the CT-0332 LAMP assay.

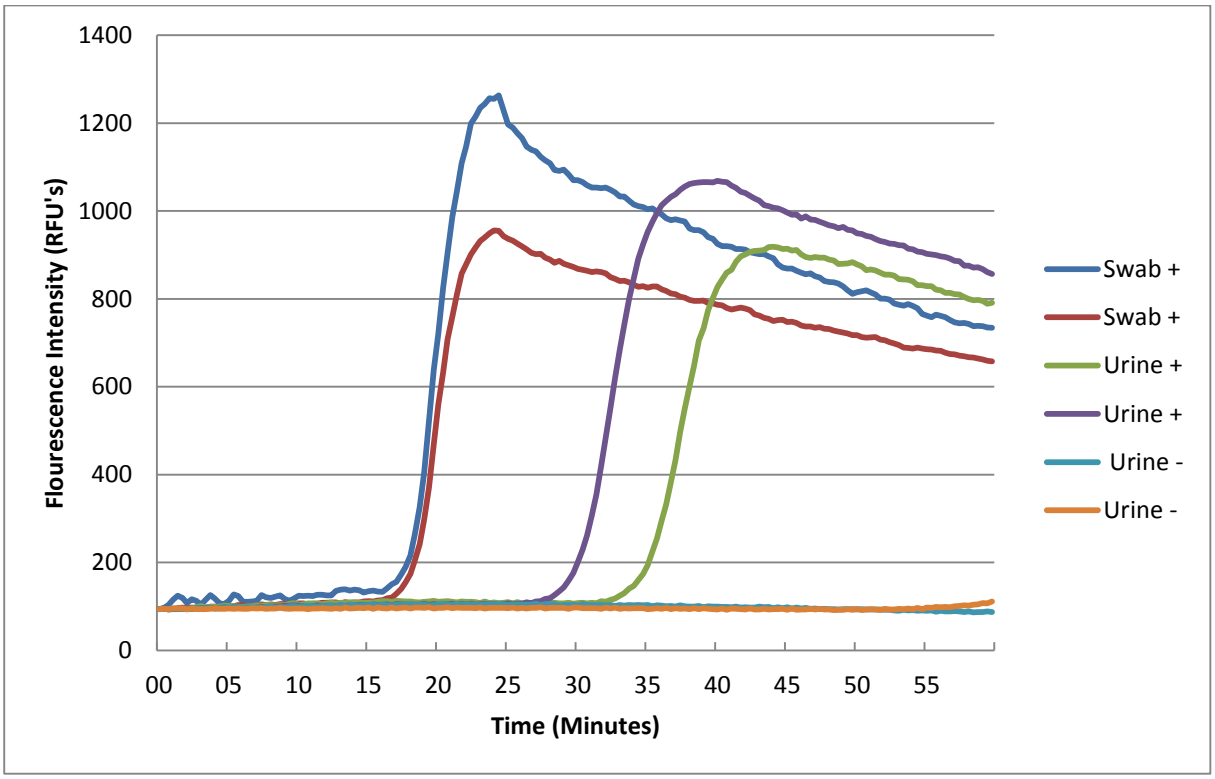

Fig.4.5. CT-0332 LAMP reaction amplification plot of nucleic acid extractions carried out using the silica bead based method, from *C. trachomatis* positive clinical endocervical swab sample (Swab +), and positive urine sample (Urine +). A *C. trachomatis* negative clinical urine sample was used as a negative control (Urine -).

The CT-0332 LAMP assay was able to identify *C. trachomatis* DNA in the positive urine and swab sample, in both duplicate extractions. The LAMP assay generated no detectable amplification from the samples determined as negative by the Aptima assay. The swab sample generated the earliest amplification, with both duplicates showing good agreement of amplification time, crossing the manually set Ct value at ~18 minutes (Fig.4.5). The urine sample generated a later amplification, indicating a lower gene copy number, and hence organism load, in this sample. There was a larger difference in time to amplification between the duplicates of the extractions from the positive urine samples, which crossed the threshold line at 29 minutes and 34 minutes, respectively.

4.3.4. The Determination of the limits of detection (LOD) of the CT-0332 LAMP assay, 16s rRNA taqman qPCR assay, and Genprobe Aptima Combi 2 assay for the detection of *C. trachomatis*.

The limit of detection (LOD) of an assay is the minimum concentration of analyte, in this case *C. trachomatis* cells, which the assay is capable of detecting. This is an important factor to determine, as the detection limit will directly impact on the assay sensitivity. It is important that the limit of detection is not greater than the minimum concentration seen in positive clinical sample, as this will lead to false-negative results when used as a diagnostic test. It is important that the *C. trachomatis* LAMP assay performs as well as other NAAT methods, as these are the benchmarks of sensitivity that the assay will be judged against.

The lowest number of *C. trachomatis* elementary bodies detectable by three nucleic acid amplification tests (NAATs) for the detection of *C. trachomatis*; 16S rRNA taqman qPCR assay, 0332 LAMP assay, and the Aptima assay (Genprobe), were determined.

Purified *C. trachomatis EB* stock was added to transport media in an Aptima assay tube (Genprobe), to a final concentration of 100,000 EB per ml. A total of 6 1:10 serial dilutions were then carried out into additional Aptima assay tubes containing transport media. The EB concentration of each tube is shown in Table 4.5.

Sample Tube	EB concentration (per ml)
1	100,000
2	10,000
3	1,000
4	100
5	10
6	1
7	0

Table.4.5.Concentration of *C. trachomatis* EBs in each sample tube

Total genomic nucleic acid was then extracted from 200µl of each sample, using the KIngFisher Ml automated RNA extraction system, in conjunction with standard extraction buffers. The sample tubes then underwent the Aptima Combi 2 CT/GC assay, ran using a Genprobe Tigris automated NAAT system. The RNA extractions were then tested for the presence of *C. trachomatis* nucleic acid via the 16SrRNA qPCR assay, carried out on an ABI 7500, and also the CT 0332 LAMP assay, carried out on a Qiagen ESEquant.

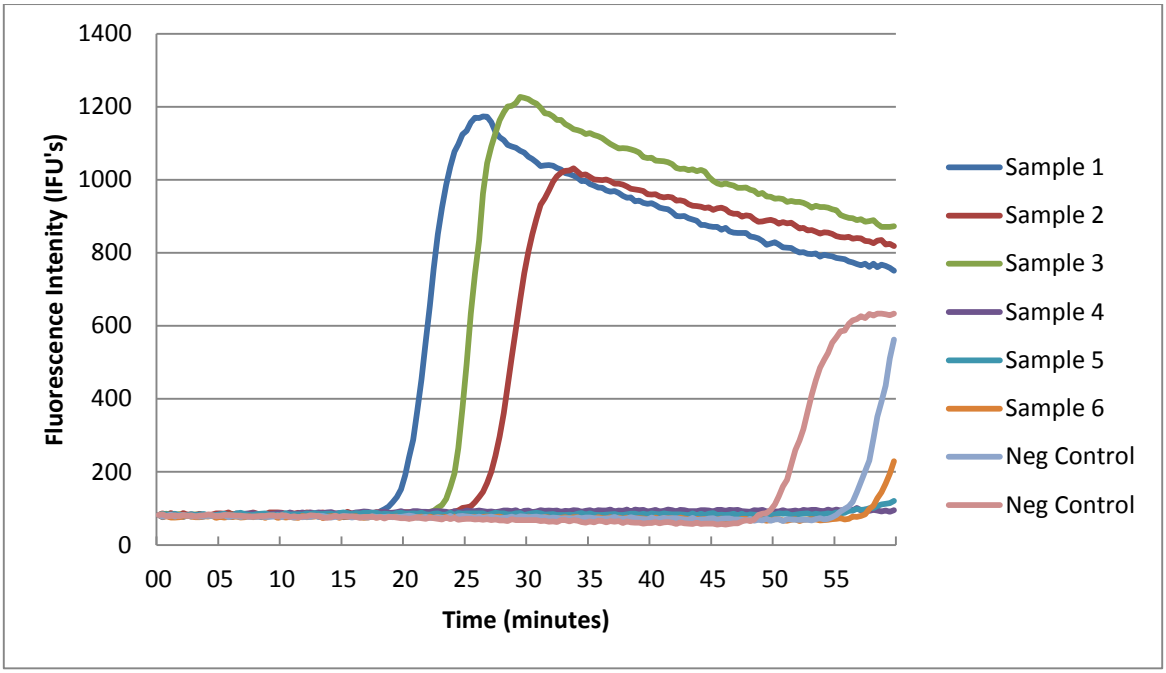

Fig.4.6. LAMP amplification plot showing the limit of detection of the CT-0332 LAMP assay. Nucleic acid extractions were carried out on 1/10 serial dilutions of a quantified *C. trachomatis* elementary body suspension (100,000 EB's per ml). Extractions were then tested for the presence of *C. trachomatis* nucleic acid using the CT-0332 assay. A *C. trachomatis* negative clinical swab sample was used as a negative control.

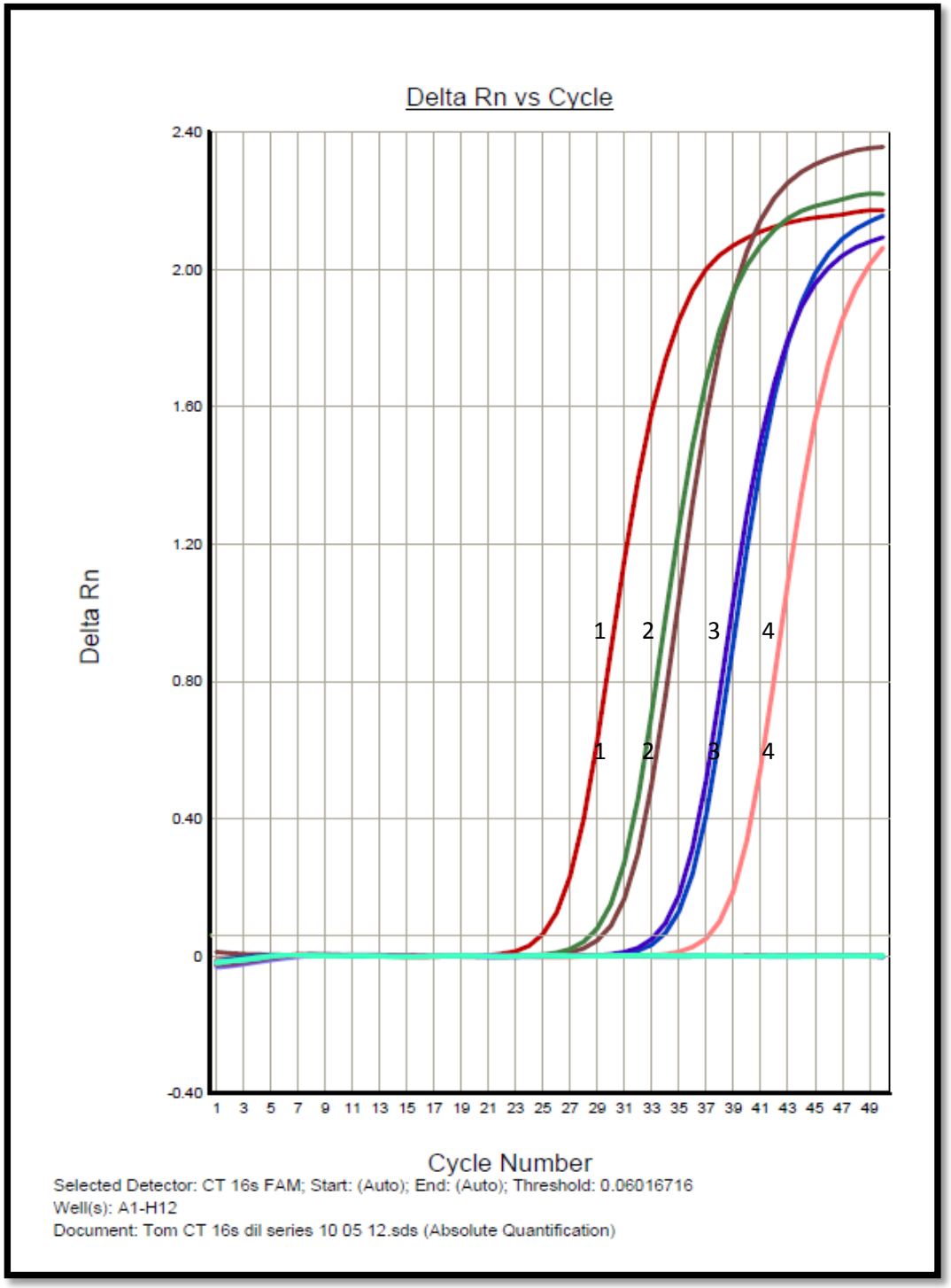

Fig.4.7. Amplification plot showing limits of detection of *C. trachomatis* 16s Taqman qPCR assay. Nucleic acid extractions were carried out on 1/10 serial dilutions of a quantified *C. trachomatis* elementary body suspension (100,000 EB's per ml). Extractions were then tested for the presence of *C. trachomatis* nucleic acid using the CT-0332 assay. A *C. trachomatis* negative clinical swab sample was used as a negative control. Line colour corresponding to sample identity shown via key.

Sample	EB count (per ml)	Total RLU (000's)	Result
CT Positive Control		1178	CT POS
1	100,000	-	-
2	10,000	1301	CT POS
3	1,000	1313	CT POS
4	100	1261	CT POS
5	10	669	CT POS
6	1	59	CT EQUIV (NEG)
7	0	27	CT NEG
Negative Control	0	6	CT NEG

Table 4.6. Table showing the limits of detection of the Genprobe Aptima *C. trachomatis* diagnostic assay, as run on a Genprobe Tigris instrument system. 1/10 serial dilutions of a quantified *C. trachomatis* elementary body suspension (100,000 EB's per ml) were prepared and processed as samples. Sample 1 was not processed due to an excessive organism load, which can cause a false negative result.

16S Taqman Assay

Sample	EB count (per ml)	Volume used in extraction	EB's used in extraction	Volume eluted	Volume amplified	Equivalent number of EBs	Results
1	100,000	200µl	20,000	75µl	5µl	1,333	POS
2	10,000	200µl	2,000	75µl	5µl	133	POS
3	1000	200µl	200	75µl	5µl	13	POS
4	100	200µl	20	75µl	5µl	1.3	P/N
5	10	200µl	2	75µl	5µl	0	NEG
6	1	200µl	0	75µl	5µl	0	NEG
7	0	200µl	0	75µl	5µl	0	NEG
Negative	0	200µl	0	75µl	5µl	0	NEG

Table 4.7. Tables showing the relation between the number of *C. trachomatis* Elementary bodies per extraction and per assay, and limit of detection for the 16s Taqman qPCR assay.

Genprobe Aptima

Sample	EB count (per ml)	Volume used in extraction	EB's used in extraction	Volume eluted	Volume amplified	Equivalent number of EBs	Results
1	100,000	1ml	100,000	1ml	1ml	100,000	-
2	10,000	1ml	10,000	1ml	1ml	10,000	POS
3	1000	1ml	1000	1ml	1ml	1000	POS
4	100	1ml	100	1ml	1ml	100	POS
5	10	1ml	10	1ml	1ml	10	POS
6	1	1ml	1	1ml	1ml	1	POS
7	0	1ml	0	1ml	1ml	0	NEG
Negative	0	1ml	0	1ml	1ml	0	NEG

Table 4.8. Tables showing the relation between the number of *C. trachomatis* Elementary bodies per extraction and per assay, and limit of detection for the Genprobe Aptima assay.

CT 0332 LAMP Assay

Sample	EB count (per ml)	Volume used in extraction	EB's used in extraction	Volume eluted	Volume amplified	Equivalent number of EBs	Results
1	100,000	200µl	20,000	75µl	3µl	800	POS
2	10,000	200µl	2,000	75µl	3µl	80	POS
3	1000	200µl	200	75µl	3µl	8	POS
4	100	200µl	20	75µl	3µl	0.8	NEG
5	10	200µl	2	75µl	3µl	0	NEG
6	1	200µl	0	75µl	3µl	0	NEG
7	0	200µl	0	75µl	3µl	0	NEG
Negative	0	200µl	0	75µl	3µl	0	NEG

Table 4.9. Tables showing the relation between the number of *C. trachomatis* Elementary bodies per extraction and per assay, and limit of detection for CT 0332 LAMP assay.

The 16s Taqman qPCR assay was able to detect a level of 1000 EB per ml of sample in both duplicate reactions (Table 4.7). This equated to 200 EB's used in the actual extraction, and an equivalent of 13 EB's quotient of nucleic acid in the actual assay tube. One of two duplicates was able to detect down to 100 EB per ml of sample, or 1.3 EB's quotient of nucleic acid per reaction tube .

The Genprobe Aptima assay was able to successfully detect 10 EBs per ml of sample (Table 4.8). The Aptima assay process involves the extraction of nucleic acid from the entire 1ml sample, increasing the chances of detection from samples with low organism loads.

The CT-0332 LAMP assay was able to detect a minimum of 1000 EB per ml of sample (Table 4.9). This involved 200 EB's being used in the actual extraction, equating to a total of 8 EB's quotient of nucleic acid present in the assay itself.

4.4. Discussion

4.4.1. Confirmation of successful *C. trachomatis* infection of McCoy cells using fluorescence microscopy

Infected McCoy cells were stained using a LIVE/DEAD backlight cell viability kit (Invitrogen). This kit contains a mix of two dyes; Syto 9, which enters all bacterial cells, irrespective of cell membrane integrity, and produces a green fluorescence; and propidium iodide, which only crosses through permeable membranes, or non-viable cells, staining with a red fluorescence. This mixture of dyes has the effect of staining viable cells green, and non-viable cells red, giving an easily read visualisation of the viability of a culture.

The harvesting of the McCoy cells, including trypsinisation, centrifugation, and air drying, has impaired their membrane integrity, leading to the uptake of propidium iodide, but not Syto 9 (Fig.4.2.). This provides a good contrast to enable the detection of *C. trachomatis* inclusion bodies surrounding the nuclei, which have taken up the Syto 9 dye, and are fluorescing green. The name "*Chlamydia*" is derived from the Greek word for cloak, after the observation that the inclusions are draped around the nuclei of infected cells, like a cloak (Kumaresan, 2005). The presence of these inclusions indicates that the infection of the McCoy cells was successful, and that replication of the *C. trachomatis* reticulate bodies has occurred. Being able to confirm this visually is very important; the alternative method, PCR, would also detect the original inoculum, and is unable to distinguish between dead or live cells. This is especially true of end-point PCR, which gives no quantifiable data on the level of DNA present. By determining via fluorescence microscopy that the infection has been successful, confirmation is given that this sample will be suitable for lysing and using to reinfect further McCoy cell cultures to maintain the culture of *C. trachomatis*.

4.4.2. Detection of *C. trachomatis* nucleic acid from *C. trachomatis* infected McCoy cell culture using CT-0332 LAMP assay.

The CT-0332 LAMP assay was able to detect *C. trachomatis* nucleic acid in all four nucleic acid extractions from cultured *C. trachomatis* elementary bodies (Fig.4.3.). This showed the generation of amplifiable nucleic acid by the silica based total nucleic acid extraction method, and showed the methods compatibility with the LAMP assay. Amplification in all 4 of the positive reactions was

detectable after 11-12 minutes, with uniform grouping of the amplification curves. The level of fluorescence intensity generated was similar between all positive reactions (340 – 370 RFUs), indicating that either each LAMP reaction generated an equal concentration of amplified product, or the V13 dye in each reaction was saturated, and giving maximum output.

The agarose gel electrophoresis of the end point reaction products (Fig 4.4) shows the characteristic ladder formation of LAMP reaction products, formed by a high concentration of nucleic acid concatamers of certain undefined lengths, between 600bp and ~100bp, containing multiple inverted repeats. Showing the presence of the characteristic bands in the LAMP reaction products via agarose gel electrophoresis confirms the reactions determined as positive by the ESEQuant are genuine positive reactions, and the amplification seen was not a product of non-specific amplification, or a false positive reaction. The negative reactions showed no amplified nucleic acid, only low molecular weight bands representing the 4 primers used in the reaction.

4.4.3. Detection of *C. trachomatis* in clinical samples using the 0332 LAMP assay in conjunction with the magnetic silica based extraction method.

The ability of the silica based nucleic acid extraction system to extract amplifiable nucleic acid from clinical samples is paramount. As well as being representative of the type of samples that will be processed by the final assay, clinical samples represent a more complex sample than those created in the lab using *C. trachomatis* culture. For example, a urine sample can include human epithelial cells, variable numbers and species of non-target bacteria and yeast cells, a range of organic compounds including creatine, uric acid and urea, a range of peptides including hormones and mucins, and a number of positive and negatively charged ions, including K^+, Na^+, Cl^-, Ca^{2+}. Concentrations of several these components are typically elevated or depressed in particular disease states, leading to a highly variable sample. Therefore it is important to test the assay on human samples to determine the extent of the effect that these factors have on assay performance.

The delay in amplification seen in extractions carried out from the urine swab sample (18, 18 minutes) in comparison to the urine sample (29, 35 minutes) could have been down to a number of factors. One possibility is that the endocervical sample contained a higher number of *C. trachomatis* cells than the urine sample, which is likely, as the average organism load is known to be higher in endocervical swab samples than urines (Wiggins *et al.*, 2009). A previous study (Wiggins *et al.*, 2009) utilised qPCR to determine organism load at varying anatomical sites, and found swab samples to

contain a mean of 10,405 organisms per ml, compared to 503 per ml in first void urine specimens. However, the organism load in clinical specimens can be highly variable, with factors such as the length of duration of infection prior to sampling, urination prior to sampling, swabbing technique and antibiotic treatment all capable of having an effect. Both sample types potentially contain PCR inhibitors; excessive mucus in endocervical swab samples has been shown to have an inhibitory effect on downstream PCR assays for *C. trachomatis* detection (Tan and Chan, 2005), and urea, present in urine samples is a known inhibitor of PCR (Khan *et al.*, 1991). The incomplete removal of these inhibitors during the extraction could also delay the amplification.

Studies have shown nucleic acid diagnostic tests to have similar sensitivities and specificities for Chlamydia infection detection when endocervical swabs or urine samples were used. (Haugland *et al.*, 2010) screened 603 women for infection, 80 of whom were positive for *C. trachomatis*. They found the sensitivity and specificity of a PCR assay to be 90.2% and 98.3% respectively when a urine sample was used. The assay carried out using endocervical swab samples was determined to have a sensitivity and specificity of 89% and 99.2% respectively. This shows that both sample types are adequate samples for nucleic acid testing, and LAMP amplification should be possible from both types of common clinical samples.

4.4.4. The Determination of the limits of detection (LOD) of the 0332 LAMP assay, 16s rRNA taqman qPCR assay, and Aptima Combo 2 (Genprobe) assay for the detection of *C. trachomatis*.

The Aptima Combo 2 (Genprobe, US) test was found to have the lowest limit of detection, successfully detecting as few as 10 EB per ml (Table 4.8). However, due to the 1ml volume used in extraction, the extracted volume contains 5 times as much target nucleic acid tests than the extractions carried out using the Kingfisher Ml with the same samples. The Aptima Combo 2 Assay is a target amplification nucleic acid probe test that utilizes target capture for the qualitative detection of *C. trachomatis* (via the 23S rRNA sequence) and *N. gonorrhoeae* from clinical swab or urine samples. Package insert specificity for *C. trachomatis* detection is given as 98.9%, which has been replicated experimentally (Levett *et al.*, 2008), making the assay one of the most sensitive commercially available diagnostic tests for *C. trachomatis* (Lowe *et al.*, 2006).

The limit of detection determined was slightly higher than seen in previous studies, which found it able to detect nucleic acids from concentrations as low as an equivalent of 0.008 EB per ml (Ikeda-Dantsuji *et al.*, 2005). This difference could be due to a higher number of dead cells present in the

purified EB stock, reducing RNA levels, or an initial overestimation of EBs in the stock. (Ikeda-Dantsuji *et al.*, 2005) used EB's purified directly from cell culture, which would have significantly higher RNA levels, resulted in a higher concentration of nucleic acid target for the assay.

The 16S Taqman assay was able to detect the sample 3 (1000 EB/ml) as positive (Fig.3.6.), which correlated to 13 EBs per assay (Table 4.7.). Of the two duplicate tests on sample 4 (100 EB/ml), one tested positive, one negative, indicating that the true LOD lies around this range. The assay is a qPCR assay, using primers targeted to the *C. trachomatis* 16S rRNA gene. The 16S rRNA genes of bacterium contain hypervariable sequences, which are typically species specific and are therefore a good target for amplification in diagnostic PCR assays. A Taqman probe provides the fluorescent signal, enabling real-time detection. Component concentrations of this assay were optimised by Bristol HPA, and the assay is used as a gold standard in their laboratory.

The 0332 LAMP assay, like the 16S Taqman PCR, was able to detect sample 3 (1000 EB/ml) (Fig.3.5.). However due to the reduced volume of extracted nucleic acid used per test, this correlated to 8 EB's per individual assay (Table 4.8.). This detection limit was found at a higher number of organisms than expected. Previous studies have reported detection limits of as low as 10 cells per ml, for organisms including *Trypanosoma brucei rhodesiense* (Njiru *et al.*, 2008), and reports of detection limits in the range of 100 organisms per ml are common, for species such as *mycobacterium tuberculosis* (Bi *et al.*, 2012), *Pneumocystis jirovecii* (Uemura *et al.*, 2008) and *Salmonella* (Chen *et al.*, 2011).

Blocker (Blocker *et al.*, 2002), found EB levels in positive urine samples ranged from 32/ml to 1,048,576/ml. An apparent bimodal distribution was displayed, with 37% of samples in a peak ranging from 32 to 1,015 EB/ml (median = 297 EB/ml), and 63% of samples in a grouping of higher concentrations, ranging from 1,086 to 218,670 EB/ml (median = 7,389 EB/ml). By scaling the LOD of the CT 0332 LAMP reaction up to a sample size of 1ml, 200 EBs would be the assays limit, relatively close to the minimum levels seen in Blocker *et al.,* (2002) (Blocker *et al.*, 2002). This could be improved further via optimisation of the primer concentration, reaction mix component concentration and extraction procedure.

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Chapter 5

Trichomonas vaginalis

5.1. Introduction

Trichomonas vaginalis is a flagellated protozoan parasite of the human genital tract and the cause of the most prevalent curable sexually transmitted disease globally, with an estimated 276.4 million cases per year, worldwide (World Health Organisation, 2012). Infections of the female genital tract

can cause a range of symptoms, including vaginitis and cervicitis (Heine and McGregor, 1993). Infections in males are generally asymptomatic, although mild urethritis or prostatitis can occur (Guenthner *et al.*, 2005). During the last decade, the discovery that *T. vaginalis* infection is associated with a range of more serious conditions, such as prostate cancer, cervical cancer, adverse pregnancy outcomes, and an increased likelihood of HIV infection, has increased the efforts to diagnose and treat patients harbouring this parasite (Bachmann *et al.*, 2011).

5.1.1. Morphology

T. vaginalis has no cystic stage in its life cycle, existing in axenic culture only as a tear drop shaped trophozoite, with an average length and width of 10µm and 7µm, respectively (Petrin *et al.*, 1998). A total of four anterior flagella provide the parasite with its characteristic twitching motility, whilst a single posterior flagellum, which forms the outer edge of the undulating membrane, runs along the length of one side of the cell, assisting in motility and the movement of extracellular nutrients towards the cytosome of the cell. The cell also possesses an axostyl, a bundle of microtubules passing through the cell along its anterior-posterior axis into the extracellular environment (Lee *et al.*, 2012), which has a function in cell attachment and mitosis (Ribeiro *et al.*, 2000). During infection, whilst in contact with host epithelial cells, the morphology of the cell is markedly different; the flagella are internalised and the cell assumes an amoeboid conformation, and adheres to the epithelial surface (Fiori et al., 1999). The cytoplasm contains a single defined nucleus, and several hydrogenosomes; primitive redox organelles, evolved from mitochondria, which produce molecular hydrogen and ATP (Schneider *et al.*, 2011).

5.1.2. Pathogenesis

Transmission occurs almost exclusively via sexual contact, although transmission via fomites has been documented, but is rarely encountered and controversial (Schwebke and Burgess, 2004). Documented suspected non-sexual transmission routes include shared bathing water (Crucitti *et al.*, 2011; Burch *et al.*, 1959), and bathing implements (Adu-sarkodie, 1995); routes that are theoretically

possible due to the ability of the parasite to survive for up to three hours in a moist environment (Krieger and Kimmig, 1995). However, these reports are difficult to definitively prove, as ruling out sexual contact relies on the reports of the individuals concerned, which may not always be reliable.

During sexual intercourse, *T. vaginalis* cells in the genital tract of the infected partner are transferred to the uninfected partner, and come in to contact with the genital epithelia. When in contact with epithelial cells, the typically ovoid *T. vaginalis* cell morphologically adjusts, assuming an amoeboid conformation (Gould *et al.*, 2013). The cells attach to the epithelial surface, with the amoeboid morphology enabling the parasite to increase the surface area contact, and interaction, with the epithelial cell. *T. vaginalis* adhesion is largely mediated by a range of iron-dependent surface adhesins (Munoz *et al.*, 2012). There are five primary surface adhesins responsible for the attachment of the parasite to the host epithelia; AP120, AP65, AP51, AP33, AP23 (Garcia and Alderete, 2007). With the exception of AP51, the genes encoding these proteins are all transcriptionally upregulated by the presence of iron, which is an essential mediator of *T. vaginalis* growth, and a key factor in virulence (Ryu *et al.*, 2001). Of these surface proteins, AP65 has been hypothesised as the most important; anti AP65 serum IgG antibodies inhibit *T. vaginalis* cytoadherance, which does not occur when the same is carried out on other adhesins (Garcia *et al.*, 2003). Interestingly, it has been shown that AP65 does not have a covalent anchor motif, and is released extracellularly, where it binds to both the *T. vaginalis* and epithelial cell surface (Garcia and Alderete, 2007). The synthesis and transport of these adhesins to the outer membrane occurs in response to the contact of the parasite with vaginal epithelial cells, in tandem with a morphological shift to the amoeboid form. After adherence, the *T. vaginalis* cells recruit further parasites to the location, forming sizeable aggregates of ameboid cells on the epithelial surface (Arroyo *et al.*, 1992). The other primary mediator of cytoadherance to the host epithelia is surface lipophosphoglycan, the most highly expressed protein on the *T. vaginalis* surface membrane (2×10^6 to 3×10^6 copies per parasite) (Fichorova *et al.*, 2006), which binds to the galectin-1 protein located on the surface of human epithelial cells (Ryan *et al.*, 2011). Site directed mutagenesis studies have shown that *T. vaginalis* cells expressing a lipophosphoglycan molecule with altered surface residues have a greatly reduced adherence and cytotoxicity to human vaginal epithelial cells, underlining the importance of this molecule for parasite attachment and virulence (Bastida-Corcuera *et al.*, 2005). The glyceraldehyde-3-phosphate dehydrogenase, GAPDH, is another protein expressed on the surface of *T. vaginalis* that has been determined to be involved in cytoadherance (Lama *et al.*, 2009).

The adherence of *T. vaginalis* to the epithelial cell surface is a crucial factor in pathogenesis; adherence of the parasite is cytotoxic, and typically results in the lysis of the host cell, and erosion of

the epithelial monolayer. This process also instigates the inflammatory response, involving the release of chemokines such as IL-8 and the recruitment of neutrophils to infected tissues (Fichorova *et al.*, 2006). Damage to the vaginal epithelial monolayer during infection is known to occur via a variety of mechanisms, and this contact dependent killing does not involve phagocytosis (Krieger *et al.*, 1985). Adherence of *T. vaginalis* to epithelial cells causes a weakening of the junctional complex between individual cells in the epithelial monolayer. This weakening is a result of a decrease in trans-epithelial electrical resistance, an increase in the gap between neighbouring cells, and also modification of the distribution of junction complex proteins, all resulting from the interaction with the parasite (Guenthner *et al.*, 2005; da Costa *et al.*, 2005).

Damage to the host epithelia is also caused by parasite mediated apoptosis of epithelial cells, which is dependent on the release of CP30 cysteine proteases (Kummer *et al.*, 2008). This group of 4 cysteine proteases are also linked to adhesion and the passage of the parasite through the mucosal barrier, making them important factors in *T. vaginalis* pathogenesis. The release of polyamine compounds as metabolic end-products by *T. vaginalis* is another cause of the cytotoxicity caused during infection. *T. vaginalis* is incapable of producing spermine metabolically, and relies on the uptake of his molecule from the extracellular environment. This occurs via an antiporter system that requires the extracellular release of putrescine (Yarlett et al., 2000), which is detectable at high levels in the secretions of *T. vaginalis* infected women (Garcia et al., 2005). Putrescine has been shown to induce apoptosis of mammalian cells *in vitro*, via increased activity of caspase-3, a key regulator of the apoptosis process (Takao et al., 2006). The inhibition of polyamine metabolism has been shown to reduce contact dependent killing of vaginal epithelial cells by 90% *in vitro*, and also inhibit *T. vaginalis* growth (Garcia et al., 2005), demonstrating the importance of polyamine production in *T. vaginalis* pathogenesis. *T. vaginalis* is also capable of the *in vitro* phagocytosis of vaginal epithelial cells, leukocytes and erythroyctes, along with commensal bacteria (Rendon-Maldonado *et al.*, 1998) and yeasts (Pereira-Neves and Benchimol, 2007) of the genital tract. Two distinct mechanisms of phagocytosis have been observed during *in vitro* studies with yeasts; a classic form of phagocytosis involving extension of pseudopodia, which then engulf the target cell, and also a more passive form, where the target cell sinks into the *T. vaginalis* membrane (Pereira-Neves and Benchimol, 2007). Phagocytosis is followed by intracellular killing in lysosomes, and provides the *T. vaginalis* cell with a source of nutrients (Francioli *et al.*, 1983). Phagoyctosis is also thought to be the primary route of horizontal gene transfer between bacteria and *T. vaginalis*, providing the parasite with an important mechanism of genetic diversification and adaptation (de Koning *et al.*, 2000). The precise mechanisms by which the *T. vaginalis* cells recognise target cells appropriate for phagocytosis is poorly understood, although non-specific mannose receptors on the *T. vaginalis*

outer membrane have been implicated in the internalisation of yeast cells (Pereira-Neves and Benchimol, 2007). Mannose binding lectins have been shown to bind Gram positive and Gram negative bacteria, as well as yeasts, protozoa and even some viruses (Klein and Kilpatrick, 2004), showing the wide range of organisms identifiable by the presence of this ligand. Mannose is also present on the surface of epithelial cells (Hanada *et al.*, 2014), leukocytes (Rodriguez-Ortega *et al.*, 1987), and erythroyctes (Lodish, 2000), so *T. vaginalis* mannose receptors may play a part in the recognition of these cell types during phagocytosis or lysis. *T. vaginalis* is known to be able to recognise erythrocytes, and is able to lyse erythrocytes both *in vitro* and *in vivo*, with haemolysis depending on adherence of the parasite (Fiori *et al.*, 1993). This is thought to provide the parasite with a source of iron, an essential nutrient for *T. vaginalis* growth (Ryu *et al.*, 2001).

5.1.3. Epidemiology and clinical presentation

The collection of meaningful epidemiological data on *T.vaginalis* infection is hampered by the fact that trichomoniasis is not currently a reportable infection, in developed or developing countries (Poole and McClelland, 2013). This means that global and local prevalence and infection rates have to be estimated from localised studies, due to the lack of case reporting data available. The most recent World Health Organisation (WHO) estimates from data collected in 2008 (World Health Organisation, 2012) indicate 276.4 million new cases per year, or 187 million adults infected at any one time. This represents a larger number of new infections than those of the next two most prevalent STI's combined, with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections estimated to number 105.7 million and 106.1 million, respectively (World Health Organisation, 2012). The prevalence and incidence of *T. vaginalis* infection is geographically variable (Table 5.1), with the highest prevalence found in Africa (20.2% of females, 2% of males), and the Americas (22% of females, 2.2% of males). The prevalence in Europe is estimated as 5.8% of females and 0.6% of Males.

WHO Region	Incidence (per 1000)		Prevalence (%)	
	Female	Male	Female	Male
Africa	146.0	164.8	20.2	2
The Americas	177.7	180.6	22.0	2.2

South East Asia	40.3	50.1	5.6	0.6
European	51.7	48.4	5.8	0.6
Eastern Mediterranean	64.0	66.1	8.0	0.8
Western Pacific	45.6	47	5.7	0.6

Table 5.1. Global incidence and prevalence of *T. vaginalis* infection, according to 2012 WHO estimates. Data taken from World Health Organisation, 2012.

The estimated incidence of *T. vaginalis* is fractionally higher in men than in women, with a male to female ratio of total cases globally of 1.14. There is an obvious discrepancy between this fairly equal relationship, and the sizeable difference in estimated prevalence rates between the sexes. The prevalence of *T. vaginalis* infection in women is around 10 times higher than in men, irrespective of geographic location. This is indicative of the self clearing nature of the infection in males, with the majority of persistent infections occurring in females (Van Der Pol, 2007). The higher likelihood of persistence in the female genital tract has been linked to the availability of iron, which increases during the menstrual cycle, providing the parasite with increased exposure to a major growth requirement (Beltrán *et al.*, 2013). The expression of various adhesion proteins promoting cytoadherance is enhanced in response to increased iron concentration (Sehgal *et al.*, 2012), and this is associated with increased virulence (Ryu *et al.*, 2001). The prevalence of *T. vaginalis* infection has been seen to differ between ethnic populations in the same geographical area, with black females in the US having a higher prevalence (10.5%; 95% CI, 8.3-13.3%) than white females (1.1%; 95% CI, 0.8-1.6%) (Miller *et al.*, 2005).

T. vaginalis infection in females is symptomatic in around 50% of cases, and around 30% of asymptomatic cases develop some symptoms in the 6 month period post infection (Rein, 1990). Common symptoms include itching and pain during intercourse, a frothy discharge, and vaginitis, which can range from mild to severe (Petrin *et al.*, 1998). In acute cases, punctate hemorrhagic spots may be present on the vaginal and cervical mucosa, a pathology referred to as colpitis macularis, or “strawberry cervix” (Swygard *et al.*, 2004). *T. vaginalis* infection has also been associated with cervicitis, urethritis and also more serious complications such as pelvic inflammatory disease (PID) (Heine and McGregor, 1993), cervical cancer (Zhang *et al.*, 1995) and infertility (El-Shazly *et al.*, 2001). The effects of *T. vaginalis* infection during pregnancy, and on pregnancy outcomes, are well

documented. Infections with *T. vaginalis* at the mid gestation point of pregnancy increases the likelihood of a preterm delivery and low birth weight (Cotch *et al.*, 1997). Interestingly, the successful treatment of *T. vaginalis* infection in pregnant women at the mid gestation point does not prevent this subsequent preterm delivery (Klebanoff *et al.*, 2001). Neonatal genital and nasopharyngeal infections have been reported, with transmission thought to occur during birth (Smith *et al.*, 2002).

Infection of the male genitourinary tract is generally asymptomatic, although mild urethritis, epididymitis (Fisher and Morton, 1969), and prostatitis can occur (Guenthner *et al.*, 2005). *T. vaginalis* colonisation of the prostate can lead to chronic infection, and is thought to be the cause of most persistent infections in males. Trichomonads have been detected in the prostatic urethra, and in the surrounding tissues of the prostate, including the glandula lumina, sub mucosa and stroma (Gardner *et al.*, 1986). Until recently, *T. vaginalis* infection in males was considered a "nuisance infection", without serious consequences (Van Der Pol, 2007). This view has been changed somewhat, with the realisation that colonisation of the prostate by *T. vaginalis* is a risk factor in the development of prostate cancer (Sutcliffe *et al.*, 2006). A study involving 673 subjects with prostate cancer, and an equal number of control subjects, found a statistically significant association between those seropositive for *T. vaginalis* antibodies, and those with prostate malignancy (Stark *et al.*, 2009). The increase in risk in developing prostate cancer has been estimated to be between 23% and 40% (Stark *et al.*, 2009; Sutcliffe *et al.*, 2006). One potential mechanism responsible for the increased likelihood of carcinogenesis seen during *T. vaginalis* infection is the host inflammatory response (Ryu *et al.*, 2004), which involves the increase production of pro-inflammatory cytokines that have been implicated in prostate malignancy (Azevedo *et al.*, 2011). *T. vaginalis* infection of has also been shown to increase the expression of the proto-oncogene *PIM1* in cultured prostate epithelial cells (Sutcliffe *et al.*, 2012). PIM1 is also known to induce the expression of another proto-oncogene, HMGA1, via the PIM1/c-MYC/HMG1 signalling cascade. Both c-MYC and HMG1 are frequently over expressed in malignant prostate cells, and their expression has been linked to increased proliferation and metastasis (Sutcliffe *et al.*, 2012). Recent work has shown that *T. vaginalis* produces a novel protein, *T. vaginalis* macrophage migration inhibitory factor (TvMIF), which has 47% sequence agreement with the pro-inflammatory cytokine Human macrophage migration inhibitory factor (HuMIF) (Twu *et al.*, 2014). TvMIF inhibits macrophage migration, causes inflammation, and activates ERK, Atk, and Bcl-2-associated death promoter phosphorylation, inhibiting with apoptosis and causing cellular proliferation. The same study demonstrates that exposure of benign and malignant prostate cells to TvMIF *in vitro* instigates growth and invasion (Twu *et al.*, 2014).

5.1.4. Relationship with other microorganisms

The presence of *T. vaginalis* in female patients can cause extensive changes in the vaginal microbiome, and trichomoniasis often occurs in tandem with bacterial vaginosis (BV) (Fichorova *et al.*, 2013), a condition involving an imbalance in the bacterial flora of the vagina causing vaginal inflammation. This imbalance commonly manifests as a reduction in overall numbers of *Lactobacillus sp*., thought to be crucial for the maintenance of vaginal health, in combination with an increase in other commensal bacteria that are usually only present in lower levels, such as *Gardnerella vaginalis*, *Mobiluncus curtisii*, *Megasphaera sp*., *Atopobium vaginae*, and *Leptotrichia sp*. (Fredricks *et al.*, 2007). The lactobacilii, predominant in women with a typical vaginal microbiota, contribute to vaginal health by releasing lactic acid, which maintains optimal vaginal pH. *T. vaginalis* grows optimally at a pH of 6 to 6.3 (Petrin *et al.*, 1998), whilst the vaginal pH in women with a lactobacilli dominated vaginal environment ranges between 2.8 and 4.2 (O'Hanlon *et al.*, 2011). The disruption of the lactobacilli community seen during trichomoniasis reduces the lactic acid released into the vaginal environment, increasing the pH, and creating more favourable conditions for *T. vaginalis* (Cudmore *et al.*, 2004). Lactobacilli also act against pathogenic organisms in the vaginal environment by out-competing them for nutrients, and also via the release of hydrogen peroxide, which is toxic to a number of potentially BV causing organisms such as *Gardnerella vaginalis* (Klebanoff *et al.*, 1991), and the production of a range of antimicrobial peptides. These peptides include lactocin B, a bacteriocin which specifically inhibits the growth of a number of bacterial species associated with BV, whilst sparing organisms that form the healthy vaginal flora (Turovskiy et al., 2009). A variety of bacteriocins have been isolated from *Lactobacillus* strains, which have been characterised as having an antimicrobial effect on a wide range of Gram positive and Gram negative bacteria (Soylak et al., 2003). It is possible that some of these antimicrobial peptides could also have an antiprotozoal effect, and that the reduction of these organisms is conducive to the survival and growth of *T. vaginalis*. BV can have serious health implications, many of which overlap with trichomoniasis; increased risk of HIV transmission (Mirmonsef *et al.*, 2012), ascending inflammatory infections and preterm birth/low birth weight pregnancy (Taylor *et al.*, 2013). The combination of T*. vaginalis* and BV associated bacteria have been shown to amplify the host immune response, including an up-regulation of chemokines such as IL-8, and a down regulation of SLPI, an enzyme which protects epithelial cells from serine proteases (Fichorova *et al.*, 2013). SLPI has virucidal effects, and the down-regulation of this enzyme, in combination with the damage caused to the

epithelial monolayer by *T. vaginalis*, could increased the likelihood of infection with sexually transmitted viral pathogens such as HIV and HPV.

There is a growing body of evidence that suggests *T. vaginalis* infection increases the chance of the acquisition, and transmission of HIV (Mavedzenge *et al.*, 2010).This is of particular concern as *T. vaginalis* is especially prevalent in regions where HIV is considered to be endemic, such as Sub-Saharan Africa, where 32 million *T. vaginalis* infections occur each year (McClelland *et al.*, 2007). Epidemiological studies have recorded an increase in the risk of HIV-1 acquisition of between 1.52 and 2.74 fold in *T. vaginalis* positive women in Sub-Saharan African countries (McClelland *et al.*, 2007; Mavedzenge *et al.*, 2010; Van Der Pol *et al.*, 2008). A similarly sized increase in the risk of transmitting HIV to a serodiscordant partner has also been recorded in *T. vaginalis* positive women in these regions (Sorvillo *et al.*, 2001). The fairly recent realisation of the impact that *T. vaginalis* prevalence has on HIV rates has hastened a greater public health response (McClelland *et al.*, 2007), and it is now recognised that the control of *T. vaginalis* could have a sizeable impact on the reduction of HIV transmission in these populations.

The exact mechanism by which *T. vaginalis* infection acts to increase the risk of contracting HIV is currently undetermined (Thurman and Doncel, 2011), although a number of theories have been suggested and tested. *T. vaginalis* infection instigates a robust mucosal immune response, involving localised inflammation and the recruitment of lymphocyctes and macrophages (Fichorova *et al.*, 2013). This increases the number of potential cells for the virus to invade and proliferate in, and would make transmission more likely in a HIV-negative individual. Additionally, in a HIV-positive individual, the increase in cells infected with the virus localised in the genital tract would aid HIV shedding during sexual contact, exposing any partners to a higher level of viral particles, facilitating transmission (Shafir *et al.*, 2009). HIV positive men with symptomatic urethritis caused by *T. vaginalis* have been shown to have a higher seminal viral load than those with either *T. vaginalis* negative, or with an asymptomatic (Hobbs *et al.*, 1999). Those with symptoms of urethritis will necessarily have the greatest level of inflammation, and the greatest levels of CD4 lymphocytes and macrophages, increasing the targets for HIV invasion. Additionally, *T. vaginalis* causes damage to the urogenital epithelia, facilitating passage of HIV to deeper layers of the epithelium, and enhancing infection (Guenthner *et al.*, 2005).

One of the more novel intermicrobial relationships of *T. vaginalis* is the association of the parasite with *Mycoplasma hominis*. This symbiotic relationship, first reported by Nielsen (Nielsen, 1975) was the first described association of two obligate human parasites. Co-infection of *T. vaginalis* with a variety of bacterial strains is common, due to the change in vaginal milieu caused by *T. vaginalis*

infection, which leads to a favourable growth environment for a number of bacterial species not normally associated with the vaginal microbiota. In the case of *M. hominis* however, the mycoplasma has been found to actually enter *T. vaginalis* cells, surviving and even proliferating internally (Vancini and Benchimol, 2008). The presence of *M. hominis* alongside T. *vaginalis* has been shown to lead to an upregulation of inflammatory cytokines expression in host macrophages *in vitro*, which would potentially increase localised inflammation *in vivo* (Fiori *et al.*, 2013). Initial concerns that infection with *M. hominis* could be related to metronidazole resistance in *T. vaginalis* appear unfounded (Butler *et al.*, 2010).

The relationship with *M. hominis* is not the only symbiotic relationship that occurs involving *T. vaginalis*. The majority of *T. vaginalis* strains encountered in human infections are infected with one or more of a family of four double stranded RNA viruses, from the genus Trichomonasvirus (TVV), family totiviridae (Parent *et al.*, 2013). The presence of this virus has been shown to increase virulence of *T. vaginalis* (Parent *et al.*, 2013), in a process hypothesised to involve the modulation of parasite gene expression (Goodman *et al.*, 2011). *T. vaginalis* borne TVV is also recognised by the host immune system via its interaction with human toll-like receptor 3 (TLR3), which instigates a pro-inflammatory cytokine cascade; a process previously linked to increased susceptibility to epithelial invasion by HIV (Fichorova *et al.*, 2012). The presence of TVV during trichomoniasis can cause up to 30 fold amplification of the immune response, increasing the severity of the infection, and risk of more serious complications such as PID (Fichorova *et al.*, 2013). It has also been demonstrated that this effect is particularly pronounced during simultaneous bacterial vaginosis (Fichorova *et al.*, 2013), highlighting that the interaction between the vaginal microbiome, protozoan parasite, associated endosimbiant TVV and human epithelial cells has a large bearing on the immune response and infection severity.

5.1.5. Diagnosis in Female patients

Trichomoniasis is the most common non-viral sexually transmitted infection (STI) worldwide, with a higher prevalence than *C. trachomatis* and *N. gonorrhoeae* infections combined (World Health Organisation, 2012). Despite this, there is no routine screening programme in place in the UK or US, apart from during pregnancy, as there is for *C. trachomatis* (Workowski and Berman, 2010; Ross,

2006). This is due in part to the higher incidence of PID and tubal infertility caused by *C. trachomatis*, and also the lower frequency of asymptomatic infection in female patients with trichomoniasis. Symptomatic patients in the UK are tested for *T. vaginalis*, with testing available in the majority of GUM clinics.

The symptoms of *T. vaginalis* infection overlap significantly with those caused by a number of other sexually transmitted pathogens, such as *N. gonorrhoea* and *Mycoplasma genitalium,* and a diagnosis from clinical presentation alone is rarely possible. The specific symptoms of trichomoniasis, such as a the typical inflamed and speckled “strawberry cervix” , and frothy discharge, only occur in a minority of around 2% of cases (Fouts and Kraus, 1980), and so cannot be relied upon as a sole indicator of the infection. Accurate diagnosis of trichomoniasis is important for the subsequent treatment of infection, as antibiotics given for general urethritis treatment such as azithromycin or doxycycline are not effective treatments for trichomoniasis (Abdolrasouli *et al.*, 2007).

Diagnosis of trichomoniasis in female patients is frequently carried out microscopically, by the examination of a “wet mount” of vaginal or cervical exudates for motile parasites. This method is very simple to carry out, fast, and cost effective, when compared with alternative diagnostic options, including culture or molecular methods (Bachmann *et al.*, 2011). Despite these advantages microscopic evaluation is not considered the optimal detection method, due to the low sensitivity afforded by this technique. Microscopy has been shown to have a sensitivity of around 60%, when compared with PCR based methods (Patil *et al.*, 2012). Microscopy is unlikely to detect low level infections, in which the organism load in the sample may be under 10^4 cells/ml, and therefore will potentially not be included in the fields examined on the slide (Garber, 2005). The sensitivity of this method decreases rapidly if delays are present between sample acquisition and examination, with a reduction in sensitivity to 20% caused by a as little as a 10 minute delay reported (Kingston *et al.*, 2003). Due to this it has been suggested that any diagnostic service which cannot guarantee the ability to test samples within an hour of acquisition should use alternative methods (Stoner *et al.*, 2013). This is of particular concern for clinics that do not operate an on-site microscopy service, and rely on the transport of samples to remote centralised laboratories for examination. This loss of sensitivity occurs due to the reduction in parasite motility, making the trichomonads difficult to identify. Due to their similar size and shape, *T. vaginalis* cells can be hard to differentiate between lymphocytes when non motile (Garber, 2005).

The culture of *T. vaginalis* from clinical samples has long been regarded as the gold standard for the diagnosis of this organism (Bachmann *et al.*, 2011). Cultures are typically maintained in a broth medium, and inoculated with swab samples taken from the vaginal canal or cervix of female

patients, or urethral discharge from male patients. Cultures can also be inoculated from urine samples, although this is not the optimal sampling method, and reduces sensitivity. Growth can be apparent in as little as 48 hours, but cultures should be incubated for at least 7 days to enable the detection from low inocula (Garber, 2005). The most common media used are variants of Diamonds (TYM) medium (Diamond, 1957), which originally contained trypticase digest, yeast extract, maltose, cysteine, ascorbic acid and sheep serum. Possibly the most frequently used variant is Diamonds TYI-S-33 medium, which also contains a source of iron, foetal bovine serum in place of sheep serum, and a vitamin 107-Tween 80 mixture (Diamond *et al.*, 1978). *T. vaginalis* is incapable of synthesising a range of macromolecules necessary for survival and growth, including purines, pyrimidines and some lipids, and relies on nutrients acquired from secretions or phagocytosed human or bacterial cells in the host genital tract. This necessitates the presence of these molecules in any culture media, and serum especially is a key component to support axenic growth of this organism (Petrin *et al.*, 1998).

The use of broth culture to diagnose *T. vaginalis* infection has a higher diagnostic sensitivity than wet mount microscopy. One study of 337 samples, including 97 positive samples, found microscopy and culture had sensitivities of 52% and 78% respectively (Wendel *et al.*, 2002). However, the use of culture to diagnose trichomonas infection does have some significant disadvantages. The week long incubation required means that culture is the diagnostic option with the longest time between sample acquisition and result. Additionally, culture is less sensitive than molecular methods, such as PCR. The use of solid media to culture *T. vaginalis* has been reported in the literature (Stary *et al.*, 2002), although is rarely used in a clinical diagnostic setting. One study found a solid modified Columbia agar medium to be more sensitive (98.4%) than a commercially available Trichomonas medium (92.1%) (Stary *et al.*, 2002).

One commercially available culture based diagnostic test is the InPouch culture system (Biomed Diagnostics, USA). This system combines both culture and microscopy to provide a diagnostic solution that offers the advantages of both methods (Sood *et al.*, 2007). It consists of a clear plastic pounch containing two conjoined chambers full of media, one of which is inoculated via a swab. The other chamber has thinner walls, and a viewing window enabling examination for any trichomonads using microscopy. This method has been shown to be more sensitive than wet-mount microscopy alone (Draper *et al.*, 1993; Sood *et al.*, 2007), and also removes the need for a pre culture transport medium, as the pouch is inoculated directly from the patient, which improves the likelihood of a successful culture (Schwebke *et al.*, 1999). The fact that microscopic evaluation of the culture can be carried out without any fluid manipulation removes the possibility of contamination and reduces the

time taken during the examination. This method is more expensive than standard culture or microscopy based methods, however (Draper *et al.*, 1993).

Serological methods for diagnosing *T. vaginalis* from vaginal secretions have been developed, but are rarely used clinically. A monoclonal antibody based enzyme-linked immunosorbant assay (ELISA) test specific to *T. vaginalis* surface peptides has been shown to offer sensitivities and specificities of 89% and 98% compared with broth culture (Lisi *et al.*, 1988). A detection limit of 100 trichomonads per ml, and a greater sensitivity than wet mount microscopy, has been demonstrated using another ELISA assay (Watt *et al.*, 1986). The Trichomonas Direct Enzyme Immunassay (California Integrated Diagnostics, US), was a commercially released ELISA test, which is no longer on the market. It relied on a mix of peroxidase labelled monoclonal antibodies to an assortment of *T. vaginalis* proteins, and was as sensitive as broth culture (Petrin *et al.*, 1998). One immunoassay currently available commercially, and the only *T. vaginalis* immunoassay currently awarded FDA approval in the US, is the OSOM Trichomonas Rapid Test (Sekisui Diagnostics, US). The test is an immunochromatographic capillary flow dipstick test, and provides a result within 10 minutes, enabling it's use at the point of care (POC). The OSOM test has a sensitivity and specificity of 82% and 97% respectively (Huppert *et al.*, 2007), making it a more sensitive diagnostic test than wet-mount microscopy, culture, and standard ELISA methods, whilst being far quicker and simpler to carry out.

Both commercial, and "in house" PCR based assays for *T. vaginalis* are available, and provide a more sensitive form of testing than the traditional methods of wet-mount microscopy and culture. Although PCR requires more highly trained staff and more expensive equipment and reagents, than alternative methods, the sizeable increase in sensitivity, coupled with a relatively short turnaround time, makes PCR based assays the optimum diagnostic method in developed countries. A range of genes have been exploited as targets for *T. vaginalis* specific PCR tests. A standard PCR assay specific to a sequence of the beta-tubulin gene was found to have a sensitivity and specificity of 97% and 98% respectively (Madico *et al.*, 1998). The same study found the sensitivities of wet mount microscopy and culture to be 36% and 70% respectively, illustrating the improvement in sensitivity offered by PCR. A study comparing the sensitivity of two real-time fluorescence resonance energy transfer (FRET) hybridisation probe based PCR assays specific to the beta-tubulin gene and 18S rRNA gene found assay sensitivities of 96% and 100% respectively (Simpson *et al.*, 2007). The *T. vaginalis* genome harbours a number of conserved repeated DNA sequences, and these are attractive targets for nucleic acid amplification tests (NAATs), as they provide a higher copy number per cell, and improve detection limits and sensitivities (Bandea *et al.*, 2013). The sequencing of the ~160 Mb *T. vaginalis* genome identified 59 common repeat families that make up ~39 Mb of the complete

sequence (Carlton *et al.*, 2007). The majority of the repeat sequences have a copy number of >100, with the average being 660 copies. Importantly, these repeats show a high level of homogeneity, with sequence variation identified between repeats of the same family in only 2.5% of repeats. This provides a stable, high copy number target for molecular assays. Single parasite detection has been demonstrated for PCR assays using these repeated sequences as targets (Kengne *et al.*, 1994), and the improved sensitivity has enable testing from non-invasive urine samples, which typically contain a lower organism load than the swab samples more frequently used (Bandea *et al.*, 2013).

The majority of NAATs available for *T. vaginalis* identification are PCR based, however novel isothermal diagnostic methods have been applied to the detection of this organism. The commercially available APTIMA *T. vaginalis* assay (Hologic Gen-Probe, US), relies on transcription mediated amplification technology, in combination with a target capture specimen processing system, to provide a highly sensitive assay for *T. vaginalis* detection (Chapin and Andrea, 2011). The assay is designed to be used on one of the automation systems available from Gen-Probe, such as the TIGRIS. The assay is approved for use in the US by the Federal Drug Administration (FDA), and is approved for use in the UK. The approval only relates to the use of the assay with a number of sample types from female patients, including urine samples, endocervical swabs, and vaginal swabs. GenProbe also manufactures AMPTIMA assays for other sexually transmitted pathogens, including *N. gonorrhoeae*, human papillomavirus (HPV), and a combined *C. trachomatis* and *N. gonorrhoeae* assay (APTIMA COMBO 2 assay). A large scale study of 933 symptomatic and asymptomatic female patients attending an STI clinic found the APTIMA assay to have the following sensitivity and specificity, respectively, in the following samples types; 100% and 99.0% for vaginal swabs, 100% and 99.4% for endocervical swabs, and 95.2% and 98.9% in urine specimens (Schwebke *et al.*, 2011).

Another commercially available molecular diagnostic test for *T. vaginalis* infection is the Affirm VPIII *Trichomonas vaginalis* assay (Becton Dickinson, US), which relies on RNA probe hybridisation to detect target DNA. The test has been shown to be more sensitive than wet mount microscopy (Brown *et al.*, 2004), but lacks sensitivity compared to NAATs, as the target DNA is not amplified before detection, which results in a higher starting copy number being required in order to generate signal. One study compared the Affirm VPIII assay with the APTIMA assay, and found sensitivities of 63.4% and 100% respectively (Chapin and Andrea, 2011). The test is fully automated, and takes 45 minutes to run, including 2 minutes “hands on time”, potentially enabling point of care testing. The BD ProbeTec Qx (Becton Dickinson, US) is another NAAT for *T. vaginalis* with FDA approval, which uses strand displacement amplification in conjunction with a fluorescent detection system, targeting a portion of the *T. vaginalis ap61-5* gene. This assay is processed on the BD Viper system, a fully

automated sample handling system capable of processing the sample through nucleic acid extraction and subsequent amplification. An evaluation of the assay determined the assay performance as being similar to the Aptima *T. vaginalis* assay, with a sensitivity and specificity of 98.3% and 99.6% respectively, over the 838 samples tested (Van der Pol et al., 2014). The planned release of a *T. vaginalis* test for the GeneXpert system (Cepheid, US) in the 2014-2015 product range has also been announced, potentially expanding the options for NAAT testing. The GeneXpert is a platform for processing real-time PCR based assays with fully automated sample preparation, amplification and detection on disposable assay-specific cartridges, providing results within 90 minutes in order to enable same-day testing and treatment (Helb *et al.*, 2010). The automated liquid handling and extraction process minimise hands-on time, to less than five minutes per specimen (Marlowe et al., 2011), and provides the high sensitivity and specificity of NAAT diagnostics without the need for highly trained laboratory staff.

POC testing for sexually transmitted infections, including trichomoniasis, could be of great benefit in the control of these diseases (Tucker *et al.*, 2013). Testing at the POC enables consultation, testing, and the provision of appropriate treatment to all be carried out in the same day, at the same site. This removes the risk of patients neglecting to return for results and medication, and also reduces the possibility of transmission by sexual contact during the delay before treatment is instigated (Tucker *et al.*, 2013). Currently, it is possible to carry out testing via wet mount microscopy at POC, although this is insensitive, low throughput, and effected by the experience and skill of the technician. Also, the immunochromatographic OSOM Trichomonas Rapid Test (Sekisui Diagnostics, US) is able to provide results within 10 minutes, is easy to use and read, and has higher sensitivity than non-molecular methods (82% compared with PCR), making it a very good option for providing a POC diagnostic (Huppert *et al.*, 2007). The current goal of POC diagnostic research is to provide the sensitivity of NAAT diagnostics, whilst eliminating hands-on processing, and decreasing the time-to-result, enabling maximally sensitive testing at the point of care (Craw and Balachandran, 2012). The majority of POC NAAT systems in development rely on an automated nucleic acid procedure, rapid target amplification, and detection of reaction products, typically by optical detection of fluorescence (Niemz *et al.*, 2011). These processes are often carried out in a disposable single use cartridge or chip, preventing contamination of the machine during sample handling (Niemz *et al.*, 2011). One such system currently in development is the Atlas Io PoC (Atlas Ltd, UK) platform, which is aiming to release a *T. vaginalis* test in 2014. The test involves automated nucleic acid extraction, followed by amplification of a multi-copy DNA repeat sequence target, and novel electrochemical endpoint detection. A small scale lab evaluation of the test, comparing its performance with that of

the APTIMA *T. vaginalis* test (Hologic Gen-Probe, USA), over 90 clinical samples, found that the sensitivity and specificity of the assay were 95.5% and 97.5% respectively (Pearce *et al.*, 2013).

The use of POC testing for trichomoniasis could be of particular benefit in sub-Saharan Africa, where both *T. vaginalis* and HIV infection are highly prevalent. The improved control of *T. vaginalis* could potentially reduce HIV transmission, and significantly impact on morbitity and mortality in the region (Johnston and Mabey, 2008). POC testing has been regarded as being particularly well suited to developing countries, as the automated POC systems reduce the need for skilled technicians, or well equipped centralised laboratories, which may not be widely available (Pai *et al.*, 2012). Additionally, the ease of transport, and lack of additional equipment needed by these systems, enables the testing of remote communities, far removed from traditional hospital based healthcare. However, concerns remain over whether the expense of POC NAAT systems will prevent their widespread use in developing countries, with studies examining the prospective cost of implicating widespread POC NAAT testing in Africa highlighting the increased cost of diagnosis (Meyer-Rath *et al.*, 2012).

5.1.6. Diagnosis in male patients

The diagnostic testing for *T. vaginalis* infection in male patients is rarely undertaken, for a number of reasons. *T. vaginalis* infection in men is rarely symptomatic, and male partners of women who have received a positive diagnosis are treated concurrently without any confirmatory testing (Schwebke and Lawing, 2002). Microscopy of urethral discharge, if present, has a poorer sensitivity with male samples than females. Culture can be undertaken from male samples, and the optimal sample type is considered to be a combination of urethral swabbing and collection of urine sediment, however, as with microscopy, sensitivity is poor (Krieger *et al.*, 1993). The low organism loads encountered in male patients mean that sensitive, molecular methods are required in order to provide an acceptable level of sensitivity, if testing for *T. vaginalis* infection is going to be carried out.

In the past *T. vaginalis* infection in males has often been considered to be a nuisance infection, due to the fact that the infection in males is self-limiting, typically clears without intervention, and the belief that infection in males rarely results in any serious sequelae (Van Der Pol, 2007). However, there is growing evidence to the contrary; that infection in males can lead to chronic colonisation (Lee *et al.*, 2012), and that it may be a causative agent in prostate cancer (Sutcliffe *et al.*, 2006). It has been shown conclusively that *T. vaginalis* increases the transmission of sexually transmitted viruses, including HIV (Sorvillo and Kerndt, 1998; Mavedzenge *et al.*, 2010), which in itself makes the

control of this organism in males a non-trivial matter. The increasing realisation that this organism is associated with disease states with high morbidity in both men and women, and can have serious detrimental effects on reproductive health has lead to an increased interest for diagnosis and treatment of infections (Soper, 2004), and the treatment of males inevitably will play a part in this process. This is driving the need for sensitive, molecular based approaches for the detection of this organism.

5.1.7. Treatment

The standard treatment for *T. vaginalis* infection, in the UK and worldwide, is a single 2g oral dose of metronidazole or tinidazole (Muzny and Schwebke, 2013). These antibiotics from the 5-nitroimidazole family exert their antimicrobial activity by disrupting the redox system of the parasite, with metabolic products of the drug binding to proteins in the thioredoxin mediated redox network and inhibiting thioredoxin reductase (Leitsch *et al.*, 2009). The antimicrobial properties of these drugs rely on reduction at the nitro group, which occurs after passive diffusion of the drug into the hydrogenosome of the cell, generating nitroradical anions, and further reduced reactive intermediates (Dunne *et al.*, 2003).Whilst in bacterial cells these nitroradicals would cause DNA damage, and cell death, the precise mechanism of by which they damage eukaryotic microorganisms is poorly understood (Kulda, 1999).

Of the available 5-nitroimidazole drugs, metronidazole is most frequently admistered, part due to the low associated cost (Sherrard *et al.*, 2014). This is despite tinidazole being considered the optimal antimicrobial agent, with numerous studies showing that tinidazole therapy has either an equal or lower failure rate than metronidazole therapy (Bachmann *et al.*, 2011). Tinidazole therpay is also better tolerated, and has lower associated toxicity than metronidazole (Nailor and Sobel, 2007). A large scale study reviewing the outcomes of female patients receiving different therapies found the clinical failure rate of those taking metronidazole to be 14.8%, compared with a 3.7% failure rate for those receiving tinidazole (Bachmann *et al.*, 2011). This is partly explained by the ~12.5 hour half-life of tinidazole being ~70% longer than that of metronidazole, and its higher serum concentration (Bachmann *et al.*, 2011), and tissue availability (Viitanen et al., 1985). The tissue availability of metronidazole also varies significantly between individuals, which is thought to account for a proportion of the treatment failures encountered (Holter et al., 1983; Kirkcaldy et al., 2012)

A major cause of treatment failure in metronidazole therapy is metronidazole resistance; a study involving 175 women who had failed at least two standard treatments of metronidazole therapy found that 115 of the 175 isolates displayed metronidazole resistance *in vitro* (Bosserman et al., 2011). Resistance to metronidazole, the antimicrobial that has historically seen the greatest use in the treatment of this organism, is more frequently encountered than tinidazole resistance. Although increased metronidazole resistance is correlated with increased tinidazole resistance, *in vitro* testing has shown the minimum inhibitory concentrations (MICs) of metronidazole are significantly higher than those of tinidazole in metronidazole resistant isolates (Crowell et al., 2003). The overall prevalence of resistance is thought to be low; a study of 538 isolates from a number of clinical sites in the US found low level metronizadole resistance in 4.3% of isolates, and no resistance to tinidazole (Kirkcaldy *et al.*, 2012). Another study, involving 467 high risk female adolescents attending STI clinics in the US found a high prevalence of T. vaginalis infection (14.4%), but a low prevalence of resistance to metronidazole (2.7%) and no tinidazole resistant isolates (Krashin et al., 2010). The prevalence of 5-nitroimidazole resistance in developing countries does not appear to be any greater than in developed countries; studies in Africa have found the prevalence to be 6% (Rukasha *et al.*, 2013). Although the prevalence of resistance is low, the number of resistant cases is still significant, due to the high incidence of *T. vaginalis* infection overall. Treatment failures due to metronidazole resistance increase the cost of therapy, due to the need for reassessment and additional treatment, and increase the risk of further transmission, if the patient resumes sexually activity in the belief that they are no longer infected.

The *in vitro* determination of metronidazole MICs has been shown to be of value in determining therapeutic dose in female patients who have experienced treatment failure, with a higher cure rate obtained in women who had been treated in accordance with recommendations based on MIC testing (Bosserman et al., 2011). MIC testing for this organism has not been standardised, but is most commonly carried out by incubating a culture of the isolate in media containing varying concentrations of the antimicrobial for up to 48 hours, and then examining microscopically for motile trichomonads (Crowell et al., 2003; Wendel and Workowski, 2007). The MIC is given as the minimum concentration at which no motile parasites are detectable. For comprehensive susceptibility testing the incubations should be carried out under both aerobic and anaerobic conditions, as the mechanisms of anaerobic and aerobic resistance differ in this organism (Upcroft and Upcroft, 2001). Anaerobic resistance is very rarely clinically encountered; metronidazole resistant isolates are generally found to employ aerobic resistance mechanisms, making this the more clinically valuable condition to assess (Cudmore et al., 2004). Culture based MIC determination is time consuming, with incubation times of up to 48 hours, and also relies on visual identification of

motile trichomonads, which is insensitive (Patil et al., 2012; Garber et al., 2005). Whilst an aerobic metronidazole MIC of >200µg/ml is strongly associated with treatment failure, isolates with MICs of 50 - 200µg/ml can vary significantly in their response to metronidazole *in vivo*, which when taken into account with the time consuming nature of susceptibility testing, has led to some clinicians questioning the usefulness of initial MIC determination of isolates, in absence of a suspected treatment failure (Sobel et al., 2001; Wendel and Workowski, 2007)

Due to the lack of effective alternative antimicrobials for *T. vaginalis* infection, the only available approved treatment for metronizadole resistant infections is to increase the dosage, to a potentially toxic and side effect inducing level, or alternatively use tinidazole (Cudmore *et al.*, 2004). In the case of repeated treatment failures with increased levels of nitroimidazole antimicrobials, the only alternative options are intravaginally administered antimicrobials such as paramomycin sulphate, furazolidone, clotrimazole or povidone iodine, which have all been used to successfully treat refractory cases of trichomoniasis (Bosserman et al., 2011; Dunne et al., 2003). Despite high efficacy *in vitro*, these treatments are associated with high failure rates, potentially due to the failure of the treatments to react extra-vaginal parasites, such as those residing in the urethra and Skene's glands (Helms et al., 2008; Muzny et al., 2013; Nanda et al., 2006). These options are only available for female patients; there are no topical treatments available for treating males (Cudmore *et al*., 2004).

Hypersensitivity to metronizadazole is encountered, and frequently occurs in tandem with tinidazole hypersensitivity, due to the structural similarity of the compounds (Helms et al., 2008). Documented adverse reactions to the administration of these drugs include fever, urticarial, angioedema and anaphylactic shock (Knowles et al., 1994; Asensio Sánchez et al., 2008). Nitroimidazole desensitization regimes, via either the oral or IV route, have been found to be effective in curing *T. vaginalis* infection in hypersensitive women. A study involving 15 hypersensitive women reported a 100% success rate associated with desensitisation therapy, with 2 of the 15 individuals reporting manageable adverse symptoms (Helms et al., 2008). Desensitization therapy is not always possible in patients with a severe nitroimidazole allergy, meaning alternative treatments must be sought, typically involving topical intravaginal medications (Muzny et al., 2012). The screening of natural products extracts for antimicrobial activity against *T. vaginalis* has yielded some promising results, with one study of Brazilian indigenous medicinal plants reporting that the aqueous extracts of one plant species had high activity against *T. vaginalis*, with an MIC of 4.0mg/mL having 100% efficacy (Brandelli *et al*., 2013). The identification of novel compounds with antimicrobial activity against *T. vaginalis* derived from natural products could potentially prove invaluable in the search for the

alternative therapies necessary to manage nitroimidazole resistant *T. vaginalis* strains, or in individuals with nitroimidazole allergy.

5.1.8. Conclusion

The pathogenic effect caused by the colonisation of the genital tract by *T. vaginalis* results from damage to the genital epithelia, which the parasite causes in a variety of ways, including mechanical damage, the secretion of apoptosis inducing proteases, the disturbance of the junctional complexes in the monolayer, and the instigation of an inflammatory response. The virulence of the infection, and disease severity, is similarly complex, and governed by a range of factors. Infection of the female genital tract is capable of significant modification of the vaginal microbiome, potentially resulting in bacterial vaginosis, worsening the symptoms of the infection (Fichorova *et al.*, 2013). As well as organisms present in the vaginal environment, *T. vaginalis* infection can be impacted by the presence of the intracellular symbionts *M. hominis* or TVV, which modulate *T. vaginalis* gene expression in a process thought to increase virulence (Fichorova *et al.*, 2013; Fiori *et al.*, 2013; Fraga *et al.*, 2012).

T. vaginalis infection is most common non-viral STI worldwide, and of the viral STI's only genital human papillomavirus (HPV) is more prevalent (Bruni *et al.*, 2010) . There are an estimated total number of 276.4 million cases per year world-wide, more than the 106.1 million new cases of *N. gonorrhoeae* infection, and 105.7 new *C. trachomatis* infections combined (World Health Organisation, 2012). *T. vaginalis* infection is a widespread, global concern, prevalent in Europe (5.8%), the Americas (22%) and Africa (20.2%). The high prevalence of *T. vaginalis* infection in Africa is of a particular concern, as the parasite has been implicated in increasing the likelihood of both becoming infected by, and transmitting, HIV (Mavedzenge *et al.*, 2010). Despite the high frequency, and ubiquitous geographic spread of this disease, it has received a much smaller public health response than the next most prevalent curable STI's, *C. trachomatis* and *N. gonorrhoeae* (Van Der Pol, 2007). This has been due in part to the consideration of trichomoniasis as a mild "nuisance" infection, compared with the more serious tubal infertility risk associated with chlamydia infections in women, and the obvious symptoms caused by gonorrhoea. Growing evidence that *T. vaginalis* infection can increase the risk of disease states associated with high morbidity, such as HIV infection, in both male and female patients is increasing the interest in the detection and treatment of this parasite. The development of sensitive NAAT tests for *T. vaginalis* has opened up the possibility of

testing asymptomatic patients, who often have low organism loads, undetectable with less sensitive diagnostic methods. In the UK, the cost of offering this service in a sexual health screen is thought to outweigh the benefit of detecting these asymptomatic infections, due to the relatively low prevalence of this organism in the general population (Ng and Ross, 2012). However, in the US, where the prevalence is much higher, this could be a viable strategy. The testing of males, who tend to have a lower organism load, by traditional methods such as microscopy or culture, is less sensitive than in females, and the increased sensitivity afforded by NAATs allows for the sensitive testing of this group. The continuing development and improvement of POC NAAT testing, and the imminent release of *T. vaginalis* assays for existing POC platforms, will provide the opportunity for the provision of sensitive rapid testing for this organism, with all the inherent benefits of testing at the POC.

Aims

- To maintain the culture of *T. vaginalis*, in order to provide a source of *T. vaginalis* DNA and cell suspensions to ourselves and also Public Health England

- To provide confirmation of the suitability of the magnetic silica based extraction system for the purification of nucleic acids from *T. vaginalis*.
- To determine the limits of detection of the *T. vaginalis* DNA rpt LAMP assay designed by PHE.

5.2. Methods

5.2.1. Media

Modified Diamonds (TYM) Media was formulated as shown in Table 5.2;

Component	Weight/volume per 100ml
Trypticase digest	2g
Yeast extract	1g
Maltose	0.5g
L-Cysteine hydrochloride	0.11g
L-Ascorbic acid	0.02g
K_2HPO_4	0.08g
KH_2PO_4	0.08g
Agar	0.05g
Inactivated foetal bovine serum	10ml

Table 5.2. Modified Diamonds (TYM) Media components

All components apart from the agar and inactivated bovine serum were dissolved into a final volume of 90ml of distilled water. The pH was adjusted to 7.2-7.4, using sodium hydroxide or hydrochloric acid. The agar was added, and the media autoclaved at 121^{o}C for 10 minutes, and allowed to cool to around 49^{o}C. A 10ml volume of foetal bovine serum was inactivated by heating to 56^{o}C, and added to the media at this point. The media was then divided between 7ml bijoux's in 7ml aliquots. The media could then be refrigerated at 4^{o}C until use. Media was stored no longer than 3 weeks.

Trichomonas Media Number 2 (TMN2) was purchased commercially from Oxoid (UK); the composition is shown in Table 5.3 (Atlas et al., 2004).

Component	Weight/volume per 100ml
Glucose	2.25g
Liver digest	1.8g
Pancreatic digest of casien	1.7g

NaCl	0.5g
Pancreatic digest of soybean meal	0.3g
K_2HPO_4	0.25g
Chloramphenicol	0.0125g
Calcium pantothenate (0.5% solution)	0.1ml
Horse serum	25ml

Table 5.3. Trichomonas Media Number 2 components

The media was supplied in individual 7ml glass bijoux's. These were stored at 4^{o}C, and used no later than the use by date on each bottle, typically around 60 days after the purchase.

5.2.2. Culture conditions

Trichomonas vaginalis (ATCC 30001) was cultured in a variety of liquid culture media, at 37^{o}C. The vessels used were 7ml Bijoux bottles, filled with 7ml of media, in order to minimise the air volume under the lid of the vessel. The lids on the bottles were tightened and sealed with parafilm.

5.2.3. Counting of cells

The bijoux or vessel containing the cells was gently mixed so as not to damage the trichomonads. An aliquot was removed from the mid-point of the culture, and the organisms were quantified using a Neubauer improved counting chamber. Any motile cells, or non motile cells exhibiting movement of the flagellae or undulating membrane were considered to be viable cells. Any trichomonads that did not fall into these categories were considered non viable. If the concentration of cells was considered too high to count, another aliquot was taken and diluted with sterile saline. Once the aliquot of cells was taken, the culture was resealed and placed back in the incubator if further required.

5.2.4. LAMP Reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), as follows; 5µl five times LAMP reaction buffer, 1µl intercalating dye (propriety dye, emission in FAM channel), 1µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 1µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers, 12µl molecular grade water. Finally 5µl of DNA sample or water was added to the reaction. Reactions were carried out in an ESEQuant Tube Scanner (Qiagen Inc., CA), at 63^{o}C for 60 minutes. Reactions were terminated by heating to 80^{o}C for 1 minute in order to denature the *Bst* polymerase. The ESEQuant Tube Scanner allows for real-time monitoring of the LAMP reactions, via optical detection of the fluorescent intercalating dye through the FAM channel. The "Tube Scanner Studio" software package was used to analyse the real time data and produce a manually set threshold line for the purposes of determining time to amplification (Ta).

5.2.5. Microscopic evaluation of *T. vaginalis* culture

Aliquots of 3µl *T. vaginalis* culture were added to glass slides, and coverslips applied. Images were obtained using an Olympus (model) inverted microscope, using the x40 lens. Images were acquired using the in-built camera system.

5.2.6. Scanning electron micrographs of *T. vaginalis*

A 1ml aliquot of *T. vaginalis* culture, containing 1 x 10^5 cells, was centrifuged at 2000 x g for 5 minutes, and the supernatant removed. Cells were washed in PBS, pH 7, and re-centrifuged under the same conditions as previously. Cells were then resuspended in 2.5% gluteraldehyde solution, and fixed for one hour at 4^{o}C. The fixed cells were then washed by centriging the sample at 2000 x g for 5 minutes, resuspending in molecular grade water, and repeating 5 times. The cells then underwent an ethanol dehydration gradient, and a 5µl aliquot of the suspension of fixed cells was added to an SEM specimen mount and coated with 15nm thick gold/palladium, then observed using a (Make/Model) SEM.

5.2.7. Growth of *T.vaginalis* in TYM media

Trichomonas vaginalis cells (ATCC 30001) were cultivated overnight in Diamonds (TYM) media. This culture was then quantified, and used to inoculate 7ml aliquots of fresh Diamonds media with a final density of 10,000 trichomonads per ml. The cultures were then incubated at 37^{o}C for a period of 120 hours. Every 24 hours, an aliquot was taken from the same 3 cultures, and the cells were counted as described. Additionally, 1 culture was taken, and the pH monitored using a pH meter. This culture was then disregarded due to the contamination risk.

5.2.8. Public Health England *T. vaginalis* DNA rpt LAMP primer set

Sequences were provided by Public Health England for their *T. vaginalis* DNA rpt LAMP primer set. The primer sequences are shown in Table 5.4.

Primer	Sequence	Length (bp)
T. vaginalis DNA rpt F3	GGTCGACAACTTTGTTGAAAG	21
T. vaginalis DNA rpt B3	CGTCATGAGTGCGTCAAT	18
T. vaginalis DNA rpt FiP	AAGACCAATGTTCGACAATAGGTTTTCTCCTTTTGTTTTGAAGTACG	47
T. vaginalis DNA rpt BiP	AGTTCGCAAAGGCAGTCCTTGTTCTCATAGTAAACCTTCACTT	43
T. vaginalis DNA rpt LoopF	GTTACTAAGCCACTTTGACCTTGA	24
T. vaginalis DNA rpt LoopB	GACAACTACAACAAATTCTTCTCCG	25

Table 5.4. Sequences of the Public Health England *T. vaginalis* DNA rpt LAMP primer sets

5.2.9. Sensitivity of the DNA rpt LAMP assay in conjunction with magnetic silica total nucleic acid extraction

T. vaginalis cells were cultured in Trichomonas Media Number 2 (Oxoid, UK) for 48 hours. The cell count of the culture was determined using an improved Neubauer counting chamber, and the culture diluted to 5 x 10^5 cells per ml. The culture was then sequentially diluted 1:10 in sterile Diamonds Media, and a total nucleic acid extraction using the Kingfisher system was carried out o 200µl of each dilution. LAMP reactions were carried out using the *T. vaginalis* DNA rpt primer set, with 5µl of each sample of extracted nucleic acid. Molecular grade water and extracted DNA from *T. vaginalis* free media were used in place of DNA as negative controls. Reaction products PCR reactions were then separated using agarose gel electrophoresis (2% w/v gel), and visualised. LAMP reactions were monitored in real-time using the ESEQuant tube scanner.

5.3. Results

5.3.1. Microscopic evaluation of *T. vaginalis* culture

T. vaginalis culture was examined microscopically in order to ensure culture viability and motility.

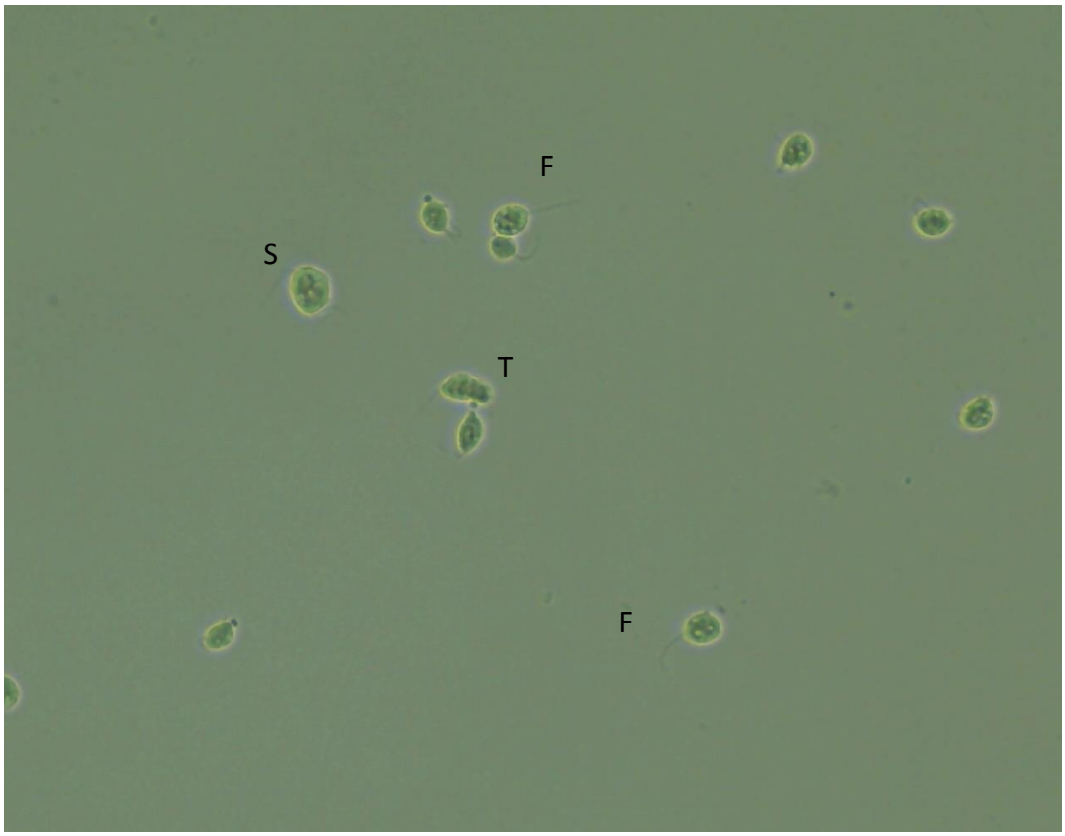

Fig.5.1. Image of motile *T. vaginalis* cells, 100x magnification. Flagella (F) can be seen. Cell morphology varies between the classic tear drop shape (T), and a more spherical form (S).

Microscopic examination of *T. vaginalis* culture in Diamonds TYM media, 24 hours post-inoculation, revealed highly motile cells (Fig.5.1.). Cell morphology varied considerably, between the typical tear-drop shape and a more circular morphology. Flagella were visible, and motile. No amoeboid cell morphology was evident. Parasite:parasite interactions are known to occur in this organism, and some cell to cell contact was occurring between the *T. vaginalis* cells.

The cultured *T. vaginalis* cells were also seen to form aggregates in Diamonds TYM media, comprising of between 5 and 20 cells (Fig.5.2.).

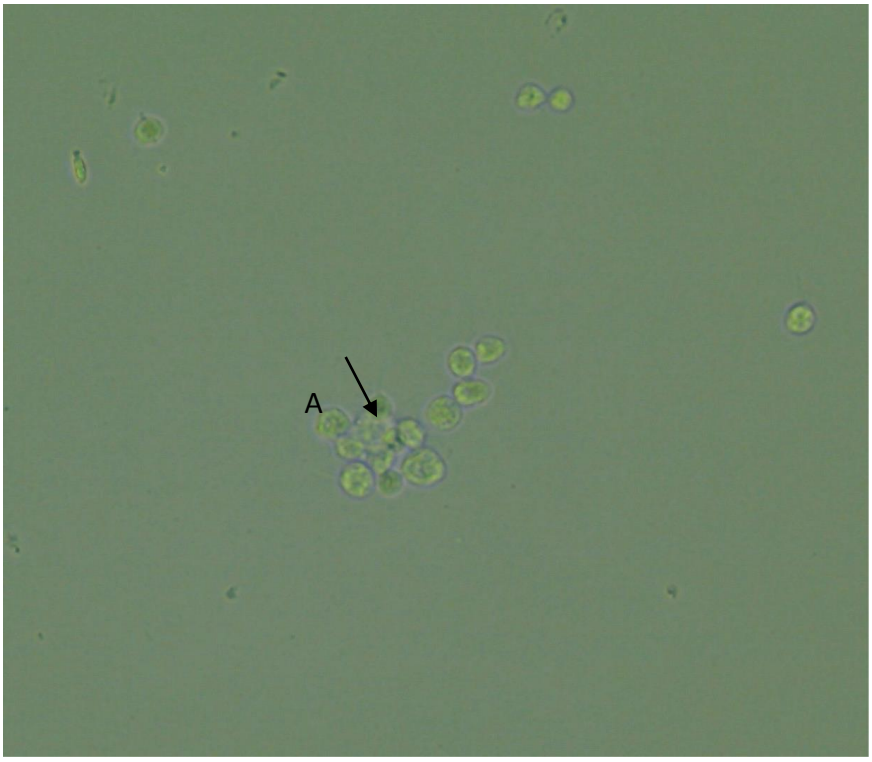

Fig.5.2. As when cultured alongside epithelial cells, aggregates (A) of *T. vaginalis* also form in axenic culture. Enlarged cell in centre of aggregate indicated by arrow.

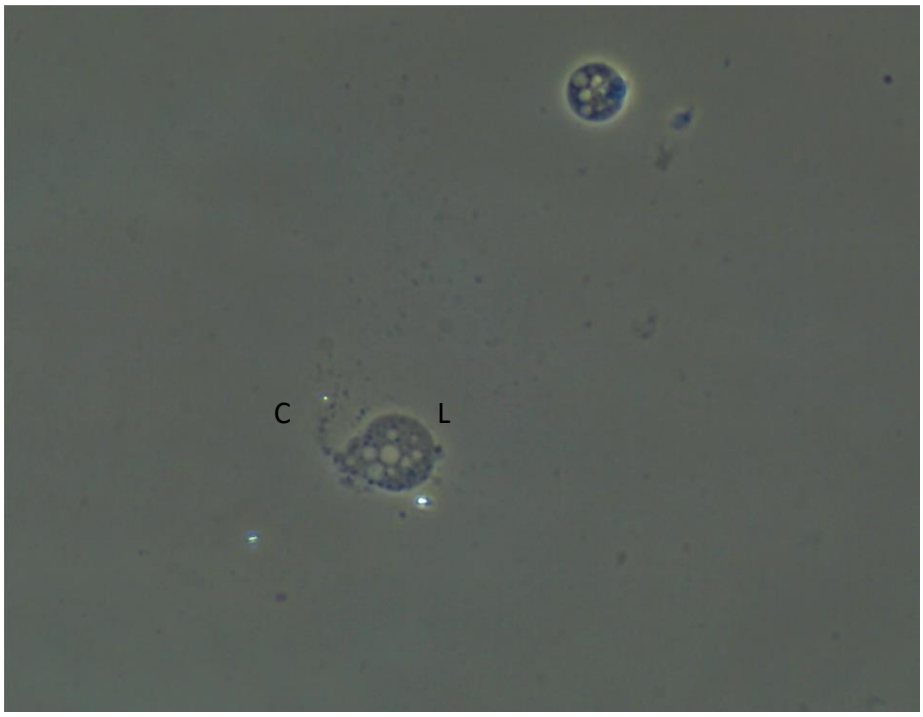

Fig.5.3. Lysis of a *T. vaginalis* cell (L). During cell death, the *T. vaginalis* cell enlarges, and the membrane integrity is lost, resulting in the ejection of the cytoplasmic contents into the extracellular environment (C).

Lysis of the *T. vaginalis* cells could be observed under the microscope in late stage (72 hours post inoculation) culture in Diamonds TYM media (Fig.5.3.). The cells enlarged in size, which was followed by the rupture of the outer cell membrane, and release of cytoplasmic contents into the media.

5.3.2. Scanning electron micrographs of *T. vaginalis*

Scanning electron microscopy (SEM) images of *T. vaginalis* cells were obtained in order to observe the morphological aspects of the cellular structure in more detail.

Fig.5.4. SEM of *T. vaginalis* cell, at a magnification of 23,000x. The flagella are visible (F), as is the origin of the undulating membrane.

Observation of the *T. vaginalis* cell using the SEM revealed the structure of the flagella, undulating membrane (Fig.5.4.) and axostyle (Fig.5.5.) in more detail.

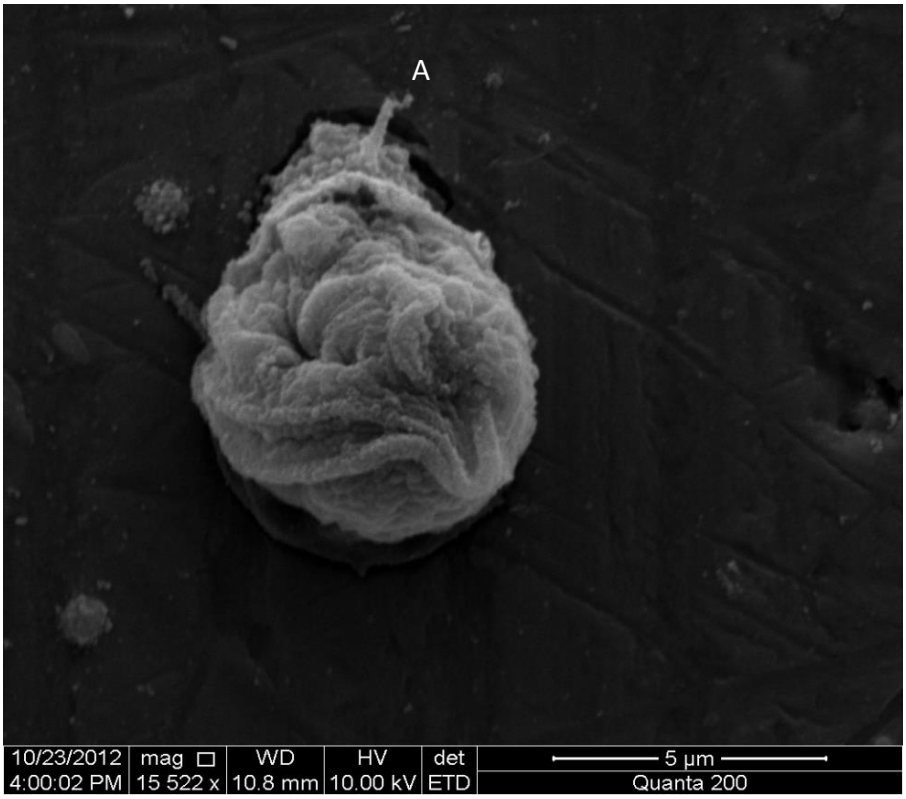

Fig.5.5. SEM of *T. vaginalis* cell, at a magnification of 15,522x. The axostyle is visible as a projection from the proximal tip of the cell.

5.3.3. Growth characteristics of *T. vaginalis*

The growth of *T. vaginalis* in Modified Diamonds Media (Fig.5.6.) shows that proliferation continued steadily for 48 hours post inoculation. During the first 48 hour period post inoculation, the generation time was ~4 hours, with the number of live cells increased from 1 x 10^4 to 1.3 x 10^6 during this period. At 72 hours the number of live cells had decreased from 1.3 x 10^6 to 7 x 10^4. By 96 hours no live trichomonads were visible. The pH shifted over the 96 hour period from 6.2 to 4.62.

After 24 hours, ~1 x 10^4 cells were non-motile, or obviously dead, representing ~5% of the total biomass. The number of dead cells increased to 6.9 x 10^5 cells, or 33% of the total cell count, after 48 hours. The pH of the media decreased during the trichomonads growth, from 6.2 (0 hours), 6.06 (24 hours), 4.95 (48 hours), 4.64 (72 hours), to 4.62 (96 hours).

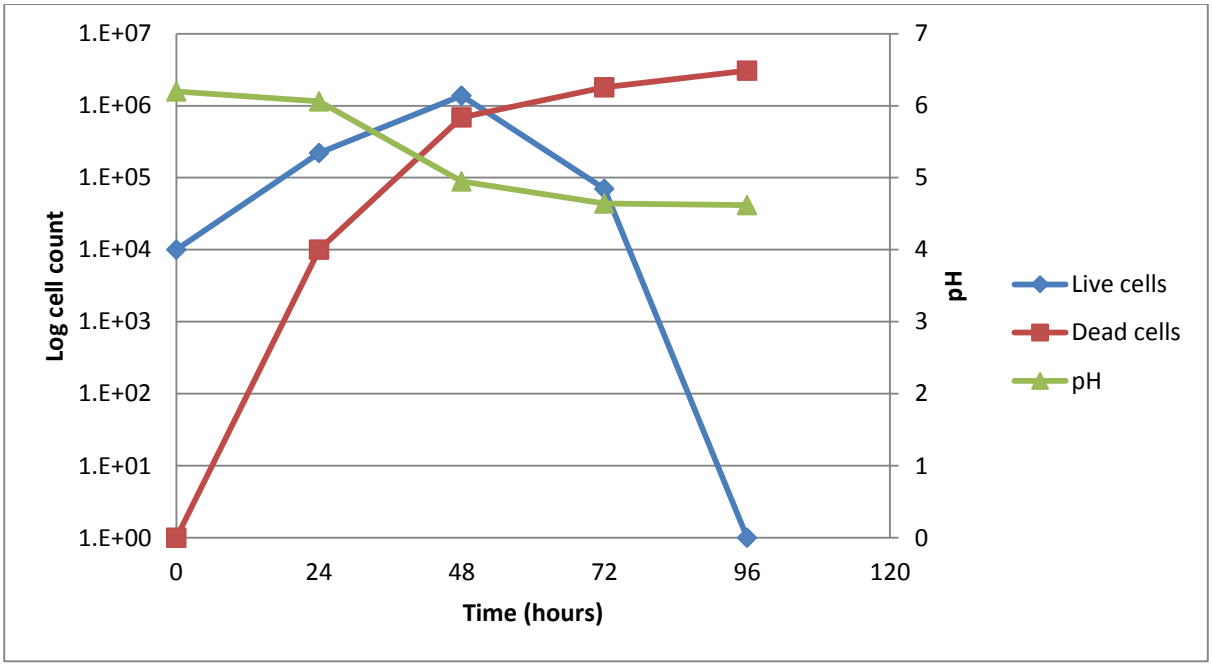

Fig.5.6. The growth of *T. vaginalis* in Modified Diamonds Media.

When cultured in TMN2, the number of motile trichomonads increased from 1 x 10^4 to 2.7 x 10^5 after 24 hours, and 2.8 x 10^6 after 48 hours. The number of live cells then decreased to 2 x 10^4 at 72 hours, and 0 at 96 hours. No non motile cells were observed at the 0 hours, 24 hours or 48 hours time points. At 72 hours post inoculation 4.7 x 10^6 dead cells were observed, decreasing to 3.85 x 10^6 dead cells after 96 hours. The pH decreased during the growth of the culture, from 6.2 (0 hours), 5.69 (24 hours), 5.7 (48 hours), 4.9 (72 hours), and 4.8 (96 hours).

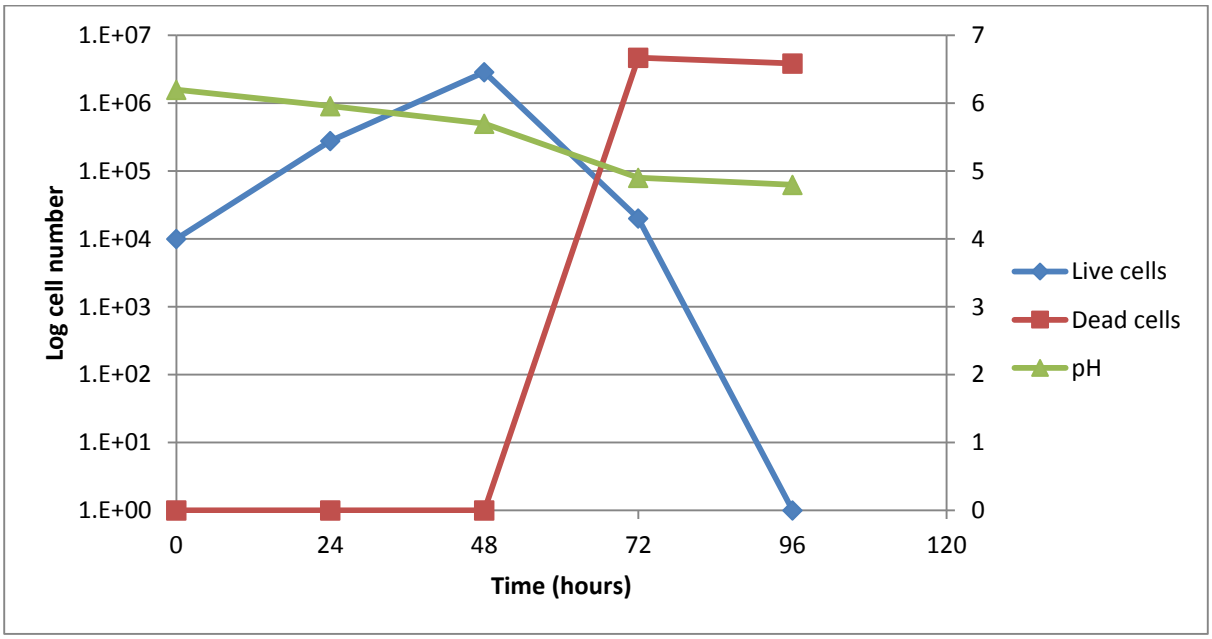

Fig.5.7. The growth of *T. vaginalis* in TMN2.

5.3.4. Sensitivity of the DNA rpt LAMP assay in conjunction with magnetic silica total nucleic acid extraction

The sensitivity of the *T. vaginalis* DNA rpt LAMP assay, in conjunction with the magnetic silica based total nucleic acid extraction method, was tested by carrying out nucleic acid extractions on 1:10 sequential dilutions of *T. vaginalis* cultured in Diamonds Media. The cell counts per extraction were $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, 10, and 1. Each extraction was assayed using the *T. vaginalis* DNA rpt LAMP assay. The reactions were monitored in real-time (Fig.5.8), and the reaction products were examined via agarose gel electrophoresis (Fig.5.9.).

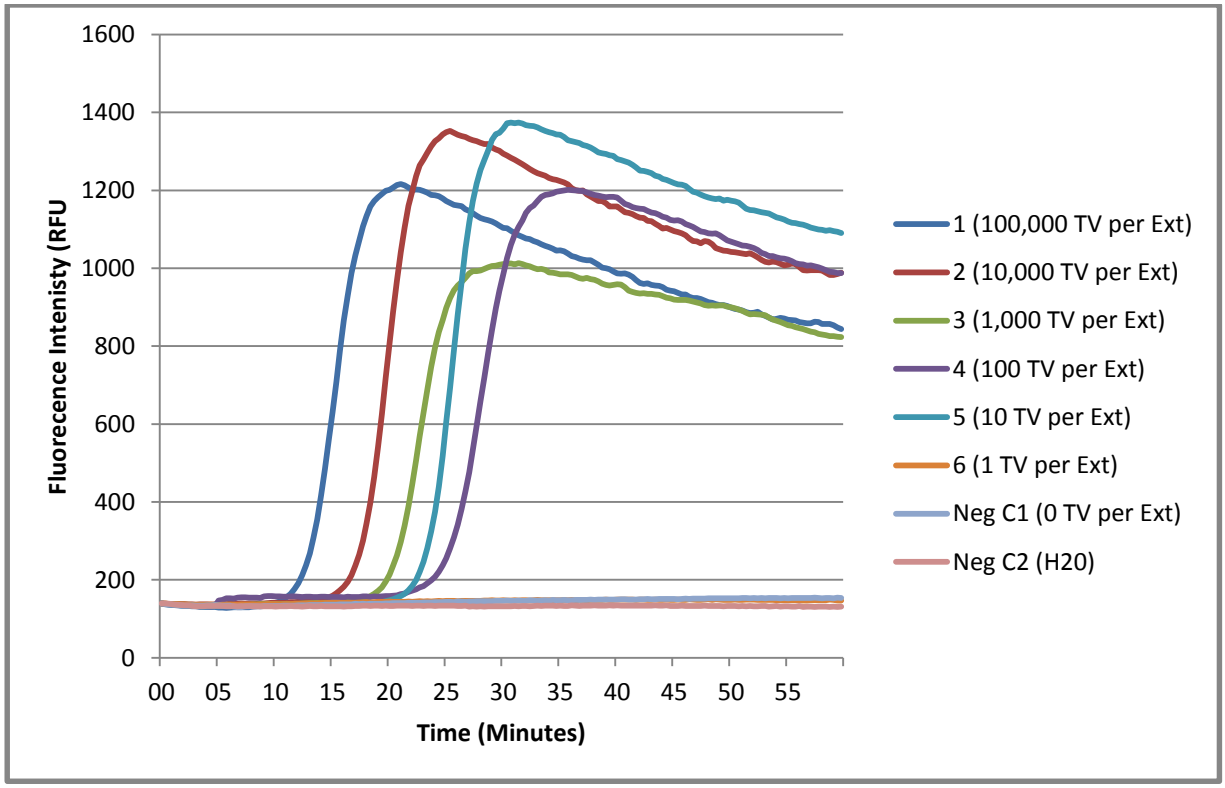

Fig.5.8. LAMP amplification plot showing the detection limits of the TV DNA rpt DL1 assay.

The extractions containing 1 x 10^5 to 10 *T. vaginalis* cells all generated an amplification in the subsequent LAMP assays. The examination of the reaction products by electrophoresis agreed with the real time amplification data, with all reactions that generated an amplification curve also generating visible characteristic ladder like reaction products when separated on the gel (Fig.5.9).

The limit of detection of the assay was found to be 10 *T. vaginalis* cells per extraction, with the LAMP reaction from a single *T. vaginalis* cell failing to generate a detectable amplification.

Both the negative control reactions did not generate an amplification, indicating primer specificity and also the absence of contamination of both the LAMP reaction and extraction reagents.

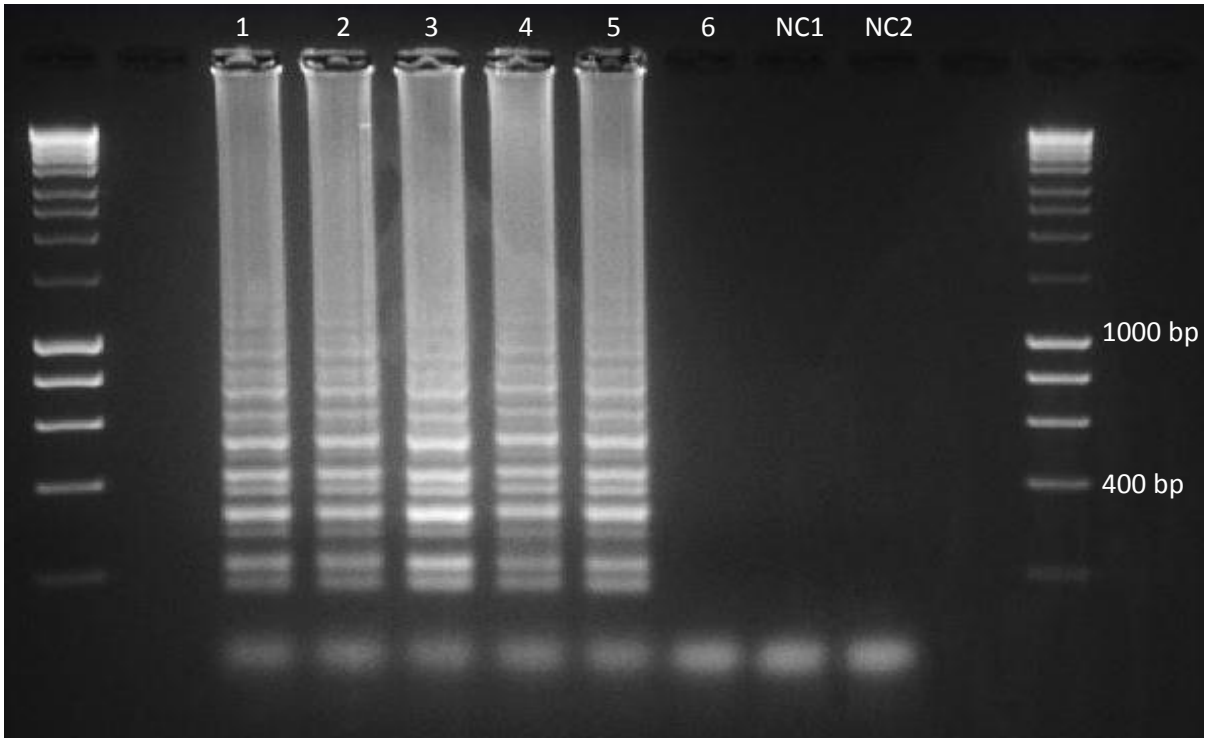

Fig.5.9. Agarose gel electrophoresis (2%) of LAMP products from the *T. vaginalis* DNA rpt LAMP assay, corresponding to the real-time data displayed in Fig.5.8.

The theoretical number of cells per reaction, worked out using the number of cells in the extraction, and elution volume, are shown in Table 5.5. The lowest number of cells per extraction that were detectable by the downstream LAMP assay was 10. This is equivalent to 0.4 cells per individual reaction, as 5µl of the 75µl of eluted nucleic acid is transferred to the LAMP assay.

Sample	TV cell count (per ml)	Volume used in extraction	Cells used in extraction	Elution Volume	Volume amplified	Equivalent number of cells	Results
1	500,000	200µl	100,000	75µl	5µl	7,000	POS
2	50,000	200µl	10,000	75µl	5µl	700	POS
3	5,000	200µl	1,000	75µl	5µl	70	POS
4	500	200µl	100	75µl	5µl	7	POS
5	50	200µl	10	75µl	5µl	0.7	POS
6	5.0	200µl	1	75µl	5µl	0.07	NEG
7	0	200µl	0	75µl	5µl	0	NEG
Negative	0	200µl	0	75µl	5µl	0	NEG

Table 5.5. Limit of detection of the *T. vaginalis* DNA rpt LAMP assay, in terms of cells per extraction and theoretical cells per reaction.

5.4. Discussion

5.4.1. Microscopic evaluation of *T. vaginalis* culture

Examination of the *T. vaginalis* culture in Diamonds TYM media at 24 hours post inoculation revealed the variable cellular morphology of the trichomonads, with some cells displaying the classic teardrop morphology commonly seen in axenic culture (Petrin *et al.*, 1998) and other cells were more spherical in structure (Fig.5.1.). Under unfavourable culture conditions, *T. vaginalis* is known to return to a more spherical form, whilst internalising the flagella and reducing motility, in a process hypothesised to conserve energy until more conditions improve (Honigberg, 1990). The spherical parasites pictured (Fig.5.1.) were still equally motile as the teardrop shaped cells, and also retained the extracellular flagella, so it unlikely these morphological changes were to the energy conserving form previously described.

Aggregates of *T. vaginalis* cells (Fig.5.2.) occur during co-culture with epithelial cells during a process in which the parasites adhere to the epithelial cells, and congregate, resulting in damage to the epithelial cells involved (Tasca, 2002). It is possible that the enlarged cell in the centre of the aggregate (indicated by arrow) is in the process of cell death, and the other *T. vaginalis* cells are involved in this process. *T. vaginalis* are known to engage in parasite:parasite interactions, involving the use of exosomes to transfer proteins between cells (Twu *et al.*, 2013), and it's also possible that it is this process that is responsible for these aggregates, and the cell to cell contact seen in fig.5.1.

By examining cultures that have started to enter in the death phase, after a period of 72 hours, it is possible to observe cell lysis (Fig.5.3.). Due to an exhaustion of nutrients in the media by this stage of the culture, the cells have internalised their flagella, ceased motility, and become more spherical in shape. There is a debate on whether these forms can be considered pseudocysts, or whether it is a stage in the process of degeneration of the *T. vaginalis* cell (Petrin *et al.*, 1998).

5.4.2. Scanning electron micrographs of *T. vaginalis*

The use of SEM enables observations of *T. vaginalis* morphology in more detail, due to the higher magnification possible with this method. It is able to clearly view the flagella and undulating membrane (Fig.5.4.) and axostyle (Fig.5.5.), which are not as easily visible using conventional microscopy. SEM has proven an invaluable tool during research into *T. vaginalis*, and important

studies on phagocytosis (Pereira-Neves and Benchimol, 2007) and cytopathogenicity (Mirhaghani and Warton, 1996) have been heavily reliant on SEM.

5.4.3. Growth characteristics of *T. vaginalis*

As an obligate human pathogen, incapable of survival and proliferation outside of the extracellular environment of the human genital epithelia, *T. vaginalis* is incapable of the synthesis of a range of macromolecules, and these need to be present in any culture media in order to support growth. The presence of serum in any media is particularly important, as this provides a source of lipids, fatty acids, and amino acids. *T. vaginalis* is also incapable of *de novo* synthesis of purines or pyrimidines, and relies on the phagocytosis of epithelial or bacterial cells to provide a source of these essential molecules *in vivo*. *T. vaginalis* is capable of releasing ectonucleotideases in order to hydrolyse extracellular nucleotides and nucleosides, bypassing phagocytosis, enabling the uptake of these molecules from serum during culture. These enzymes have been demonstrated to be significantly up-regulated in low serum culture conditions (Frasson *et al.*, 2012). Successful culture of the organism also relies on the availability of iron in the culture media; growth of the parasite in media containing excess iron is three times greater than that achieved in iron-limited media (Lehker and Alderete, 1992). Undefined concentrations of iron will be present in the serum, liver digest and yeast extract used in the Diamonds Media, or Trichomonas Media Number 2 (TMN2) (Table.5.2.).

The most significant difference between the two media tested, Diamonds Media and TMN2, was the concentration of serum, which was 10% and 25%, respectively. TMN2 supported the growth of a maximum cell density of 2.9×10^6 cells per ml, whereas Diamonds media supported the maximum cell density of 1.4×10^6 cells, representing a 52% decrease. This is likely to be due to the reduced serum concentration in the Diamonds Media increasing the generation time, and reducing overall growth. There are intrinsic benefits to the use of media with a lower concentration of serum; serum is expensive, and has a finite shelf life. Additionally, *T. vaginalis* grows more slowly in a low serum media, and therefore exhausts the other nutrients in the media more slowly, enabling a sustained period of growth. This is not desired for a diagnostic culture procedure, but is useful for the long term propagation of cells in a research setting. Although a lower cell density was reached in the Diamonds Media, cell viability was not retained for any longer, with no motile cells observed in either media after 96 hours. A higher number of motile cells were observed in the Diamonds Media after 72 hours; 7×10^4 per ml, compared with 2×10^4 per ml in the TMN2.

The Diamonds media contains 1g per 100ml of yeast extract (Table.5.1.), which has been shown to enhance growth of *T. vaginalis* in serum free media, but have less effect in the presence of serum (Smith et al., 1982).

During the growth of *T. vaginalis* in Diamonds TYM, the largest increases in biomass occurred between 24 and 48 hours post inoculation, with the cell count per increasing from 2.2 x 10^5 to 1.3 x 10^6 per ml during this period (Fig. 5.6.). This was accompanied by the largest shift in pH; from 6.06 to 4.95. In TMN 2, the growth during this period was greater, with the number of trichomonads per ml increasing from 2.7 x 10^5 to 2.9 x 10^6 (Fig.5.7.). Despite this, the accompanying pH change was less pronounced, with a shift from 5.96 to 5.7 occurring between these time points. The reduced pH change could be a possibly explanation for the large reduction in cell death that occurred in this media; no non motile cells were observed at 48 hours post inoculation in TMN 2, compared with 6.9 x 10^5 in the Diamonds media. *T. vaginalis* is known to be sensitive to acidic pH, and the pH reduction generated during batch culture is known to be detrimental to the organism (Lehker and Alderete, 1992). This pH sensitivity is apparent in vivo; During the colonization of the vagina by *T. vaginalis*, the pH is raised from the usual level of around pH 4 to as high as pH 7, in order to provide optimal conditions for the parasites growth (Cudmore *et al.*, 2004). The parasite achieves this by the disruption of the commensal lactobacilli community present in the vagina (Cudmore *et al.*, 2004).

The absence of cell death seen during the first 48 hours post inoculation in the TMN2 media makes this media better suited for further experiments to determine the limits of detection of the assay. The non-motile cells make the accurate determination of cell count more difficult, as cells may have degraded before the count takes place, releasing nucleic acid into the media, which would then be picked up by downstream testing by LAMP. This would could potentially cause an artificial increase in assay sensitivity, as a larger amount of target nucleic acid would be present in the sample than the cell count would suggest.

5.4.4. Sensitivity of the DNA rpt LAMP assay in conjunction with magnetic silica total nucleic acid extraction

The sensitivity of the DNA rpt LAMP assay was determined in conjunction with the magnetic silica extraction method, which would be used in the final test. This was decided to be a more accurate assessment of the combined protocols performance, than by measuring the extracted DNA spectrophotometrically and then testing the assay against the DNA dilutions. This was compounded

by the lack of access to a spectrophotometer capable of accurately measuring DNA concentration in small volumes at the time. It was found that the minimum number of parasites detectable were 10 per 200µl extraction (Fig.5.8.), which is equivalent to 50 per ml of sample, or 0.7 per extraction (Table.5.5.). Separation of reaction products via electrophoresis enabled the visualisation of the characteristic ladder like DNA fragments, in all of the reactions determined positive using real-time fluorescence detection (Fig.5.9.). There was no obvious difference in the level of product generated between the reactions. The sensitivity of the DNA rpt LAMP assay, in conjunction with the silica extraction method is in agreement with previous molecular tests. The combination of a simple chelex based extraction method with an 18S rRNA PCR assay was found to have a sensitivity equivalent to 1 cell per 25µl reaction (Mayta *et al.*, 2000). A commercially available combination of spin column based extraction kit and PCR assay (Urine-Based Trichomonas vaginalis Detection Kit, Norgen Biotek Corp, Canada), for the extraction of DNA and subsequent detection of *T. vaginalis* in urine, has a detection limit of 100 cells per ml; twofold higher than the DNA rpt LAMP assay. Other commercial PCR assays have been determined as having a detection limit of 10 copies per reaction (LightMix Kit Trichomonas vaginalis, Tib MolBiol, Germany).

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Final Summary

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The extraction method described in the first chapter of this thesis proved to be a robust and rapid system for the extraction of nucleic acid from both urine and swab samples. The system was able to withstand concentrations of inhibitors such as BSA, non-target bacteria, and red blood cells at higher concentrations than would be expected in STI samples. The system was modelled using the Kingfisher ML automated extraction system, which enables the magnetic transfer of beads between various buffer wells, in a process that fairly closely mimics the magnetic movements of the beads on the LabDisk. There are some differences between the systems however, and it is possible that more beads will be lost during the process on the disk, as the movement of the beads between the buffers is more complex, as the beads need to be guided through the channels in liquid suspension. Additionally, the pre-elution drying step on the LabDisk may be more problematic, as it is taking place in an enclosed chamber, rather than open air. Heat is being applied to assist in the drying process, but this is something that will require further optimisation once the extraction method is utilised in the LAbDisk. The application of the extraction methodology, and reagent formulations described in this thesis, will be combined with the LabDisk that has been designed to house these reagents. This will be carried out by MAST group Ltd, prior to the clinical evaluation of the test.

The *N. gonorrhoea* ORF1 LAMP assay is the first report of a LAMP assay designed for this organism, and would enable the use of sensitive NAAT diagnostics without the need for thermal cycling equipment. The remarkable tolerance of the assay to urea, demonstrated in this work, shows that unprocessed urine samples could be used without the need for complex DNA extraction methodologies. Therefore, this assay may be of particular use in the developing world, where access to the materials needed for PCR testing can be limited. LAMP assays for leishmaniasis, tuberculosis and malaria have all been successfully applied in the developing world, illustrating the suitability of LAMP for this environment. The availability of sensitive molecular diagnostics for *N. gonorrhoeae* is of increasing importance, due to the rapid development and spread of multi drug resistance in this organism.

The *M. genitalium pdhD* LAMP assay reported in this thesis is potentially a welcome addition to the diagnostic options for this organism, which is undetectable by culture and serological tests, and have solely relied upon PCR. The assay was as sensitive as the PCR assays that were used as a comparison, whist being faster and being more amenable for application in a decentralised setting.

Whilst the analytical sensitivity of the assays was determined during the work presented in this thesis, in order to thoroughly validate the assays prior to clinical use it would be necessary to repeat

these experiments with a greater number of dilutions and replicates. A suggested experimental plan for a robust evaluation would include the testing of between ten and 16 dilutions, with between five and ten replicates per dilution. This would enable a full probit analysis to be carried out, to determine an accurate LOD.

A significant step towards the goal of a fully functioning diagnostic platform will be the integration of the nucleic acid extraction process with the chosen LAMP assays on the LabDisk, which is currently being undertaken at MAST Group Ltd. Once the automated system is operational, it will be evaluated against a gold standard test for each organism. This will provide data on the sensitivity, specificity, and positive and negative predictive value of the test, which are the qualities that will determine whether the platform is of clinical use, and recommended for application in a healthcare setting.

If the test performs well in these clinical evaluations, and is considered sufficiently sensitive to act as a first line molecular test, it could see clinical use in GUM clinics, GP surgeries, and other sites where STI consultations occur. This would enable same day diagnosis and treatment, reducing the non-return rate, and the risk that positive patients infect further people during this time. This would act to save the time of healthcare practitioners and could have a cost benefit to healthcare providers.

One obstacle to the uptake of point of care tests such as these is the training of the users, who often have little laboratory experience, and also the QA of results. The responsibility for these practices will reside with the end user, who will need to instigate proficiency testing of staff, and also ensure that the system is delivering quality results, most likely through the use of external quality assessment providers such as the United Kingdom National External Quality Assessment Services (UK NEQAS). Organisations such as this provide blind samples for testing, to ensure the diagnostic accuracy of the service, making sure that the information generated is sound enough to base therapeutic decisions on.