

Loop-Mediated Isothermal Amplification (LAMP) Test for the Detection of *Neisseria gonorrhoeae* in Urine Samples and the Tolerance of the Assay to the Presence of Urea.

Running title; *N. gonorrhoeae* LAMP assay tolerance to urea

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Abstract

A loop mediated isothermal amplification (LAMP) assay for ORF1 of *Neisseria gonorrhoeae* was able to tolerate urea concentrations of 1.8M and below, compared with a PCR assay which was functional at concentrations of <100mM. The LAMP assay was as sensitive as the PCR assay, whilst faster and simpler to perform.

Gonorrhoea is the second most common sexually transmitted infection in the developed world (1) with 106 million incidences of gonorrhoea infection reported yearly worldwide (2), representing a significant global health burden.

Nucleic acid amplification testing (NAAT) for gonorrhoea is becoming more widespread. 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 (3).

LAMP is a novel nucleic acid amplification method, designed to amplify target nucleic acid in a highly specific and rapid manner, under isothermal conditions (4). The LAMP reaction requires a constant temperature of ~63°C, and can be easily carried out in a water bath (5). LAMP assays enable a more rapid diagnosis than PCR based tests, offer improved sensitivities (6,7), and have a higher tolerance for inhibitors present in a number of clinical samples, including faeces and blood (8).

Diagnosis of genital *Neisseria gonorrhoeae* infection by NAAT is carried out from a variety of sample types, including invasive swab samples and urine specimens. Urea, present in urine, is a known inhibitor of PCR at concentrations over 50mM (9). Urea prevents the non-covalent bonding of the polymerase enzyme, and also interferes with primer annealing, hence inhibiting amplification (10). Diagnostically this leads to an increase in the likelihood of false-negative results and a reduction in assay sensitivity (11). Although the concentration of urea in adult human urine is highly variable, a previous study (9) found the average concentration of urea to be ~300mM, above the minimum concentration needed for PCR inhibition. The most simple methods for nucleic acid extraction, such as the preparation of a crude lysate, chelex based methods, or proteinase K digestion, are unsuitable for processing urine samples for PCR, due to their inability to remove the inhibitory urea, and the use of more complex extraction methods is recommended (12).

In the present study, the minimum concentration of urea inhibitory to LAMP was determined, in order to test the suitability of the LAMP assay for testing urine samples with simple extraction methods. The detection of *N.gonorrhoeae* DNA from spiked urine samples was used to test this principle.

N.gonorrhoeae (ATCC 19424) was cultured on chocolate agar (blood agar base, Sigma Aldrich, UK; defibrinated horse blood, TCS, UK) at 37°C with 5% CO₂.

LAMP primers were designed for ORF1 of the glutamine synthetase (*glnA*) gene (Genbank accession number M84113) of *N. gonorrhoeae*. This region offers excellent diagnostic sensitivity as a PCR target for this organism (13). LAMP primers were designed using PrimerExplorer Version 4

(<http://primerexplorer.jp/e/>). ORF1 LAMP primer sequences were as follows: F3, 5' –TGG TCG GTG CTT CAA AGT G- 3'; B3, 5'- GCA CGT CCA CCA ATC CAT T-3'; FiP, 5'-_CAA ACA CGC CAA AGC CCT GAA CGC ACG AGG CGT TTG TAG G-3'; BiP, 5'- TGT AGT AGA GCG CGG TAT CGG ACG GTC AAA ACCT GTT CGC A-3'. PCR primers specific to the same target were designed using Primer3 (<http://frodo.wi.mit.edu/>). ORF1 PCR primer sequences were as follows: F, 5'-GAC GTA TTT CAT ATC TTG GG-3'; R, 5'-GGT GAA CAT TTT GGA AG-3'. Primer specificities were checked using BLAST (<http://blast.ncbi.nlm.nih.gov/>).

DNA extractions from *N. gonorrhoeae* cell suspensions were carried out using a QIAmp DNA mini kit (QIAGEN Inc., USA). 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 \times 665 Da/bp $\times 1.67 \times 10^{-24}$ g/Da) (14).

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 molecular grade 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. Thermal cycling was carried out in an ABI 2720 instrument (Applied Biosystems, UK). The reaction product was a 139bp fragment.

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK), according to the manufacturer's instructions. Final primer concentrations were 0.2µM F3 and B3, and 1.6µM BiP and FiP. Reactions were monitored in real-time using an ESEQuant Tube Scanner (Qiagen Inc., UK). Reactions were terminated by heating to 80°C for 1 minute. The detection of reaction products from the PCR and LAMP reactions was carried out by electrophoresis, using 2% w/v agarose gels with UV transillumination. For endpoint visual detection, the 1µl intercalating dye was replaced with 1µl of calcein (fluorescent detection reagent; Eiken Chemical Co., Japan).

As a small scale test of the specificity of the ORF1 LAMP and PCR reactions, reactions were set up for each assay, containing 0.1µg of DNA from the following organisms; *C. trachomatis* (lab strain, serovar L2), *Trichomonas vaginalis* (ATCC 30001) and *Pseudomonas aeruginosa* (ATCC 15692). These organisms are all causes of urethritis. No amplification was seen, confirming the high levels of primer specificity.

The limit of detection of both the *N. gonorrhoeae* ORF1 PCR and LAMP assays was found to be 20 copies of the *N. gonorrhoeae* genome (Table. 1.). Real-time monitoring showed that the time to

amplification was shorter with an increased starting concentration of template, with the reaction containing 2×10^5 copies showing the earliest amplification, crossing the manually set threshold line at 17 minutes (Table. 1.) The latest time to amplification, 27.3 minutes, was seen in the reaction containing 20 copies. The time to amplification was within 2 minutes during all 3 replications of the experiment.

To determine the minimum concentration of urea inhibitory to both the ORF1 LAMP and PCR assays, LAMP and PCR reactions, including 0.1 μ g of *N.gonorrhoeae* DNA, were formulated with the following concentrations of urea; 1mM, 10mM, 100mM, 1M, 2M. Additional ORF1 LAMP reactions were carried out with 1M, 1.2M, 1.4M, 1.6M, 1.8M and 2M urea to define the sensitivity of the reaction at these higher levels. Molecular grade urea powder (Sigma, UK) was dissolved at 30°C in molecular grade water to 8.5M, then filter sterilised, diluted appropriately, and added in place of water to the LAMP reaction. The ORF1 PCR reaction tolerated a urea concentration of 10mM, whereas a concentration of 100mM proved inhibitory (Fig.1.A.). The LAMP assay detected *N.gonorrhoeae* DNA at urea concentrations of 1.8M and below (Fig.1.B.), demonstrating the increased robustness of the LAMP assay to urea mediated inhibition.

The application of LAMP for the direct detection of *N. gonorrhoeae* DNA from urine samples was demonstrated by carrying out ORF1 LAMP reactions on 5 μ l samples of both molecular grade water and human urine spiked with 2×10^3 copies, 2×10^2 copies, 20 copies, 2 copies and 0 copies of the *N.gonorrhoeae* genome. The reactions contained 1 μ l of calcein to enable visual detection (15). The urine used was taken from one individual over a 12 hour period, stored at 6°C and used no later than 24 hours after excretion. The sensitivity of the LAMP reaction was not reduced by the presence of urine, with a minimum of 20 copies detectable in urine or water. Positive reactions underwent an orange to green colour change, which was unambiguous in natural light. The negative reactions remained orange, whether urine or water was used.

The ORF1 LAMP assay could detect the minimum copy number detectable by PCR, and required less time. The inclusion of calcein allows for the direct detection of ≥ 20 genome copies from urine samples, after a 1 hour time period, and without requiring any specialist equipment. The ability of the LAMP reaction to withstand higher levels of urea than those found in human urine enables 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. This makes the ORF1 LAMP assay, in conjunction with calcein, well suited to the sensitive detection of *N. gonorrhoeae* directly from urine samples in a resource poor setting.

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References

1. **Newman LM, Moran JS, Workowski, KA** . 2007. Update on the management of gonorrhoeae in adults in the United States. Clin. Infect. Dis **44**:s84-s101.
2. **World Health Organisation**. 2012. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. Geneva, Switzerland: World Health Organisation
3. **Luijt DS, Bos PAJ, van Zwet AA, Van Voorst Vader PC, Schirm J**. 2005. Comparison of COBAS AMPLICOR *Neisseria gonorrhoeae* PCR, including confirmation with *N. gonorrhoeae*-specific 16S rRNA PCR, with traditional culture. J.Clin. Microbiol. **43**:1445-1447.
4. **Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T**. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. **28**:E63
5. **Njiru ZK, Mikosza AS, Matovu E, Enyaru JC, Ouma JO, Kibona SN, Thompson RC, Ndung'u JM**. 2008. African trypanosomiasis: sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int. J. Parasitol. **38**:589-599.
6. **Kaneko H, Iida T, Aoki K, Ohno S, Suzutani T**. 2005. Sensitive and rapid detection of herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification. J.Clin. Microbiol. **43**:3290-3296.
7. **Nakao R, Stromdahl E, Magona J, Faburay B, Namangala B, Malele I, Inoue N, Geysen D, Kajino K, Jongejan F, Sugimoto C**. 2010. Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Ehrlichia ruminantium*. BMC Microbiol. **10**:296.
8. **Francois P, Tangomo M, Hibbs J, Bonetti EJ, Boehme CC, Notomi T, Perkins MD, Schrenzel J**. 2011. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. FEMS Immunol. Med. Mic. **62**:41-48.
9. **Khan G, Kangro HO, Coates PJ, Heath RB**. 1991. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. J. Clin. Pathol. **44**:360-365.
10. **Hedman J, Radstrom P**. 2013. Overcoming inhibition in real-time diagnostic PCR. Methods Mol. Biol. **943**:17-48.
11. **Chernesky MA, Jang, D., Sellors, J., Luinstra, K., Chong, S., Castriciano, .S, Mahony, J.B.** 1997. Urinary inhibitors of polymerase chain reaction and ligase chain reaction and testing of multiple specimens may contribute to lower assay sensitivities for diagnosing *Chlamydia trachomatis* infected women. Mol. Cell. Probes. **11**: 243-249.

12. **A. Behzadbehbahani PEK, P.J. Vallely, G.M. Cleator.** 1997. Detection of BK virus in urine by polymerase chain reaction: a comparison of DNA extraction methods. *J. Virol. Methods.* **67**:161-166
13. **Chaudhry U, Saluja D.** 2002. Detection of *Neisseria gonorrhoeae* by PCR using orf1 gene as target. *Sex. Transm. Infect.* **78**:72.
14. **Geraats-Peters CW, Brouwers M, Schneeberger PM, van der Zanden AG, Bruisten SM, Weers-Pothoff G, Boel CH, van den Brule AJ, Harmsen HG, Hermans MH.** 2005. Specific and sensitive detection of *Neisseria gonorrhoeae* in clinical specimens by real-time PCR. *J.Clin. Microbiol.* **43**:5653-5659.
15. **Tomita N, Mori Y, Kanda H, Notomi T.** 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Protoc.* **3**:877-882.

Figure legends

Table 1. Sensitivity of ORF1 LAMP and PCR assay

Average time to amplification of ORF1 LAMP reactions containing 2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 , 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. ORF1 PCR reactions were carried out from the same DNA dilutions, and the products were separated using electrophoresis and then visualised.

Fig.1.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 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.

Fig.1.B.

Agarose gel electrophoresis of reaction products from LAMP reactions 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 (B2).

Tables and Figures

Table.1. Sensitivity of ORF1 LAMP and PCR assay

<i>N. gonorrhoeae</i> genome copy number	LAMP Time to amplification (minutes)	PCR Result
2×10^5	17	+
2×10^4	18.6	+
2×10^3	20.7	+
2×10^2	25	+
20	27.3	+
2	No amplification	-
0	No amplification	-

Table.1. Average time to amplification of ORF1 LAMP reactions containing 2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 , 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 5 μ l of water instead of DNA was used as a negative control. ORF1 PCR reactions were carried out from the same DNA dilutions, and the products were separated using electrophoresis and then visualised.

A)

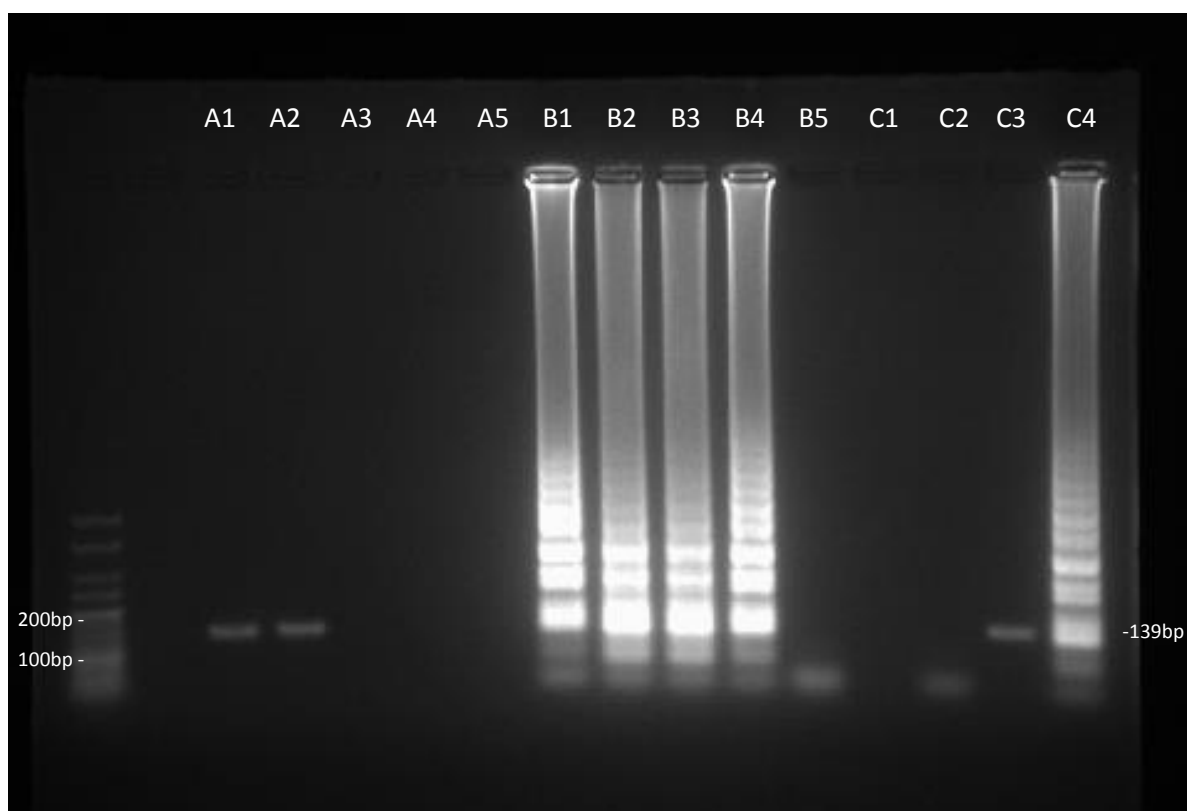


Fig.1.A. Tolerance of the ORF1 PCR assay and ORF1 LAMP assay to urea; agarose gel electrophoresis of ORF1 PCR (lanes A1-A5) and LAMP (lanes B1 – B5) reaction products from reactions with urea concentrations of 1mM (lanes A1, B1), 10mM (lanes A2, B2), 100Mm (lanes A3, B3), 1M (lanes A4, B4), 2M (A5, B5). 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)

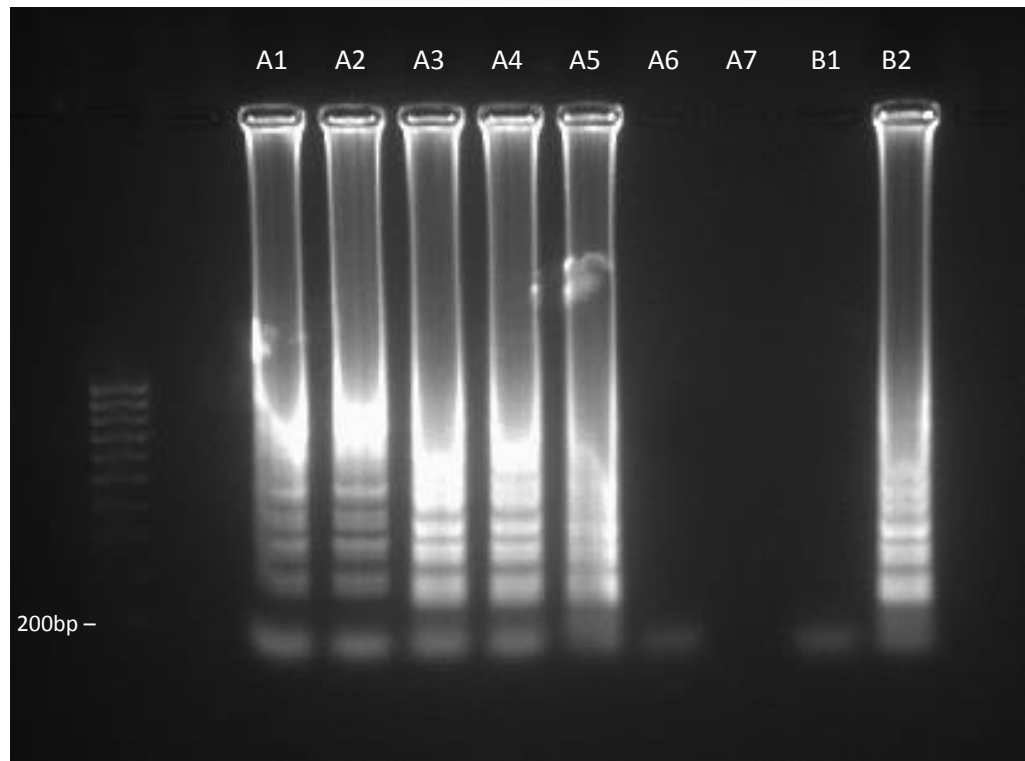


Fig.1.B. Agarose gel electrophoresis of reaction products from LAMP reactions with urea concentrations of 1M (lane A1), 1.2M (lane A2), 1.4M (lane A3), 1.6M (lane A4), 1.8M (lane A5), 2M (lane A6). Positive reactions contained 0.1 μ g of *N.gonorrhoeae* DNA. A LAMP reaction (B1) containing 5 μ l of water instead of DNA was used as a negative control. A LAMP reaction containing no urea was carried out as a positive control (B2).