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Research Paper

Development of a qPCR assay for the quantification of canine autosomal DNA recovered from livestock attacks

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ABSTRACT

The absence of a standardised method to quantify canine DNA recovered from livestock attacks leaves forensic providers without an important quality control step to help support their decision making. Typically used to normalise the amount of DNA for STR amplification, modern forensic DNA quantification approaches use qPCR of target genes and can also include an Internal Positive Controls (IPC) to determine the presence of PCR inhibitors. The co-amplification of livestock DNA alongside canine DNA has meant that previously developed qPCR methods are not suitable for use so a standardised approach is needed. This research describes the development of a Taq-man multiplex qPCR assay that simultaneously quantifies the autosomal MC1R and Y-specific SRY gene to determine the concentration of canine DNA recovered from attacked livestock. Data suggests that the method is robust and reproducible with no significant difference in the standard curves produced from multiple runs or from different DNA standards derived from different canines. Assay sensitivity of between 15 and 31 pg is consistent with other forensic quantification assays and also in line with the sensitivity of the two tested canine STR kits, Canine Genotype 2.1 Kit and CaDNAP Panels 1 and 2. The assay is highly specific to canines when tested against 163 different dogs representing 33 different breeds and no cross-amplification of non-target species' DNA was observed even from livestock DNA tested at 31.25 ng/μl. This strongly suggests that any DNA detected on evidence collected from attacked livestock is canine. The assay also shows robust tolerance to common livestock inhibitors continuing to amplify when inhibitor-spiked DNA samples were tested. Both mixed and inhibited DNA samples underwent STR typing using two canine forensic STR kits with data showing the Canine Genotype 2.1 Kit displaying pronounced cross-amplification of livestock DNA and and/or extensive PCR inhibition leading to the complete loss of amplification when using this kit. Conversely the CaDNAP Panels 1 and 2 showed little cross-amplification of livestock DNA and improved inhibitor tolerance suggesting that this approach was better suited for the analysis of livestock attack samples. Findings are discussed and the impact of the observations on future work in this area are explored.

1. Introduction

The collection and analysis of canine DNA during the course of a criminal investigation is well established. Whether to link a suspect to a murder through their pet [1], determine the identity of a dog at an illegal dog fight [2], or confirm that DNA recovered from a deceased badger belongs to a suspected badger baiter's dog [3], the forensic casework employing canine DNA typing are wide ranging. Typically focussed on establishing a link between a sample collected at a crime scene (Query sample) and a reference sample (Known sample) from an individual dog, these methods employ either STR typing or mtDNA sequencing to first establish that the Q and K profiles match before establishing the probability of the match through established calculations [4–6]. As such, the evidential analysis pipeline closely follows the human forensic DNA pipeline with one important difference; there is no common qPCR method for canine DNA quantification. Quantification of autosomal DNA prior to STR amplification is a key quality assurance step in the forensic workflow and provides both quantitative and qualitative data on the evidence sample. Quantitative data typically relates to the concertation of DNA recovered (ng/μl), while qualitative data

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may be used to infer if the sample contains PCR inhibitors or degraded DNA [7]. The absence of DNA quantification often results in the unnecessary processing of samples that do not contain sufficient DNA or require the repeated electrophoreses of amplified samples on a genetic analyser to ensure STR peak balance is suitable for interpretation. Both are time consuming and expensive and can be mitigated through the adoption of qPCR quantification methods.

Existing research into the development of such qPCR assays for canines have looked at both SYBR green detection of SINE elements and Taq-man probe detection of MC1R [8,9] with data showing a good limit of detection and target specificity. Despite encouraging research data, current forensic providers in the United Kingdom have not adopted a single approach. Some laboratories initially perform mtDNA DNA amplification of the canine control region following methods outlined in [10] and only attempt subsequent STR typing if a confirmatory mtDNA band is observed after agarose gel electrophoresis. This method is very sensitive due to the use of multi-copy gene fragments and can yield informative mtDNA data, but does not account for copy number and rate of degradation differences between the mitochondrial genome and the autosomal genome required for STR profiling. Other forensic service providers use proprietary melt curve analysis techniques [i.e. 11] of the 12S mtDNA gene which are unavailable to other laboratories. Other methods include spectrophotomic quantification of total DNA (e.g. Nanodrop) which due to instrumental limits of detection, accuracy and poor species specificity may only be viable when enough single source canine DNA has been collected and therefore unsuitable for forensic application. The issue with these current approaches is that they make assumptions about the integrity of the sample and accurate determination of autosomal DNA concentration is not a common part of the canine forensic STR pipeline.

As noted above, the crimes where canine DNA may be of evidential value are wide ranging and involve both humans and wildlife species. One particular criminal act that is receiving increasing attention in the United Kingdom is that of livestock worrying [12]. With annual attacks in the 1000 s, the ability to collect DNA from livestock and match back to an individual suspect dog through STR profiling is a key forensic tool in such criminal investigations. This research looks to develop a robust qPCR assay that can be used to quantify canine DNA recovered from deceased livestock for subsequent STR profiling using one of the two existing STR assays [13,14] and with wider application of the method expected in wildlife and human related attacks.

2. Methods

2.1. Assay design

The assay was designed to allow the simultaneous detection and quantification of canine autosomal DNA (MC1R gene), sex determining region (SRY) and an Internal Positive Control (IPC) region based on similar human forensic qPCR assays [15,16]. Primer and Taq-man probe sequences for canine amplification and detection previously published in the literature [8,9] were first screened to assess their suitability for inclusion in the assay. Early screening data showed that the published MC1R primer combination co amplified humans and livestock while the synthetic IPC template fragment size from [17] was too small to allow effective probe design. Therefore, new primers and Taq-man probes for the MC1R gene and SRY regions were designed using NCBI primer design software [18] and underwent extensive wet lab validation alongside IPC primers, probe and EXTREmer IPC template oligo (Table 1).

Multiplex qPCR reactions were performed in 20 μl final volumes using Taq-Path™ ProAmp™ Multiplex Master Mix (Applied Biosystems) allowing 4 μl DNA input volume for DNA standard or unknown sample, with final reaction oligo concentrations reported in Table 1. Thermal cycling was performed on a RotorGene 5plex HRM (Qiagen) instrument using the following 2-step protocol; 10 min polymerase activation at

Table 1
Canine qPCR assay oligos, concentrations and analysis settings for multi-locus quantification using Hydrolysis probes with Internal Positive Control.

Locus	Oligo	Primer Sequence	Fragment Size (bp)	RotorGene Q 5plex HRM Analysis Settings
MC1R	Forward	5′-CGCCCATGTATTACTTCATCTGTTGCC-3′	163	**Filter Data•** Light Smoothing – check• Remove Non Amolified Curves with Fluorescence change < 7 %• Remove Non Amolified Curves with reaction efficiency < -100 %• Crop cycles – check• Remove date before 1 cycles• Remove date after 35 cycles **Normalization•** Dynamic Tube – check• Use noise slope correction – check• Ignore first cycles at 0• Adjust take-off point – check• If take-off point < 15, use take-off cycle 20
	Reverse	5′-CAGATGAGCACGTCAATGATGTC-3′		
	Probe	[FAM] 5′-TCGTCACGCTCACCAGCAGGTCG-3′ [BHQ1]		
SRY	Forward	5′- CAGCGGTACAACAAAATGCCT-3′	106	
	Reverse	5′-TCTCTACCGTTTCCTCCGCT-3′		
	Probe	[TEX] 5′-CGGAGAAAGTCTTCCGACCGTTGGACGGAC-3′ [BHQ2]		
IPC	Forward	5′-CGCGAGATACACTGCCAGAA-3′	92	
	Reverse	5′-GACCACAGCCAGATTAAATTTACCA-3′		
	Probe	[JOE] 5′- TCCGCGTGATTACGAGTCCGTATCG-3′ [BHQ1]		
	IPC Template	5′AACGCGAGATACACTGCCAGAAAGCCAATCCGCGTGATTACGAGTCCGTATCGCCTGAGCCAGTGTGGTAAATTTAATCTGGCTGTGGTCAA3′		

95 °C; followed by 40 cycles of 95 °C denature for 15 sec and 60 °C anneal/extend for 1 min. Where stated, quantified samples underwent downstream STR analysis using the commercially available Canine Genotype 2.1 Kit following manufacturers recommended conditions [13] and the CaDNAP STR kit Panels 1 and 2 [14]. Samples that underwent STR typing were analysed on a SeqStudio (Applied Biosystems) using LIZ600 size standard, long fragment analysis, 7 s injection, 1200 injection voltage, and 4200 run voltage.

2.2. Study samples

Samples for validation studies were sourced from pets with the owner's permission following ethical approval. DNA was collected from pets using a cotton buccal swab and swabbing the jowls for 30 s. For the canine specificity study, extracted DNA samples previously used to generate UK allele frequency data [19] were provided. Tissue from representative livestock species were sourced from commercial supermarkets while tissue samples from other relevant species of interest were provided by SASA Wildlife Forensic Laboratory. Human DNA from swabs were collected from within the research team. All tissue and swab samples underwent extraction using the DNA Investigator Kit (Qiagen Ltd) following the manufacturer's conditions. Post extraction, samples first underwent spectrophotomic DNA quantification using Nanodrop (Thermofisher Ltd) before being normalised to specific concentrations depending on the validation study described below.

There is currently no commercially available canine DNA at high enough concentrations to use to generate standards curve for qPCR. However, the use of DNA extracted from a single male canine donor provides an alternative source of DNA for forensic laboratories to use as a 'standard'. The 'within-dog' repeatability of this approach was investigated by collecting seven swabs obtained from a single donor dog at different times across a three month period. All swabs underwent DNA extraction and Nanodrop quantification before creating a series of DNA standards at the following concentrations; 2 ng/μl; 400 pg/μl; 80 pg/μl; 16 pg/μl with the standard curves compared. The 'between-dog' reproducibility of the approach was inferred through independent amplifications from three additional different male canine DNA samples. Accuracy was assessed by quantifying the canine control DNA sample MDCK.1 provided with the commercial Canine Genotype 2.1 kit supplied at 250 pg/μl. All standards were amplified in duplicate to create a standard curve with the standards also undergoing STR amplification using both the Canine Genotype 2.1 kit and the CaDNAP Panels 1 and 2 as described above.

2.3. Assay performance testing

The limit of detection (LOD) of the qPCR assay was assessed by amplifying DNA from a single male and single female canine sample in duplicate across a range of concentrations (500 pg/μl, 250 pg/μl; 125 pg/μl; 62.5 pg/μl; 31.25 pg/μl; 15.63 pg/μl; 7.5 pg/μl; 3.5 pg/μl; 1.75 pg/μl). The LOD was determined as the point by which a sample failed to break the cycle threshold (ct) during PCR as detected using the Qiagen Q-REX Absolute Quantification software.

To ensure the assay amplified across a range of canine breeds, 163 samples representing 33 different pure breeds, 25 different known cross-breeds and 7 different mongrels were tested (Supplemental Data 1). Relevant species of livestock including sheep (*Ovis aries*), cattle (*Bos taurus*), goat (*Capra hircus*), pig (*Sus scrofa*), chicken (*Gallus gallus*), turkey (*Meleagris spp*), wildlife species including Eurasian badger (*Meles meles*), Fox (*Vulpes vulpes*), Carrion crow (*Corvus corone*) and human were assessed alongside canine samples for cross amplification in the laboratory (n = 2 for each species) at a concentration of 2 ng/μl.

Mixed species samples were assessed in order to mimic the effect of recovering a small amount of canine DNA amongst a much higher background of livestock DNA. The likely upper limit of contaminating livestock DNA was determined from average DNA concentrations obtained by swabbing raw meat samples (n = 6) of each lamb, beef, and goat tissue for 30 s before undergoing DNA extraction. The average DNA concentrations obtained were lamb = 9.2 ng/μl S.D = 2.3; beef = 4.7 ng/μl S.D = 0.8; goat = 13.9 ng/μl S.D = 5.4. The likely lower limit of canine DNA was determined as between 62.5 pg (LOD of the Canine Genotype 2.1 Kit) and 100 pg (LOD of CaDNAP Panels 1 and 2). These limits were used to create mixed canine:livestock samples in the ratios of 1 in 100 (62.5 pg/μl canine:6.25 ng/μl livestock) and 1 in 500 (62.5 pg/μl canine:31.25 ng/μl livestock) with 4 μl of the mixed DNA sample being used for qPCR. Single source canine DNA and single source livestock DNA samples were also prepared at the same concentrations with all samples undergoing qPCR (n = 3) before STR amplification using both Canine Genotype 2.1 Kit and CaDNAP Panels 1 and 2 as described above.

To explore the effect of PCR inhibition on canine DNA quantification wool was chosen as a representative substrate due to casework reports of inhibition associated with this sample type. Mock attack samples were prepared by spiking three inch tufts of naturally shed sheep wool with canine saliva from a single dog. Saliva was obtained by throwing a rubber ball to a dog and half the ball was wiped over the wool to represent a bite (n = 6). Canine saliva was recovered from spiked wool by swabbing following standard collection practices and subject to qPCR with two representative samples being selected for subsequent STR typing using both Canine Genotype 2.1 Kit and CaDNAP Panels 1 and 2 as described above. Negative control samples (un-spiked wool) were also prepared.

2.4. Data analysis

All qPCR data was analysed using Q-Rex software and Absolute Quantification Plugin software with software settings reported in Table 1. STR data was analysed using GeneMapper 7 with allele peaks identified by automatic allele bin calling developed for each STR panel. Quality control checks of LIZ 600 size standard and positive and negative control samples was performed. After passing control checks three STR loci (FH3313, FH2017, FH2361) in the Canine Genotype 2.1 Kit were automatically removed for the remaining analysis due to previous recommendations regarding their inclusion in forensic identity testing [19]. Single source canine DNA samples were scored using the above method while mixed canine:livestock DNA samples were first manually inspected with off ladder noise, artefacts, and dye blobs removed. Where suspected artefacts were observed within the allelic bin windows, peak morphology, heterozygote balance and stutter ratios were used in exclusion criteria. Genotypes from mixed canine:livestock samples were determined through reference to the single source livestock profile and subsequent exclusion of livestock artefact peaks from the mixed profile. These data were processed blind without reference to the single source canine genotype. All data was exported to excel and analysed.

3. Results and discussion

The data reported below describes the development of a Taq-man based qPCR assay for the detection and quantification of canine DNA recovered from worried livestock. The data shows clear amplification of canine DNA and fluorescence detection of all three probes through qPCR with no amplification of the negative control (Figure 1). The MC1R gene (green channel) and SRY gene (orange channel) both show a delayed ct as the concentration of the DNA standards decrease as expected. Across seven independent amplifications of the canine DNA standard there was consistent amplification for the MC1R and SRY loci (Table 2). Standard deviation never exceeded 0.74 cycles for MC1R and 0.67 cycles for SRY suggesting that the collection, extraction and Nanodrop quantification of DNA from a single donor canine allowed reproducible standard curves to be generated. After Bonferoni correction, regression analysis using Minitab 21.4 [20] showed no significant difference between the seven independent runs for the y-intercept and for the slope of the standard curve for both the MC1R and SRY marker (Table 2). The data also shows

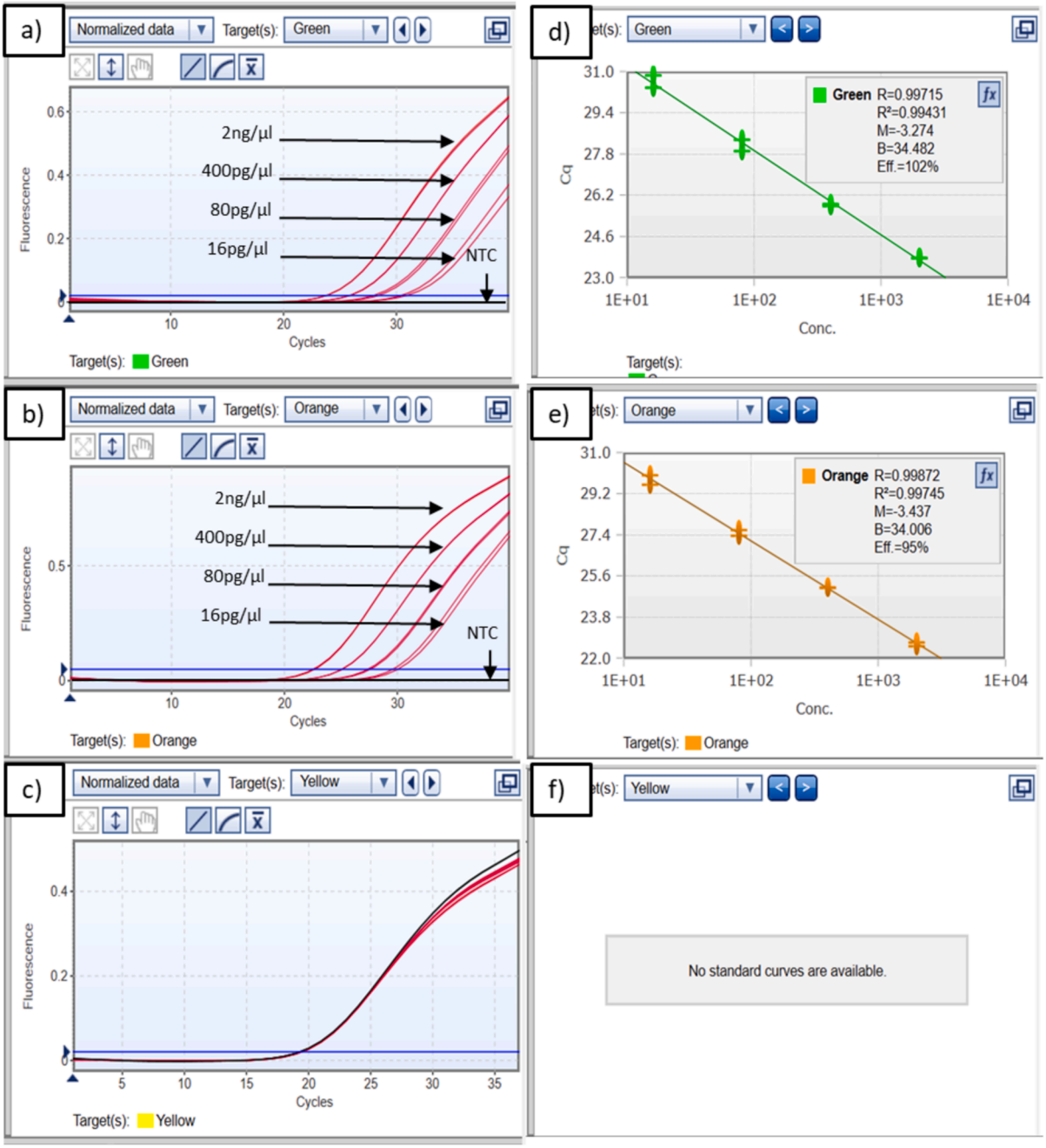

Fig. 1. Example amplification plots generated from Qiagen Q-Rex software showing a) increasing fluorescence for the MC1R gene (green channel), b) SRY gene (orange channel), and c) IPC target (yellow channel). The ct values from the amplification data were used to generate associated standard curves of d) MC1R gene and e) SRY gene to quantify unknown samples. No standard curve is generated from the IPC target (f). Negative control samples (NTC) show no amplification in the MC1R and SRY but show amplification of the IPC target.

no significant difference between the four different individual canine donors for the y-intercept and for the slope of the standard curve for both the MC1R and SRY marker (Table 2). Together these data support the idea that laboratories can use locally sourced volunteer male dog DNA in lieu of a commercial DNA standard, although the development of a standardised cell line that can be shared amongst practitioner laboratories should be strongly considered. The data also shows consistent ct values for the IPC template across different standard DNA concentrations suggesting there is little impact of canine DNA concentration on IPC amplification efficiency. However, the IPC showed signs of increasing ct over the eight week experimental period (week 0 average ct = 18.67, S.D = 0.35, week 8 average ct = 24.20, S.D = 0.11) suggesting that the EXTREmer IPC template was slowly degrading over time when stored in the fridge. This is a commonly observed issue with oligo storage and possible mitigation measures include buffering the IPC template with a higher concentration of non-target DNA, such as salmon sperm DNA, or simply freezing working stock aliquots of the IPC oligo.

The accuracy of the qPCR method was assessed by quantifying the concentration of the MDCK.1 Cell Line (250 pg/µl) which returned an average concentration of 124 pg/µl (S.D = 26.5) across four

Table 2
Table showing average ct values for DNA standards at each concentration for MC1R and SRY loci together with low variation in IPC ct.

Study	Donor Sample ID	Average ct MC1R (Green)						Average ct SRY (Orange)							IPC (Yellow)	
		2 ng/µl	400 pg/µl	80 pg/µl	16 pg/µl	R^2	Y-intercept p-value	Slope p = value	2 ng/µl	400 pg/µl	80 pg/µl	16 pg/µl	R^2	Y-intercept p-value	Slope p = value	Average cq	Std Dev
Seven independent runs of one sample	001	24.18	26.45	29.10	31.42	***0.998***	–	–	22.20	24.47	26.94	29.35	***0.999***	–	–	18.67	0.35
	001	24.38	26.68	29.20	31.02	***0.996***	***0.943***	***0.859***	21.62	23.78	26.45	28.00	***0.991***	***0.323***	***0.844***	19.43	0.07
	001	23.79	25.85	28.16	30.64	***0.994***	***0.380***	***0.806***	22.62	25.11	27.51	29.83	***0.994***	***0.527***	***0.943***	21.35	0.06
	001	22.90	24.96	27.15	29.38	***0.995***	***0.045***	***0.710***	22.51	24.66	27.11	29.02	***0.991***	***0.969***	***0.826***	21.52	0.07
	001	24.78	27.05	29.22	31.23	***0.996***	***0.908***	***0.755***	22.07	24.26	26.37	28.30	***0.998***	***0.441***	***0.718***	22.27	0.15
	001	24.57	26.95	29.20	30.88	***0.994***	***0.953***	***0.766***	21.91	24.40	26.45	28.34	***0.993***	***0.513***	***0.825***	23.82	0.05
	001	24.36	26.61	29.11	31.58	***0.999***	***0.913***	***0.961***	22.70	25.06	27.21	29.65	***0.999***	***0.687***	***0.920***	24.20	0.11
	Average	24.14	26.36	28.73	30.88	***0.996***	–	–	22.23	24.53	26.86	28.93	***0.995***	–	–	–	–
	Std Dev	0.58	0.68	0.74	0.68	***0.002***	–	–	0.37	0.43	0.41	0.67	***0.003***	–	–	–	–
One run of four independent samples	001	24.45	26.76	28.90	31.39	***0.991***	–	–	22.56	24.90	26.82	29.43	***0.989***	–	–	24.46	0.49
	003	25.42	27.88	30.28	32.47	***0.995***	***0.209***	***0.897***	23.50	25.85	28.03	30.41	***0.998***	***0.256***	***0.943***	24.33	0.20
	004	25.47	27.65	29.75	32.45	***0.993***	***0.333***	***0.965***	23.49	25.81	27.86	30.05	***0.993***	***0.366***	***0.955***	24.57	0.12
	007	25.12	27.34	29.52	31.57	***0.996***	***0.653***	***0.884***	23.10	25.28	27.46	29.78	***0.998***	***0.622***	***0.968***	24.37	0.10
	Average	25.11	27.40	29.61	31.97	***0.994***	–	–	23.16	25.46	27.54	29.92	***0.995***	–	–	–	–
	Std Dev	0.41	0.42	0.49	0.49	***0.002***	–	–	0.38	0.39	0.47	0.36	***0.004***	–	–	–	–

independent amplifications. Despite the calculated concentration being half the expected value the method is still considered acceptable as the data is within the informative range for supporting casework management decisions. This is supported by the results of the STR typing of the DNA standards (Fig. 2) that shows full profiles were observed until 80 pg and with peak heights showing a steady reduction as less DNA is introduced into the Canine Genotype 2.1 kit and CaDNAP Panels 1 and 2. Heterozygote peak balance also gets progressively worse with allelic dropout observed at 16 pg DNA input for both typing kits. The data presented shows strong support that the qPCR standards used correlate well with the dynamic range required for STR typing in both commonly used forensic canine STR kits [13,14].

The sensitivity of the qPCR assay was assessed in both male and female canine samples. Data shows that both replicate samples of the male were able to detect the MC1R and SRY target at 31.25 pg/µl. No amplification of this sample was observed below this concentration, despite amplification of the DNA standard at 16 pg/µl, suggesting that the male sample used in the sensitivity study was overly diluted during preparation. The female sample showed detection of the MC1R target at 15.63 pg/µl with no amplification of the SRY marker observed, as expected. The limit of detection (LOD) observed is not as sensitive as the 5 pg previously reported in the literature for other canine qPCR approaches [8]. The difference in reported LOD may result from reduced PCR efficiency in the current method. Reduced PCR efficiency may be due to an increased fragment length (72 bp fragment in the previous study compared to 163 bp fragment in this study) to ensure species specificity, the co-amplification of multiple targets that occurs in multiplex assays or simply PCR formulation differences between studies. Despite the reduced LOD, the assay has the required level of sensitivity for its intended purpose and is more sensitive than the stated LOD of the STR kits tested.

Data generated from the analysis of 163 canine DNA samples showed that all breeds tested amplified with only three instances of non-amplification (two unknown cross-breeds and one Staffordshire Bull Terrier). These three samples were subject to STR typing using both STR kits, all of which failed to produce a single STR profile, suggesting these samples were genuinely negative, possibly resulting from poor sample recovery or unsuccessful DNA extraction. Non-target species of livestock (sheep, cattle, goat, pig, chicken, and turkey) showed no amplification of either MC1R or SRY when 2 ng/µl species DNA was added to the assay and no amplification was observed in other non-target species including human, badger and carrion crow. Amplification was observed in both MC1R and SRY for fox. This result is unsurprising given the species relatedness to canines and the previously observed amplification of canine STRs in this species [13]. As a possible scavenger of deceased livestock there is a potential that fox DNA will be collected at site and mistakenly assumed to be canine through qPCR. Amplification of fox DNA would also be an issue if the approach was used to investigate illegal fox hunting, although there is no possibility that the resulting fox STR profile will be mistaken for the suspect dog profile and consequently the amplification of fox DNA is not considered to cause a significant problem, especially in livestock worrying cases.

Higher concentrations of single source livestock DNA at 6.25 ng/µl and 31.25 ng/µl were also assessed with no amplification observed in the MC1R and SRY targets for cattle, pork, turkey or chicken (Fig. 3). Amplification past 35 cycles was observed in the MC1R target for both single source sheep and goat at 31.25 ng/µl and past 38 cycles for sheep and goat at 6.25 ng/µl. For this reason the analysis parameters specified in Table 1 exclude data after 35 cycles, effectively nullifying amplification of DNA past 35 cycles whether it be from canine or livestock. This cycle cropping method does not change the LOD reported above for canine DNA, which remains between 15.63 pg/µl and 31.25 pg/ul. The high level of specificity observed in the single source samples was also observed when canine:livestock samples were mixed in ratios of 1:100 (62.5 pg/µl canine: 6.25 ng/µl livestock) and 1:500 (62.5 pg/µl canine: 31.25 ng/µl livestock) (Fig. 3). The data shows little difference in cycle

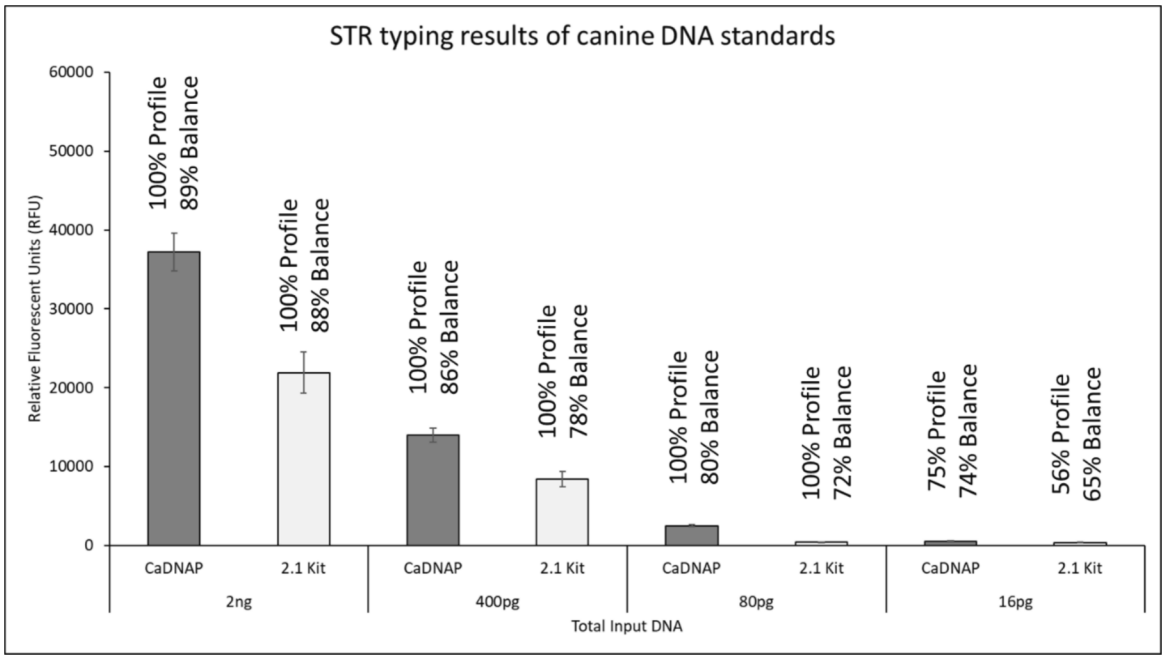

Fig. 2. STR data from the DNA standards for 2 ng input DNA, 400 pg input DNA, 80 pg input DNA and 16 pg input DNA. Data shows reducing peak heights and heterozygote balance leading to loss of alleles at 16 pg input DNA.s.

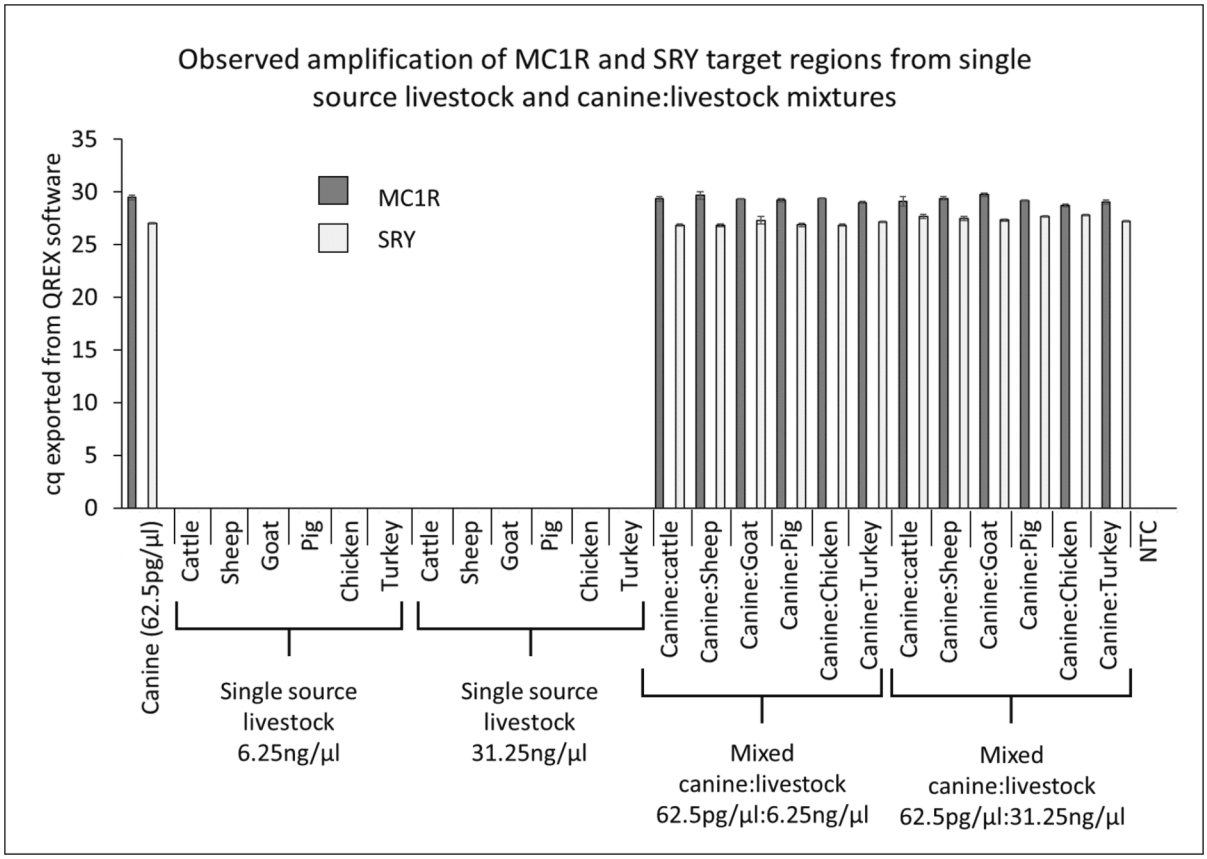

Fig. 3. Amplification of single source canine DNA (62.5 pg/µl), single source livestock DNA (6.25 ng/µl and 31.25 ng/µl) and 1 in 100 (62.5 pg/µl:6.25 ng/µl) and 1 in 500 (62.5 pg/µl:31.25 ng/µ) canine:livestock DNA mixtures for MC1R (Dark Grey) and SRY (Light Grey). The total volume of DNA within each PCR is 4 µl so total input DNA amounts are four times higher than the stated concentrations.

Table 3
Positions of spurious non-target artefact peaks observed in single source livestock at 31.25 ng.

		Relative Position of on-ladder and off-ladder spurious artefact peaks					
		Sheep (*Ovis aries*)	Cattle (*Bos taurus*)	Goat (*Capra hircus*)	Pig (*Sus scrofa*)	Chicken (*Gallus gallus*)	Turkey (*Meleagris spp*)
Canine Genotype 2.1 Kit	PEZ02	106, 127	126, 130, 131	106, 123, 127, 134, 137	126, 127	119, 133, 135, 137, 139	132
	ZFXY	–	–	158	158	–	–
	PEZ17	196, 203, 207, 212, 213, 225	197, 215,	197, 203, 205, 206, 212, 214	212, 216, 219, 224, 227	201, 205	221
	FH2309	357, 382, 386, 401, 416	372, 423	355, 387	359, 361, 377, 387	342, 362	348, 362, 364, 375, 403
	PEZ05	104, 115	93, 95	92, 101, 104, 109, 112, 115	93, 105, 108, 112	95, 109, 111	113
	FH2001	130, 133, 142, 143, 145, 149, 154, 158, 160	121, 126, 130, 139, 144, 148, 152, 160	125, 130, 138, 150, 153, 159	124, 125, 134, 136, 140	122, 125, 131, 135, 144, 145, 150	120, 126, 137, 145, 148, 157
	FH2328	179, 198, 199	178, 180, 191, 195, 198, 205	173, 177, 179, 180, 197, 199, 206	179, 181, 184, 187, 191, 203, 204, 210	176, 194, 211	173, 192, 196, 208
	FH2004	236, 252, 257, 285, 309	239, 252, 259, 263, 266, 296, 305	244, 246, 253, 257, 263, 268	242, 252, 261, 268, 274, 276, 279a, 281, 297, 301, 303, 305	241, 246, 250, 252, 263, 294, 305, 315	243, 249, 253, 260, 274, 275, 298
	PEZ21	86, 90, 97, 99, 100	90, 96	83, 86, 87, 90, 91, 99, 101	94, 99	84, 97	102
	FH2054	149	145, 152, 154, 167	149, 152, 154, 163, 168, 174	153, 156, 158	147, 152	144, 164, 173
	FH3377	195, 214, 216, 249, 258, 263, 272, 283, 292, 230, 303	193, 196, 213, 237, 234, 304	204, 212, 214, 217, 223, 247, 252, 258, 263, 266, 270, 271, 278, 292	200, 210, 215, 281, 287, 297	194, 208, 210, 229, 245, 279, 289, 291	200, 202, 204, 211, 216, 232, 249, 253, 258, 277
	FH2107	349, 353, 361, 381, 400, 415	346, 363a, 379, 401, 420	352, 361, 369, 376, 378, 391, 400, 408, 413	363, 423	357, 378	345, 359, 399, 423
	FH2088	99, 101, 131	134	108, 109, 126	94, 100, 128, 131	101, 120, 133, 135	107, 122, 122, 128
	vWF.X	157	154, 167	153, 160, 174	156, 177	163	163, 166
	FH2010	–	227	225	228	242	233
	PEZ16	–	304	281, 294	293, 316	296a	295, 301
CaDNAP Panel 1	FH2087	–	–	112	112, 134	127	112
	FH2611	–	206	180, 223	–	–	199, 203
	FH2613	–	103	–	108	96, 113	–
	PEZ6	–	–	–	–	–	–
	FH2508	–	–	–	–	–	–
	FH2361	–	–	–	–	149	–
	C38	–	118	122	118, 122	–	–
CaDNAP Panel 2	FH2087	–	–	–	–	–	131
	WILMS-TF	–	196	–	–	–	–
	FH2054	–	144, 148	–	144	148	–
	PEZ15	–	–	–	–	–	–
	SRY	–	88	–	89	–	89
	Amelogenin	110, 132	119	96, 110, 132	132	–	–
	FH2137	177, 191	195	177	162	–	–
	PEZ3	–	–	–	146, 154	–	–
	FH2328	–	196a, 216	177	176, 179, 182, 187, 188, 194, 198a, 200, 204, 208, 210a	–	–

Peak positions in Repeat Units provided for the Canine Genotype 2.1 Kit and in base pairs (bp) for CaDNAP. [a] denotes two peaks (n and n-1) were observed.

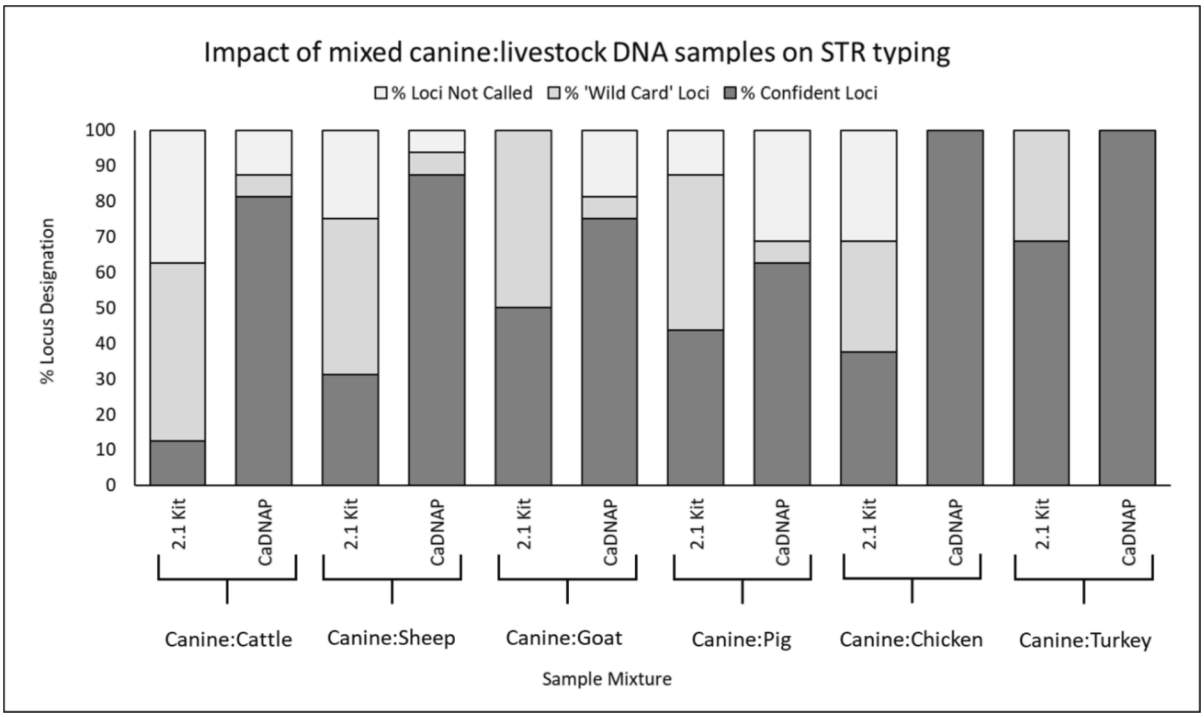

Fig. 4. STR profile quality for Canine Genotype 2.1 Kit and CaDNAP Panels based on interpretation of mixed canine:livestock DNA. Loci not called = failed due to locus dropout or presence of non-target livestock peaks. Wildcard Loci = where one allele was clearly visible but the other potentially obscured by non-target livestock peaks. Confident loci = all loci where two heterozygote canine alleles could be identified or where one homozygote canine allele could be identified with no non-target livestock peaks in the size range.

threshold (ct) for canine DNA at 62.5 pg/μl whether it is single source or mixed. This suggests that any amplification of livestock DNA is having very little effect on the ability to quantify the canine DNA.

Together the mixed canine:livestock qPCR data suggest that this method could be used to triage livestock attack samples to prevent the false progression of DNA samples that do not contain canine DNA. Such a method would offer cost savings to the investigating authority providing the data correlates with the downstream generation of a STR profile of the canine [21]. When the single source canine DNA (62.5 pg/μl) underwent STR typing a clean canine DNA profile was obtained across all loci for both the Canine Genotype 2.1 Kit and the CaDNAP Panels 1 and 2. However, when the single source livestock DNA (31.25 ng/μl) underwent STR typing the resulting data showed evidence of both on-ladder and off-ladder spurious amplification from all livestock species (Table 3). Non-target livestock amplification was very pronounced in the Canine Genotype 2.1 Kit with peaks varying in both height and peak morphology (Supplemental Data 2). Such data disagrees with the validation data from the Canine Genotype 2.1 Kit [13] which states that no livestock cross-amplification peaks occurred in the non-target species, although the previous study did not test livestock DNA at the high concentrations specified in this study. The extent of the interference from the livestock DNA when using the Canine Genotype 2.1 Kit was so prominent that interpretation of the canine:livestock mixed samples were only possible through peak subtraction. This approach first identified the fragment sizes of each non-target peak present in the single source livestock sample before removing peaks located at these sizes from the canine:livestock mixed sample. In doing so, partial canine STR profiles were obtained with some locus alleles still fully obscured by livestock peaks. In such instances only loci with two clearly defined visible alleles could be called with confidence while loci with a single visible peak were given a 'wildcard' designation due to the presence of multiple livestock on-ladder artefact peaks preventing the identification of the second peak or preventing the confirmation of the single homozygote peak (Fig. 4).

The peak subtraction method has the potential to be a usable method for forensic laboratories conducting casework although further assessment, optimisation and validation is required. It also requires that first responders collect a reference swab from the deceased livestock which is not currently performed.

STR data quality was substantially improved when using the CaDNAP Panels 1 and 2 which only showed minimal evidence of livestock artefact peaks (Supplemental Data 3, Table 3). This allowed greater confidence in calling both heterozygote and homozygote alleles at a number of different loci with only three loci (FH2137, PEZ3, FH2328) in CaDNAP Panel 2 showing signs of non-target amplification that prevented successful calls. Four loci were given the 'wildcard' designation and only one of these due to the second allele being obscured due to the presence of livestock peaks. The other three instances were due to allelic dropout that occurred at the 62.5 pg canine DNA input. Together the data shows that the CaDNAP Panels 1 and 2 produced sustainably cleaner STR profiles than the Canine Genotype 2.1 Kit suggesting that forensic laboratories should consider switching to this STR Kit. While this method is widely used across Europe, UK forensic labs interested in using CaDNAP panels for livestock attack casework will require time to adopt and internally validate this method. Such a move would not invalidate the use of the Canine Genotype 2.1 Kit for other purposes, such as pet authentication in theft cases, but would specifically strengthen results associated with livestock attacks. Alternatively, robust cell separation methods or mixture deconvolution approaches should be developed in order to generate informative data when using the Canine Genotype 2.1 Kit. Research into the separation of canine epithelial cells present in saliva from livestock white blood cells (the likely main contributor to non-target peaks) is likely to be fruitful due to the prior use in forensic analysis of cell separation approaches including laser capture microscopy [22] and the separation of suspect and victim cells through differential extraction [23]. There are also a large number of approaches, such as cell sorting, that are currently used in biomedical applications and are only beginning to be explored for forensic

applications [24].

To assess the impact of PCR inhibition on the qPCR and downstream STR data, DNA was extracted from the six wool samples using a swab (average = 0.2 ng/μl; SD = 0.05). No inhibition was observed during qPCR from these samples in respect to the ct of the Internal Positive Control (IPC) and all samples provided a canine DNA concentration within the acceptable margins for STR amplification using both STR kits. However, when the two representative samples underwent STR profiling using the Canine Genotype 2.1 Kit both samples failed, while both amplified when using the CaDNAP Panels 1 and 2. To confirm that the failed STR amplification was due to the presence of inhibitors in the wool, 1 μl DNA extract from the negative control wool (unspiked with canine DNA) sample was included in a positive control STR reaction and this completely prevented amplification of all STRs (data not shown). This observation has ramifications for all forensic work that uses the Canine Genotyping 2.1 Kit and suggests that laboratories that offer canine testing forensic services should consider moving to the alternative CaDNAP panel of markers when dealing with inhibited and potentially mixed samples. The fact that inhibition was not observed using the qPCR assay suggests it is more tolerant to inhibition than the Canine Genotype 2.1 kit, likely due to i) fewer loci requiring amplification, ii) the different polymerases used in each kit (Dual-Lock™ Taq DNA for qPCR verses Phusion Hot Start DNA Polymerase for STR amplification), and iii) different buffer components with the Taq-Path™ ProAmp™ Multiplex Master Mix specifically developed for samples with high levels of inhibitors [25]. To circumvent PCR inhibition while still using the Canine Genotype 2.1 Kit strategies include sample dilution, switching to alternative enzymes or using a DNA extraction kit specific for inhibited samples [26], all of which need addressing in further research.

4. Summary

This research reports the developmental validation of a novel qPCR assay for use in the quantification of canine DNA recovered from livestock attacks. The assay is shown to be reproducible and capable of providing an accurate quantification through the use of a volunteer male canine buccal swabs as a standard. Whilst this is acceptable in the short term, future research should look to develop and share a DNA standard derived from a cell line with collaborative proficiency testing schemes following the efforts of the International CaDNAP profiling group [27]. The qPCR assay is shown to be sensitive with a limit of detection between 15.63 and 31.25 pg/μl and is highly species specific when tested across 163 canine samples from 33 breeds. Furthermore, the assay shows no amplification of other key livestock species even at high DNA amounts, supporting suitability of this assay for quantifying canine DNA from canine:livestock mixed samples. While the application of this qPCR method should improve data quality when working with canine forensic samples, this research has also highlighted the limitations of the current commercial Canine Genotype 2.1 kit when faced with livestock mixed and inhibited samples and suggests that the CaDNAP Panel of STR markers has higher inhibitor tolerance and lower non-target amplification. While the Canine Genotype 2.1 Kit is still suitable for single-source canine DNA typing, UK forensic providers may want to consider adopting the CaDNAP panel for specific use in livestock attacks, which are estimated in the thousands per year. For this to occur, UK specific canine allele frequencies for all the CaDNAP loci will need to be developed and published, although work in this area is pending. Additional research should also consider the inclusion of a mtDNA marker within the multiplex so laboratories can decide which analysis route (STR or mtDNA) to choose. In doing so the canine DNA testing pipeline will become similar to the human DNA pipeline in terms of quality and robustness which will support the investigation of livestock attacks and other wildlife crimes in the UK and elsewhere.

5. Ethics statement

Ethical approval for the collection of samples from deceased livestock was secured by North Wales Police. All livestock keepers and participants were provided with a Study Information Sheet and Participant Consent Form following best practice. LJMU University Research Ethics Approval was not required due to the samples being from deceased livestock. Ethical approval for the collection of canine saliva and buccal swabs was obtained by LJMU PBSREC (PBS/2022-23/13). The authors have legal permission to publish the manuscript and all associated data

CRediT authorship contribution statement

N. Dawnay: Conceptualization, Methodology, Writing – original draft, Supervision. **P. Riley:** Methodology. **L. Dawnay:** Visualization, Writing – review & editing, Project administration. **R. Ogden:** Resources. **S. McColl:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scijus.2024.10.003.

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