Validation of a multiplex-tandem RT-PCR for the detection of bovine respiratory disease complex using Scottish bovine lung samples

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ABSTRACT

The welfare and economic impact of bovine respiratory disease complex (BRDC), and its associated antibiotic usage, are major challenges to cattle rearing and beef cattle finishing industries. Accurate pathogen diagnosis is important to undertake appropriate treatment and long-term management strategies, such as vaccine selection. Conventional diagnostic approaches have several limitations including high costs, long turnaround times and difficulty in test interpretation, which could delay treatment decisions and lead to unnecessary animal losses. We describe the validation of a multiplex-tandem (MT) reverse transcription-polymerase chain reaction (RT-PCR) for the detection of seven common pathogens associated with BRDC. This test has the potential to advance pathogen identification and to overcome many of the limitations of current testing methods. It requires a single sample and results are obtained quickly and not influenced by prior antimicrobial therapy or overgrowth of contaminating organisms. We demonstrated a test specificity of 100% and sensitivity ranging from 93.5% to 100% for these seven common pathogens. This test will be a useful addition to advance BRDC investigation and diagnosis.

Introduction

Bovine respiratory disease complex (BRDC) affects beef and dairy herds worldwide and is a major economic and welfare concern. Associated economic costs include production losses (long and short term), treatment costs (direct and indirect), increased mortality rates and consequent replacement costs. Bartram et al. (2017) estimated the total lifetime cost to range from UK £ 262 to £ 772[1] per affected animal depending on the type of production system. Antibiotics licensed for BRDC are among those used most frequently on beef rearer and finisher units (Humphry et al., 2021) and in Canadian feedlot cattle, notable levels of antimicrobial resistance have been found in *Mannheimia haemolytica* and *Pasteurella multocida* (Timsit et al., 2017). Treatment failure rates are high with many cases becoming chronic, negatively impacting welfare standards of cattle on farm and reducing staff morale (Booker and Lubbers, 2020). BRDC occurrence is influenced by a range of factors, primarily animal immune function, environmental conditions and presence of a range of specific viral (bovine respiratory syncytial virus, BRSV; bovine herpesvirus 1, BoHV 1; bovine parainfluenza virus 3,

BoPI3) and bacterial (*M. haemolytica, P. multocida, Mycoplasma bovis* and *Histophilus somni)* pathogens. Mixed infections are frequent and pathogen involvement can rarely be distinguished based on clinical presentation alone (Lowie et al., 2021).

Accurate identification of the pathogens involved in an outbreak of respiratory disease is an important step in reducing the impact of disease by informing appropriate treatment plans and suitable vaccine selection. The conventional diagnostic approach is based on submitting guarded nasopharyngeal swabs (from live animals) or lung samples (from post mortem cases) in transport medium for viruses and *Mycoplasma* polymerase chain reaction (PCR) and for selective and conventional culture medium for *Mycoplasma* and other bacterial pathogens respectively. This conventional approach has several limitations such as the need to obtain multiple samples from a single animal, which requires handling facilities and veterinary time and has the potential to exacerbate clinical signs when handling sick animals. Secondly, antimicrobial therapy administered before sampling is likely to affect bacterial culture results (false negative results) and any delay between sampling and laboratory receipt of the swabs may result in growth of contaminant bacteria.

Finally, the bacteria involved in BRDC are also commensals of the respiratory tract (Griffin et al., 2010), and thus the interpretation of results that lack quantitative analysis can be challenging.

Bell et al. (2014) showed that polymerase-chain reaction techniques can be successfully used to detect pathogens involved in BRDC. A multiplex-tandem (MT) reverse transcription-polymerase-chain reaction (RT-PCR), commonly known as MT-PCR, for the detection of the seven common pathogens associated with BRDC (BRSV, BoHV-1, BoPI3, *M. haemolytica*, *P. multocida*, *M. bovis* and *H. somni*) was developed, which has the potential to advance pathogen identification in BRDC and to overcome many of the limitations of current testing methods. The MT-PCR requires only a single sample from each affected animal (lung tissue from dead animals). Results are obtained quickly, are semi-quantitative and are not influenced by prior antimicrobial therapy, or by competitive growth or overgrowth of organisms. While partial analytical validation was completed by the manufacturer during test development, full independent analytical and field validation to estimate test characteristics has not previously been carried out, thus limiting clinical interpretation of test results. The aims of this paper are to describe the analytical and diagnostic test characteristics of this new MT-PCR test and to estimate the optimum diagnostic cut-off points for its use.

Materials and methods

Analytical assay design

Primer design and assay parameters were provided by the assay and instrument manufacturer (AusDiagnostics Pty. Ltd). Specific primers for this panel (AusDiagnostics Pty. Ltd., catalogue no. 78149) were designed to target the internal regions of the following pathogens: BoHV-1, BoPI3, BRSV, BoCoV, *Mycoplasma bovis, H. somni, P. multocida* and *M. haemolytica,* as well as a sample adequacy control (mammalian β-actin) and an internal assay control.

The primer sequences are considered commercially sensitive by the assay manufacturer and were not disclosed. Suitability of the assay design for the viral targets was demonstrated in our laboratory (Virus Surveillance Unit at the Moredun Research Institute) by using samples ($n = 217$) previously shown positive in our in-house quantitative (q)RT-PCR (Thonur et al., 2012). These samples included unfiltered tissue homogenates (trachea, larynx, lung and liver), nasopharyngeal swabs, respiratory aspirates, nasal discharges, broncho-alveolar lavages (BAL), and viral isolates (Table 1). Initial assay design for the bacterial targets

Table 1
Summary of 231 samples used for initial virological validation. Multiple positivity was observed in some samples. The results refer to in-house qPCR assays which represented the standard reference tests.

Target for validation	Samples positive for one virus (*n*)	Samples positive for two or more viruses (*n*)	Clinical material or viral isolates
BoHV-1	57	16	Tissues[a], respiratory swabs and aspirates, nasal discharges
BRSV	17	2	Tissues, respiratory swabs, BAL
BoPI3	4	10	Tissues, respiratory swabs
BoCoV	88	23	Tissues, respiratory swabs, BAL
Total positive	166	51	
Negative (*n* = 14)	0	0	Tissues, respiratory swabs, BAL

BoHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BoPI3, bovine parainfluenza virus 3; BoCoV, bovine coronavirus; BAL, bronchoalveolar lavage.
[a]Tissues included unfiltered homogenates of trachea, larynx, lung and liver.

was performed in silico on sequences available from the NCBI database (Sayers et al., 2022).

Analytical assay validation

Parallel testing (classical PCRs vs MT-PCR) was carried out for both viral and bacterial targets. For the viral targets, nucleic acids were extracted from the 217 positive and 14 negative archival samples (Table 1) and tested using the in-house qRT-PCRs, a coronavirus qRT-PCR (Izzo et al., 2012) and the MT-PCR assay. Positive and discordant samples were sequenced by the manufacturer. The results of the parallel testing were used to calculate sensitivity and specificity of the MT-PCR assay.

Validation of the bacterial targets was performed by the manufacturer in collaboration with an undisclosed veterinary diagnostic laboratory in the UK. Nucleic acids from the following bacterial species were tested: *Mycoplasma bovis* ($n = 138$), *H. somni* ($n = 133$), *P. multocida* ($n = 165$) and *M. haemolytica* ($n = 167$). As for the viral target validation, positive and discordant samples were sequenced by the manufacturer. Sensitivity and specificity values for viral and bacterial targets are presented in the results section.

MT-PCR assay

The MT-PCR assay was performed using the Mini-Plex system (AusDiagnostics Pty. Ltd.), consisting of a Mini-Plex 12 System liquid handling robot (AusDiagnostics Pty. Ltd.) and a DTprime Real-Time PCR thermocycler (DNA-technology). A schematic diagram of the process is presented in Fig. 1.

The assay consisted of a multiplex/parallel plex nested RT-PCR. Five microliters of total nucleic acids were initially reverse transcribed into cDNA, then amplified simultaneously with all the external primers in the panel for 15 cycles (Step 1, multiplex stage, Fig. 1, left panel). The amplified product was then diluted (1:121 ratio) and 10 μL transferred by the liquid handler to each well of a 96-well plate (Step 2, parallel plex stage, Fig. 1, right panel) where single PCR reactions (one for each target gene) were carried out independently. The Step 2 PCR was SYBRgreen-based and was followed by automated cycle threshold (CT) quantification and melting curve analysis to confirm target identification. The second PCR was run for 30 cycles.

Positive (pooled clinical samples), negative (PBS) and sample adequacy controls were included in every run and instrument performance was monitored automatically. The sample adequacy controls (mammalian β-actin and the internal control "spike") confirmed the suitability of each nucleic acid extract for PCR (absence of sample inhibition). The analytical threshold value for negative samples was set at $CT \geq 45$ for each target. Based on predefined parameters, the software assigned a CT value to the positive reactions. CT values were calculated as follows: cycles from Step 1 ($n = 15$) plus cycles for Step 2 ($n = 30$), less nominal cycles lost by dilution. The efficiency of the PCR reaction was verified by the internal control (spike) and a semi-quantification was achieved by using this internal control to normalize the CTs of the target genes of interest as described in Szewczuk et al. (2010). Multiple infections were found to be common among the single samples. In such cases, the relative contribution of each pathogen was inferred by comparing the respective CTs. The limit of detection (LoD), as the number of gene copies/mL, was determined by the manufacturer using serial dilutions of plasmids, with 16 replicates per dilution tested, and found to be 250–1750 copies/ mL. Specificity was calculated as MT-PCR negative/true negative and sensitivity as test positive/true positive for each pathogen, using the in-house assays as reference standard.

Samples for field validation

A total of 323 bovine lung samples were collected between 2020 and 2021 from carcasses originating from commercial Scottish farms,
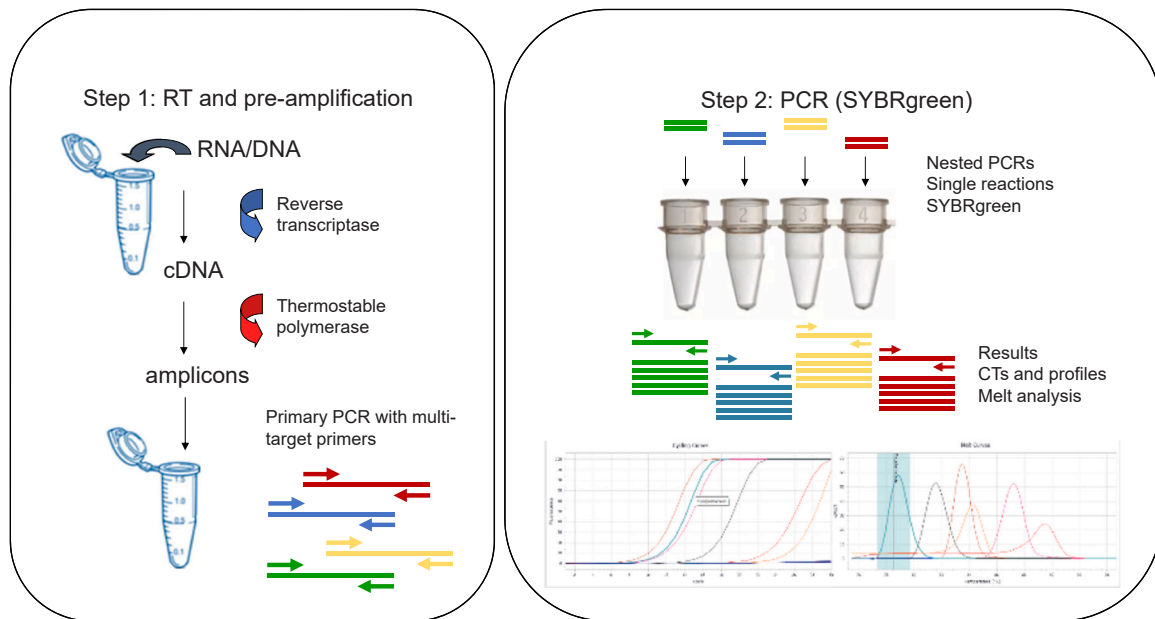
Fig. 1. Schematic representation of the multiplex-tandem PCR (MT-PCR) process. The left panel depicts the reverse transcription (RT) step followed by the primary PCR with combined multi target primers. The right panel shows the secondary nested PCRs (one for each target), typical cycle threshold (CT) profiles and the melt analysis.
(modified from AusDiagnostics, 2018)

including both beef ($n = 254$) and dairy ($n = 69$) breeds, and from a range of ages (< 2 years old, $n = 285$; > 2 years old, $n = 38$).

Positive control samples ($n = 299$) were taken from bovine carcasses that were submitted for post mortem examination. They were used as positive controls only if the following criteria were met: (1) a history of respiratory disease; (2) visible or palpable lesions consistent with pneumonia were present and (3) lung tissue collected from these carcasses was positive for at least one pathogen following a conventional test protocol. In the positive control group, no differentiation was made between animals that had received treatment for pneumonia before death and those that had not.

Negative control samples ($n = 24$) were taken from bovine carcasses submitted for post mortem examination or to an abattoir at the end of their productive life. They were used as negative controls only if the following criteria were met: (1) no clinical history of pneumonia; (2) upon gross examination there was no visible or palpable lesions consistent with pneumonia; and (3) no respiratory pathogens were identified following a conventional testing protocol.

Sample collection

All lung samples were collected from the cranioventral lung lobes. The lung tissue surface was seared using a heated pallet knife before a sterile scalpel was used to incise the lung tissue. An Amies charcoal transport medium swab was then used to swab the cut surface. Three sections of lung tissue measuring $0.5 - 1$ cm^3 were collected from the same area, avoiding the seared surface tissue from each carcass. Two pieces of lung tissue were stored in virus transport medium (VTM) and one in Eaton's broth.

Testing

All samples were processed using the following conventional testing protocol. A Quantifast Multiplex RT-PCR system (Qiagen) was used to detect the presence or absence of RNA and DNA from target respiratory viruses: BoHV-1, BRSV and BoPI3. Samples were deemed positive for one or more of the viruses if the CT value was ≤ 35 and negative if a CT value could not be determined. A single piece of lung tissue stored in

VTM was used for this analysis.

The presence or absence of *Mycoplasma bovis* DNA was determined using the VetMAX RT-PCR *M. bovis* kit (Life Technologies/Thermo Fisher Scientific). *M. bovis* was considered present if a CT value of ≤ 37 was obtained, and absent if a CT value was undeterminable. A single piece of lung stored in Eaton's broth was used for this analysis.

Amies charcoal transport medium swabs were used to inoculate Columbia Agar with 5% sheep blood (Oxoid PB0123A or equivalent), Columbia Agar with chocolated horse blood (Oxoid PB0124A or equivalent) and MacConkey Agar (Oxoid PO0148A or equivalent). Plates were incubated at 37 °C for 48 h before initial examination. Bacteria of the Pasteurellaceae family were identified using standard biochemical techniques (refer Supplementary Table S1, Supplementary material). All reference standard testing was carried out in a laboratory with accreditation from the United Kingdom Accreditation Services (UKAS), in accordance with the internationally recognised ISO/IEC 17025 standard for laboratory competence.

Whilst bovine coronavirus was included in the analytical analysis, it was not included in the validation. This was due to the lack of a commercial or widely used equivalent conventional test and its uncertain role in the pathogenesis of BRDC.

Estimation of an optimum cut-off point for diagnostic samples

We explored the distribution of CT values from positive control samples to determine whether an alternative cut-off point to the one provided by the manufacturer (CT$\geq$45) might offer a diagnostic advantage. Firstly, we visually compared the empirical distributions of CT values for individual pathogens as obtained by kernel density estimation (KDE) (Silverman, 1986). Next, pairwise differences between pathogens were statistically tested for significance using the Wilcoxon rank sum test (Wilcoxon, 1945). The resulting *P*-values were adjusted for multiple comparisons by the method of Benjamini and Hochberg (1995). The observed data were bootstrapped (1000 resamples generated) to obtain an estimate of the 97.5% quantile of each pathogen CT value distribution (point estimated obtained by median value over bootstrap resamples), and this was used to provide a pathogen-specific upper CT negativity threshold. To determine an appropriate value for a single

negative threshold value (that could be applied to all pathogens and simplify interpretative guidelines), a 97.5% quantile estimate was equally obtained considering all CT value distributions jointly. The resultant test characteristics at individual pathogen level were re-calculated using this alternative single cut-off CT value.

Results

Validation of assay design for viral and bacterial targets

Initially, the results from the assay design study, shown as percentage sensitivity and specificity, indicated agreement between the MT-PCR and the reference assays, with the exception of the PCR for BoHV-1 (sensitivity estimated at 74.6%), which required assay redesign (primers and cycle number changes) to increase sensitivity. The final sensitivity and specificity values, calculated by the manufacturer and published in the corresponding Instruction for Use (IFU) are reported in Table 2.

Initial diagnostic characteristics, field evaluation (CT>45 cut-off)

From the 299 positive control and 24 negative control samples used in the analysis, and applying a CT value of > 45 as the negative cut-off for the MT-PCR, the results of the initial comparison between these and conventional testing on positive samples were in full agreement, giving a test sensitivity of 100% for all pathogens. Test specificity was 100% for five pathogens, the exceptions being *H. somni* and *P. multocida* where one negative sample for each pathogen was incorrectly identified as positive, with CT values of 30.46 and 32.31, respectively. This resulted in a specificity estimate of 96% for both *H. somni* and *P. multocida*.

Estimating optimal cut-off point

Table 3 summarises the MT-PCR CT value distribution from positive control samples, upper negativity threshold estimate (97.5% quantile, $Q_{97.5}$) and the sensitivity and specificity when an upper negativity cut-off of 30.06 was applied for each individual pathogen and for all pathogens considered collectively.

Kernel density estimates of the empirical distributions are shown and compared in Fig. 2. There were multiple statistically significant differences ($P < 0.05$) in the CT distributions between the pathogens (P values ranged between 5.7×10^{-7} and 0.046) when investigated using the Wilcoxon rank sum test.

The estimates of the 97.5% quantile of each distribution are shown in Table 3. Comparing overall estimates, the negative threshold for 'all pathogens' was 30.06. The lowest individual negative thresholds were those of *H. somni* (26.41) and BRSV (27.32) while the highest were *M. haemolytica* (32.65) and BoHV-1 (31.03). Applying a negative cut-off of 30.06 led to reduced test sensitivity in five of the seven pathogens

Table 2
Sensitivity and specificity values obtained for the MT-PCR assay following initial analytical validation performed by the manufacturer in collaboration with our laboratory.

Assay	Sensitivity %	Specificity %
BoHV-1	100	98.14
BoPI3	92.3	100.0
BRSV	100.0	99.4
BoCoV	76.5	100.0
Mycoplasma bovis	100.0	100.0
Histophilus somni	100.0	92.2
Pasteurella multocida	100.0	100.0
Mannheimia haemolytica	100.0	100.0

MT-PCR, multiplex-tandem PCR; BoHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BoPI3, bovine parainfluenza virus 3; BoCoV, bovine coronavirus

while specificity was increased to 100% in all pathogens.

Discussion

This study reports an analytical evaluation of a novel multiplex PCR for the diagnosis of bovine respiratory disease complex and provides estimates of the test characteristics to aid accurate interpretation of the results. The findings agreed with conventional testing at a positive cut-off of both CT< 45 (as recommended by the kit manufacturer) and at our proposed alternative cut-off of CT≤ 30.06.

Initial validation of the MT-PCR assay at an analytical level showed similar sensitivity and specificity when compared to the reference standard assays already in use in different laboratories. Laboratory validation was carried out in collaboration with the instrument and kit manufacturer and an undisclosed UK private veterinary testing laboratory and in compliance with the following standards: IVD Directive 98/79/EC, Annex I A.3 and Annex III.3; EN 13612: 2002 Performance evaluation of in vitro diagnostic medical devices and ISO 13485: 2016 Medical devices, Quality management systems, Requirements for regulatory purposes. The assay Instruction for use (IFU) are available online (AusDiagnostics, 2018) and the test is available in the UK.

Preliminary validation studies indicated the need for a change in primer design and PCR conditions to address the low sensitivity for BoHV-1. BoHV-1 is not generally present as a commensal (Muylkens et al., 2007) and its detection, even at low levels, is significant. Assay redesign resulted in an increase in sensitivity for BoHV-1, whereas assay redesign was not carried out for BoCoV since this virus has only been associated with mild respiratory disease (coughing, rhinitis) and pneumonia in young calves (Cho et al., 2001), or is a possible co-factor in severe cases of BRDC (Storz et al., 2000). Serological and virological positivity for BoCoV seems to be widespread in cattle herds, especially in young animals (Berge and Vertenten, 2022).

Obtaining a reliable negative control group was a challenge to the study design due to the high occurrence rate of sub-clinical respiratory disease in cattle populations. We prioritised strict criteria for the inclusion of negative controls in the study to maximise confidence of them being truly negative for presence of any respiratory pathogen. A negative control sample size of 24 samples was used in the current study. Selecting an appropriate sample size requires an estimation of the variability in the population being studied. When such variability is expected to be high, an increased number of samples is required to ensure accurate statistical estimates representative of the population. However, if the variability was zero, then even a single sample would be representative of the entire population. The detection rate (variance) of bacteria and viruses associated with respiratory disease is known to be extremely low when using culture-based or molecular techniques (Chai et al., 2022). We therefore estimated that variance in healthy lung samples would be very low in the present study, and that results from 24 samples would be representative of a healthy cattle population. The results obtained are supportive of this, as reflected in the narrow confidence interval estimates for specificity obtained, and we found no evidence that the underlying variability would require a larger sample size.

Presence of non-pathogenic bacteria of the Pasteurellaceae family which cross react with the MT-PCR assay may present a risk to reliability of the test. We did not detect such organisms in either our negative or positive control samples and therefore have not specifically assessed the risk of cross reactions leading to false positive results. The frequency of occurrence of non-pathogenic Pasteurellaceae in bovine lungs from the UK cattle population is considered extremely low. Data derived from all bovine lung samples cultured at our laboratories between March 2020 and March 2022 ($n = 1375$) indicated that only five samples (0.4%) had a potentially non-pathogenic member of the Pasteurellaceae present (*Mannheimia varigena* in all five cases). Therefore, any cross-reactions from such organisms will have minimal impact on the specificity estimates reported here and for clinical samples from the UK. As with any

Table 3
Summary of the distribution of cycle threshold (CT) values (overall and per pathogen), upper negativity threshold estimate (97.5% quantile) and test sensitivity (Se) and specificity (Sp) for all pathogens when an upper negativity cut-off value of CT 30.06 was applied.

Pathogen	Min	Q1	Mean	SD	Median	Q3	Max	Estimate $Q_{97.5}$	Se (%) at 30.06 cut-off	Sp (%) at 30.06 cut-off
All	6.92	15.81	19.46	5.17	19.23	23.16	33.64	30.06	97	100
BoHV-1	10.21	19.69	22.78	5.42	23.17	26.42	32.70	31.03	93.5	100
BRSV	9.71	14.36	17.62	5.03	15.63	21.58	28.45	27.32	100	100
BoPI3	6.92	15.25	19.19	7.31	19.97	24.65	30.28	29.16	90.9	100
Mycoplasma bovis	6.92	12.46	16.80	5.60	15.59	20.65	33.42	28.30	98.6	100
Haemophilus somni	11.79	16.80	19.90	4.23	19.22	23.33	28.97	26.41	100	100
Pasteurella multocida	14.88	19.30	21.56	3.30	21.64	23.96	30.61	27.72	97	100
Mannheimia haemolytica	11.02	16.36	19.30	4.59	18.48	21.44	33.64	32.65	95	100

SD, Standard deviation; Q1, first quartile; Q3, third quartile; BoHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BoPI3, bovine parainfluenza virus 3.
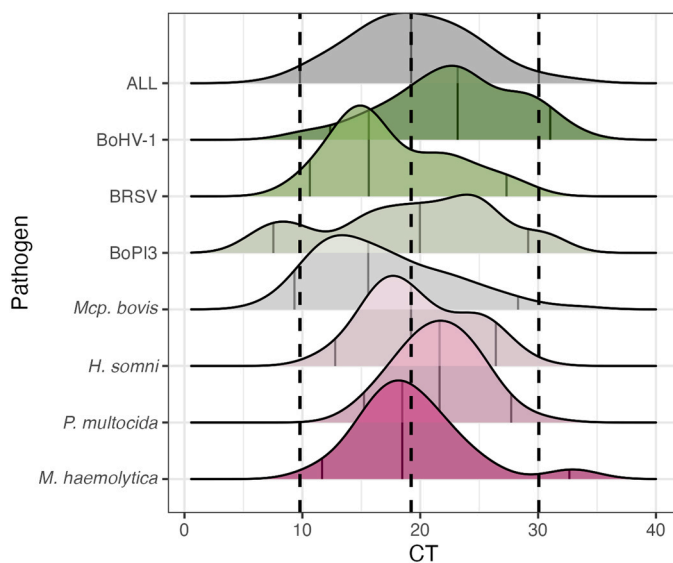

Fig. 2. Estimated empirical density distributions of cycle threshold (CT) values (overall and per pathogen). Vertical lines indicate location of 1st, 2nd and 3rd quartiles of the overall (dashed lines) and pathogen-specific (solid lines) CT distributions. BoHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BoPI3, bovine parainfluenza virus 3; Mcp. bovis, Mycoplasma bovis; H. somni, Histophilus somni; P. multocida, Pasteurella multocida; M. haemolytica, Mannheimia haemolytica.

test, unexpected or unusual results should be subject to further investigation using conventional microbiological techniques.

An initial CT cut-off of 45, based on absence of detection of signal, was proposed by the manufacturer. To assess the relevance of cut-offs to clinical disease and post mortem findings, we used the distribution of results from positive control samples to evaluate an alternative CT cut-off value that optimises the specificity of this test. A high specificity is desirable where a test is being used to confirm disease and such tests can lead to better economic outcomes in cases of BRDC through reduced mortality (Theurer et al., 2015).

Based on the present study a positive cut-off of CT≤ 30.06 appears optimal for use for application in UK cases of BRDC. CT values between the optimised cut-off of 30.06 and 45 should be reported with a note of caution as to their involvement in disease, due to the high sensitivity of the assay which might detect commensals as well as pathogenic organisms. This is particularly relevant where samples are being taken antemortem and suggests that validation needs to be carried out on field samples from live animals as CT values may possibly fluctuate over time. We evaluated the impact on test characteristics of utilising individual cut-off points for each pathogen. While individual pathogen cut-offs may offer a marginal gain in test characteristics, this introduces substantial complexity in test interpretation. The simplicity of a single cut-off is more useful than the marginal gain of individual cut-offs for each pathogen.

While further work is required for field validation on live animals, the MT-PCR described here offers many advantages over conventional testing, including ease of sample collection in the field and transport, lower overall cost for completing the testing, faster test turnaround time and production of a semi-quantitative result. However, there are several advantages of continuing conventional testing in selected cases such as in-vitro antimicrobial sensitivity testing for surveillance purposes, and whole genome sequencing for monitoring virulence factors and informing future vaccine development (Griffin et al., 2010; Shirbroun, 2020). Furthermore, autogenous vaccines can be created for bacterial isolates where no alternative is available. Conventional testing is therefore likely to be valuable for a proportion of BRDC cases.

Conclusions

We investigated the analytical and diagnostic test characteristics of a MT-PCR test for the identification of several pathogens involved in BRDC. To simplify test interpretation, we identified a single CT cut-off value that optimises the specificity of this test without significantly reducing its sensitivity. This test will be a useful addition to advance the investigation and diagnosis of BRDC, and aspects such as pathogenesis, vaccination and treatment.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. Ausdiagnostic played no role in the above study design nor in the collection, analysis and interpretation of data of the field validation, or in the decision to submit the manuscript for publication.

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Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2023.106058.

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