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A multiplex real-time PCR assay for the detection and differentiation of five bovine pinkeye pathogens

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ABSTRACT

Infectious bovine keratoconjunctivitis (IBK), also known as pinkeye, is one of the most common eye diseases in cattle. Several pathogens have been associated with IBK cases, however, *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and bovine herpesvirus type 1 (BHV-1) are most frequently observed. A multiplex real-time PCR assay using two reactions was developed for the detection and differentiation of these five pathogens. Detection sensitivities of the multiplex assays were compared to singleplex reactions testing for the same targets. Correlation coefficients (R^2) of > 0.99, and PCR efficiencies between 92 and 106% were demonstrated in all singleplex and multiplex real-time PCR reactions. The limits of detection (LOD) of multiplex assays for *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 were 19, 23, 25, 24 and 26 copies per reaction, respectively. No cross amplification was observed for specificity testing of 179 IBK positive clinical samples and 55 non-target clinical samples. Percentage of clinical samples positive for *Mycoplasma bovoculi*, *Moraxella bovoculi*, *Moraxella bovis*, BHV-1 and *Mycoplasma bovis* were 88.8% (159/179), 75.9% (136/179), 60.3% (108/179), 11.7% (21/179) and 10.0% (18/179), respectively. *Moraxella bovis*, *Moraxella bovoculi* and *Mycoplasma bovoculi* were more prevalent than *Mycoplasma bovis* and BHV-1 in IBK samples collected from animals in this study population. Our data indicates that the multiplex real-time PCR panel assay is highly sensitive and highly specific for the detection and differentiation of the five major pathogens associated with bovine pinkeye.

1. Introduction

Infectious bovine keratoconjunctivitis (IBK), commonly known as 'pinkeye', is a highly contagious disease among bovine populations that can spread rapidly within a herd through direct contact, nasal or ocular discharges and via insect vectors when the integrity of the cornea is compromised (Kopecky et al., 1986; O'Connor et al., 2012). All cattle breeds are considered susceptible, although a lower incidence of IBK has been reported in Brahman cattle and cattle with more periocular pigmentation (Angelos, 2010). IBK can produce ocular discharge, epiphora, mild conjunctivitis and corneal opacity, resulting in transitory blindness in most cases. However, IBK outbreaks may result in more severe clinical signs, including infection of the cornea that may lead to ulceration and perforation of the eye (Fernandez-Aguilar et al., 2017). Most animals recover from infection, however permanent corneal scarring and vision loss have been associated with IBK. Considerable economic impact has been attributed to IBK, particularly due to reduced weight gain in calves at weaning and high costs associated with antibiotic treatment (Fernandez-Aguilar et al., 2017). Calves with IBK usually weigh an average of 6.8–13.6 kg less than unaffected pasture mates at weaning (Funk et al., 2009). Lower performance in post-weaning cattle has been documented, including reduced average daily gain, 365th day weight, and final weight (Ali et al., 2012; O'Connor et al., 2011; Pugh Jr. et al., 1986). Additionally, the drug cost for treatment, decreased market value due to corneal scarring, loss of value of show and breeding stock, and reduced milk production from dairy

Abbreviations: IBK, Infectious bovine keratoconjunctivitis; BHV-1, Bovine herpesvirus type 1; mqPCR, Multiplex quantitative real-time PCR; LOD, Limit of detection.

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Table 1
Primers and probes information for real-time PCR target genes.

Panel	Primers/probes	Sequence (5′- 3′)	Amplicon size (bp)	Accession number
Panel 1	Mora bovis-F	GGTGACGACCGCTTGTTT	63	EF436233.1
	Mora bovis-R	ATCATCGCCTTCATCTCCAG		This study
	Mora bovis-Pr	5′- Texas Red- CGATCGTTGCCTTTACCACC -3′		
	Mora bovo-F	GGTGATATTTATCATGAAGTTGTGAAA	88	KP410780.1
	Mora bovo-R	TYTCAATTCATAATCACGATACTCAAG		This study
	Mora bovo-Pr	5′-VIC-CCAAGATACTGCGGTAGGTAAACG-3′		
	18S-F	GGAGTATGGTTGCAAAGCTGA	100	DQ222453.1
	18S-R	GGTGAGGTTTCCCGTGTTG		(Bai et al., 2018)
	18S-Pr	5′-Cy5-AAGGAATTGACGGAAGGGCA-BHQ2–3′		
Panel 2	Myco bovis-F	GCTGATGGCGGTATACAACA	110	KX772802.1
	Myco bovis-R	GCTTTGGTTTTGTGAAACTC		This study
	Myco bovis-Pr	5′- MAX-CGCTTAAAACGCTTAATATAAACATCC-3′		
	Myco bovo-F	AGCTATGGCAGGGGACAAC	66	JQ390390
	Myco bovo-R	CCACGTTCAATGTCTTTACGG		This study
	Myco bovo-Pr	5′-FAM- TGGTGTTCTACTCCGTGG-3′		
	BHV-1-F	TCGCGCACTGAGCAGAC	86	KR230748.1
	BHV-1-R	CCGTCGGTTGTAGAAAGCAG		This study
	BHV-1-Pr	5′- Texas Red-CCCGGGCTGGTAAACCTG-3′		

Note: F: Forward primer; R: Reverse primer; Pr: Probe; FAM: Fluorescein, or Fluorescein amidite; Texas Red: sulforhodamine 101 acid chloride; MAX: Rhodamine (HEX or VIC equivalent); VIC: 2′-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein; Cy5: Cyanine 5; BHQ: Black-Hole Quencher. All sequence accessions listed in the table are submitted by other researchers. They are listed as a representative accession from which the primers and probes were designed.

animals make this disease a significant economic and animal welfare consideration (O'Connor et al., 2012).

Over the last 50 years, numerous epidemiological observations and experimental challenge models have implicated *Moraxella bovis* as the major causal agent of IBK (Gould et al., 2013; O'Connor et al., 2012). A major virulence factor of *Moraxella bovis* is a secreted cytotoxin shown to reproduce IBK lesions in calves (Angelos et al., 2007; Farias et al., 2015). *Moraxella bovoculi*, a newly described species has been isolated and characterized in association with IBK in the absence of *Moraxella bovis* (Loy and Brodersen, 2014). *Mycoplasma* spp. have also been frequently reported as causal agents of bovine IBK, with *Mycoplasma bovoculi* being most prevalent (Li et al., 2015; Pinho et al., 2012; Schnee et al., 2015). One IBK outbreak was attributed to the synergistic action of *Mycoplasma bovoculi* and *Mycoplasma bovis*, following the possible predisposing effect of bovine respiratory disease (Levisohn et al., 2004). Several studies have also suggested that bovine herpesvirus-1 (BHV-1) can also cause bovine IBK (Pugh Jr. et al., 1970). It was experimentally demonstrated that BHV-1 serves as a predisposing factor for IBK after conjunctival inoculation of pure BHV-1 cultures caused conjunctivitis and blepharitis. Inoculation of BHV-1 cultures did not cause keratitis that can be produced by inoculation with pure cultures of *Moraxella bovis* (Pugh Jr. et al., 1970; Zbrun et al., 2011).

Considering that several pathogens, alone or in combination, may be associated with individual cases of IBK, it is necessary to develop a sensitive and specific detection method for the most common IBK associated agents. Although treatment is not pathogen dependent, pathogen identification is very important to guide the selection and application of appropriate vaccines including the possible need to pursue autogenous vaccine productions. Therefore, the objective of this study was to develop a multiplex real-time PCR (mqPCR) assay for the detection and differentiation of *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 from cattle, and to evaluate the prevalence of these five pathogens during an outbreak of naturally occurring IBK in cattle.

2. Materials and methods

2.1. Primers and probes

All available gene sequences of *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi*, and Bovine herpesvirus 1 were downloaded from GenBank database (https://www.ncbi.nlm.nih.gov/genbank/), and aligned and analyzed to identify conserved regions that could serve as potential targets. Primers and probes (Table 1) were selected using the online PCR design tool, Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). An 18S rRNA gene conserved in the bovine genome (Bai et al., 2018) was used as an internal control to monitor nucleic acid extractions and potential PCR inhibitions. All primers and probes were checked for potential formation of secondary structures using Auto-Dimer 1.0 software (Vallone and Butler, 2004). Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

2.2. Construction of recombinant plasmids for use as positive amplification controls

Fragments (300–500 bp) encompassing the qPCR target region of *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 were amplified. The gene segment of *Mycoplasma bovoculi* was synthesized by Genewiz (South Plainfield, NJ, USA). Amplified PCR products were cloned into pCR2.1 plasmid using the TOPO TA Cloning kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol with minor modifications. The ligation reaction for each target was mixed gently and incubated for 5–15 min at room temperature. For each ligation reaction, 1 vial of Mix &Go cells (50 μL) was removed from -80 °C storage and thawed on ice. Four microliters of ligation reaction was added, then the tube was immediately placed on ice for 5 min. Two-hundred microliters of S.O.C. medium was added, then the tube was incubated at 37 °C with shaking (300 rpm) for 1 h. Two volumes of the transformation mixtures, 20 μL and 100 μL, were spread-plated onto pre-warmed ampicillin-containing Luria-Bertani (LB) plates. The plates were incubated at 37 °C for 14–16 h. For each construct, 2–6 white colonies were picked for confirmation by PCR then subsequent secondary confirmation using DNA sequencing.

2.3. Assay formulation and optimization

Two mqPCR reactions were used: *Moraxella bovis*, *Moraxella bovoculi*, and 18S rRNA internal control were multiplexed into subpanel 1 and *Mycoplasma bovis*, *Mycoplasma bovoculi* and bovine BHV-1 into subpanel 2. Each mqPCR reaction was performed in a 20 μL volume that contained 10 μL of 2× iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 0.5 μL of each primer pair at 10 pM/μL, 0.5 μL of each probe at 10 pM/μL, 5 μL of DNA, and nuclease-free water to achieve a final reaction

volume of 20 μL. Reactions were run on a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules CA) using the following assay running conditions: initial denaturation for 10 min at 95 °C followed by 45 cycles of denaturation for 15 s at 95 °C, and combined annealing and extension for 60 s at 60 °C. The 60 °C optimal annealing/extension temperature was determined through a temperature gradient analysis.

2.4. Analytical sensitivity

Standard curves were generated by testing cloned plasmids of each pathogen in single and multiplex reactions. Plasmid DNA used to generate standard curves was also quantified using a NanoDrop 2000 (Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions. To mimic a clinical testing condition, each plasmid was serially diluted in a bovine DNA extracted from bovine tissue homogenates (~Ct 20 by 18S rRNA PCR) to supply with naturally occurring 18S rRNA internal control template, and each dilution was tested in triplicate. Correlation coefficients and PCR amplification efficiencies were determined using the CFX Manager software (BioRad, Hercules CA, USA). The Ct values were plotted with log dilutions. The slope of the linear regression was used to determine PCR amplification efficiencies (E) using the following equation: $E = 10^{(-1/slope)}$-1. Standard curves were also used to determine the limit of detection (LOD) for each target. To increase the accuracy of each LOD, 2-fold serial dilutions were prepared from the last 10-fold dilution that still generated a positive signal, then tested in triplicate by real-time PCR. Final LOD was defined as the lowest concentration of each plasmid DNA that yielded a positive signal in all three replications. The lowest detectable copy number for each assay was calculated using the following equation (Huang et al., 2009; Shi et al., 2016):

$$Plasmid\ copies/\mu L = \frac{(6.02 \times 10^{23}) \times (X\ ng/\mu L \times 10^{-9})}{Plasmid\ length\ (bp) \times 660}$$

2.5. Assay specificity

To evaluate the assay's specificity, PCR products of these field samples positive for the five IBK pathogens were sequenced and searched in GenBank database for comparative matches. Furthermore, the assay specificity was also analyzed by testing 55 non-target samples or cultures positive for non-pinkeye pathogens were also used for specificity analysis. These non-target samples or cultures include bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCoV), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Bibersteinia trehalosi*, bovine viral diarrhea virus (BVDV), bovine coronavirus (BCoV), bovine rotavirus group A (RVA), *Acinetobacter indicus, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes*, *Cryptosporidia* spp. and *Neospora caninum*. 11 different serotype of *Salmonella* culture samples including Anatum, Mbandaka, Montevideo, Kentucky, Newport, Orion va.15+ 34+, Muenster, Anatum var. 15+, 34+, Muenster var. 15+, 34+, Schwarzengrund, Senftenberg and Orion var. 15+, and 8 different serogroups of *Escherichia coli* culture samples including O26, O103, O104, O157, O121, O145, O45 and O111 serogroups. Assay's intra-assay specificity was evaluated with five pools of the target plasmids that were created with one of the five targets absent from each pool. Each pool was then tested by a singleplex qPCR using primers and probes corresponding to the absent target.

2.6. Assay sensitivity on diagnostic samples

A total of 1,791 bovine ocular swab samples from cattle with clinical signs of IBK mainly collected in the state of Kansas were tested using this multiplex real-time PCR panel. Eight eye swabs from asymptomatic animals were also collected to serve as negative samples for validation. The swab samples were transferred into 1 mL of 1× PBS and briefly vortexed. DNA was extracted from the supernatant using Qiagen (Valencia, CA, USA) QIAamp Viral RNA Mini Kit. Selected clinical samples that were positive by multiplex real-time PCR were confirmed by DNA sequencing.

3. Results

3.1. PCR amplification efficiencies and correlation coefficients of the standard curves

Analytical sensitivities of each individual assay were determined by standard curves using plasmids as templates. The results are shown in Fig. 1. The correlation coefficients (R^2) for each of the five singleplex assays were all > 0.99, and PCR amplification efficiencies were between 90.8% and 105.3%. Standard curve results from each subpanel of the two multiplex real-time PCRs are shown in Fig. 2. Subpanel 1, that includes *Moraxella bovis*, *Moraxella bovuculi*, and the 18S rRNA internal control, had R^2 > 0.99 for both targets with PCR amplification efficiencies of 91.7% for *Moraxella bovis* and 96.2% for *Moraxella bovuculi*. Similarly, for the subpanel 2 multiplex real-time PCR that includes *Mycoplasma bovis Mycoplasma bovoculi*, and BHV-1, R^2 values were all > 0.99 and PCR amplification efficiencies were 95.1%, 98.8% and 90.3% for the three pathogens, respectively (Table 2).

3.2. The limit of detection of the PCR assay

The copy number for each target was detected using a serial dilution of the plasmids. Results indicate that this assay has a high sensitivity in detecting these five pathogens. The average singleplex PCR detection limits for *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 were 20, 21, 22, 23 and 26 copies per reaction, respectively (Table 2). The average multiplex PCR detection limits for *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 were 19, 23, 25, 24 and 26 copies per reaction, respectively, which are nearly identical to those generated by singleplex reactions.

3.3. Analytical specificity

PCR products of field samples (n = 179) positive for IBK pathogens were submitted for DNA sequencing. No non-specific positive signals were observed in the 179 clinical samples. The intra-assay specificity analysis of the five non-target plasmid pools did not result in any non-target cross amplification. The assay specificity was also analyzed by testing 55 non-target positive samples. Again, no non-specific positive signals were observed in the 55 non-IBK clinical samples.

3.4. Prevalence of the five pathogens in IBK samples

The percentage of positive samples for *Mycoplasma bovoculi*, *Moraxella bovoculi*, *Moraxella bovis*, BHV-1 and *Mycoplasma bovis* were: 88.8% (159/179), 75.9% (136/179), 60.3% (108/179), 11.7% (21/179) and 7.6% (9.5/179), respectively (Fig. 3A). Among the 179 positive clinical samples, 95 (53.0%) samples were positive for both *Moraxella bovis* and *Moraxella bovoculi*; 100 (55.8%) samples were positive for both *Moraxella bovis* and *Mycoplasma bovoculi*; 129 (72.0%) samples were positive for both *Moraxella bovoculi* and *Mycoplasma bovoculi* and 93 (51.9%) samples were positive for *Moraxella bovis*, *Moraxella bovoculi* and *Mycoplasma bovoculi* (Fig. 3B). Results suggest that *Moraxella bovis*, *Moraxella bovoculi* and *Mycoplasma bovoculi* have a higher prevalence than *Mycoplasma bovis* and BHV-1 in IBK samples collected from this study population.

4. Discussion

A variety of pathogens have been associated with cases of IBK, however, causative agents most frequently include *Moraxella bovis*, *Moraxella bovoculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1.

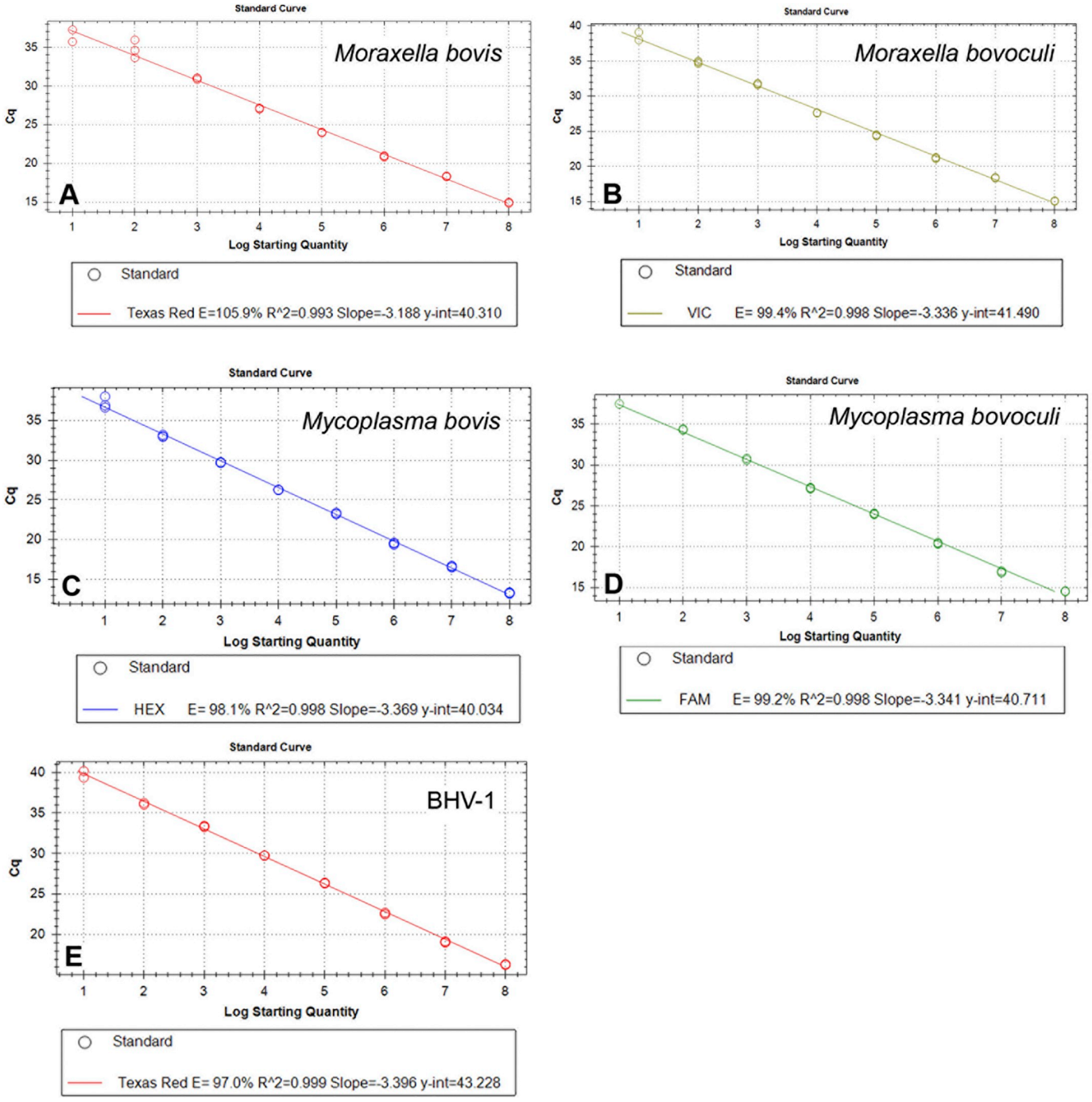

Fig. 1. Standard curves of singleplex real-time PCR reactions. The X-axis represents log concentration (copies of plasmids/reaction), and the Y-axis represents cycle threshold (Ct) values. Panel A represents: *Moraxella bovis*; B: *Moraxella bovuculi;* C: *Mycoplasma bovis*; D: *Mycoplasma bovoculi*; and E: BHV-1. Abbreviations: E: PCR amplification efficiency, R^2: Correlation coefficient of the linear regression; Cq: quantification cycle, equivalent to Ct. y-int (Y-intercept) is the Cq value when X value is zero.

Moraxella bovis was long considered the sole causative agent of IBK, since it was most often isolated from clinical cases (Henson and Grumbles, 1960; O'Connor et al., 2012). Recently, another *Moraxella* species, *Moraxella bovoculi,* was isolated from calves with IBK (Angelos et al., 2007). During a drug efficacy trial conducted in northern California, IBK-affected eyes of dairy and beef calves were cultured, and in most calves, hemolytic Gram-negative cocci but not *Moraxella bovis* were isolated. In a few cases, both *Moraxella bovis* and hemolytic Gram-negative cocci were isolated (Dueger et al., 2004). The hemolytic Gram-negative cocci were subsequently characterized; biochemical and molecular data showed that these isolates were distinct from *Moraxella bovis* and *Moraxella ovis* and warranted their classification as a novel species named *Moraxella bovoculi* (Angelos et al., 2007). The frequency of co-isolations may indicate that *Moraxella bovoculi* may be serving as an opportunistic agent or colonizing eyes in conjunction with *Moraxella bovis*, which has been shown to experimentally induce characteristic corneal ulcers in gnotobiotic calves (Rogers et al., 1987). In this study, clinical sample data supports that *Moraxella bovis* and *Moraxella bovoculi* have a high prevalence, some are with very high concentrations, in these IBK samples (Zheng et al., 2019, submitted).

Mycoplasma spp. is also highly prevalent in the conjunctivae of cattle with IBK, with *Mycoplasma bovoculi* being the most commonly identified

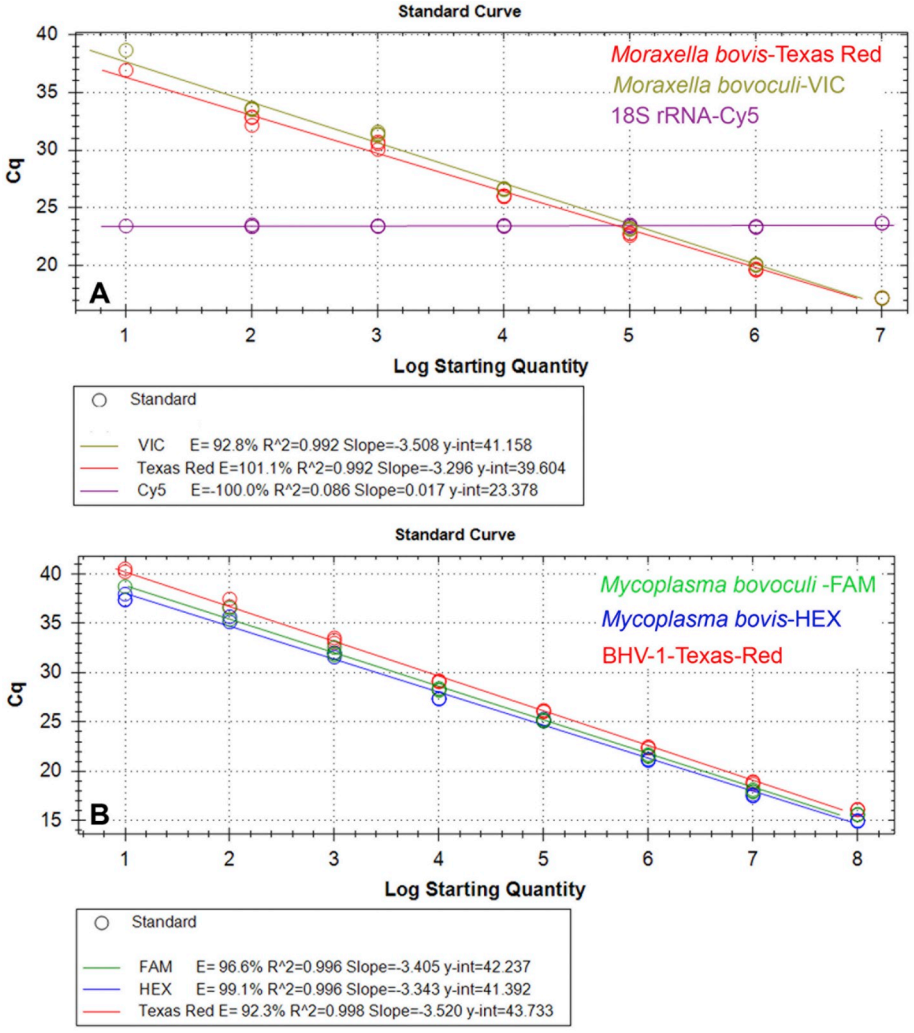

Fig. 2. Standard curves of multiplex real-time PCR reactions. The X-axis represents log concentration (copies of plasmids/reaction), and the Y-axis represents cycle threshold (Ct) values. Panel A: Standard curves of subpanel 1 including *Moraxella bovis* (Texas Red), *Moraxella bovuculi* (HEX), and 18S rRNA (CY5, a constant concentration was used). Panel B: Standard curves of subpanel 2 including *Mycoplasma bovoculi* (FAM), *Mycoplasma bovis* (HEX) and BHV-1 (Texas-Red). Abbreviations: E: PCR amplification efficiencies, R^2: correlation coefficients; Cq: quantification cycle. y-int (Y-intercept) is the Cq value when X value is zero. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analytical comparison of PCR amplification efficiencies, correlation coefficients and limit of detection between singleplex and multiplex reactions.

Target	PCR reaction format	PCR amplification efficiency (%)	Correlation coefficient (R^2)	Limit of detection (copy/reaction)
Moraxella bovis	singleplex	105.9	0.993	20
	multiplex	101.1	0.992	19
Moraxella bovoculi	singleplex	99.4	0.998	21
	multiplex	92.8	0.992	23
Mycoplasma bovis	singleplex	98.1	0.998	22
	multiplex	99.1	0.996	25
Mycoplasma bovoculi	singleplex	99.2	0.998	23
	multiplex	96.6	0.996	24
Bovine herpes virus 1	singleplex	97.0	0.999	26
	multiplex	92.3	0.998	26

Note: PCR amplification efficiency (E) is calculated by $E = 10^{(-1/slope)} - 1$.

species (Schnee et al., 2015; Vilei et al., 2007). Whether *Mycoplasma bovoculi* alone can cause clinical IBK, or act as a predisposing factor enhancing the effects of *Mycoplasma* spp. is currently unclear. Herds with a high prevalence of *Mycoplasma bovoculi* are more predisposed to acute IBK, possibly due to this pathogen facilitating enhanced infection with *Moraxella* spp. (Schnee et al., 2015). An outbreak of IBK has even been attributed to the synergistic action of *Mycoplasma bovoculi* and *Mycoplasma bovis*, following the possible predisposing effects of bovine respiratory disease (Levisohn et al., 2004). However, doubts have also been raised over the infective potential of *Mycoplasma bovoculi* to cause IBK in the absence of pathogenic *Moraxella* spp. In the past, classical culture methods were used for epidemiological investigations of *Mycoplasma* spp. infections (Vilei et al., 2007). However, the unique biochemical properties of *Mycoplasma* spp. have made it difficult to culture the bacteria (Becker et al., 2012), which may have impacted the prevalence data in these studies. PCR may circumvent some of these diagnostic challenges, since it offers a sensitive, rapid, robust and high-throughput application for detection. Our data suggests that *Mycoplasma bovoculi* has a higher prevalence than *Mycoplasma bovis* in IBK samples collected from this study population. Selected samples that were positive by the real-time PCR were sequence-confirmed to further support the PCR data.

Another significant infectious agent associated with IBK is BHV-1, which also causes respiratory tract disease in cattle. It was experimentally demonstrated that BHV-1 serves as a predisposing factor for IBK after conjunctival inoculation of pure BHV-1 cultures caused conjunctivitis and blepharitis. Inoculation of BHV-1 cultures did not cause keratitis which can be produced by inoculation with pure cultures of *Moraxella bovis* (Pugh Jr. et al., 1970; Zbrun et al., 2011). High concentrations of BHV-1 have been shown to shed primarily by respiration and ocular secretions from infected animals, making it easily transmittable among animals (Zbrun et al., 2011). However, the virus can also be transmitted indirectly through people and equipment (Pugh Jr. et al., 1970). Our data show that animals with clinical IBK lesions had a low prevalence (10.0%) of BHV-1 infection.

In conclusion, we have developed a multiplex real-time PCR panel assay for the detection and differentiation of *Moraxella bovis*, *Moraxella*

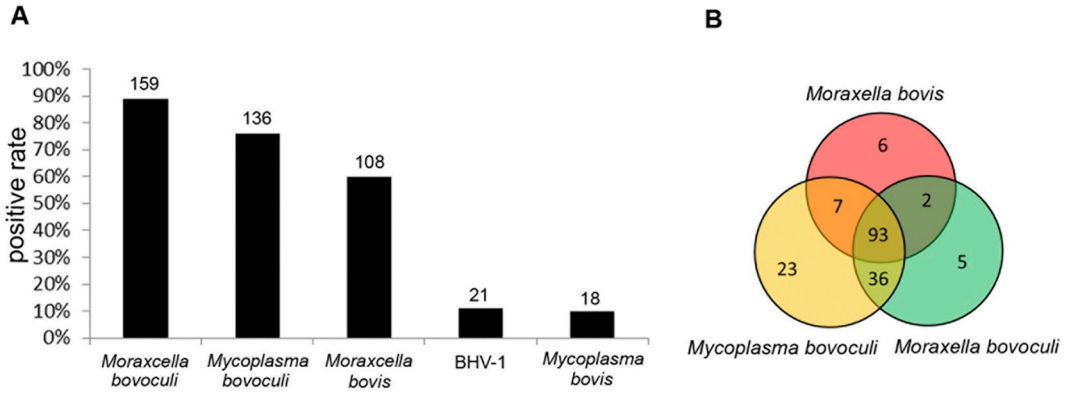

Fig. 3. The prevalence of the five pathogens in IBK samples. Panel A: Prevalence of the five pathogens from the 179 bovine ocular swab samples that were subjected to multiplex real-time PCR testing. Panel B: Co-infections among the three most prevalent IBK pathogens in the 179 positive clinical samples: 95 (53.0%) samples were positive for both *Moraxella bovis* and *Moraxella bovoculi*; 100 (55.8%) samples were positive for both *Moraxella bovis* and *Mycoplasma bovoculi*; 129 (72.0%) samples were positive for both *Moraxella bovoculi* and *Mycoplasma bovoculi* and 93 (51.9%) samples were positive for *Moraxella bovis*, *Moraxella bovoculi* and *Mycoplasma bovoculi*.

bovoculi, *Mycoplasma bovis*, *Mycoplasma bovoculi* and BHV-1 from cattle. Results from this study suggest that *Moraxella bovis*, *Moraxella bovoculi* and *Mycoplasma bovoculi* have a higher prevalence than *Mycoplasma bovis* and BHV-1 in bovine IBK samples collected from the state of Kansas. This assay may serve as a viable alternative or supplemental detection method for traditional culture methods.

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Conflicts of interest

Authors declare that there is no competing interest to this research.

References

Ali, A.A., O'Neill, C.J., Thomson, P.C., Kadarmideen, H.N., 2012. Genetic parameters of infectious bovine keratoconjunctivitis and its relationship with weight and parasite infestations in Australian tropical Bos taurus cattle. Genet. Sel. Evol. 44, 22.

Angelos, J.A., 2010. Moraxella bovoculi and infectious bovine keratoconjunctivitis: cause or coincidence? Vet. Clin. North Am. Food Anim. Pract. 26, 73–78 (table of contents).

Angelos, J.A., Spinks, P.Q., Ball, L.M., George, L.W., 2007. Moraxella bovoculi sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. Int. J. Syst. Evol. Microbiol. 57, 789–795.

Bai, J., Trinetta, V., Shi, X., Noll, L.W., Magossi, G., Zheng, W., Porter, E.P., Cernicchiaro, N., Renter, D.G., Nagaraja, T.G., 2018. A multiplex real-time PCR assay, based on invA and pagC genes, for the detection and quantification of Salmonella enterica from cattle lymph nodes. J. Microbiol. Methods 148, 110–116.

Becker, C.A.M., Ramos, F., Sellal, E., Moine, S., Poumarat, F., Tardy, F., 2012. Development of a multiplex real-time PCR for contagious agalactia diagnosis in small ruminants. J. Microbiol. Methods 90, 73–79.

Dueger, E.L., George, L.W., Angelos, J.A., Tankersley, N.S., Luiz, K.M., Meyer, J.A., Portis, E.S., Lucas, M.J., 2004. Efficacy of a long-acting formulation of ceftiofur crystalline-free acid for the treatment of naturally occurring infectious bovine keratoconjunctivitis. Am. J. Vet. Res. 65, 1185–1188.

Farias, L.D., Maboni, G., Matter, L.B., Scherer, C.F., Libardoni, F., de Vargas, A.C., 2015. Phylogenetic analysis and genetic diversity of 3′ region of rtxA gene from geographically diverse strains of Moraxella bovis, Moraxella bovoculi and Moraxella ovis. Vet. Microbiol. 178, 283–287.

Fernandez-Aguilar, X., Rossi, L., Cabezon, O., Giorgino, A., Victoriano Llopis, I., Frey, J., Lopez-Olvera, J.R., 2017. Infectious keratoconjunctivitis and occurrence of Mycoplasma conjunctivae and Chlamydiaceae in small domestic ruminants from Central Karakoram, Pakistan. Vet. Record 181, 237.

Funk, L., O'Connor, A.M., Maroney, M., Engelken, T., Cooper, V.L., Kinyon, J., Plummer, P., 2009. A randomized and blinded field trial to assess the efficacy of an autogenous vaccine to prevent naturally occurring infectious bovine keratoconjunctivis (IBK) in beef calves. Vaccine 27, 4585–4590.

Gould, S., Dewell, R., Tofflemire, K., Whitley, R.D., Millman, S.T., Opriessnig, T., Rosenbusch, R., Trujillo, J., O'Connor, A.M., 2013. Randomized blinded challenge study to assess association between Moraxella bovoculi and infectious bovine Keratoconjunctivitis in dairy calves. Vet. Microbiol. 164, 108–115.

Henson, J.B., Grumbles, L.C., 1960. Infectious bovine keratoconjunctivitis. I. Etiology. Am. J. Vet. Res. 21, 761–766.

Huang, Y.L., Pang, V.F., Pan, C.H., Chen, T.H., Jong, M.H., Huang, T.S., Jeng, C.R., 2009. Development of a reverse transcription multiplex real-time PCR for the detection and genotyping of classical swine fever virus. J. Virol. Methods 160, 111–118.

Kopecky, K.E., Pugh Jr., G.W., McDonald, T.J., 1986. Infectious bovine keratoconjunctivitis: contact transmission. Am. J. Vet. Res. 47, 622–624.

Levisohn, S., Garazi, S., Gerchman, I., Brenner, J., 2004. Diagnosis of a mixed mycoplasma infection associated with a severe outbreak of bovine pinkeye in young calves. J. Vet. Diagn. Investig., vol. 16, 579–581 American Association of Veterinary Laboratory Diagnosticians, Inc.

Li, J.H., Zhang, J.X., Zhang, N., Zhang, Y.W., Wu, W.X., Li, J.X., 2015. Development of a replicative plasmid for gene expression in Mycoplasma bovis. J. Microbiol. Methods 108, 12–18.

Loy, J.D., Brodersen, B.W., 2014. Moraxella spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: a retrospective study of case submissions from 2010 to 2013. J. Vet. Diagn. Investig., vol. 26, 761–768 American Association of Veterinary Laboratory Diagnosticians, Inc.

O'Connor, A.M., Brace, S., Gould, S., Dewell, R., Engelken, T., 2011. A randomized clinical trial evaluating a farm-of-origin autogenous Moraxella bovis vaccine to control infectious bovine keratoconjunctivis (pinkeye) in beef cattle. J. Vet. Intern. Med. 25, 1447–1453.

O'Connor, A.M., Shen, H.G., Wang, C., Opriessnig, T., 2012. Descriptive epidemiology of Moraxella bovis, Moraxella bovoculi and Moraxella ovis in beef calves with naturally occurring infectious bovine keratoconjunctivitis (Pinkeye). Vet. Microbiol. 155, 374–380.

Pinho, L., Thompson, G., Rosenbusch, R., Carvalheira, J., 2012. Genotyping of mycoplasma bovis isolates using multiple-locus variable-number tandem-repeat analysis. J. Microbiol. Methods 88, 377–385.

Pugh Jr., G.W., Hughes, D.E., Packer, R.A., 1970. Bovine infectious keratoconjunctivitis: interactions of Moraxella bovis and infectious bovine rhinotracheitis virus. Am. J. Vet. Res. 31, 653–662.

Pugh Jr., G.W., McDonald, T.J., Kopecky, K.E., Kvasnicka, W.G., 1986. Infectious bovine keratoconjunctivitis: comparison of infection, signs of disease and weight gain in vaccinated versus nonvaccinated purebred Hereford heifer calves. Can. J. Vet. Res., vol. 50, 259–264.

Rogers, D.G., Cheville, N.F., Pugh Jr., G.W., 1987. Pathogenesis of corneal lesions caused by Moraxella bovis in gnotobiotic calves. Vet. Pathol. 24, 287–295.

Schnee, C., Heller, M., Schubert, E., Sachse, K., 2015. Point prevalence of infection with Mycoplasma bovoculi and Moraxella spp. in cattle at different stages of infectious bovine keratoconjunctivitis. Vet. J. 203, 92–96.

Shi, X., Liu, X., Wang, Q., Das, A., Ma, G., Xu, L., Sun, Q., Peddireddi, L., Jia, W., Liu, Y., Anderson, G., Bai, J., Shi, J., 2016. A multiplex real-time PCR panel assay for simultaneous detection and differentiation of 12 common swine viruses. J. Virol. Methods 236, 258–265.

Vallone, P.M., Butler, J.M., 2004. AutoDimer: a screening tool for primer-dimer and hairpin structures. BioTechniques 37, 226–231.

Vilei, E.M., Bonvin-Klotz, L., Zimmermann, L., Ryser-Degiorgis, M.P., Giacometti, M., Frey, J., 2007. Validation and diagnostic efficacy of a TaqMan real-time PCR for the detection of Mycoplasma conjunctivae in the eyes of infected Caprinae. J. Microbiol. Methods 70, 384–386.

Zbrun, M.V., Zielinski, G.C., Piscitelli, H.C., Descarga, C., Urbani, L.A., 2011. Dynamics of Moraxella bovis infection and humoral immune response to bovine herpes virus type 1 during a natural outbreak of infectious bovine keratoconjunctivitis in beef calves. J. Vet. Sci. 12, 347–352.

Zheng W., Noll L., Porter E., Stoy C., Lu N., Wang Y., Liu X., Purvis T., Peddireddi L., Lubbers B., Hanzlicek G., Henningson J., Liu Z., Bai J. Real-time PCR assay threshold cycle data of bovine ocular swab samples indicate high concentrations of pinkeye-associated bacteria in diseased cattle eyes. Data in Brief. Submitted.