Development of multiplex real-time PCR assay for the detection of Brucella *spp.,* Leptospira *spp. and* Campylobacter foetus

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Summary
Abortion among dairy cattle is one of the major causes of economic losses in the livestock industry. This study describes a 1-step multiplex real-time polymerase chain reaction (PCR) to detect *Brucella* spp., *Leptospira* spp. and *Campylobacter foetus*, these are significant bacteria commonly implicated in bovine abortion. ß-actin was added to the same PCR reaction as an internal control to detect any extraction failure or PCR inhibition. The detection limit of multiplex real-time PCR using purified DNA from cultured organisms was set to 5 fg for *Leptospira* spp. and *C. foetus* and to 50 fg for *Brucella* spp. The multiplex real-time PCR did not produce any non-specific amplification when tested with different strains of the 3 pathogens. This multiplex real-time PCR provides a valuable tool for diagnosis, simultaneous and rapid detection for the 3 pathogens causing abortion in bovine.

Sviluppo di un test multiplex real-time PCR per il rilevamento di Brucella *spp.,* Leptospira *spp. e* Campylobacter foetus

Parole chiave
Aborto,
Bovini,
Brucella spp.,
Leptospira spp.,
Campylobacter foetus,
Multiplex real-time PCR.

Riassunto
L'aborto è una delle principali cause di danno economico per l'allevamento di bovini. Questo articolo descrive l'uso di 1-step multiplex real-time PCR (polymerase chain reactions) per l'identificazione di *Brucella* spp., *Leptospira* spp. e *Campylobacter foetus*, tre batteri comunemente associati all'aborto nei bovini. Il rinvenimento di ß-actina come controllo interno nello stesso esame PCR dimostra la validità di questo test. In questo studio, il *detection limit* per il multiplex real-time PCR condotto usando DNA purificato ottenuto da organismi da cultura è stato fissato a 5 fg per *Leptospira* spp. e *C. foetus* e a 50 fg per *Brucella* spp. Il test non ha prodotto alcuna amplificazione non specifica quando è stato usato con diversi ceppi dei 3 patogeni. Il multiplex real-time PCR usato in questo studio si è rivelato uno strumento utile per la diagnosi e per il rilevamento simultaneo e rapido dei 3 patogeni.

Introduction

Infectious abortion is a significant cause of reproductive failure and significant economic losses for the cattle industry (De Vries 2009). Under optimal laboratory conditions, etiologic diagnosis is achieved in 23.3 to 45.5% of the cases (Anderson 2007). The risk of abortion depends on several factors, ranging from infectious, toxic, endocrine, physical to nutritional causes, a variety of infectious agents have also been reported to cause bovine abortion throughout the world, agents such as *Brucella abortus*, *Leptospira* spp., *Campylobacter foetus*, *Hammondia heydorni, Neospora caninum, Toxoplasma gondi, Coxiella burnetti, Chlamydophila psittaci, Mycoplasma bovis, Mycoplasma bovigenitalium, Ureaplasma diversum,*

Bovine viral diarrhoea virus and *Bovine herpesvirus-1* (Tramuta *et al.* 2011). Brucellosis, Leptospirosis and Campylobacteriosis, in particular, are serious diseases causing abortion in cattle. They have been reported worldwide and affect both wild and domestic animals (Henegariu *et al.* 1997, Iraola *et al.* 2012, Skirrow *et al.* 1994). The importance of these diseases is increasing due to several reasons: fast spreading, difficulty of control and prevention, time consuming, and the cost of the treatment. These diseases have also critical consequences on the trading of livestock and animal products, as such they also inhibit social and economic development of breeders (Dieffenbach and Dveksler 1995).

Brucellosis causes abortions in the second half of gestation (usually about the seventh month) and 80% of incidence on unvaccinated cattle. The organisms enter via mucous membranes and invade the udder, lymph nodes, and uterus, causing a placentitis, which may be acute or chronic (Smith and Ficht 1990).

Laboratory detection of *Brucella* spp. is mainly based on cultural isolation, a labor intensive process which has been associated with a high risk of laboratory acquired infections (Miller *et al.* 1997).

Leptospirosis is identified as 1 of the emerging infectious disease and affects virtually all mammals, including humans (Palaniapann *et al.* 2005). Up to 30% of a dairy herd may abort during an outbreak of Leptospirosis. The incidence of Leptospirosis is highest during Summer, being it facilitated by heavy rain and floods. The microscopic agglutination test (MAT) is considered the gold standard seromethod for detection of Leptospirosis, but it does not permit early diagnosis and has low sensitivity (Xue *et al.* 2008).

Campylobacter foetus may be found in the genital tract and may cause sporadic abortion in cattle. It is an infectious venereal disease, which leads to reproductive complications, such as infertility, lowered pregnancy rate, and abortion. Bacteriological analyses, such as culture isolation and biochemical tests are useful for evaluating different samples, even those with low bacterial counts (Iraola *et al.* 2012). Although these methods are well standardized and extensively used, they are laborious and time consuming, thus making them disadvantageous when processing large scale samples or delivering a fast diagnosis (Van der Graaf-van Bloois *et al.* 2013). In addition, *C. foetus* is labile and requires special techniques to be isolated, with a low percentage of success rate (Skirrow et al. 1994).

Efficient diagnosis requires a complete diagnostic protocol associated with submission of appropriate specimens and clinical history.

Several PCR protocols have been recently developed for identification of infectious agents in aborted bovine foetuses, including *Brucella abortus* (Baily *et al.* 1992, Bricker *et al.* 2003), *Leptospira* spp. (Heinemann *et al.* 1999, Richtzenhain *et al.* 2002), and *C. foetus* (Van der Graaf-van Bloois *et al.* 2013). Unfortunately, these PCR methods have either been questioned with regards to their sensitivity and/or specificity and for using more laborious intensive and less sensitive gel-based assays (Reisberg *et al.* 2013).

However, the multiplex real-time PCR method is advantageous for detecting more than 1 target DNA sequences in a single reaction. This direct method is generally applied for the detection of specific bacteria from the hosts (Dieffenbach and Dveksler 1995).

The purpose of this study was to develop multiplex real-time PCR for detection of 3 pathogens in a single reaction with high sensitivity and specificity using a DNA template extracted directly from tissue samples. The specificity and sensitivity of the multiplex real-time PCR were tested and the results were compared with those of single PCR detection.

Material and methods

Bacterial strains

The reference strains of *Brucella* spp. and *C. foetus* spp. were obtained from Friedrich-Loeffler-Institute (FLI, Jena, Germany). The *Leptospira* spp. strains were obtained from the strain collection of the Veterinary Laboratories agency (VLA, Berlin, Germany). The *Leptospira* spp. strains also included the 10 strains used in routine MAT testing for diagnosis of Leptospirosis (Table I).

Genomic DNA extraction

Total DNA was extracted from both tissue specimens and different bacterial isolates using High pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to manufacture's protocol. The extracted DNA was stored at -20 °C for further use. DNA concentration of stock solutions was measured by spectrophotometer (DU640 Photometer, Beckman Coulter GmbH, Krefeld, Germany) at 260 and 280 nm.

Primers and probes of multiplex real-time PCR

The primers and probes used for the multiplex assay are shown in Table II. For *Brucella* spp. identification, the primers and probe target the bcsp31 gene was used as described by Probert and colleagues (Probert *et al.* 2004). Previously described primers and probes were used for *Leptospira* spp. target LipL32 gene

Table I. *Reference strains of* Brucella *spp.,* Leptospira *spp. and* Campylobacter foetus*, the strains have been obtained from Friedrich-Loeffler-Institute (FLI, Jena, Germany), the Veterinary Laboratories agency (VLA, Berlin, Germany).*

Serovar	Strain	Multiplex real-time PCR		
		***Brucella* spp.**	***Leptospira* spp.**	***C. foetus***
Leptospira borgpetersenii serovar *ballum*	Mus 127	No Cq	Positive	No Cq
Leptospira interrogans serovar *icterohemorrhagia*	RGA (ref. strain, FLI)	No Cq	Positive	No Cq
Leptospira interrogans serovar *bataviae*	Swart	No Cq	Positive	No Cq
Leptospira interrogans serovar *bratislava*	Jez Bratislava	No Cq	Positive	No Cq
Leptospira interrogans serovar *canicola*	Hond Utrecht	No Cq	Positive	No Cq
Leptospira interrogans serovar *copenhageni*	M 20	No Cq	Positive	No Cq
Leptospira kirschneri serovar *grippotyphosa*	Moskva 5	No Cq	Positive	No Cq
Leptospira interrogans serovar *hardjo*	Hardjoprajitno	No Cq	Positive	No Cq
Leptospira interrogans serovar *pomona*	Pomona	No Cq	Positive	No Cq
Leptospira interrogans serovar *saxkoebing*	Perepelitsin	No Cq	Positive	No Cq
Leptospira borgpetersenii serovar *sejroe*	M 84	No Cq	Positive	No Cq
Leptospira australis	PK 8/12	No Cq	Positive	No Cq
Leptospira copenhageni	PK 9/12	No Cq	Positive	No Cq
Leptospira rerassovi	PK13/12	No Cq	Positive	No Cq
Leptospira hardjo	PK 11/12	No Cq	Positive	No Cq
Leptospira grippotyphosa	PK 10/12	No Cq	Positive	No Cq
Leptospira pomona	PK 12/12	No Cq	Positive	No Cq
Brucella suis	CS (2013), swine	Positive	No Cq	No Cq
Brucella microti	Ref strain CCM4915	Positive	No Cq	No Cq
Brucella ceti	Ref strain NCTC 12891	Positive	No Cq	No Cq
Brucella pinnipedialis	Ref strain NCTC 12890	Positive	No Cq	No Cq
Brucella abortus	Cs (2013), cattle	Positive	No Cq	No Cq
Campylobacter foetus ssp. *veneralis*	13CS0045 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0046 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0047 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0048 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0049 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0050 (ref. strain, FLI)	No Cq	No Cq	Positive
	13CS0051 (ref. strain, FLI)	No Cq	No Cq	Positive
	NCTC010354	No Cq	No Cq	Positive
Campylobacter foetus ssp. *foetus*	12CS0290 (ref. strain, FLI)	No Cq	No Cq	Positive
	12CS0291 (ref. strain, FLI)	No Cq	No Cq	Positive
	11CS0001 (ref. strain, FLI)	No Cq	No Cq	Positive
	DSMZ 5361	No Cq	No Cq	Positive
Chlamydia abortus	Cs 26 (2004), sheep, abortion	No Cq	No Cq	No Cq
Coxiella burnetti	Cs 1928 (2008), sheep, spleen	No Cq	No Cq	No Cq
Escherichia coli	Ref- DNA, JLU Giessen (1996)	No Cq	No Cq	No Cq
Listeria monocytogenes	Field isolate(1998), sheep	No Cq	No Cq	No Cq
Mycoplasma bovis	Cs 3654/79 (2005), cattle, milk	No Cq	No Cq	No Cq
Neospora caninum	Cs 472/06 (2006), cattle, abortion	No Cq	No Cq	No Cq
Pasteurella multocida	Cs 500/1 (2001), cattle, lung	No Cq	No Cq	No Cq

FLI = Friedrich-Loeffler-Institute; JLU= Justus-Liebig University; Cs = Clinical samples; Cq = quantification cycle; DSMZ = designation of type and reference strains of the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; NCTC = National collection of type culture.

(Stoddard *et al.* 2009) and *C. foetus* target nahE gene (Van der Graaf-van Bloois *et al.* 2013), respectively. To monitor the conditions and to detect PCR inhibitors, an internal control based on the housekeeping gene beta-actin (Wernike *et al.* 2011) was integrated into the multiplex real-time PCR.

Multiplex real-time PCR conditions

Multiplex real-time PCR was conducted in 25 µl reaction volume. For a single reaction, the PCR mastermix contained 2.0 µl of each primer-probe mix (100 pmol/µl), 2.0 µl of each primer-probe mix of ß-actin (100 pmol/µl), 0.5 µl RNase-free water and 12.5 µl of QuantiTect Multiplex No Rox MasterMix (Qiagen, Hilden, Germany); finally 4.0 µl template was added. All primers were delivered by Biotez (Berlin, Germany) and probes by Eurogentec (Serating, Belgium).

The PCR reaction was performed on Stratagene Mx3005 with the following protocol: initial denaturation and activation of Taq-polymerase for 15 minutes at 95°C, followed by 45 cycles of 1 minute at 94°C, 90 seconds minutes at 60°C and finally 30 seconds at 40 °C for cooling.

Evaluation of multiplex real-time PCR

The detection limit of the multiplex real-time PCR was evaluated by 10-fold serial dilution of a DNA of the reference strain (*Brucella* abortus 544, *Leptospira interrogans* serovar *icterohemorrhagiae* and *Campylobacter foetus* subsp. *veneralis* 13CS0047). Serial dilutions were freshly prepared from stock solutions with sterile water in a broad range from 5 ng to 5 fg/µl. These dilutions were examined 3-fold with the following calculation of mean values and standard deviation (SD).

To determine the specificity of the optimized multiplex real-time PCR, 16 *Leptospira* spp. strains, 5 *Brucella* spp. strains and 12 *C. foetus* spp. strains and other closely related bacteria were used as shown in Table I.

To study the diagnostic sensitivity of multiplex real-time PCR protocol, 29 positive-*Brucella* and 12 positive-*Leptospira* samples of an aborted foetus and placenta were tested. These samples have also been examined by conventional PCR (Baily *et al.* 1992) and nested PCR (Lester and LeFebvre 2003) by State Agency of Agriculture, Food Safety Institute, Rostock, Germany.

Results

Integrity of clinical samples

A ß-actin signal was detected in all the clinical samples tested via multiplex real-time PCR, indicating no evidence of extraction failure or PCR inhibition.

Detection limit of multiplex real-time PCR

The detection limit of multiplex real-time PCR was determined by using a 10-fold serial dilution of the DNA of a reference strain ranging from 5 ng to 5 fg/µl. The reproducible detection limits were set at 50 fg for in *Brucella*-PCR, while the detection limits for the *Leptospira* spp. and *C. foetus* spp. were set at 5 fg of DNA were detected. The SDs of the cycle threshold (CT) values ranged between 0.1 and 0.5 (*Brucella* spp.), 0.3 and 1.8 (*Leptospira* spp.), 0.1 and 0.4 (*C. foetus* spp.), as shown in Table III.

The detection limit of the multiplex real-time PCR was compared with single-target PCR for *Brucella* spp., *Leptospira* spp. and *C. foetus* spp. based on the same serial dilution of the DNA of

Table II. *Oligonucleotide sequences of the primers and probes used in this study.*

Target	Oligo name	Sequences	Reference
Brucella	Brucc sp-F	5`-GCT CGG TTG CCA ATA TCA ATG C-3´	Probert *et al*. 2004
	Brucc spp-R	5`-GGG TAA AGC GTC GCC AGA A-3´	
	Probe	FAM 5`-AAA TCT TCC ACC TTG CCC TTG CCA TCA-Rox	
Leptospira	LipL32-45F	AAGCATTACCGCTTGTGGTG	Stoddard *et al*. 2009
	LipL32-Rb	GAACTCCCATTTCAGCGAT	
	LipL32-189P	FAM - AAAGCCAGGACAAGCGCCG – Cy5	
Campylobacter foetus	nahE-F	5´-TGT TAT GGT GAT CAA AAT AGC TGT TG -3´	Van der Graaf-van Bloois *et al*. 2013
	nahE-R	5´-GAG CTG TTT TTA TGG CTA CTC TTT TTT TA -3`	
	Probe	Hex - AAAGCCAGGACAAGCGCCG – MGB	
ß-actin	ACT-1005-F	5´- CAG CAC AAT GAA GAT CAA GAT CAT C-3´	Wernike *et al*. 2011
	ACT-1035-R	5´- CGG ACT CAT CGT ACT CCT GCT T-3´	
	ACT-1081	FAM- TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1	

the 3 reference pathogens. The detection limits of single real-time PCR of the pathogens were lower than in muliplex real-time PCR as 500 fg DNA with *Brucella* spp. was detected and 50 fg DNA in the case of *Leptospira* spp. and *C. foetus*, as shown in Figure 1.

Specificity of multiplex real-time PCR

The performance of the multiplex real-time PCR with different reference strains of the 3 pathogens demonstrated neither non-specific reactions nor any inter-assay cross amplification. The result showed positive amplification only with the integrated strains in multiplex real-time PCR, as shown in Table I.

Analysis of clinical samples

The performance of the multiplex real-time PCR assay in clinical samples was evaluated on different positive organs material obtained from aborted animals. The results showed detectable ct-values with all positive 29 *Brucella* and 12 *Leptospira* clinical samples. Consequently, the optimized multiplex real-time PCR was able to simultaneously detect all 3 pathogens in 1 reaction.

Discussion

Abortion in cattle is a multifactorial disease, often caused by different infectious agents. In most cases, traditional methods are not sufficient to detect the cause of the abortion, as they are often inconclusive and time consuming. Furthermore, each agent may be identified by a specific technique, such as isolation, immunohistochemistry or immunofluorescence and serology (Anderson 2007). At the same time, antigen-antibody interactions can be complicated by non-specific interactions, and false positive from vaccinated animals with high levels of circulating antibodies can be misdiagnosed as active infections.

There are, however, several single and multiplex PCR studies that have been conducted so to detect some of these pathogens (Vasconcellos *et al.* 2002, Van der Graaf-van Bloois *et al.* 2013).

Previous studies have also demonstrated the successful application of single PCR using specific primer pairs for the detection of *Brucella* spp. in bacterial culture or blood samples (Baily *et al.* 1992),

Table III. *Analytical sensitivity of multiplex real-time PCR with quantified DNA of the three strains (*Brucella *spp.,* Leptospira *spp.,* Campylobacter foetus*) obtained from Friedrich-Loeffler-Institute (FLI, Jena, Germany), the Veterinary Laboratories agency (VLA, Berlin, Germany).*

DNA per PCR reaction	*Brucella* spp.		*Leptospira* spp.		*Campylobacter foetus*	
	mean	SD	mean	SD	mean	SD
5 ng	20.5	0.1	19.5	1.6	19.3	0.3
500 pg	24.3	0.3	22.5	1.8	22.3	0.4
50 pg	28	0.3	25.2	1.3	25.5	0.4
5 pg	32	0.1	28.5	0.3	28.7	0.4
500 fg	35	0.3	32.11	0.8	32.1	0.1
50 fg	39.3	0.5	35	0.8	36.7	0.4
5 fg	No Ct		38.6	0.5	38.8	0.2

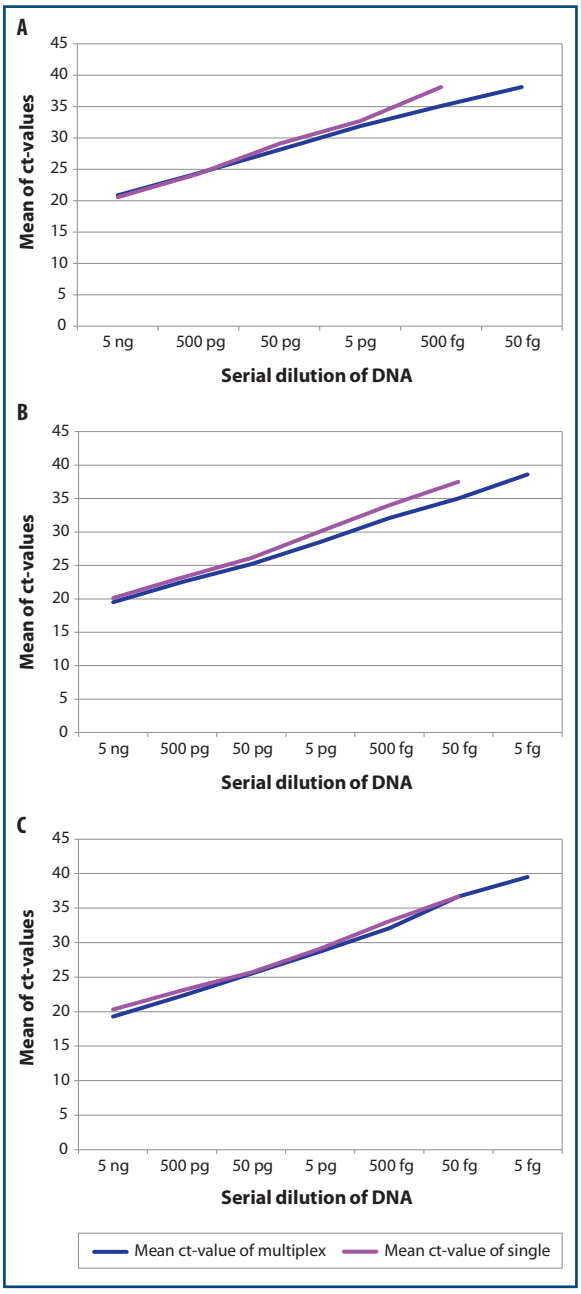

Figure 1. *Comparison of the standard curves of the single and multiplex real-time PCR assay for the detection of (A)* Brucella *spp., (B)* Leptospira *spp. and (C)* Campylobacter foetus *using strains obtained from the from Friedrich-Loeffler-Institute (FLI, Jena, Germany), the Veterinary Laboratories agency (VLA, Berlin, Germany).*

the detection of *Leptospira* spp. in clinical samples (Merien *et al.* 1992), and the detection of *C. foetus* spp. (Van der Graaf-van Bloois *et al.* 2013).

Nonetheless, single PCR only allows for the detection of nucleic acid from 1 specific pathogen at time. Recent attempts to use individual PCR for the simultaneous detection of the 3 abortive pathogens in bovine described in this study have proved to be relatively costly and time-consuming.

The multiplex PCR has the advantage of simultaneous detection of different pathogens and has been proven to be sensitive, specific and cost effective. It can also be useful in diagnosis, screening and surveillance of flocks (Henegariu *et al.* 1997).

The detection limit of the multiplex and single PCR assays was evaluated by testing 10-fold serial dilutions of reference strains of the 3 pathogens. The efficiency and the detection limit of the PCR were not affected by multiplexing the reaction. The reproducible detection limit was 5 fg for *Leptospira* spp. and *C. foetus* spp., while the detection limit for *Brucella* spp. was 50 fg. The detection limit of single PCR (500 fg for *Brucella* spp. and 50 fg for *Leptospira* spp., and *C. foetus*), was lower than multiplex real-time PCR limit (Figure 1).

In general, the multiplex real-time was better than triplex real-time PCR, which has been used in a multiplex format for rapid confirmation of *Brucella* species to detect 150 pg of DNA (Probert *et al.* 2004).

In addition, multiplex conventional PCR for detection of *C. foetus* spp. capable of detecting 5 ng/µl (Iraola *et al.* 2012) and for pathogenic leptospires targeting LipL32 capable of detecting as low as 2.5 pg have already been reported in the literature (Bhure *et al.* 2003). Previous studies also report multiplex PCR for detection of *Leptospira* spp. and *Brucella* spp. capable of detecting 100 fg for *Brucella* and 1 pg for *Leptospira* spp. (Kim *et al.* 2006) and 17 pg for both pathogens (Bhure *et al.* 2003).

Two or more target sequences present in different amount in the same foetal tissue may cause false negative results when concerning the less prevalent agent. Such problem can potentially affect all multiplexes targeting molecular assays. However, the data obtained in this study by using the set of multiplex PCR assays were in perfect accordance with those observed by the same PCR assay run in simplex, thus demonstrating the high reliability and sensitivity of the proposed panel.

According to previous studies (Probert *et al.* 2004, Stoddard *et al.* 2009, Van der Graaf-van Bloois *et al.* 2013), the primers and probe used in multiplex real-time PCR were specific for *Brucella* spp., *Leptospira* spp. and *C. foetus* spp. and were applicable for all species and subspecies among those 3 bacterial genera. The results of multiplex real-time PCR with different reference strains of 3 pathogens showed 100% specificity for the 3 bacteria and no crossing reaction between them.

The inhibition of PCR can be a puzzling factor, because unknown inhibitors may be released from tissue in DNA extraction. The presence of inhibitors could be affecting the sensitivity of PCR especially for various tissue samples (Lester and LaFebvre 2003, Navarro *et al.* 2002).

The results of multiplex real time PCR from previous examined positive field samples for *Brucella* and *Leptospira* were positive in all samples. Furthermore, a ß-actin signal was detected in all clinical samples tested via the multiplex real-time PCR indicating no evidence of extraction failure or PCR inhibition.

In conclusion, the set of multiplex real-time PCR assays described in this study showed a way of simultaneously detecting the 3 important infectious agents associated with abortion in cattle. Application of this panel of multiplex real-time PCR is simpler, less expensive, and faster than the use of single PCR assays.

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