Development and validation of duplex real-time PCR for simultaneous detection of Brucella *and bovine alphaherpesvirus from clinical specimens*

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Diagnosis,
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Real-time PCR.

Summary
A duplex real-time PCR was developed and validated for the simultaneous detection of *Brucella* and bovine alphaherpesvirus-1 (BoHV-1) from bovine clinical specimens. The *bcsp31* gene of *Brucella* and *gB* gene of BoHV-1 were used as targets in the assay. The limit of detection for BoHV-1 was 0.03 $TCID_{50}$ of virus and 10 plasmid copies containing the target gene while for *Brucella* it was 4.1×10^1 CFUs. Intra-assay and inter-assay values showed high repeatability and reproducibility of the assay. The diagnostic sensitivity (dsn) and diagnostic specificity (dsp) of the duplex assay were determined by screening 443 clinical specimens and comparing the results with the respective individual assays. The dsn and dsp for detection of *Brucella* were found to be 95.24% and 95.65%, respectively whereas for BoHV-1, the dsn (100%) and dsp (99.47%) were slightly higher. The duplex assay had a very good degree of agreement with the respective individual real-time PCR test {kappa value 0.97 for *Brucella* and 0.95 for BoHV-1}. The results of the current study suggest that the duplex assay would be a cost-effective and time-saving alternative for the individual real-time PCR assay for the detection of *Brucella* and BoHV-1.

Introduction

Bovine brucellosis is an economically important zoonotic disease of cattle and buffaloes caused by *Brucella abortus*, less frequently by *B. melitensis* and rarely by *B. suis*. It has been recognized as a major cause of contagious abortion in cattle and may account for 80% incidence in naive herds (Radostits *et al.* 2007, Selim *et al.* 2014). The disease is characterized by abortion in last trimester of gestation, retained placenta and impaired fertility in the cow (OIE 2016a). The disease is usually asymptomatic after the first abortion. Infected animals often become chronic carriers and continue to shed bacteria in milk and uterine discharges (CFSPH 2009).

Infectious bovine rhinotracheitis (IBR)/ infectious pustular vulvovaginitis (IPV) is caused by bovine alphaherpesvirus-1 (BoHV-1) belonging to the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae* and order *Herpesvirales* (Murphy *et al.* 1999, Muylekns *et al.* 2007). The disease is characterized by rhinotracheitis, pustular vulvovaginitis, balanoposthitis, conjunctivitis, enteritis, encephalitis, decreased milk production, weight loss and abortion (Muylekns *et al.* 2007). IBR/IPV is the major cause of viral abortion in bovines and it may account for 5-60% of abortions in non-vaccinated herds (Tibary 2016). Following primary infection, the virus becomes latent in the sensory neurons of trigeminal ganglia. During stress/ immune suppression, the virus reactivation occurs and the virions are secreted through nasal secretion, lachrymal secretion, genital secretion, semen, etc. (Kutish *et al.* 1990, Jones *et al.* 2006).

Both bovine brucellosis and IBR/IPV are endemic

in India and cause huge economic loss to the dairy industry (Trangadia *et al.* 2010, Mukherjee *et al.* 2015, Krishnamoorthy *et al.* 2016). Quick, accurate and comprehensive diagnosis of pathogenic agents in a herd is of utmost importance to initiate control and preventive actions. The conventional diagnostic methods consist of serological tests and isolation of the causative agent. The bacteriological isolation of *Brucella* is still considered the gold standard but is poorly sensitive, labour intensive, with long turnaround time and requires skilled personnel. Isolation of *Brucella* is bio hazardous and requires biosafety level III facility. Serological tests that detect antibodies cannot identifty the early phase of infection and require paired sera for confirmation. Hence, the development and combination of fast, quantitative and accurate molecular tests based on PCR are strongly reccommended.

Both *Brucella* and BoHV-1 cause reproductive disorders in dairy animals including abortion, establish latent/ chronic carrier status in the animals and are excreted in nasal, vaginal and lacrimal secretions and also in semen of infected bulls. A duplex assay targeting both the pathogens will be an effective tool in abortion investigations and also in determining the infection status of the animals in the herd aiding implementation of effective disease control. In this study, a duplex real-time PCR for simultaneous detection of *Brucella* and BoHV-1 from clinical samples was standardized. Further, attempts were made to validate the assay as per the guidelines of the OIE (OIE 2016b).

Materials and methods

Reference sample

Virus

GUK57/2007 strain (BoHV-1.1 genotype) stored at the repository of National Diary Development Board Research and Development (NDDB R&D) laboratory, Hyderabad, was used as reference virus. The titer of the virus was $2 \times 10^{5.0}$ $TCID_{50}$/ml (Reed and Muench 1938).

Bacteria

Brucella abortus strain 19 (vaccine strain) was used as reference strain. The bacterial count was determined by pour plate method and the bacterial load was found to be 4.1×10^7 CFUs/ml.

Plasmid and DNA

A plasmid construct containing the target 97bp of BoHV-1 glycoprotein B (*gB*) gene cloned into pDRIVE cloning vector (Rana *et al.* 2011) was used as positive control in the real-time PCR assay for BoHV-1 detection. The DNA extracted from the *Brucella abortus* strain 19 was used as positive control for *Brucella* real-time PCR reaction.

Preparation of mock specimens

Nasal and vaginal swab samples collected from IBR and *Brucella* sero-negative animals and tested negative in real-time PCR were used as known negative samples. Aliquots of these known negative samples were pooled to obtain a large volume of uniform sample matrix. The pooled sample matrix was further tested by real-time PCR to confirm the absence of BoHV-1 and *Brucella*. An aliquot of this sample matrix was spiked with a fresh culture of *Brucella abortus* strain19 (4.1×10^7 CFU/ml) and serial ten-fold dilutions were prepared. Similarly, serial ten-fold dilutions of BoHV-1 virus ($2 \times 10^{5.0}$ $TCID_{50}$/ml) were prepared in sample matrix.

Extraction of DNA from samples

The QIAmp® blood DNA mini kit (Qiagen, Germany) was used for extraction of DNA from the nasal and vaginal swabs. The manufacturer's protocol was followed albeit addition of 2 µL of carrier RNA to the samples before extraction for increasing the DNA yield. A sample volume of 200 µL was used for DNA extraction and elution was made in 100 µL of buffer AE. The eluted DNA was stored at - 20 °C till further use. The DNA from abortive tissues was extracted by QIAmp® cador® pathogen mini kit (Qiagen, Germany) as per the manufacturer's protocol.

Real-time PCR for detection of BoHV-1 and *Brucella*

DNA extracted from swabs and abortive tissues were processed by real-time PCR for detection of BoHV-1 and *Brucella*. The uniplex assay for detection of BoHV-1 used primers and probes targeting *gB* gene of BoHV-1 (Wang *et al.* 2007) and the test was performed as per the protocol described earlier (Rana *et al.* 2011, Sarangi *et al.* 2018). The uniplex real-time PCR for *Brucella* targeting *bcsp31* gene was performed as per the established protocol (Mukherjee *et al.* 2015). The duplex real-time PCR assay used the same primers and probes combination, targeting *bcsp31* and *gB* gene, as in the respective individual (uniplex) assays with a change in the reporter dye for *bcsp31* probe. The Premix Ex Taq (TaKaRa Bio) was used for reaction set-up and real-time PCR was carried out in 7,300 Real-time PCR system (Applied Biosystems) or Rotor-Gene Q (Qiagen). The duplex real-time

Table I. *Bacteria and viruses used to evaluate the specificity of the duplex real-time PCR .*

Name the of the species	Source /Origin	Amplification in FAM channel (BoHV-1)	Amplification in HEX channel (*Brucella*)
Brucella abortus 544	ATCC (23448)	Negative	Positive
Brucella abortus S19	Vaccine strain (USDA/Mar. 98)	Negative	Positive
Brucella isolate 1 from placenta	Field strain (NDDB)	Negative	Positive
Brucella isolate 1 from milk	Field strain (NDDB)	Negative	Positive
Brucella isolate 2 from milk	Field strain (NDDB)	Negative	Positive
Brucella isolate 3 from milk	Field strain (NDDB)	Negative	Positive
Brucella isolate 4 from milk	Field strain (NDDB)	Negative	Positive
Positive plasmid harbouring *gB* gene of BoHV-1	NDDB	Positive	Negative
Bovine alphaherpes virus isolate 1	Field strain (NDDB)	Positive	Negative
Bovine alphaherpes virus isolate 2	Field strain (NDDB)	Positive	Negative
Bovine alphaherpes virus isolate 3	Field strain (NDDB)	Positive	Negative
Escherichia coli	ATCC (51299)	Negative	Negative
Staphylococcus aureus	ATCC (25923)	Negative	Negative
Klebsiella pneumonia	ATCC (700603)	Negative	Negative
Listeria monocytogenes	ATCC (19111)	Negative	Negative
Pseudomonas aeruginosa	ATCC (27853)	Negative	Negative
Campylobactor foetus	ATCC (27374)	Negative	Negative
Trichomonas foetus	ATCC (30003)	Negative	Negative

PCR reaction contained 4.5 µM of primers, 4 µM of probes of both the targets and ROX as passive reference dye. The thermal profile used in the uniplex as well as in the duplex real-time PCR was 95 °C for 30 seconds (s), followed by 45 cycles of 5 s at 95 °C and 35 s of 60 °C. The threshold level was usually set manually at the starting of exponential phase of the positive controls which were higher than that of background. The cut-off used in the respective uniplex assays were also retained for the duplex assay. Any sample showing a cycle threshold (Ct) value less than 40 in FAM fluorophore detection channel was regarded as positive for BoHV-1. Similarly, any sample showing a Ct value less than 38 in HEX fluorophore detection channel was regarded as positive for *Brucella* DNA.

Determination of limit of dilution (LOD) /analytical sensitivity of the assay

The LOD of the assays was determined by spiking experiment. DNA was extracted from the mock specimens (serially diluted spiked sample) and was tested in quadruplicate by both uniplex and duplex real-time PCR assay. The highest dilution of BoHV-1 and *Brucella,* which was showing positive amplification in all the replicates, was considered as the LOD (probability point 100%). The LOD was confirmed by testing 10 replicates of this highest dilution sample.

Determination of analytical specificity of the assay

The analytical specificity of the assay was determined by using DNA extracted from pathogens that might be present in the nasal swabs/ vaginal swabs or aborted tissues of bovine which may cause similar clinical signs to Brucellosis and IBR/IPV. The microorganisms included in the analytical specificity study are listed in Table I.

Repeatability and interference assays

Reference test samples for the determination of repeatability and interference assay were prepared by mixing DNA extracted from the mock specimens with various concentrations of *Brucella* and BoHV-1.

Inter-assay and intra-assay variations were also evaluated. The combinations of the two pathogens were tested in both duplex as well as with the uniplex real-time PCR assays, in triplicates (intra-assay variation) and for three consecutive days (inter-assay variation). Positive controls and negative controls were included in the tests.

Determination of diagnostic sensitivity and specificity

The diagnostic sensitivity (dsn) and diagnostic specificity (dsp) were determined for diagnosis of

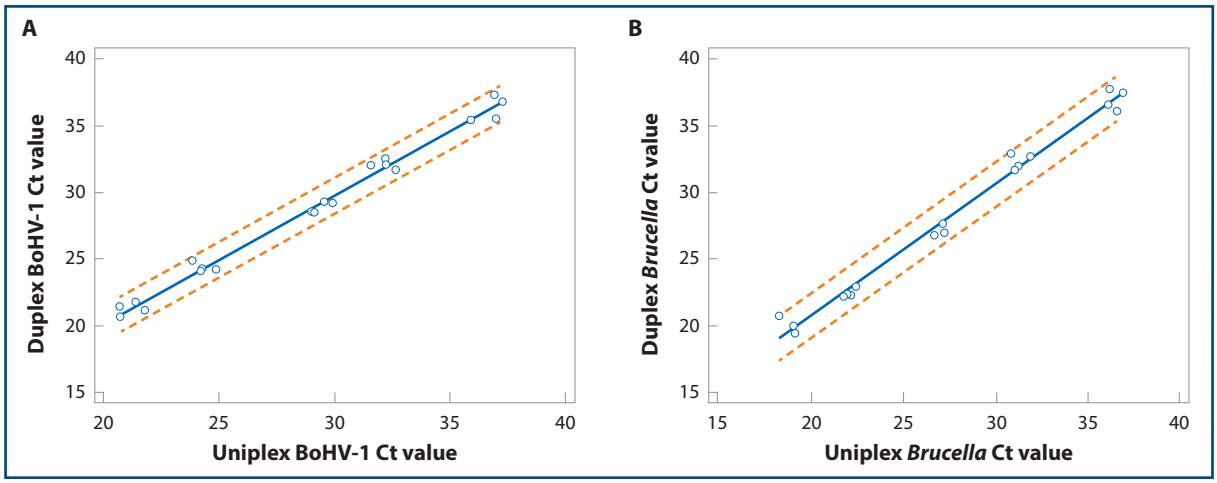

Figure 1. *Correlation of Ct values of uniplex and duplex real-time PCRs for detection of BoHV-1 (***A***) and* Brucella *(***B***).*

Brucella and BoHV-1 by both uniplex and duplex real-time PCRs from 443 clinical specimens. The samples were tested in duplicate and any replicate showing positive amplification (Ct values < 40 for BoHV-1 and < 38 for *Brucella*) was considered positive. The degree of agreement between these two tests was calculated by using Kappa statistics (Altman 1991, Fleiss *et al.* 2003).

Results and discussion

Individual real-time PCR assay have been validated and routinely used in our laboratory for detection of BoHV-1 and *Brucella* from clinical samples (Rana *et al.* 2011, Mukherjee *et al.* 2015).

In this study, a duplex real-time PCR was optimized for the detection of *Brucella* and BoHV-1 by using the same primers and probe combination, targeting *bcsp31* and *gB* gene respectively, as in the respective uniplex assays with a change in the reporter dye for *bcsp31* probe. The duplex real-time PCR was validated by comparing its results with the respective uniplex real-time PCR.

Initially, 6-FAM and HEX fluorophores were evaluated for their suitability in the individual real-time PCR reactions for both BoHV-1 and *Brucella*. The correlation coefficient between the results obtained in 6-FAM and HEX for detection of *Brucella* was 0.997 (95% CI: 0.990 to 0.999), whereas for BoHV-1 it was 0.97 (95% CI: 0.927 to 0.989). However, the Ct value obtained with HEX fluorophore for BoHV-1 was comparatively higher

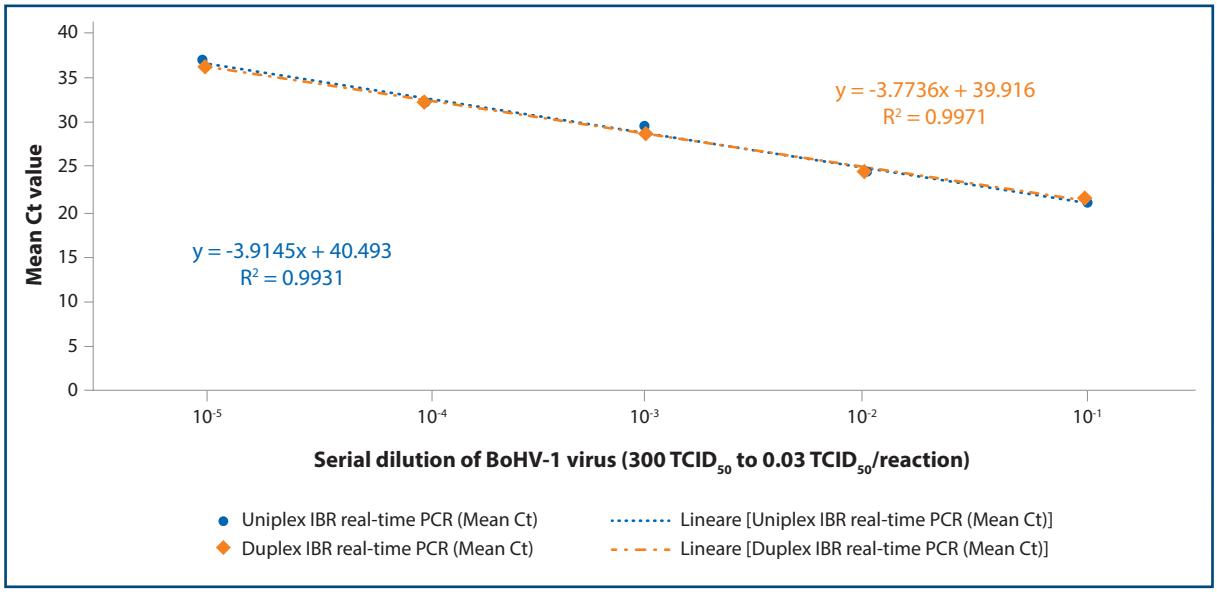

Figure 2. *Comparison of standard curve of uniplex and duplex real-time PCR assay for detection of BoHV-1 using serial dilution of BoHV-1 [GUK57/2007 (BoHV-1.1 genotype)] strain infected cell culture supernatant*

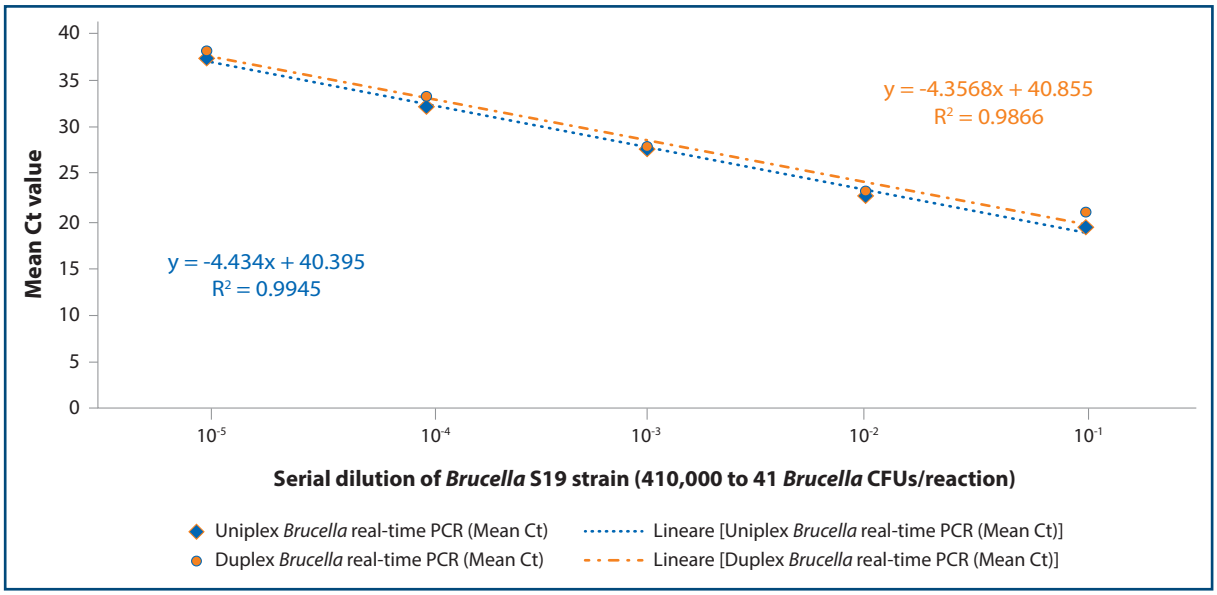

Figure 3. *Comparison of standard curve of uniplex and duplex real-time PCR assay for detection of* Brucella *using serial dilution of S19 reference strain.*

than the 6-FAM fluorophore. Therefore, 6-FAM labelled probe was retained for BoHV-1 and HEX labelled probe was used for *Brucella*.

Limit of detection (LOD) was carried out to measure the analytical sensitivity of the assay. Serial ten-fold dilutions of the mock specimens spiked with the respective organism were used. The highest dilution at which amplification was observed in all four replicates was considered as the LOD (100% probability point). The LOD for detection of BoHV-1 was found to be 0.03 $TCID_{50}$ per reaction in both uniplex and duplex real-time PCR (Figure 1A and 2). Similarly, the LOD for *Brucella* was determined to be 41 CFUs/reaction in both uniplex as well as duplex real-time PCR (Figure 1B and 3). The LODs for the duplex assay were further confirmed by testing 10 replicates of the dilution series containing 0.03 $TCID_{50}$ virus per reaction of BoHV-1 and 41 CFUs of *Brucella* per reaction.

The analytical specificity study suggested that the duplex assay was able to detect only *Brucella* and BoHV-1 isolates but did not amplify DNA from any of other micro flora included in the study viz., *E. coli, Staphylococcus* spp., *Listeria monocytogenes, Pseudomonas aeruginosa, Klebsiella pneumonia, Campylobacter foetus* and *Trichomonas foetus*. The RNA viruses were not included in the specificity test as no reverse transcriptase step was involved. Further, primers and probes used in the current study were taken from published works which already investigated the specificity (Wang *et al.* 2007, Rana *et al.* 2011, Mukherjee *et al.* 2015). The primers and probe used for amplification of *gB* gene of BoHV-1 could successfully amplify BoHV-1.1, BoHV-1.2 and BoHV-5 and no cross-reaction was reported with BVDV, PI3, BSRV and human herpesvirus 1-5 (Wang *et al.* 2007).

One major drawback encountered in the multiplex PCR is the reduced efficiency at detecting the less abundant pathogen in mixed infections. Although there were minor differences in the Ct values obtained in the mixed sample versus the samples from BoHV-1 alone, there was no noticeable difference in amplification efficiency of BoHV-1 even with the

Table II. *Intra-assay and inter-assay variations observed in individual (uniplex) and duplex real-time PCR for detection of BoHV-1 and* Brucella. Data presented in co-efficient of variation in mean Ct values obtained in real-time PCR; samples of each combination were tested in triplicate.

***Brucella* and BoHV-1 combination**		**Inter-assay**				**Intra-assay**			
***Brucella* concentration**	**IBR concentration**	**Uniplex BoHV-1**	**Duplex BoHV-1**	**Uniplex *Brucella***	**Duplex *Brucella***	**Uniplex BoHV-1**	**Duplex BoHV-1**	**Uniplex *Brucella***	**Duplex *Brucella***
10^{-2}	10^{-2}	0.45-1.89	0.57-0.92	0.10-2.33	0.57-0.92	1.98	2.09	2.84	4.76
10^{-2}	10^{-5}	0.55-1.52	1.79-3.57	0.19-1.25	1.79-2.59	2.26	5.61	5.56	10.24
10^{-5}	10^{-2}	0.12-0.89	0.15-0.41	0.53-2.75	0.15-0.41	0.88	1.94	1.35	10.37
10^{-5}	10^{-5}	0.59-2.07	0.57-3.14	0.47-3.08	0.57-3.24	4.44	3.78	2.90	7.07
Brucella PC	IBR PC	1.64-2.33	0.40-2.11	0.40-2.11	0.0-1.45	1.27	2.49	1.34	1.01

Table III. *Effect of different amounts of target pathogen on the sensitivity of the duplex real-time PCR.* All the combinations were tested in triplicates and the average Ct value is presented.

DNA concentration	Ct value in Uniplex BoHV-1	Ct value in Duplex BoHV-1	Ct value in Uniplex *Brucella*	Ct value in Duplex *Brucella*
High - High		25.23		26.69
High - Medium	24.38	25.73	22.44	23.36
High - Low		26.09		22.60
Medium - High		32.14		37.10
Medium - Medium	31.38	31.72	29.94	31.23
Medium - Low		31.89		30.03
Low - High		36.58		41.45
Low - Medium	35.17	35.02	35.55	38.89
Low - Low		35.38		37.08

presence of a high concentration of *Brucella* DNA in the reaction (Table III). Although, the amplification efficiency of *Brucella* was reduced in presence of high concentration of BoHV-1 (> 2.3 $TCID_{50}$ per reaction), *Brucella* DNA with concentration higher than 410 CFUs could be successfully amplified (Table III). In actual field scenario, the possibility of obtaining such high concentration of BoHV-1 in swabs is remote, and hence the interference in the detection of *Brucella* is negligible. Further, during screening of 443 field samples, the presence of DNA of both the pathogens were detected in 7 samples by the respective uniplex assays. The duplex real-time PCR could also detect all the 7 cases suggesting in none of the cases, the dominant amplification of one analyte competitively inhibited amplification of the another analyte.

Inter-assay and intra-assay analyses were performed. All the combination of DNA mixture were tested in triplicates for three consecutive days. The coefficient of variation (CV) in terms of Ct values were found to be similar for the duplex and the respective uniplex assays (Table II) and were within the acceptable range suggesting that the tests are repeatable. The inter-run and intra-run CV of IBR uniplex real-time PCR was earlier reported to be 0.81-1.02 and 0.51-1.37, respectively (Wang *et al.* 2007).

The correlation between uniplex and duplex real-time PCR for detection of *Brucella* and BoHV-1 was evaluated by scatter diagram using the data obtained from the LOD experiment. There was high correlation between the duplex assay and the uniplex assays [correlation coefficient (r) ≥ 0.99 between the assays]. To evaluate the day to day variation, a histogram was plotted for Ct values obtained in the uniplex and the duplex real-time PCR for positive controls tested on 16 different days. Standard deviations of 1.64, 1.76, 2.0, and 1.34 were observed for the uniplex *Brucella*, uniplex BoHV-1, duplex *Brucella* and duplex BoHV-1 real-time PCR, respectively, which is within an acceptable range.

Bland-Altman plot was used to simultaneously display and analyze the results obtained in uniplex and duplex PCR reactions carried out on each sample. The Ct values obtained in the analytical sensitivity and repeatability study were included in the analysis. Results suggest that, on average, the Ct values obtained in duplex real-time PCR for BoHV-1 is 0.15 more than the uniplex BoHV-1 real-time PCR method. Similarly, on an average, the Ct values obtained in duplex real-time PCR for *Brucella* is 0.07 less than the uniplex *Brucella* real-time PCR method.

The presence of inhibitors in the sample can result in false-negative results in PCR based assays. However, the majority of the samples tested in this study were nasal and vaginal swabs collected in virus transport media which are not usually known to contain PCR inhibitors (Buckwalter *et al.* 2014). Hence, the need for inclusion of an inhibition control in this qualitative duplex real-time PCR assay was not considered but yet suggested for testing samples containing PCR inhibitors.

The dsn and dsp were calculated by screening DNA extracted from 443 samples. The results of the uniplex and duplex real-time PCR for *Brucella* and BoHV-1 are presented in Table IV. The dsn and dsp of the duplex real-time PCR for the detection of *Brucella* were recorded as 95.24% (95% CI: 76.18% to 99.88%) and 100.00 % (95% CI: 99.13% to 100.00%), respectively. The dsn and dsp for the detection of BoHV-1 were 95.65% (95% CI: 87.82% to 99.09%) and 99.47% (95% CI: 98.08% to 99.94%), respectively. The degree of agreement between the duplex and the respective uniplex assays was 0.974 (95% CI: 0.92 to 1.00) and 0.957 (95% CI: 0.92 to 0.99) for *Brucella* and BoHV-1, respectively. The disagreement between the test methods occurred at very low analyte concentrations (Ct value > 38 for BoHV-1 and > 37.5 for *Brucella*) resulting in amplification in either duplex and the respective uniplex.

The duplex real-time PCR developed and validated in the present study could be a cost effective and time saving alternative for routine diagnostic use over the individual real-time PCR assays.

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