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COMPARATIVE EVALUATION OF REAL-TIME PCR WITH CONVENTIONAL PCR ASSAY FOR DIAGNOSIS OF BRUCELLOSIS IN RUMINANTS

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ABSTRACT

Brucellosis is zoonotic and highly infectious disease which not only causes the economic losses in ruminants but also infect the humans. In the present study, we compared the molecular techniques of PCR versions and clinical specimens for the diagnosis of brucellosis in ruminants. Blood and serum samples were collected from 692 cattle, 798 buffalo, 471 sheep and 960 goats from two areas; Kasur and Sheikhupura. After serological screening with Rose Bengal antigen, the seropositive serum samples of 73 cattle, 61 buffalo, 91 sheep and 118 goats were subjected to Real-time PCR and Conventional PCR for comparison. Real-time PCR detected significantly ($P \leq 0.01$) more *Brucella abortus* (66 cattle, 53 buffalo) and *Brucella melitensis* (59 sheep, 81 goats) in large and small ruminants, respectively in the serum samples of seropositive ruminants than conventional PCR (45 cattle, 37 buffalo, 34 sheep and 47 goats). The *Chi*-square analysis confirmed significantly ($P \leq 0.01$) more detection of *B. abortus* (66 cattle, 53 buffalo) and *B. melitensis* (59 sheep, 81 goats) in serum samples specimens of cattle, buffalo and sheep, goats, respectively than their respective blood samples (48 cattle, 39 buffalo, 44 sheep and 63 goats) in seropositive ruminants. It can be concluded that real-time PCR assay is more sensitive and reliable method for the diagnosis of brucellosis, that serum is the optimal specimen for the diagnosis of brucellosis by Real-time PCR.

Key words: Brucellosis, *B. abortus, B. melitensis,* Buffalo, Cattle, PCR, RBPT, Molecular diagnosis.

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INTRODUCTION

Brucellosis is an important zoonotic disease affecting both large and small ruminants. Control and eradication of the disease is imperative from public health point of view. But from the last few years its prevalence in Pakistan is increasing day by day (Ali *et al*., 2015). In developed countries it has been eradicated but it is still present in developing and tropical countries (Pappas *et al*., 2006). To decrease the economic losses due to brucellosis, accurate, safe and more sensitive molecular diagnostic techniques play a significant role for the eradication and control of brucellosis in ruminants (Habtamu *et al.,* 2013; Verma *et al.,* 2014). There are several diagnostic tests available for brucellosis, including serological, culture and molecular based assays. Culture methods entail a living host and are both time consuming and hazardous for laboratory workers (Navarro *et al*., 2004). Diagnosis of brucellosis in small and large ruminants by serological techniques is not recommended (Nielsen *et al*., 2004). As it can lead to cross-reaction and are sub-sensitive and unspecific with other pathogens including *Escherichia coli* O: 157, *Salmonella*, *Yersinia enterocolitica* and other *Brucella* spp. (Cventik *et al*., 2004). The traditional method for the diagnosis of *Brucella* spp. is based on phenotypic characters, but it accompanies a high risk of laboratory-acquired contaminations and are time consuming (Navarro *et al*., 2004). Thus, in order to expedite these difficulties, and seeing the high degrees of genetic similarity of different species of *Brucella*, there are numerous molecular based diagnostic assays that are more convenient, faster, safer, easier and accurate such as PCR, real time PCR, LAMP, sequencing and others (Scott *et al*., 2007; Foster *et al*., 2008; Karthik *et al.,* 2014b). Molecular based assays have been applied for the diagnosis of *Brucella* in a wide variety of clinical specimens as accurate and sensitive techniques (Karthik *et al.,* 2014a). For other fastidious bacterial pathogens, molecular based techniques offers an alternative way of diagnosing brucellosis. Genomic based amplification techniques, such as PCR is a highly sensitive and specific assay that can eliminate the limitations of conventional techniques (Karthik *et al.,* 2016). Limited studies have addressed direct detection of *Brucella* species in preferred clinical specimens of ruminants for diagnostic purposes (Khan and Zahoor, 2018). In present study, our aim was to compare a more sensitive diagnostic PCR

assay and define the optimal clinical specimen for the diagnosis of brucellosis. For this purpose, peripheral blood samples from seropositive ruminants, i.e., whole blood and serum, were examined and tested by real-time PCR and conventional PCR to compare their utility as rapid and sensitive diagnostics for brucellosis in ruminants. So that appropriate adoptive measures could be taken for the control of brucellosis.

MATERIALS AND METHODS

Serum and blood samples were collected from small and large ruminants. After serological screening only blood and serum samples of seropositive animals were stored. DNA was extracted from these samples. In first part of experiment sensitivity of real-time PCR was compared with conventional PCR for the diagnosis of brucellosis from serum samples. While in second part of experiment serum samples were compared with blood samples for the diagnosis of brucellosis by most sensitive molecular technique; Real-time PCR.

Sampling: The blood samples were collected in both EDTA and without anticoagulant added BD Vacutainer® from 692 cattle, 798 buffalo, 471 sheep and 960 goats from two areas; Kasur (Latitude: 31.0896 °N, Longitude: 74.1240° E) and Sheikhupura (Latitude: 31.6243°N, Longitude: 74.1240° E) because previously brucellosis has been reported from Sheikhupura and Lahore districts (Ahmed *et al*., 2017). The samples were collected from the animals having age between 2 to 8 years, no vaccination of brucellosis and history of abortion at farm. The serum was separated from non EDTA added vacutainers and stored. GIS (Geographic information system) mapping was developed by using *QGIS® software 2.18.9 version* (Quantum geographic information system) for recording the sampling areas.

Screening of samples: All the serum samples were screened by Rose-Bengal Plate Test (RBPT). Antigen was procured from Veterinary Research Institute (VRI), Lahore, Pakistan. Serum sample of 30µL was mixed and agitated for four minutes with an equal quantity of antigen on glass slide. Serum samples were considered positive after agglutination (Ali *et al*., 2015). After screening all serum and respective blood samples of seropositive animals were subjected to further molecular investigation.

DNA extraction: DNA was extracted from blood and serum samples of seropositive animals by using Exgene™ SV-mini Kit (GeneALL® Biotechnology Co. Ltd, Songpa-gu, Korea) according to manufacturer's instruction. The extracted genome concentration was tested by using Nano Drop. Genomic samples were stored at -20 °C. DNA samples were analyzed by species specific primers of *B. abortus* and *B. melitensis* for conventional PCR as given in Table 1. While for Real-time PCR the primers with probe which are used are given in Table 2.

Table 1. Species specific primers for conventional PCR (Bricker and Halling 1994).

Specie	Primers	Sequence (5' to 3')	Target	Product
B. abortus	Forward	GACGAACGGAATTTTTCCAATCCC	IS711/*alk*B	498 bp
	Reverse	TGCCGATCACTTAAGGGCCTTCAT		
B. melitensis	Forward	AAATCGCGTCCTTGCTGGTCTGA	IS711/*alk*B	731bp
	Reverse	TGCCGATCACTTAAGGGCCTTCAT		

Table. 2. Species specific primers and probe for Real-time PCR (Probert *et al*., 2004).

Specie	Primers/Probe	Sequence (5' to 3')	Target	5 Fluorophore/3 quencher
B. abortus	Forward	GCGGCTTTTCTATCACGGTATTC	*alkB*	HEX/BHQ1
	Reverse	CATGCGCTATGATCTGGTTACG		
	Probe	CGCTCATGCTCGCCAGACTTCAATG		
B. melitensis	Forward	AACAAGCGGCACCCCTAAAA	*alkB*	Texas Red/BHQ2
	Reverse	CATGCGCTATGATCTGGTTACG		
	Probe	CAGGAGTGTTTCGGCTCAGAATAATCCACA		

Amplification by conventional PCR: GeneAmp™ PCR master mix (GeneALL® Biotechnology Co. Ltd, Songpa-gu, Korea) was used in master mix of reaction mixture. Reaction mixture of 20 µL containing 10µL of master mix, 1µL each forward and reverse primers having 10 pmol/µL, 2 µL genome of interest and 6 µL Nuclease-free water was subjected to PCR. DNA of *Brucella* reference strains (BA 544 and BM-16M) obtained from Veterinary Research Institute (VRI), Lahore, Pakistan was used as positive control. Nuclease-free water was used as negative control (NC). Amplification of reaction was done in 96 well microplate thermocycler (Thermo

Fisher Scientific Inc., Agilent Technologies, Santa Clara USA) having cycling conditions as follows; initial decontamination at 50°C for 5 minutes then initial denaturation at 95°C for 10 minutes followed by 40 cycles each consisting denaturation at 95°C for 30 seconds and annealing at 60°C for 30 seconds followed by extension at 72°C for 1 minute. Final extension of amplification was done at 72°C for 5 minute.

Amplification by real-time PCR: Amplification of reaction mixture was performed by using prepared Real-Amp™ TaqMan qPCR master mix (Cat# 801-020, GeneALL® Biotechnology Co. Ltd, Songpa-gu, Korea). A reaction mixture of 20µL containing 10µL of master mix, 0.5µL (200nmol) of each forward and reverse primers, 1µL (100nmol) of probe, 1µL of DNA and 7µL of nuclease free water were used for amplification. The cycle threshold (*Ct*-value) below 40 was considered as positive. The PCR reaction was optimized for standard concentrations DNA, primers and probe. The known concentration of DNA standards were provided by VRI, Lahore. PCR conditions was followed and reaction mixture composition was prepared (Probert *et al*., 2004). Amplification of desired DNA was done in 72-well Rotor-Gene®Q real-time PCR cycler (Qiagen Q-Rex Software 2.3.11.4.9-Windows platforms).Initial denaturation at 94°C for 10 minutes followed by 40 cycles of each consisting denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Final extension was done at 72°C for 7 minutes. Double stranded PCR product was detected by fluorescent dye associated with Probe at each extension step. An amplification curve of PCR product was analyzed and recorded through computerized software. Holding temperature was 4°C till further testing.

Statistical analysis: Standard error sample proportion was calculated by using SE_P formula as given $SPe = \sqrt{(P(1-P)/n)}$ and statistical analysis using *chi* square by using *SPSS* for Windows version 20, *SPSS Inc*., Chicago, IL, USA (Statistical Package for Social Science). $P \leq 0.01$ was considered as significant.

RESULTS

Blood samples and serum samples were collected from cattle, buffalo, sheep and goats. All serum samples were subjected to serological screening for brucellosis by Rose Bengal antigen. The precipitation reaction confirmed the seropositive animals. Through serological screening we found out the seroprevalences of 10.54% ± 0.0116, 7.644% ± 0.0093, 19.32% ± 0.289 and 12.29% ± 0.0105 in cattle, buffalo, sheep and goats, respectively. In first part of experiment only these seropositive samples were subjected to real-time PCR and conventional PCR for assessing the comparative efficacy of these molecular techniques.

Real-time PCR detected significantly ($P \leq 0.01$) more *B. abortus* in cattle and buffalo. Real-time PCR detected 90%±0.039 and 86%±0.044 *B. abortus* than conventional PCR that detected 61%±0.057 and 60%±0.062 in seropositive cattle and buffalo, respectively. Significantly ($P \leq 0.01$) more *B. melitensis* were detected in sheep and goats by real-time PCR. *B. melitensis* was detected 64%±0.050 and 68%±0.042 by real-time PCR as compare to conventional PCR that detected 37%±0.050 and 39%±0.044 in seropositive sheep and goats respectively. The above mentioned results confirmed that real-time PCR is more sensitive than conventional PCR. These results are shown in table 3. Conventional PCR results of *B. abortus* are shown in figure 1 while overall amplification of real-time PCR is shown in figure 2.

Table 3. Comparison of real-time PCR with conventional PCR for the detection of brucellosis in ruminants.

***Brucella* species**	**Animals**	**Results**		***Chi*-square analysis**	
		Real-time PCR	**Conventional PCR**	***Chi*-square (χ^2)**	***P≤ 0.01***
B. abortus	Cattle	66 (90%±0.039)	45 (61%±0.057)	16.573	0.000
	Buffalo	53 (86%±0.044)	37(60%±0.062)	10.844	0.001
B. melitensis	Sheep	59 (64%±0.050)	34 (37%±0.050)	17.339	0.000
	Goat	81 (68%±0.042)	47 (39%±0.044)	19.735	0.000

In second part of experiment serum and blood samples were compared by real-time PCR for the diagnosis of brucellosis in small and large ruminants. After serological screening the serum and respective blood samples of seropositive cattle, buffalo, sheep and goats were subjected to real-time PCR. Real-time PCR detected 90%±0.035 and 86%±0.044 *B. abortus* in serum samples than 65%±0.055 and63%±0061 *B. abortus* in blood samples of seropositive cattle and buffalo respectively. Similarly real-time PCR detected 64%±0.050 and 68%±0.042 *B. melitensis* in serum samples than 48%±0.052 and 53%±0.045 *B. melitensis* in blood samples of seropositive cattle and buffalo respectively. Comparison through *chi*-square have confirmed the significantly (P≤ 0.01) more *B. abortus* and *B. melitensis* detection in serum samples of large and small ruminants respectively. The results of second part of experiment are presented in table.

Table 4. Comparison of serum samples with blood for the detection brucellosis in ruminants through real-time PCR.

Brucella species	Animals	Real-time PCR		*Chi*-square analysis	
		Serum	Blood	*Chi*-square (χ^2)	*P≤ 0.01*
B. abortus	Cattle	66 (90%±0.035)	48 (65%±0.055)	12.967	0.000
	Buffalo	53 (86%±0.044)	39(63%±0061)	8.664	0.003
B. melitensis	Sheep	59 (64%±0.050)	44 (48%±0.052)	5.033	0.025
	Goat	81 (68%±0.042)	63 (53%±0.045)	5.772	0.016

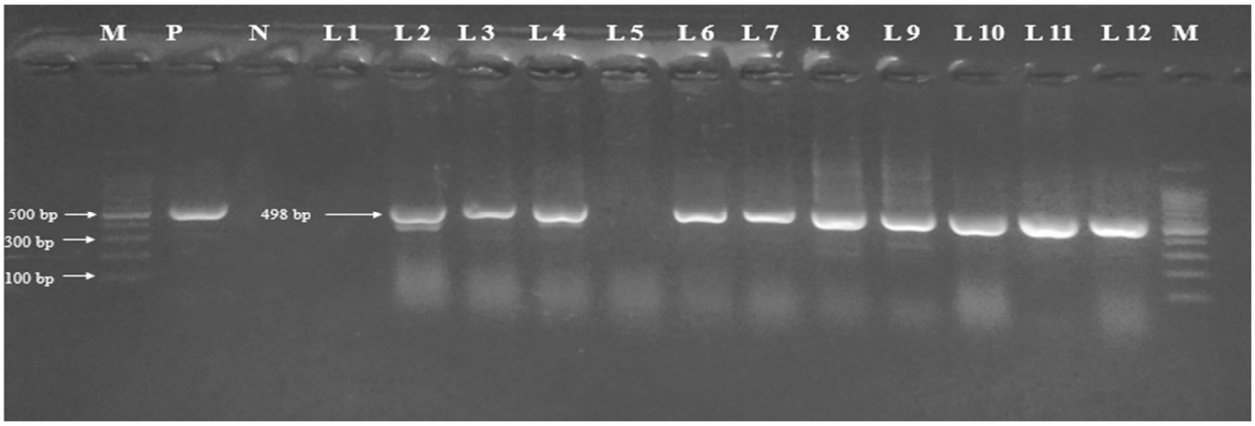

Figure 3. Electrophoretic pattern of PCR products 498 bp on 1.5% agarose gel stained with Ethidium bromide. Lane M is standard DNA marker, Lane P is positive control, Lane N is negative control, Lane-2,3,4,6,7,8,9,10,11,12 is positive PCR products of *B. abortus* 498 bp and Lane 1 and 5 is negative PCR products for *B. abortus*.

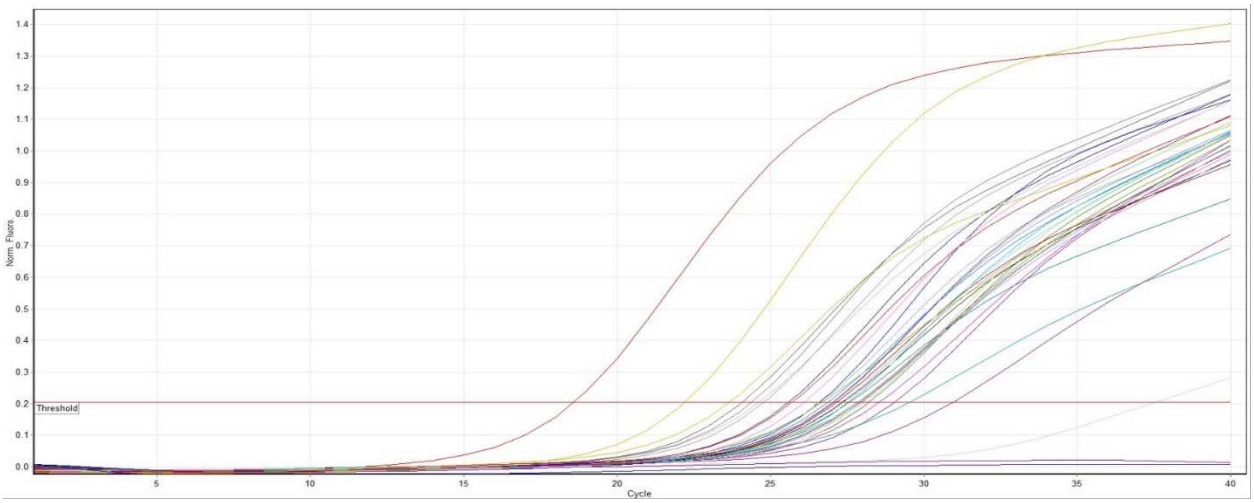

Figure 4. Overall real-time amplification results

DISCUSSION

The results of the first part of experiment presented in table 3 reflected that real-time PCR assay is more accurate, reliable and rapid test for detection and differentiation of *B. abortus* and *B. melitensis* in serum samples of small and large ruminants as compared to conventional PCR. While the second part of experiment mentioned in table 4 the serum sample was found to be the preferred specimen for the diagnosis of brucellosis as compared to blood samples. Isolation and bacterial culture is still considered as gold standard tests for the diagnosis of brucellosis but are cumbersome and hazardous procedures (Wareth *et al*., 2014). The rapid, more sensitive and safe method of diagnosis is based on molecular tests and their application are increasing during recent years (Akhtar *et al*., 2010). Molecular assays can detect both live and dead bacteria even in highly

contaminated samples as compared to culture method which cannot detect dead and contaminated samples (Saminathan *et al.,* 2016). In the present study, we compared the two molecular techniques. We compared the real-time PCR assay with conventional PCRs and found out that real-time assay is more sensitive than conventional type of PCR assay. Previously, real-time has also been used as more sensitive technique for the diagnosis of brucellosis in Pakistan (Ali *et al*., 2015). In Egypt, real-time PCR has also been proved to more and better technique for diagnosis from milk samples for brucellosis (Wareth *et al*., 2014). Sola *et al*. (2014) also proved the real-time PCR assay to be more efficient for the detection of *Brucella* from the visceral organs of slaughtered animals. The present research work is also in agreement with studies of Sidor *et al*. (2013) who proved multiplex real time PCR to be more sensitivity as compared to conventional assays, and also used this technique for the detection of *Brucella* species from the blood, tissue and faecal specimens of marine mammals. Dehkordi *et al*. (2012) compared the *Taq*Man probe real-time PCR assay with single-step conventional PCR assay for the diagnosis of brucellosis. In this study, both the *B. melitensis* and *B. abortus* were detected from the abomasal contents of aborted fetuses of goats, sheep, cattle, buffalo and camel. The sensitivity and specificity of real-time PCR assay were proved to be 100% in comparison to single step conventional PCR. This higher sensitivity is because of elimination of post PCR amplification steps in real-time PCR assay, while conventional assay requires to run the amplified products on Gel (Redkar *et al*., 2001).In real-time PCR reaction amplification can be visualized through computerized generated software consisting of log and lag phase. Even cycle threshold can been seen through computer software. But in conventional PCR we can confirm the reaction after completion of cycles of amplification. There could be error during amplification reaction. Even after successful amplification there could be error during post PCR steps. Post PCR steps include Gel preparation, Ethidium bromide inclusion, Gel Electrophoresis and visualization through UV illuminator which are time consuming and labour intensive (Yang *et al*., 2007). So real-time PCR assay can also be used for large number of reactions but conventional PCR could be for less number of samples (Dehkordi *et al*., 2012).

In second part of experiment, we compared the clinical specimens of blood and serum for diagnosis of brucellosis. We found that the serum was more suitable specimen as compared to blood for diagnosis of *Brucella* infection. We observed more cases in serum to be positive as compared to blood. Previously, assay sensitivity for serum samples was found 94% as compared to 61% of blood samples (Zerva *et al.,* 2001). While we also detected *B. abortus* in 90% serum samples as compare to 65% of blood samples in cattle. It can be concluded that, real-time PCR assay is more reliable and sensitive method for diagnosis of brucellosis. It can also be concluded that serum is the preferred and optimal clinical specimen for the diagnosis of brucellosis by real-time PCR assay.

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