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RESEARCH ARTICLE

Molecular Detection of Brucellosis, Leptospirosis and Campylobacteriosis by Multiplex PCR and Screening by ELISA Assays in Buffalo Breeding Bulls

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ABSTRACT

Abortion in buffalo-breeding industry is one of the key roots of economic losses in the livestock sector. *Brucella* spp., *Leptospira* spp. and *Campylobacter fetus* are main pathogens because of the potential impact on veterinary and human health. This study assessed the relative performance of ELISA and multiplex PCR (mPCR) assay using different primer sets for the detection of venereal pathogens. Variety of samples (blood, urine, preputial washings and semen) were collected from a total of 160 buffalo breeding bulls of Semen Production Unit, Qadirabad and private dairy farms in Punjab Province of Pakistan. Serum and total genomic DNA was extracted for molecular detection by ELISA and PCR respectively. This study comprised 9 sets of primers to optimize multiplex with respect to melting temperature and crosslinking among primers. ELISA assay for *Brucella* was 2.5%, whereas for *Leptospira* and *Campylobacter fetus* it was 1.88% for each. With respect to development of triplex and duplex PCR, results of our assay were completely consistent with monoplex PCR using a combination of newly designed and reported primers. All ELISA suspected samples found negative for *Brucella*, *Leptospira* and *C. fetus* when tested with both monoplex and triplex PCR. This multiplex PCR assay provides a valuable complementary tool in routine, simultaneous and robust detection of these three genital diseases and revealing epidemiological facts about abortion measures in buffalo.

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INTRODUCTION

Utmost part of the history of veterinary science was committed to main public health constrains for the control of various emerging and re-emerging zoonotic pathogens. Infectious abortion is a momentous cause of reproductive failure and considerable economic losses for the dairy industry (Mittal *et al*., 2018). There is dire need of bull screening against genital infections as compared to dam due to its status of half herd and may contribute to inseminate even many cows by producing thousand doses of semen and by dilution, these doses can be used maintaining continuity in generations. Brucellosis, leptospirosis and campylobacteriosis are common venereal diseases responsible for contagious genital infections and abortion in voluminous mammal host species around the world (Osunla and Okoh, 2017).

Bacteriological analyses of these veterinary pathogens is primarily rely on biochemical tests, cultural isolation and phenotypic characterization, a labor

demanding practices which has been accompanied with a high risk of laboratory acquired infections (Anderson, 2007; Mohamad *et al*., 2019). On the other hand, several conventional serological methods including ELISA have been used for diagnosis of brucellosis, leptospirosis and campylobacteriosis in humans and domestic animals (Jindan *et al*., 2019). However, presence of cross-reactions especially within species creates problems for serological diagnosis. Moreover, biochemical and serological identification (Oktay *et al.*, 2005) is limited only to the genus level (Kulkarni *et al.*, 2002). Hence, to overcome these problems, nucleic acid amplification has been tremendously exploited for the routine and rapid confirmation for the incidence of these pathogens.

Various real-time and conventional monoplex PCR methods have been well-established so far. Real-time PCR assay requires high-priced chemicals and therefore is discouraged for routine diagnostic practices particularly in rural dairy farms. PCR detection of *Brucella* isolates at species level has been more critical and thus, aims to target the specific integration of IS711 components within the genome for the particular *Brucella* strains (Scott *et al.*, 2007). For *C. fetus* PCR assay, Carbon Starvation Protein A (*cstA*) gene has been widely used as a genetic marker for screening of infections in bovine (Hum *et al.*, 1997). Most of these methods rely on monoplex PCR, which mainly focused on single marker amplification. By making use of similar PCR molecular targets, in this study with triplex approach, we have designed a triplex assay that allows rapid and simultaneous detection of *Brucella* spp., *Leptospira* spp. and *C. fetus* isolates in a single PCR assay.

This multiplex PCR (mPCR) assay is a rapid, simple and practical tool for identification of three infectious agents commonly associated with genital transmission and offers an effective alternative approach than conventional serological and biochemical-based assays. In our area of study, understanding the incidence of venereal infections because of semen transplantation is important veterinary issue linked with abortions and production losses. To our best knowledge, there is no mPCR method that uses combination of designed carbon starvation protein A, insertion sequences and ribosomal sequences for the simultaneous detection of three major venereal infectious agents, viz., *Brucella*, *Leptospira* and *C. fetus* species implicated in breeding bulls.

MATERIALS AND METHODS

Sampling: The blood samples from jugular vein, urine, semen and preputial washings were collected from 160 apparently healthy Nili Ravi bulls reared and maintained in SPU and Private Farms of Punjab in Pakistan. Serum was also recovered for blood samples in separate tubes for ELISA assay.

Approval of animal ethics committee: Blood samples of bulls were collected by a competent veterinary surgeon with the agreement of the animal head of SPU division and in accordance with the sanction guidelines from animal ethics committee, UVAS.

Bacterial strains: A total of four reference strains were used in this study. *Leptospira interrogans* (n=1) as well as *Campylobacter* (n=2) named as *Campylobacter fetus* and *Campylobacter fetus venerealis* (*Cfv*) were obtained from Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University (Ithaca, New York, USA). The strain of *Brucella abortus* was kindly provided by Department of Pathology, UVAS, Lahore, Pakistan from clinical sources.

Genomic DNA extraction: Qiagen columns were used for the extraction of DNA from samples collected from breeding bulls. DNA concentration of stock solutions was measured by dsDNA HS assay kit (Invitrogen by Thermo Fischer Scientific, USA).

***Brucella* antibody ELISA:** *Brucella* Ab 2.0 ELISA kit (PrioCHECK™, Spain) test results were obtained by reading optical density (OD) at 450nm and expressed as a ratio of corrected OD of test sample and a corrected OD of reference serum. As per manufacturer's instructions, an animal serum was considered positive for brucellosis if percentage positivity (PP) >40% specifies *Brucella* antibodies are present in the sera sample. Although, using the sera controls the PP value was estimated using the formula:

$$PP = \left(\frac{OD_{450} \cdot test\ sample}{Mean\ OD_{450} \cdot Positive\ Control} \right) \times 100$$

***Leptospira hardjo* ELISA:** *Leptospira hardjo* antibody test kit (PrioCHECK™, Netherlands) was used to screen the buffalo sera response for *Leptospira interrogans* serovar *hardjo* (*L. hardjo*) antibodies with indirect ELISA. The OD_{450} of all samples was expressed as PP relative to the mean OD_{450} of the positive control. Animals sera were considered positive for *L. hardjo* antibodies as proposed by the manufacturer if they had PP equal or greater than 45%, negative if PP less than 20% and ambiguous if between 20 and 45%.

$$Corrected\ OD_{450}\ of\ test\ sample\ =\ test\ sample\ OD - mean\ OD\ of\ negative\ control$$

$$PP = \left(\frac{corrected\ OD_{450}\ test\ sample}{corrected\ OD_{450}\ reference\ serum} \right) \times 100$$

***C. fetus* ELISA:** Bovine vibriosis antibody ELISA Kit (International Immuno-Diagnostics, USA) was according to manufacturer's protocol. Greater OD values than the cut-off value indicates that significant amounts of *Campylobacter* antigens were detected. Animals' sera were considered positive if they had PP equal or greater than 20%.

Primer designing: Six primers (2 for each disease) were designed from sequences submitted to GenBank with NCBI primer designing software. The nucleic acid targets for *C. fetus*, *Leptospira* and *Brucella* identification are relevant to those stated by Fukunga *et al*. (1990), Hum *et al*. (1997) and Redkar *et al*. (2001) respectively. However, the primers to these targets were redesigned for the multiplex format.

mPCR design: The mPCR assay was developed using a combination of newly designed and previously reported primers in consideration of the size of the PCR product and annealing temperature. These oligonucleotide sets were previously described in the literature but had never been used simultaneously. The final 20 µl mPCR mixture comprised of 1 µl of DNA extract from each bacterial strain, 0.15 mM primer Det_F/Det_R, 0.25 mM primer 16S_F1/16S_R1 and 0.2 mM primer *Vibrio*_F1/ *Vibrio*_R1 and 1X AmpliTaqGold Master Mix (Thermo Fischer Scientific, USA). Amplification was carried out in Mastercycler Pro (Eppendorf) using the following cycling conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 56.7°C for 30 s, for 72°C for 1 min and final extension at 72°C for 5 min. Samples were held at 4°C prior to analysis. Each reaction product was visualized using UV transilluminator after staining with the ethidium bromide.

RESULTS

In ELISA Based assay, a total of 160 buffaloes sera were sampled from SPU (n=148) and private dairy farms (n-=12). Ten (6.25%) of the 160 sera were positive to these three genital pathogens, including 4 (2.5%) against *Brucella*, whereas *Leptospira* and *Campylobacter* had lower test positive animals with 3 scoring for each (1.88%). *Brucella*, *Leptospira* and *Campylobacter* ELISA OD values are presented in Fig. 1. The distribution of *Brucella* and *Campylobacter* antibody ELISA OD values suggests a large negative population with small test positive sera. The *L. hardjo* ELISA does not propose a clear distinction between the test positive and negative animals at the manufacturers' cut-off. Four sera (2.7%) from SPU region examined scored positive for *Brucella* antibodies, however three (2.02%) samples were seropositive for *Leptospira* and *Campylobacter*. Seropositive results for SPU division and accumulated score were estimated for each of the three infections and are given in Table 2. No seropositive reactions to the private dairy farm animals were detected in this study.

As we sampled sera from apparently healthy animals, in an expected negative population using ELISA test, we would therefore estimate to see 10 tests positive out of 160. It might be because of presence of post infection antibodies in animal sera. To assess ELISA results, extractions were performed from all possible active pathogens sites including semen, urine and preputial washings. To detect real-time infection, all suspected samples identified by ELISA were tested with all nine oligonucleotide primer sets to assess the efficacy of the ELISA assay over PCR. All suspected samples found negative for *Brucella*, *Leptospira* and *C. fetus* when tested with both monoplex and triplex PCR, whereas all positive controls scored positive to PCR and gave OD above the cutoff value.

The mPCR assay was standardized using genomic DNA extracted from previously cultured control strains. Based on the result outcomes, duplex and triplex PCR was established by assembling the selected oligonucleotide sets in different ways which correctly amplified all corresponding markers (Fig. 2-6). To evaluate the specificity of each individual primer set used in this study, a number of closely related pathogenic organisms (*B. burgdorferi*, *B. hermsii*, *Treponema pallidum*, *T. denticola*, *Escherichia coli*, *Staphylococcus aureus*, *Agrobacterium* spp., *S. aurantia*, and *Mycobacterium tuberculosis*) were tested for PCR and no amplification products were recovered by agarose gel electrophoresis (data not shown).

Table 1: Nine sets of oligonucleotide primers for rapid and simultaneous detection of *Brucella* spp., *Leptospira* spp. and *Campylobacter fetus*

Target	Targeted Region	Reference accession number	Oligo Name	Sequence (5′→3′)	Tm (°C)	Amplicon size (bp)	Gene Location (bp)	Reference
Brucella	IS711 Insertion Element	CP026005.1	*Brucella*_F1	GAATCGCCTTAACAAGCGGC	60.5	223	621930-621949	This study
			*Brucella*_R1	AGAAAGTGCTTCGTCACGCT	58.4		622152-622132	
			*Brucella*_F2	GCCTGATCAAAATCGCGTCC	60.5	264	621693-621712	This study
			*Brucella*_R2	TAGGGGTGCCGCTTGTTAAG	60.5		621956-621937	
			Det_F	AGAATAATCCACAGAAGGTAGAG	59.3	403	621980-622002	(Navarro *et al.*, 2006)
			Det_R	ATCCAAGGTCAATCCAACAC	56.4		622383-622363	
Leptospira	16S Ribosomal RNA	NR_134067.1	16S_F1	CGGGAGGCAGCAGTTAAGAA	60.5	848	297-316	This study
			16S_R1	AAGGCCATGAGGACTTGACG	60.5		1144-1125	
			16S_F2	GGCGCGTCTTAAACATGCAA	58.4	309	8-27	This study
			16S_R2	TTCTTAACTGCTGCCTCCCG	60.5		316-297	
			16S_F	GGCGGCGCGTCTTAAACATG	62.5	331	5-24	(Merien *et al.*, 1992)
			16S_R	TTCCCCCCATTGAGCAAGATT	59.4		335-315	
Campylobacter fetus	Carbon Starvation Protein A (*cstA*)	AY158813.1	*Vibrio*_F1	CAGGTGGTGCCCCTACATTT	60.5	576	105-124	This study
			*Vibrio*_R1	CTATGGCTGCTGGATCGGTT	60.5		680-661	
			*Vibrio*_F2	ATATGCGTTACTGGCTGGGG	60.5	333	344-363	This study
			*Vibrio*_R2	GGCTGCTGGATCGGTTAGAG	62.5		676-657	
			*Vibrio*_F	GGTAGCCGCAGCTGCTAAGAT	63.3	359	1-21	(Hum *et al.*, 1997)
			*Vibrio*_R	AGCCAGTAACGCATATTATAGTAG	60.1		359-336	(Yamazaki-Matsune *et al.*, 2007)

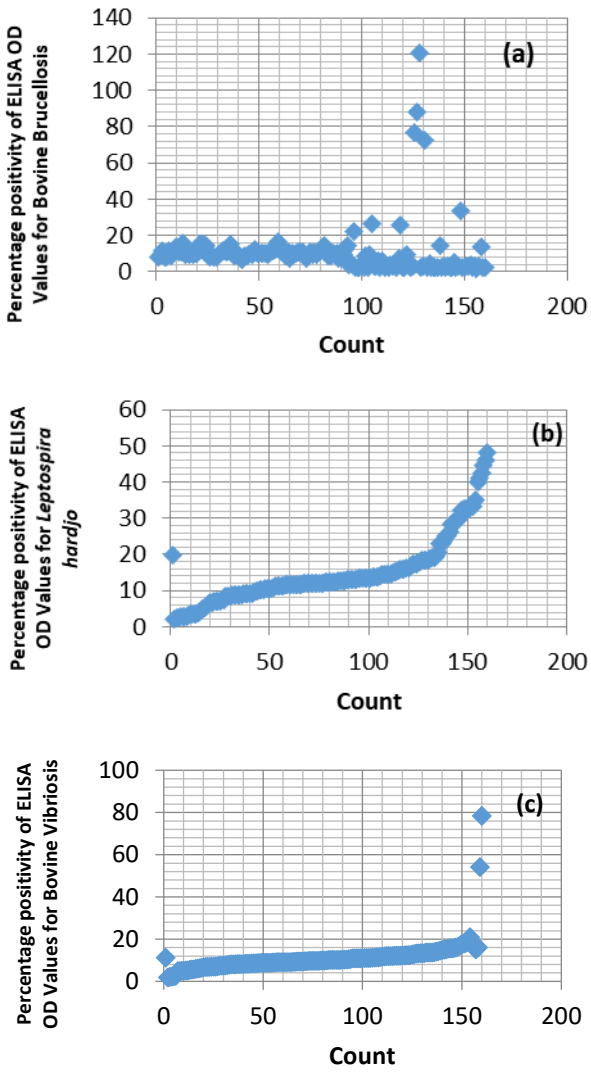

Fig. 1: Graphical representation of percentage positivity of optical density values (OD) for *Brucella* (a), *Leptospira* (b) and *Campylobacter* ELISA.

Initially duplex PCR assays in all possible combinations including *Leptospira* spp. and *C. fetus* (Fig. 2 and Fig. 3a); *Leptospira* spp. and *Brucella* spp. (Fig. 4a); *Brucella* spp. and *C. fetus* (Fig. 5) were examined and optimized. In Fig. 2, reaction mixture R1 and reaction mixture R2 shows varying combinations of primer concentrations in their duplex PCRs. R1 has higher concentration of 16S_F1/16S_R1 primers whereas R2 has equal concentration of both primers. Amplification of all two species are succeed only in R1 due to adding a higher concentration of the primers used for the generation of the long PCR products relative to the concentration of the small PCR fragment primers. One third of the R2 amplicons have not shown any product for the *Leptospira* PCR. It could be due to higher annealing temperature and excessive amplifications of shorter fragments in beginning of the reaction. For all other duplex and triplex PCR assays R1 was considered as standard reaction mixture. The triplex PCR assay included one set of previously reported primer for *Brucella* and two sets of novel primers for *Leptospira* and *C. fetus*. The primer set Det_F/Det_R amplifies a 403-bp region of IS711 insertion element; 16S_F1/16S_R1 amplifies an 848-bp region of 16S ribosomal RNA and *Vibrio*_F1/*Vibrio*_R1 amplifies a 576-bp region of *cstA* gene (Fig. 6a). Each primer pair participated in triplex PCR assays was specific for the targeted gene of the predicted and distinctly discriminated in size by gel electrophoresis for each of the three infectious pathogens.

Sensitivity of all multiplex primers assays (Det_F/ Det_R, 16S_F1/16S_R1, and *Vibrio*_F1/ *Vibrio*_R1) was evaluated by using serial dilutions of DNA template (10^{1}-10^{-5}) with the strains used for positive control. As a result of mPCR, it was obtained that the sensitivity of this method was 10^{-5}, thus it was considered as a specific and sensitive method for DNA detection of *Brucella* sp., *Leptospira* sp., and *C. fetus* in all multiplex combinations (Fig. 3b, 4b, 5 and 6b).

Table 2: Sample location from Province Punjab, Pakistan and ELISA results of sera of buffaloes against Brucellosis, Leptospirosis and Campylobacteriosis

Sampling area	Number of samples	Number of positive samples (%)		
		Brucella	*Leptospira*	*Campylobacter fetus*
Semen Production Unit (SPU) Qadirabad	148	4 (2.7)	3 (2.02)	3 (2.02)
Private Farm	12	-	-	-
Total	160	4 (2.5)	3 (1.88)	3 (1.88)

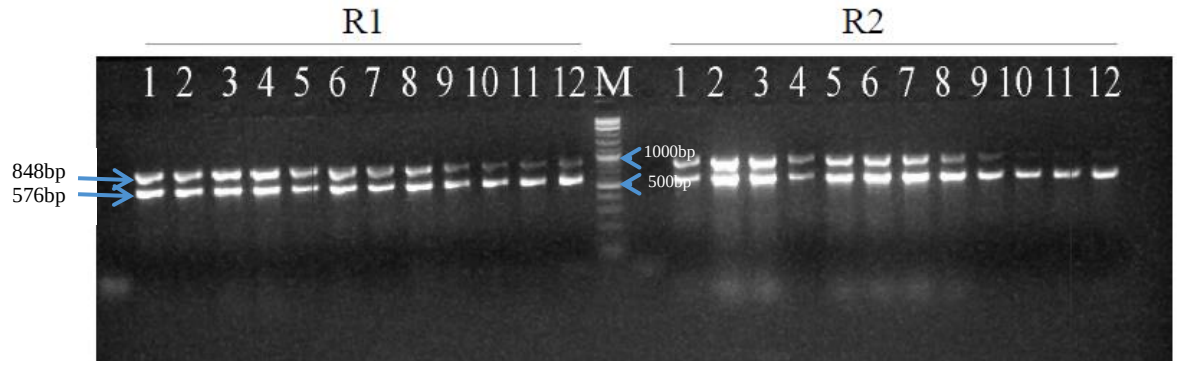

Fig. 2: mPCR optimization for *Campylobacter fetus* and *Leptospira* spp. Specific amplicon is approximately 848-bp for *Leptospira* species (16S_F1/16S_R1) and 576-bp for *C. fetus* (*Vibrio*_F1/*Vibrio*_R1). Lanes 1-12 shows gradient programming ranging from 55°C to 65°C. Lane M: 100-bp molecular weight marker, R1 Lanes 1-12: *C. fetus* and *Leptospira* spp. Positive control with reaction mixture 1 having high concentration of long PCR fragment primer pair; R2 Lanes 1-12: *C. fetus* and *Leptospira* spp. positive control with reaction mixture 2 having equal concentration of both primer pairs.

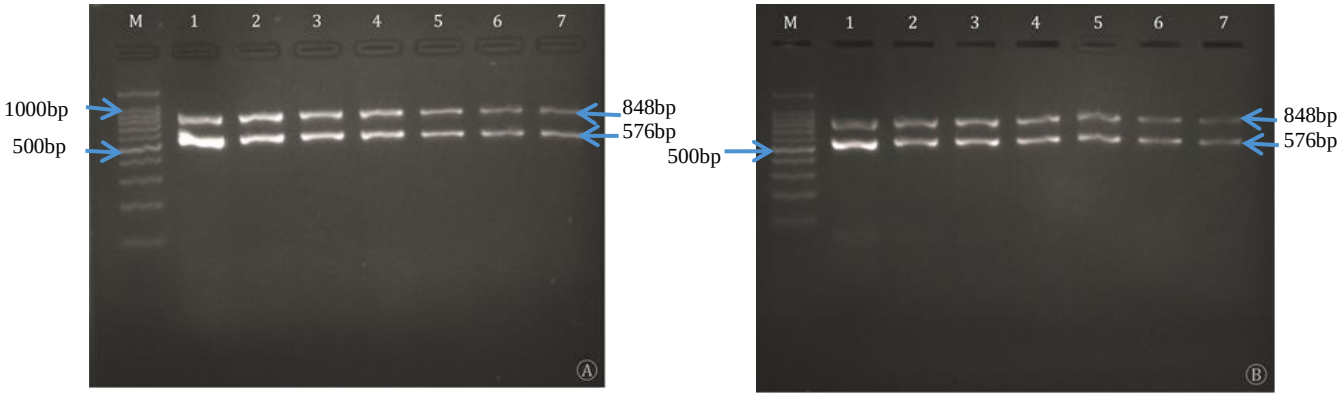

Fig. 3: (a): Amplification products obtained by the set of multiplex polymerase chain reaction (mPCR) assays for simultaneous identification of *Campylobacter fetus* and *Leptospira* spp. (PCR products of *C. fetus*: 576-bp (*Vibrio*_F1/*Vibrio*_R1); and PCR products of *Leptospira* spp.: 848-bp (16S_F1/16S_R1)). Lane M: 100–base pair DNA ladder; Lanes 1-7 shows gradient programming ranging from 55°C to 65°C. (b): Sensitivity test of multiplex polymerase chain reaction (mPCR) assays for simultaneous identification of *Campylobacter fetus* and *Leptospira* spp. (PCR products of *C. fetus*: 576-bp (*Vibrio*_F1/*Vibrio*_R1); and PCR products of *Leptospira* spp.: 848-bp (16S_F1/16S_R1)). Lane M: 100–base pair DNA ladder; Lanes 1-7 shows dilutions ranging from 10^{1}-10^{-5}.

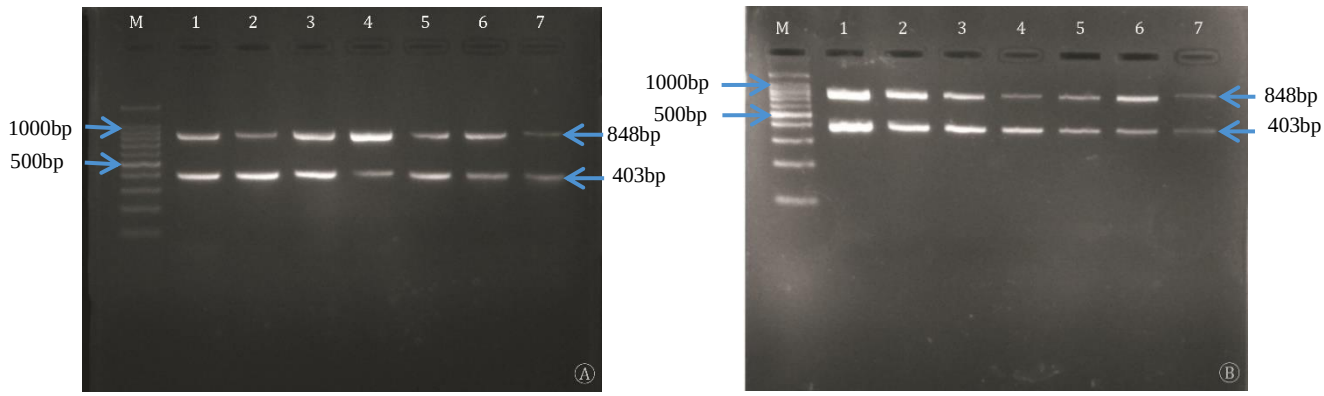

Fig. 4: (a): mPCR optimization for *Leptospira* spp. and *Brucella* spp. Specific amplicon is approximately 848-bp for *Leptospira* species (16S_F1/16S_R1) and 403-bp for *Brucella* spp. (Det_F/Det_R). Lane M: 100-bp molecular weight marker; Lanes 1-7 shows gradient programming ranging from 55°C to 65°C. (b): Sensitivity of mPCR for *Leptospira* spp. and *Brucella* spp. Specific amplicon is approximately 848-bp for *Leptospira* species (16S_F1/16S_R1) and 403-bp for *Brucella* spp. (Det_F/Det_R). Lane M: 100-bp molecular weight marker; Lanes 1-7 shows dilutions ranging from 10^{1}-10^{-5}.

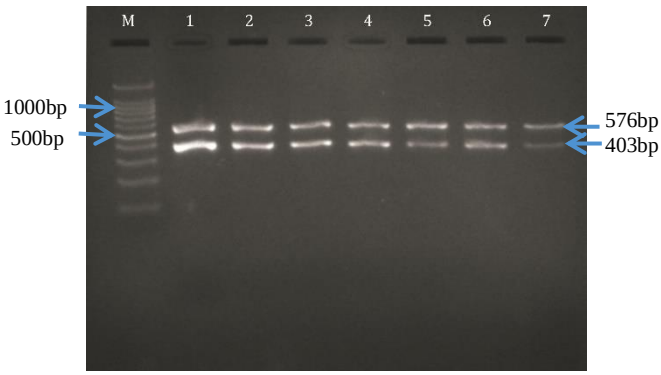

Fig. 5: mPCR sensitivity for *Campylobacter fetus* and *Brucella* spp. Specific amplicon is approximately 576-bp for *C. fetus* (*Vibrio*_F1/*Vibrio*_R1) and 403-bp for *Brucella* spp. (Det_F/Det_R). Lane M: 100-bp molecular weight marker; Lanes 1-7 shows dilutions ranging from 10^{1} -10^{-5}.

DISCUSSION

Abortion in livestock is a multifactorial disorder that usually caused by different genital infection causing pathogens. Most of these genital diseases have been eradicated in Sweden, Norway, Lithuania, Finland, Australia and New Zealand to reduce its influence on economy and international trade of animal products (Fensterbank, 1987). In contrary, these agents continue to sustain one of the major issues in several developing countries where it might have crucial impact. Earlier, ELISA was proved as an effective diagnostic test to estimate vaginal anti *C. fetus* antibodies. However at the meantime, non-specific antigen-antibody interactions and moreover false positive product with acute density of circulating antibodies in vaccinated organisms can be misdiagnosed as an active syndrome (Saadat *et al*., 2011). Previously, cross-reactivity among *Campylobacter* subspecies have been reported; even though, ELISA was considered as more specific and sensitive than agglutination tests (Grohn and Genigeorgis, 1985). In the three last decades, mostly PCR-based methods have been reported for the detection of *Brucella* spp., *Leptospira* spp. and *C. fetus*. Our results indicate that misidentification of *Brucella* spp., *Leptospira* spp. and *C. fetus* isolates in routine diagnostic laboratories may be relatively common. This study assessed the relative performance of ELISA and multiplex PCR (mPCR) assay using different primer sets for the detection of venereal pathogens.

Interestingly SPU, Qadirabad has a well-defined veterinary infrastructure where semen supplies to whole of the Punjab, Pakistan dairy farms for artificially insemination (AI) purpose through 160 breeding bulls. These bull ensures the utilization of semen for AI in majority of buffalo breeding federations (Barth and Waldner, 2002). We have

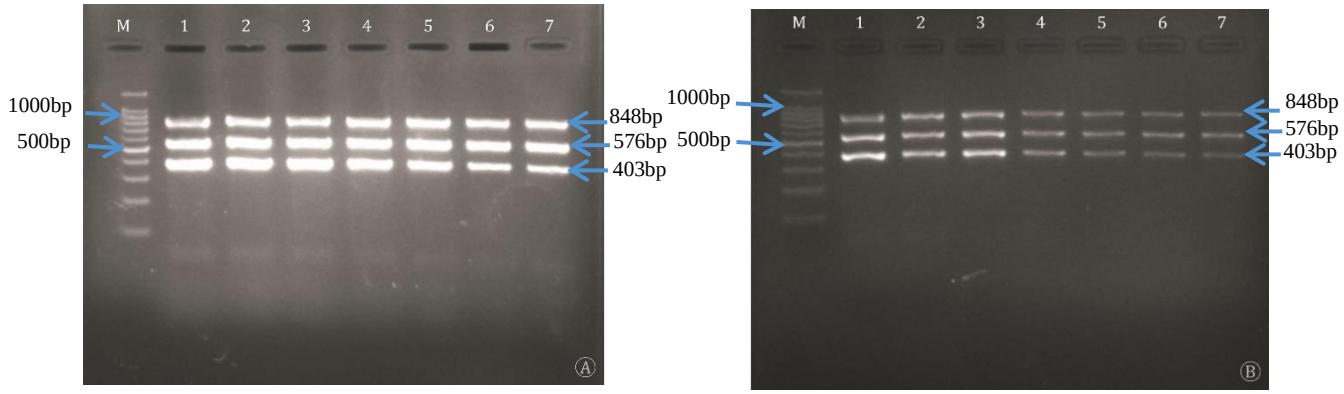

Fig. 6: (a): PCR products obtained by multiplex PCR assay for identification of the *Brucella* spp., *Campylobacter fetus* and *Leptospira* spp. Lane M: DNA size marker; Lanes 1-7 shows gradient programming ranging from 55.6°C to 59.1°C; *Brucella* spp.: 403-bp (Det_F/Det_R); *C. fetus*: 576-bp (*Vibrio*_F1/*Vibrio*_R1); and *Leptospira* spp.: 848-bp (16S_F1/16S_R1). (b): Amplification products obtained by the set of multiplex polymerase chain reaction (mPCR) assays for simultaneous identification of *Brucella* spp., *Campylobacter fetus* and *Leptospira* spp. (PCR products of *Brucella* spp.: 403-bp (Det_F/Det_R); PCR products of *C. fetus*: 576-bp (*Vibrio*_F1/*Vibrio*_R1); and PCR products of *Leptospira* spp.: 848-bp (16S_F1/16S_R1). Lane M: 100–base pair DNA ladder; Lanes 1-7 shows dilutions ranging from 10^{1}-10^{-5}.

evaluated the occurrence of these three pathogens using equally, a traditional method which permits ELISA assay and a PCR based strategy that allows integration of different primers up to multi-level design with high specificity and sensitivity. We showed that 98.12% of the analysed samples were negative for the ELISA of both *C. fetus* and *Leptospira* and 97.05% for *Brucella* in the community analyzed. To assist in the resolution of the discrepant ELISA results the experiment was repeated twice, with identical results. The less incidence rate was surprising based on previous case reports from the literature. The high frequency of negative samples observed in our work might be due to factors such as awareness and improved husbandry, climate, geographical area and density of animal kept in the area may act for this disparity and may explain the variety of the results observed by the researchers (Bharti *et al.*, 2003). This serological and molecular co-analysis of exposure to *Brucella* spp., *Leptospira* spp. and *C. fetus* is the first assessment study from well-described breeding bull population from SPU, Qadirabad.

Case studies from the literature of these three genital infections propose a very inconstant predominance at individual across study areas. Many examination studies have been conducted, mainly in government and private livestock farms of Punjab Province in Pakistan, where *Brucella* seroprevalence ranging from 1.6 to 35% (Muhammad *et al.*, 2018), although these estimations might be greatly affected by the diagnostic methods, geographical origin and sampling procedures. There was previously found a few number of published case reports of leptospirosis in livestock populations about 4.66 to 20.72% (Ijaz *et al.*, 2018). There are also two occurrence reports of *Campylobacter* species in beef with 10.9 to 15.5% have been noticed (Nisar *et al.*, 2018). Molecular based studies in buffalo in various Asian countries report overall brucellosis prevalence of 10.4 to 7.1% in Bangladesh (Sarker *et al.*, 2016); for leptospirosis 12.2% in Iran (Hajikolaei *et al.*, 2016); for *C. fetus*, 26.5% of 94 tested samples were *C. fetus* subsp. *fetus* (*Cff*) positive in Japan (Giacoboni *et al.*, 1993) and 12.6% in Iran (Hosseinzadeh *et al.*, 2013). Similar reports with low prevalence like our study have also noticed from other geographical regions where no *C. fetus* was found in 120 bovine samples from Calcutta, India (Chattopadhyay *et al.*, 2001) and in Brazil 97% of the analyzed samples were negative for the amplification of both 16S rRNA and LipL32 genes for leptospirosis (Van der Graaf-van Bloois *et al.*, 2013).

Although with few PCR approaches, both single and multiplex with variety of models have been previously adapted to estimate the disease load of pathogens targeted in our assay. These molecular assays have also revealed the successful application of PCR methods using specific oligonucleotides against *Brucella* spp. in blood samples or bacterial culture, *Leptospira* spp. in body fluids or clinical samples (Tabibnejad *et al.*, 2016) and *C. fetus* in wide range of clinical tests. In recent time real-time PCR based method has progressively gained more popularity for identification of fastidious bacterial species, but it is not well applied for routine application owing to comparatively still pricey for a low budget and small diagnostic laboratory, exclusively in the developing countries where infection is predominant. Therefore, conventional PCR assay is still a prime choice (Tramuta *et al.*, 2011) since this practice is not technically greatly demanding and consumes only few commercially accessible reagents. While the second, established for the estimation of various infectious agents in five mPCR assays, has shown less aptitude to discriminate three amplicons sizes with less than 200 bp and was not enough competent to identify *Brucella abortus* and *Brucella melitensis* in one reaction. Furthermore, *C. fetus* primer used in both studies has been revealed to be inappropriate for the detection of *C. fetus* subsp. *fetus* and may fail to recognize few atypical strains (Iraola *et al.*, 2016). We therefore established a new mPCR assay that target highly stable genomic regions with assembly of novel primer pairs for *C. fetus* and genus leptospira along those already proposed for screening and surveillance of brucellosis.

A *C. fetus* oligonucleotides were newly designed; owing to their perfect specificity for sequence of *cstA* marker (Hum *et al.*, 1997). We adopted a *Leptospira* genus primers based on 16S rRNA nucleotide sequence to permit an increase in Tm and to reduce the cross homology with closely related bacterial species. A *Brucella* primer pairs were obtained from the sequence of putative IS711 gene on the basis of high specificity of this

set. Although this PCR assay cannot differentiate among biovars from the same species without nucleotide sequencing, our primer sets were specific to targeted region and all the strains and biovars from the same isolates gave the same profile.

In conclusion, specificity of selected primers for each bacterial isolates declared that all ELISA suspected animals were negative by PCR in all collected samples. In addition, specificity of these oligonucleotides has been confirmed against taxonomic neighbor species and reveals that our assay is highly specific for corresponding isolates. Thus, it is proposed that these oligonucleotide sets may also be suitable for the direct identification of these isolates. To enable such a powerful tool to control of these infections, the practical impact of our proposed assay to environmental microbiology and veterinary use must not be challenging. This multiplex molecular assay propose a comprehensive aproach to facilitate for the detection of three corresponding genital pathogens in bovine.

Authors contributions: SI, WS, MI conceived the idea and collected variety of samples and extracted DNA from breeding bulls. MY and SAMB granted the sanction for collection of samples and provided needful data for this study. MMH and ZIQ perfomed ELISA on sera. SI, AAB, MIR and MYZ designed all primers and optimised conditions for developing duplex and triplex PCR. SI, WS, MIR and RA drafted the manuscript wth critical input from KA, AN, YFC and MA. YFC and RA provided all four reference strains for this study. All authors read and approved the final manuscript. There is no conflict of interest among authors.

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REFERENCES

Anderson ML, 2007. Infectious causes of bovine abortion during mid- to late-gestation. Theriogenology 68:474-86.

Barth AD and Waldner CL, 2002. Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. Can Vet J 43:274-84.

Bharti AR, Nally JE, Ricaldi JN, *et al.*, 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3:757-71.

Chattopadhyay UK, Rashid M, Sur SK, *et al.*, 2001. The occurrence of Campylobacteriosis in domestic animals and their handlers in and around Calcutta. J Med Microbiol 50:933-4.

Fensterbank R, 1987. Brucellosis in cattle, sheep and goats. OIE Tech Ser 6:9-35.

Giacoboni GI, Itoh K, Hirayama K, *et al.*, 1993. Comparison of fecal *Campylobacter* in calves and cattle of different ages and areas in Japan. J Vet Med Sci 55:555-9.

Grohn K and Genigeorgis C, 1985. Adaption of ELISA for the detection of *Campylobacter* antibodies and its application in seroepidemiological studies in sheep and cattle herds. Acta Vet Scand 26:30-48.

Hajikolaei MRH, Nikvand AA, Ghadrdanmashhadl AR, *et al.*, 2016. Existence of *Leptospira interrogans* in kidney and shedding from urine and relationship with histopathological and serological findings in water buffaloes (*Bubalus bubalis*). Rev Med Vet 167:269-373.

Hosseinzadeh S, Kafi M and Pour-Teimouri M, 2013. PCR detection of *Campylobacter fetus* subspecies *venerealis* in smegma samples collected from dairy cattle in Fars, Iran. Vet Res Forum 4:227-31.

Hum S, Quinn K, Brunner J, *et al.*, 1997. Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. Aust Vet J 75:827-31.

Ijaz M, Farooqi SH, Aqib AI, *et al.*, 2018. Sero-epidemiology of bovine Leptospirosis and associated risk factors in a flood affected zone of Pakistan. Pak Vet J 38:179-83.

Iraola G, Perez R, Betancor L, *et al.*, 2016. A novel real-time PCR assay for quantitative detection of *Campylobacter fetus* based on ribosomal sequences. BMC Vet Res 12:286.

Jindan RA, Saleem N, Shafi A, *et al.*, 2019. Clinical interpretation of detection of IgM anti-Brucella antibody in the absence of IgG and vice versa; a diagnostic challenge for clinicians. Pol J Microbiol 68:51-7.

Kulkarni SP, Lever S, Logan JM, *et al.*, 2002. Detection of *Campylobacter* species: a comparison of culture and polymerase chain reaction based methods. J Clin Pathol 55:749-53.

Merien F, Amouriaux P, Perolat P, *et al.*, 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 30:2219-24.

Mittal M, Sharma V, Nehra K, *et al.*, 2018. Abortions in an organized dairy farm from North India reveal the possibility of breed susceptibility to Bovine Brucellosis. One Health 5:1-5.

Mohamad N, Amal MNA, Saad MZ, *et al.*, 2019. Virulence-associated genes and antibiotic resistance patterns of *Vibrio* spp. isolated from cultured marine fishes in Malaysia. BMC Vet Res 15:176.

Muhammad I, Ijaz A, Muhammad SK, *et al.*, 2018. Seroprevalance of *Brucella abortus* in cattle and buffaloes in district Rajanpur, Punjab, Pakistan. Pure Appl Biol 7:556-64.

Navarro E, Segura JC, Castano MJ, *et al.*, 2006. Use of real-time quantitative polymerase chain reaction to monitor the evolution of *Brucella melitensis* DNA load during therapy and post-therapy follow-up in patients with brucellosis. Clin Infect Dis 42:1266-73.

Nisar M, Ahmad MUD, Mushtaq MH, *et al.*, 2018. Occurrence of *Campylobacter* in retail meat in Lahore, Pakistan. Acta Trop 185:42-5.

Oktay G, Salih O and Sahin M, 2005. Seroprevalence of Brucellosis and Leptospirosis in aborted dairy cows. Turk J Vet Anim 29:359-66.

Osunla CA and Okoh AI, 2017. Vibrio pathogens: A public health concern in rural water resources in Sub-Saharan Africa. Int J Environ Res Public Health 14:1188-215.

Saadat M, Mohamad JK, Sadegh R, *et al.*, 2011. Detection of *Brucella* spp. and *Leptospira* spp. by multiplex polymerase chain reaction (PCR) from aborted bovine, ovine and caprine fetuses in Iran. Afr J Microbiol Res 5:4627-30.

Sarker MAS, Sarker RR, Begum MM, *et al.*, 2016. Seroprevalence and molecular diagnosis of *Brucella abortus* and *Brucella melitensis* in Bangladesh. Afr J Microbiol Res 14:221-6.

Scott JC, Koylass MS, Stubberfield MR, *et al.*, 2007. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. Appl Environ Microbiol 73:7331.

Tabibnejad M, Alikhani MY, Arjomandzadegan M, *et al.*, 2016. The optimization of molecular detection of clinical isolates of Brucella in blood cultures by eryd transcriptase gene for confirmation of culture-negative samples. Iran Red Crescent Med J 18:e23879.

Tramuta C, Lacerenza D, Zoppi S, *et al.*, 2011. Development of a set of multiplex standard polymerase chain reaction assays for the identification of infectious agents from aborted bovine clinical samples. J Vet Diagn Invest 23:657-64.

Van der Graaf-van Bloois L, Van Bergen MA, Van der Wal FJ, *et al.*, 2013. Evaluation of molecular assays for identification *Campylobacter fetus* species and subspecies and development of a *C. fetus* specific real-time PCR assay. J Microbiol Meth 95:93-7.

Yamazaki-Matsune W, Taguchi M, Seto K, *et al.*, 2007. Development of a multiplex PCR assay for identification of *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis*. J Med Microbiol 56:1467-73.