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

Saheer Islam1,4, Wasim Shehzad1*, Anna Arshad Bajwa1, Muhammad Imran1, Muhammad Yasir Zahoor1, Muhammad Abdullah2, Muhammad Imran Rashid3, Kamran Ashraf3, Yung-Fu Chang4, Asif Nadeem1, Muhammad Younas5, Sayed Aamir Mehmoon Bukhari2, Muhammad Muzzammil Hassan6, Zafar Iqbal Qureshi6* and Raheela Akhtar7

1Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan; 2Department of Livestock Production, University of Veterinary and Animal Sciences, Pattoki, 553050, Pakistan; 3Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan; 4Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, 14853, USA; 5Semen Production Unit, Qadirabad, District Sahiwal 57000, Pakistan; 6Department of Theriogenology, University of Agriculture, Faisalabad, 38000, Pakistan; 7Department of Pathology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan
*Corresponding author: wasim.shehzad@uvas.edu.pk

ARTICLE HISTORY (19-203) May 14, 2019
Revised: July 04, 2019
Accepted: July 09, 2019
Published online: August 15, 2019

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 Campylobacter 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.

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 (Okтай 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 multiplex 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 multiplex 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 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:

\[ PP = \left( \frac{OD_{450 \text{ test sample}}}{Mean \ OD_{450 \text{ : Positive Control}}} \right) \times 100 \]

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

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

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

<table>
<thead>
<tr>
<th>Target</th>
<th>Targeted Region</th>
<th>Reference accession number</th>
<th>Oligo Name</th>
<th>Sequence (5’→3’)</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
<th>Gene Location (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brucella</strong></td>
<td></td>
<td>CP026005.1</td>
<td>Brucella_F1</td>
<td>GAATGCGCTTTAACAAGGCGG</td>
<td>60.5</td>
<td>223</td>
<td>621930-621990</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brucella_R1</td>
<td>AGAAAGTGCTTCTGCACGGTCT</td>
<td>58.4</td>
<td>622152-622132</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brucella_F2</td>
<td>GCCTGATCAAAAATCAGGGCTCC</td>
<td>60.5</td>
<td>264</td>
<td>621693-621712</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brucella_R2</td>
<td>TAGGGGTGCCGCTTGTGTAAG</td>
<td>60.5</td>
<td>621956-621937</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Det_F</td>
<td>AGAATAATCACCAGAGGTAGAG</td>
<td>59.3</td>
<td>403</td>
<td>621980-622002</td>
<td>(Navarro et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Det_R</td>
<td>ATCCAAGGCTAATCCAAACAC</td>
<td>56.4</td>
<td>622383-622363</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leptospira</strong></td>
<td></td>
<td>NR_134067.1</td>
<td>16S_F1</td>
<td>CCGGAGGCAGCAGGATGGA</td>
<td>60.5</td>
<td>848</td>
<td>297-316</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16S_R1</td>
<td>AAGGCAGCTAGGAGCTTGGACG</td>
<td>60.5</td>
<td>1142-1144</td>
<td>1125</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16S_F2</td>
<td>GGGCCTTCAAAACGCTAGAA</td>
<td>58.4</td>
<td>309</td>
<td>8-27</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16S_R2</td>
<td>TTCTTAATCGCTGCCTCCCG</td>
<td>60.5</td>
<td>316-297</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16S_F</td>
<td>GGGCAGGCGTCTTAAACATG</td>
<td>60.5</td>
<td>331</td>
<td>5-24</td>
<td>(Merien et al., 1992)</td>
</tr>
<tr>
<td><strong>Campylobacter</strong></td>
<td></td>
<td>AY158813.1</td>
<td>Vibrio_F1</td>
<td>CAGGTGTCGCCCTCCCCTACATT</td>
<td>60.5</td>
<td>576</td>
<td>105-124</td>
<td>This study</td>
</tr>
<tr>
<td>fetus</td>
<td>Protein A</td>
<td></td>
<td>Vibrio_R1</td>
<td>CTATGCGTCCTGGATGCTTGGT</td>
<td>60.5</td>
<td>680-661</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cstA)</td>
<td></td>
<td>Vibrio_F2</td>
<td>ATATGCGTTCTGGCTGGG</td>
<td>60.5</td>
<td>333</td>
<td>344-363</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vibrio_F</td>
<td>GGCTGCTGGATGCTTCTAGAG</td>
<td>62.5</td>
<td>676-657</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vibrio_R</td>
<td>AGCCAGTAAAGGCTATATAGTAG</td>
<td>60.1</td>
<td>359-336</td>
<td></td>
<td>(Yamazaki-Matsune et al., 2007)</td>
</tr>
</tbody>
</table>
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$^7$-10$^1$) 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

<table>
<thead>
<tr>
<th>Sampling area</th>
<th>Number of samples</th>
<th>Number of positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen Production Unit (S PU) Qadirabad</td>
<td>148</td>
<td>4 (2.7)</td>
</tr>
<tr>
<td>Private Farm</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>4 (2.5)</td>
</tr>
</tbody>
</table>

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

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.
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
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

**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; Brucelle spp.: 403-bp (Det_F/Dec_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/Dec_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-bp base pair DNA ladder; Lanes 1-7 shows dilutions ranging from 10^{-10}.
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 with 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.

Acknowledgements: This work was supported by Punjab Agricultural Research Board (PARB), Lahore [Project No-492] and Higher Education Commission (HEC) Pakistan.

REFERENCES