

Development of a multiplex PCR assay for detection of different bacterial pathogens associated with reproductive disorders in cattle and buffaloes

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ABSTRACT

The current study was undertaken to develop a multiplex PCR assay for detection of *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma* spp. associated with reproductive disorders in cattle and buffaloes. Four pairs of oligonucleotide primers chosen to amplify target DNA regions viz. 31kDa MEM protein in *Brucella*, 16S rRNA gene in *Leptospira*, *hlyA* gene in *Listeria* and 16S rDNA in *Mycoplasma* spp. produced amplicon sizes of 223-bp, 331 bp, 456 bp and 270 bp respectively. No amplification was observed when the multiplex PCR was tested against commonly prevalent and related species of bacteria. The sensitivity of the multiplex PCR assay was 116 pg for *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma* spp. The developed assay was tested for direct detection of the agents in 30 clinical samples of uterine discharges and foetal stomach contents collected from aborted animals and animals with reproductive disorders. By multiplex PCR, out of 30 samples, nine samples of uterine discharges were positive only for *Mycoplasma* and 1 sample of foetal stomach content was positive for *Brucella*, *Leptospira* and *Mycoplasma*. The multiplex PCR described here appeared to be a rapid and ideal method for detection of all four organisms simultaneously in a single tube reaction and hence can be used for routine diagnostics.

Key words: *Brucella*, *Leptospira*, *Listeria*, Multiplex PCR, *Mycoplasma*

Reproductive disorders in cattle and buffaloes such as abortions, repeat breeding, infertility and lower reproduction account for huge economic losses to the animal health industry (Yoo 2010). The disorders develop slowly over a period of time with no or little signs shown by the affected animals and hence usually go unnoticed in a herd. Though some of the affected animals especially males never show any signs of the disease and usually appear healthy, still, they pose a great threat to other animals in a herd because they carry the disease organism. Reproductive disorders due to *Brucella* spp., *Leptospira* serovars, *Listeria monocytogenes* and *Mycoplasma bovis* have been reported in the literature (Radostits *et al.* 1994). Though reproductive disorders can occur due to many other reasons, but, infectious agents are the cause of abortion in about 90% of the cases in animals (Silva *et al.* 2010). Bovine brucellosis and leptospirosis are distributed worldwide and reproductive disorders such as late term abortions and premature births may be the only clinical signs of these diseases in pregnant animals (Tramuta *et al.* 2011). Although *Mycoplasma* spp. have been thought to be relatively uncommon cause of abortion, there are reports of

Mycoplasma bovis to be associated with infertility as well as abortion (Pfutzner and Sachse 1996). Multiple abortions in a herd caused by *Listeria monocytogenes* are often associated with high bacterial numbers found in poor quality or spoiled silage on dairy farms (Ernest 2009). Abortions due to *Listeria* spp. occur approximately one week after exposure, and occur most commonly during the last trimester of pregnancy, although they may occur as early as the 4th month of gestation.

Molecular diagnosis based on multiplex polymerase chain reaction (PCR) has been successfully described for the detection of various agents associated with reproductive disorders in cattle and buffaloes. Multiplex PCR assay (mPCR) is a kind of technique where multiple target DNA sequences can be detected in a single reaction by using multiple primer pairs in a reaction mixture (Richtzenhain *et al.* 2002). Multiplex PCR has been developed for detection of *Brucella* and *Leptospira* spp from aborted bovine fetuses (Richtzenhain *et al.* 2002), for detection and differentiation of *Brucella*, *Leptospira* and *Campylobacter fetus* (Tramuta *et al.* 2011), for detection of *Brucella* and *Salmonella abortus ovis* (Sharifzadeh *et al.* 2008). So, with an aim for improvement in the direct diagnosis, the present study was conducted with the objective to develop multiplex PCR for simultaneous detection of *Brucella*, *Listeria*, *Leptospira* and *Mycoplasma* associated with reproductive disorders in cattle and buffaloes.

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MATERIALS AND METHODS

Bacterial strains: The standard reference bacterial strains *Brucella abortus* S-99, *Mycoplasma* spp., *Listeria monocytogenes*, and *Leptospira* spp. were used. In addition, a number of other commonly prevalent and cross reacting bacteria like *Salmonella*, *E.coli*, *Pseudomonas*, *Proteus*, *Mycobacterium*, *Pasteurella*, *Staphylococcus*, *Streptococcus*, *Campylobacter fetus*. were also taken for evaluations of specificity

DNA extraction: Genomic DNA from all the organisms except *Mycoplasma* and *Leptospira* was extracted by bacterial genomic DNA purification kit following the manufacturer's instructions. DNA of *Mycoplasma* and *Leptospira* were obtained from IVRI, Izatnagar.

Primers used: After carefully studying the genes and primers which have been used so far for detection, oligonucleotide primers specific for 31kDa MEM protein in *Brucella*, 16S rRNA gene in *Leptospira*, *hlyA* gene in *Listeria* and 16S rDNA in *Mycoplasma* spp. were selected from the literature. The primers were evaluated for their specificity before selection and were analysed using BLAST software. The mPCR assay was developed by using the following primers: 5'-TGGCTCGGTTGCCAATATCAA-3'(r) and 5'-CGCGCTTGCCTTTCAGGTCTG-3'(f) for *Brucella* (Tramuta *et al.* 2011); 5'-GGCGGCGCGTCTTAAACATG-3'(f) and 5'-TTCCCCCATTGAA GCAAGATT-3'(r) for *Leptospira* (Tramuta *et al.* 2011); 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3'(r) and 5'-GCA ACG TAT CCT CCA GAG TGA TCG-3'(f) for *Listeria* (Paziak-Domanska *et al.* 1999) and 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'(f) and 5'-GGGAGCAAACAGGATTAGATACCCT-3'(r) for *Mycoplasma* (Marois *et al.* 2000).

Multiplex PCR assay: Monoplex PCRs was carried out for testing individual primer pairs for each of the four organisms before proceeding with the multiplex PCR. In each phase of standardization of multiplex PCR, concentration of primers in the cocktail, annealing temperatures, concentration of *Taq* polymerase and $MgCl_2$ was optimised. Gradient PCR was carried out with annealing temperatures ranging from 59°C–61°C keeping in view the T_m values of each individual primer. The primer cocktail of all four primer pairs for multiplex PCR assay was adjusted to the concentration of 20pmol/ μ l each for *Leptospira* and *Listeria* and 12pmol/ μ l each for *Brucella* and *Mycoplasma*. mPCR reactions were performed in 25 μ l reaction volume consisting of 1XPCR buffer, 0.4mM dNTP mix, 1.5mM $MgCl_2$, primer cocktail, 2U of *Taq* polymerase, 5 μ l of DNA template and nuclease free water to make final volume 25 μ l. Cycling parameters were initial denaturation at 94°C for 5 min, followed by 30 cycles each of 94°C for 1 min (denaturation), 61°C for 1 min (annealing) and 72°C for 1 min (extension), and a final extension of 72°C for 5 min. PCR amplified products were separated using electrophoresis in 1.8% agarose gel containing ethidium bromide for 1.5 hr. at 80V and visualised under gel documentation system and photographed.

Specificity evaluation of mPCR: The standardised mPCR was assessed for its specificity by testing some commonly prevalent and cross reacting bacterial species with the primers used in this study. Specificity was determined against field isolates/ DNA of *Salmonella*, *E.coli*, *Proteus*, *Mycobacterium*, *Pasteurella*, *Staphylococcus*, *Streptococcus*, *Campylobacter fetus* and *Pseudomonas*. PCR ingredients and conditions were same as described earlier for multiplex PCR.

Sensitivity evaluation of multiplex PCR assay: DNA from *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma* was quantified in nanodrop (Thermo scientific) and cocktail was made containing equal quantities of DNA of each organism. Serial tenfold dilutions of pooled DNA was carried out to determine the detection limit of multiplex PCR assay.

Application of multiplex PCR assay on clinical samples: A total of thirty clinical samples of uterine discharges (22) and foetal stomach contents (8) were collected from repeat breeding cattle and buffaloes and from cases of abortion. DNA was extracted directly from the clinical samples by bacterial genomic DNA purification kit and used for mPCR.

RESULTS AND DISCUSSION

Reproductive disorders in cattle and buffaloes, caused by a variety of infectious agents continue to be a problem for animals throughout the world leading to severe economic losses. Though there are a number of other factors (toxic, endocrine, physical trauma or nutritional deficiencies) that can lead to reproductive disorders in cattle and buffaloes, but infectious agents are one of the most important cause (Tramuta *et al.* 2011, Yoo 2010). Specific diagnosis of the etiological agent in reproductive diseases is highly desirable since control measures vary depending on the agent involved. Direct methods of diagnosis of the infectious agents based on bacteriological isolation are usually employed, but they are time consuming, difficult, dangerous and require expert personnel (Kirkbride 1990,

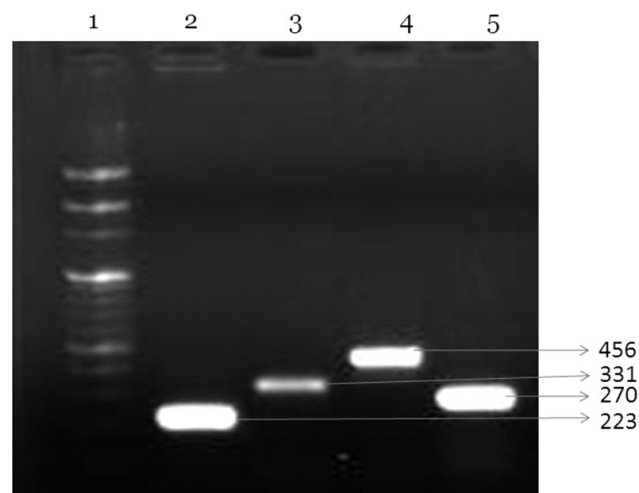


Fig. 1. Gel electrophoresis of monoplex PCR amplified fragments from *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma* (Lane 1-100 bp plus DNA ladder (Fermentas); Lane 2- *Brucella*; Lane 3-*Leptospira*; Lane 4-*Listeria*; Lane 5-*Mycoplasma*).

Nielsen and Duncan 1990, Faine *et al.* 1999). Diagnosis by PCR is a promising option and useful method for detection of the organisms. In this study, in monoplex PCRs, carried out separately for each bacterium, specific amplicon sizes of 331-bp for *Leptospira* spp., 270-bp for *Mycoplasma* spp., 456-bp for *Listeria* spp. and 223-bp for *Brucella* spp. were obtained (Fig.1). Since diagnosis by monoplex PCR allows the detection of a single bacterium at a time, hence, monoplex PCR is relatively costly and time consuming.

Multiplex PCR assay is a technique for the amplification of multiple target DNA sequences in a single tube PCR experiment. Multiplex PCR offers a considerable potential for saving of time and effort without affecting the utility of the test. Several monoplex and multiplex PCR assays have been successfully described for the detection of *Brucella*, *Listeria*, *Leptospira* and *Mycoplasma* spp. (Richtzenhain *et al.* 2002, Kaur *et al.* 2007, Rawool *et al.* 2007, Sharifzadeh *et al.* 2008, Silva *et al.* 2009, Tramuta *et al.* 2011, Dehkordi and Taghizadeh 2012, Hamali *et al.* 2012, Parin and Kirkan 2012, Moustakas *et al.* 2013, Sai Arun Batra *et al.* 2013, Sharma *et al.* 2013) but there is no assay that can simultaneously identify all the four agents. Using already reported primers, a multiplex assay was established for the simultaneous detection of *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma*. Standardisation of multiplex PCR requires careful optimisation of all the ingredients along with the PCR conditions. Standardisation of the primer cocktail is important since the inclusion of many primer pairs can lead to primer dimerization and mis-priming. The concentration of primers for multiplex assay was optimized

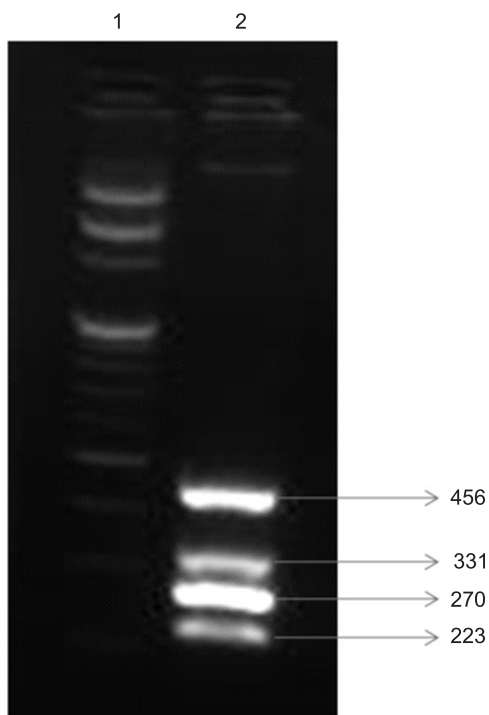


Fig. 2. Gel electrophoresis of multiplex PCR amplified fragments from *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma* (Lane 1-100 bp plus DNA ladder (Fermentas); Lane 2- *Brucella*, *Mycoplasma*, *Leptospira* and *Listeria*).

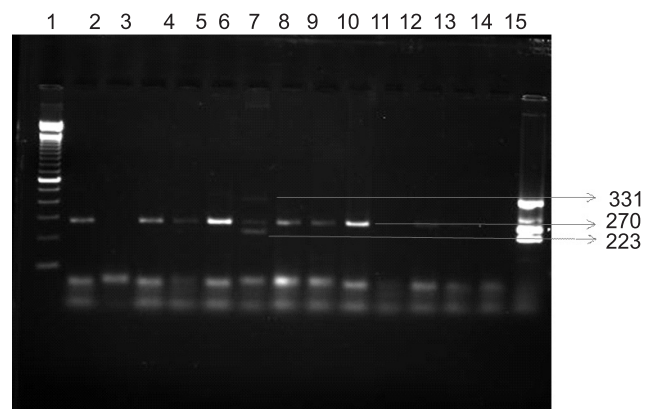


Fig. 3. Gel electrophoresis of multiplex PCR amplified fragments from DNA extracted directly from clinical samples (Lane 1-100bp DNA Ladder (invitrogen); Lane 2, 4, 5, 6, 8, 9, 10, 12, 13- *Mycoplasma*; Lane 7- *Brucella*, *Mycoplasma*; Lane 7-*Brucella*, *Mycoplasma*, and *Leptospira*; Lane 3-negative control; Lane 11 and Lane 14-negative sample; Lane 15-positive control).

after changing the concentration of individual primers in the cocktail. Different concentrations of primers from 10pmol/μl to 25pmol/μl were tested. Finally, in the standardised protocol, the primers for *Listeria* and *Leptospira* gave optimum amplification at 20pmol/μl whereas the primers for *Mycoplasma* and *Brucella* gave amplification at concentration of 12pmol/μl.

In addition, the concentration of *Taq*, $MgCl_2$, adequate concentration and ratio of primer to DNA is important. The concentration of $MgCl_2$ (25mM) and *Taq* was optimised (1.5 mM, 2mM and 2.5 mM) and (1U, 1.5U, 2U) respectively and best results were obtained at 1.5 mM of $MgCl_2$ and 2U of *Taq* polymerase. Gradient annealing temperature studies revealed satisfactory amplification for all the primers at 59°C, 60°C and 61°C but faint band was observed for *Leptospira* at 59°C. Primers targeting specific genes in *Brucella*, *Mycoplasma*, *Leptospira* and *Listeria* gave optimum amplification at 60°C, but some non specific reactions were obtained. Hence, for the multiplex PCR, 61°C was chosen as the optimum temperature for annealing.

The multiplex assay was capable of detecting *Brucella* spp., *Mycoplasma* spp., *Listeria* spp. and *Leptospira* spp. simultaneously from genomic bacterial DNA cocktail in a single tube PCR reaction. Each primer pair amplified DNA fragments specific for the corresponding gene and of the predicted size (331-bp for *Leptospira* spp., 270-bp for *Mycoplasma* spp., 456-bp for *Listeria* spp. and 223-bp for *Brucella* spp.) (Fig. 2). The sharpness and intensity of the amplified DNA fragments in monoplex PCR assay was more as compared to that obtained in multiplex PCR. This could be because of the reason that in uniplex PCR where only single gene is targeted, we can use the optimal conc. of $MgCl_2$, *Taq*, annealing temperature according to the specific primer, but, in case of multiplex PCR, the ingredients as well as PCR conditions should suit all primer pairs and target genes and so may not be optimal for each individual pair of primers. Similar observations have been

reported (Moustacas *et al.* 2013).

No amplified product was observed when multiplex PCR was evaluated for specificity by testing the DNA of other organisms. This showed that the developed mPCR assay had 100% specificity for the chosen organisms.

When applied on 10- fold serially diluted pooled DNA samples, the sensitivity of the assay was 11.6pg for *Brucella*, *Leptospira*, *Listeria* and *Mycoplasma*.

This assay was applied for direct detection of these four organisms in clinical samples of uterine discharges and foetal stomach contents. Out of 30 samples of foetal stomach contents and uterine discharges, 9 samples of uterine discharges were positive for *Mycoplasma* (Fig.3). Numerous studies have demonstrated the presence of *Mycoplasma* in genital tracts of healthy and diseased cows (Langford 1975, Nakamura *et al.* 1977, Petit *et al.* 2008). The presence of *Mycoplasma bovis* in 43% of males, in cervico vaginal mucus of 47% of females, 25% of foetuses and 11% of placentas in cattle has been demonstrated (Trichard and Jacobsz 1985). *Mycoplasma* has been found in cows with low fertility in which no other cause of infertility was identified (Kirkbride 1987). Higher isolation rates of *Mycoplasma* from infertile cows have been reported (Panangala *et al.* 1978). *M. bovis* was detected in the uterus of postpartum cows (Mohammed *et al.* 2013) with an incidence of 7.4% and might be associated with cytological endometritis in postpartum dairy cows. In the present study, one sample of fetal stomach content was positive for *Brucella*, *Leptospira* and *Mycoplasma* by mPCR (Fig.3). Similarly, infection by both *Brucella* and *Leptospira* spp. has earlier been observed in 7 out of 11 aborted fetuses of bovine by multiplex PCR (Dehkordi and Taghizadeh 2012)

It may be concluded that simultaneous detection of three different organisms in a single sample could be demonstrated by using the developed multiplex PCR assay. Hence, the diagnostic efficiency of monoplex PCR, time duration and costs can be improved by development of a multiplex PCR.

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