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Detection and differentiation of mycoplasma from bacteria and fungi by polymerase chain reaction

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Mycoplasma, the smallest self-replicating, prokaryote causes various disease conditions, viz. contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), mastitis, arthritis, infectious keratoconjunctivitis, contagious agalactia and genital disorders in different domestic animals causing heavy economic losses (Nicolet 1994, Gunning and Shephered 1996). It is also present as a common contaminant in cell cultures used for viral propagation (Barile 1979).

The routine detection of mycoplasmas is generally carried out by isolation of the organisms and serological tests. The isolation of the organisms is very tedious, laborious and time consuming (Sachse *et al.* 1993), while the serological tests are not very sensitive and specific. Moreover, a higher antibody titre is also required for serological detection, which is generally not present in subclinical and chronic infections (Simecka *et al.* 1992). Recent development of molecular techniques, viz. polymerase chain reaction (PCR), restriction endonuclease analysis, DNA probes, have overcome this problem to a great extent. Among these techniques, PCR is the most sensitive and can detect the subclinical and chronic infections, when organisms are present in a very low number (Kuppeveld *et al.* 1992, Johansson *et al.* 1996 and Ghadersohi *et al.* 1999). The present study was intended to detect the different strains of mycoplasma and also differentiate these organisms from bacteria and fungi by specific PCR assay.

The 14 strains of mycoplasma, 18 strains of bacteria and 3 strains of fungi used in this study are listed in Table 1. These mycoplasma strains were procured from the National Referral Laboratory on Mycoplasma while bacterial and fungal strains from Division of Bacteriology and Mycology, IVRI, Izatnagar. The strains of *Mycoplasma mycoides* cluster viz. *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *mycoides* SC, *M. mycoides* subsp. *mycoides* LC, *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *M.* sp.

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Table 1. Mycoplasma, bacterial and fungal strains

Mycoplasma strains	
1.	*M. mycoides* subsp. *capri* (PG3) NCTC 10137
2.	*M. mycoides* subsp. *capri* (Bhuj)
3.	*M. mycoides* subsp. *mycoides* LC (NCVP -2/99)
4.	*M. mycoides* subsp. *mycoides* LC (Y-Goat) NCTC 11706
5.	*M. mycoides* subsp. *mycoides* SC (PG1) NCTC 10114
6.	*M. mycoides* subsp. *mycoides* SC (NCVP - 1/86)
7.	*M. capricolum* subsp. *capricolum* (California kid) NCTC 10154
8.	*M. capricolum* subsp. *carpricolum* (BMET)
9.	*M. capricolum* subsp. *capripneumoniae* (F.38) NCTC 10192
10.	*M.* sp. bovine group 7 (PG 50) NCTC 10133
11.	*M. bovis* (NC 317)
12.	*M. agalactiae* (RPNS 216)
13.	*M. agalactiae* (RPNS 200)
14.	*Acholeplasma laidlawii* (NC 313)
Bacterial and fungal strains	
1.	*Pasteurella multocida* (B: 2) P52
2.	*Pasteurella multocida* (A: 1)
3.	*Pasteurella multocida* (D: 1)
4.	*Pasteurella multocida* (F: 3)
5.	*Staphylococcus* spp.
6.	*Streptococcus pyogenes*
7.	*Corynebacterium pyogenes*
8.	*Bacillus cereus*
9.	*Pseudomonas* spp.
10.	*Escherichia coli*
11.	*Enterobacter agglomerans*
12.	*Listeria monocytogenes*
13.	*Klebsiella pneumoniae*
14.	*Salmonella* Typhimurium
15.	*Salmonella* Abortus equi
16.	*Salmonella* Lille
17.	*Salmonella* Cholerae suis
18.	*Salmonella* Neighton
19.	*Aspergillus flavus*
20.	*Aspergillus fumigatus*
21.	*Aspergillus parasiticus*

bovine group 7 and *Acholeplasma laidlawii* were grown in mycoplasma liquid media as described by Carmichael *et al.* (1972) whereas, B medium (Ernö and Stipkovits 1973) was used for the growth of *M. bovis* and *M.agalactiae.* However, all the bacterial strains used in present study were grown in BHI broth while the species of *Aspergillus* in Sabouraud's dextrose agar medium.

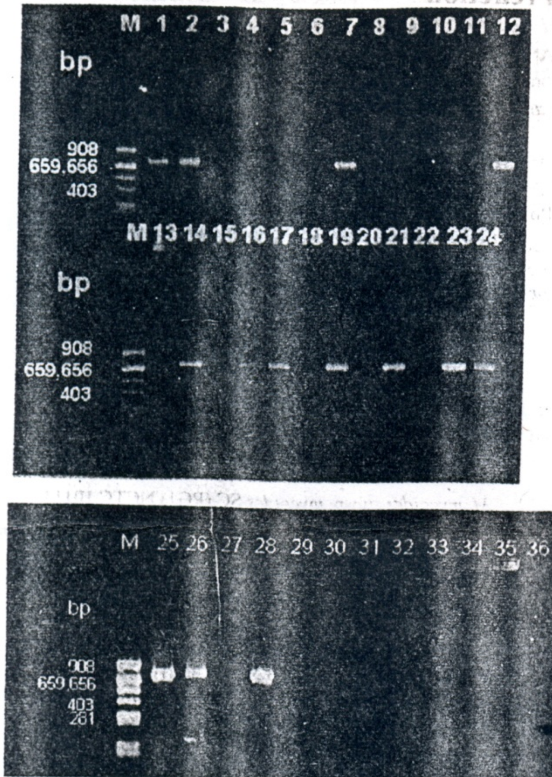

Fig. 1. Mycoplasma group specific PCR using primer GPO-1/ MGSO: Lane M: Marker (pBR 322 DNA digested with *Alu* I), Lane 1: *M. mycoides* subsp. *mycoides* SC (PG1), Lane 2: *M. mycoides* subsp. *mycoides* SC (NCVP - 1/86), Lane 3: *Pasteurella multocida* (B: 2), Lane 4: *P. multocida* (A: 1), Lane 5: *P. multocida* (D: 1), Lane 6: *P. multocida* (F: 3), Lane 7: *M. bovis* (NC 317), Lane 8: *Staphylococcus* sp., Lane 9: *Streptococcus pyogenes*, Lane 10: *Corynebacterium pyogenes*, Lane 11: *Bacillus cereus*, Lane 12: *M.* sp. bovine group 7 (PG 50), Lane 13: *Pseudomonas* sp., Lane 14: *M. capricolum* subsp. *capripneumoniae* (F-38), Lane 15: *E. coli*, Lane 16: *M. mycoides* subsp. *mycoides* LC (Y-Goat), Lane 17: *M. mycoides* subsp. *mycoides* LC (NCVP - 2/99), Lane 18: *Enterobacter agglomerans*, Lane 19: *M. mycoides* subsp. *capri* (PG 3), Lane 20: *Listeria monocytogenes*, Lane 21: *M. mycoides* subsp. *capri* (Bhuj), Lane 22: *Klebsiella pneumoniae*, Lane 23: *M. capricolum* subsp. *capricolum* (Calif. kid), Lane 24: *M. capricolum* subsp. *capricolum* (BMET), Lane 25: *M. agalactiae* (RPNS 216), Lane 26: *M. agalactiae* (RPNS 200), Lane 27: *Salmonella* Typhimurium, Lane 28: *Acholeplasma laidlawii* (NC 313), Lane 29: *Salmonella* Abortus equii, Lane 30: *Salmonella* Lille, Lane 31: *Salmonella* Neighton, Lane 33: *A. flavus*, Lane 34: *A. fumigatus*, Lane 35: *A. parasiticus*, Lane 36: Negative control (No DNA).

Isolation of genomic DNA

The genomic DNA of all the strains were isolated as per the protocol of Wilson (1987) and the concentration of genomic DNA was finally adjusted to 10 ng/μl of DNA.

PCR assay

The PCR assay was performed using the genomic DNA of mycoplasma, acholeplasma, bacteria and lysate of fungi as template along with mycoplasma group specific primers GPO-1 [5'-ACT CCT ACG GGA GGC AGC AGTA - 3'] and MGSO [5' - TGC ACC ATC TGT CAC TCT GTT AAC CTC - 3'] (Kuppeveld *et al.* 1992). The PCR reaction was performed in a thermocycler using a programme consisting of an initial denaturation of 94° C for 2 min, followed by 30 cycles of denaturation (94° C for 45 sec), annealing (55° C for 1 min) and extension (72° C for 1 min). Final extension was carried out at 72° C for 5 min.

The PCR products were detected on 1.4% agarose gel electrophoresis along with the DNA marker (pBR 322 digested with *Alu* I enzyme) and then visualised and photographed under UV-gel documentation system. The sensitivity of the PCR assay was estimated using different 10-fold dilutions of template DNA from 100 ng to 100 fg.

In the present study, all the 14 strains of mycoplasma and acholeplasma yielded a 715 bp long fragment of 16S rRNA gene with GPO-1 and MGSO primers while none of the strains of bacteria and fungi yielded any amplified product (Fig. 1).

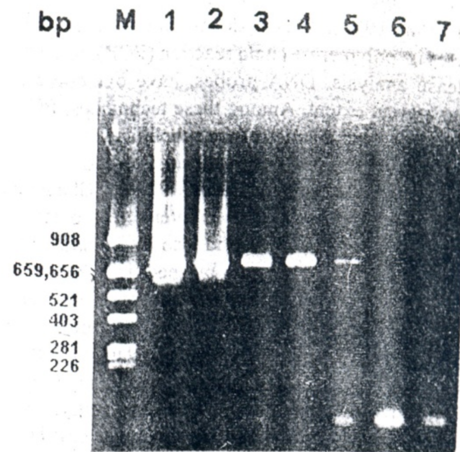

Fig. 2. Sensitivity of PCR assay using different dilutions of template DNA. Lane M: Marker (pBR 322 DNA digested with *Alu* I enzyme), Lane 1 : 100 ng template DNA, Lane 2 : 10 ng template DNA, Lane 3 : 1ng template DNA, Lane 4 : 100 pg template DNA, Lane 5 : 10 pg template DNA, Lane 6 : 1 pg template DNA, Lane 7 : 100 fg template DNA.

These results are in agreement with the findings of Kuppeveld *et al.* (1992), who designed these primers based on 16S rRNA sequence for detection of mycoplasma, acholeplasma, spiroplasma and ureaplasma. The present findings also revealed that this PCR assay can be very well utilised for detection of mycoplasma from field materials which are normally contaminated with bacteria and fungi. Further, it will also be helpful in primary screening of mycoplasma in various infections including the cell cultures, which are very prone to mycoplasma and acholeplasma infections.

The sensitivity of the PCR assay was nearly 10 pg of template DNA (Fig. 2). It indicated that this assay is very sensitive and can be used in case of chronic and subclinical conditions, where very less number of mycoplasmal organisms are present.

Polymerase chain reaction assay was used in the present study for detection of various species of mycoplasma and acholeplasma by using mycoplasma group specific primers GPO-1 and MGSO based on 16S rRNA gene yielded 715 bp amplified product only in the strains of mycoplasma and acholeplasma without any cross-amplification with any other bacterial and fungal strains.

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