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PCR-BASED DIFFERENTION OF *Mycoplasma bovis* FROM OTHER MYCOPLASMAS

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Mycoplasma bovis is associated with a large number of disease conditions viz. mastitis, arthritis, calf pneumonia, infectious kerato-conjunctivitis and genital disorders in bovines (Jasper, 1977; Bocklisch *et al*., 1986; Howard *et al*., 1987 and Gunning and Shephered, 1996). Due to lack of effective vaccines and antimicrobial therapy, *M. bovis* infections remain a significant problem resulting in extensive economic losses to cattle industry. The routine diagnostic procedures viz. isolation of the agent and serological tests are tedious and time consuming. Moreover, extreme serological cross-reactions are also observed with other mycoplasmas, particularly the *M. agalactiae* (Simecka *et al*., 1992). In recent years, the PCR has been employed as a suitable assay for specific detection of *M. bovis*, even from chronic and subclinical cases with an additional advantage of its differentiation from *M. agalactiae*, which is difficult by conventional tests (Johansson *et al*., 1996). The present study was undertaken to defferentiate *M. bovis* strains from other very closely related mycoplasmas by species specific PCR assay.

Ten strains of *Mycoplasma* spp.and one strain of *Acholeplasma laidlawii* used in this study were obtained from National Referral Laboratory on Mycoplasma, Division of Bacteriology & Mycology, IVRI, Izatnagar (Table 1). The specially enriched B medium (Ernϕ and Stipkovits, 1973) was used for the growth of *M. bovis*, *M. bovirhinis*, *M. bovigenitalium* and *M. alkalescens* strains, whereas, the strains of *M. mycoides* subsp. *mycoides* SC, *M. sp. bovine* group 7, *M. agalactiae* and *Acholeplasma laidlawii* were grown in MBHS-L liquid medium (Carmichael *et al*., 1972). The genomic DNA from each of the strains was extracted as described earlier (Wilson, 1987), the concentration has been determined by taking O.D. at 260 nm and then diluted in autoclaved distilled water to have 10 ng of DNA/µl.

PCR was carried out using *M. bovis* specific primers MboF2[5'-GAA GAA AA GT AGC ATA GGA AAT GAT -3'] and MboR2[5'-CGT CGT CCC CAC CTT CCT CCC G-3'] (Johansson *et al*., 1996). A 20 ng of genomic DNA of each strain was used as a template in 50 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.08% nonidet P40; 200 µM dNTP mixture, 20 p mole of each primer (MboF2 and Mbo R2) and 1 unit of Taq DNA polymerase (MBI Fermentas). The reaction mixture was subjected to an initial denaturation at 94°C for 2 minutes followed by 30 cycles of amplification with 94°C (denaturation) for 45 seconds, 65°C (annealing) for 1 minute and 72°C (extension) for 1 minute. Final extension was carried out at 72°C for 5 minutes.

Another PCR was carried out using a different set of *M. bovis* specific primers viz. PpMB 920-1 [5'-GGC TCT CAT TAA GAA TGT C-3'] and PpMB 920-2[5'-TTT TAG CTC TTT TTG AAC AAA T-3'] (Hotzel *et al*., 1993 & 1996). The reaction mixture similar to that of previous PCR was prepared along with the primers i.e. PpMB 920-1 and PpMB 920-2 while the programme was carried out with initial denaturation of 94°C for 2 minutes; 30 cycles of denaturation (94°C for 30 seconds), primer annealing (48°C for 45 seconds) and primer extension (72°C for 90 seconds) followed by final extension at 72°C for 5 minutes.

The PCR products were analysed by agarose gel electrophoresis on 1.4% agarose gel ethidium bromide (0.3 µg/ml). A 10 µl of each PCR product was run separately along with the suitable DNA marker at 7 volts/cm for 1 hour 30 minute in 0.5 X TBE buffer (45 mM Tris, 45 mM boric acid and 2 mM EDTA, pH 8.0). The gel was visualized and photographed under UV gel documentation system.

Both the strains of *M. bovis* viz. NC 317 and 23 K showed the amplified product (734 bp) when screened by MboF2 and MboR2 primers while the other nine strains of bovine mycoplasma and acholeplasma failed to (Fig. 1). Johansson *et al*. (1996) reported similar results using type strains of 14 different *Mycoplasma* species viz. *M. agalactiae, M. bovis, M. arginini, M. bovigenitalium, M. bovoculi, M. californicum, M. canadense, M. capricolum* subsp. *capripneumoniae, M. fermentans, M. meleagridis, M. mycoides* subsp. *mycoides* LC type, *M. mycoides* subsp. *mycoides* SC type, *M. ovipneumoniae* and *M. putrefaciens.* However, the other PCR assay using another set of primers PpMB 920-1 and PpMB 920-2 yielded an amplified product of 2kbp only in both the strains of *M. bovis* (NC 317 and 23 K) without any cross-amplification with other nine strains of mycoplasma and acholeplasma (Fig. 2). These results support the findings of Hotzel *et al.* (1993 and 1996) who detected similar amplified product in *M. bovis* strain which was isolated from nasal swab and cow milk.

The present findings of both the PCR assay also revealed no cross reactivity even with very closely related *M. agalactiae*, although, *M. bovis* and *M. agalactiae* had only 8 nucleotide difference in 16S rRNA gene (Mattsson *et al.*, 1994). So, both the

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Table 1. STRAINS

1.	*M. bovis* (NC 317)
2.	*M. bovis* (23 K)
3.	*M. bovirhinis* (NC 448)
4.	*M. bovigenitalium* (NC 58)
5.	*M. mycoides* subsp. *mycoides* type SC (PG 1)
6.	*M. mycoides* subsp. *mycoides* type SC (NCVP-1/86)
7.	*M.* sp. bovine group 7 (PG 50)
8.	*M. alkalescens* (NC 242)
9.	*M. agalactiae* (RPNS 216)
10.	*M. agalactiae* (RPNS 200)
11.	*Acholeplasma laidlawii* (NC 313)

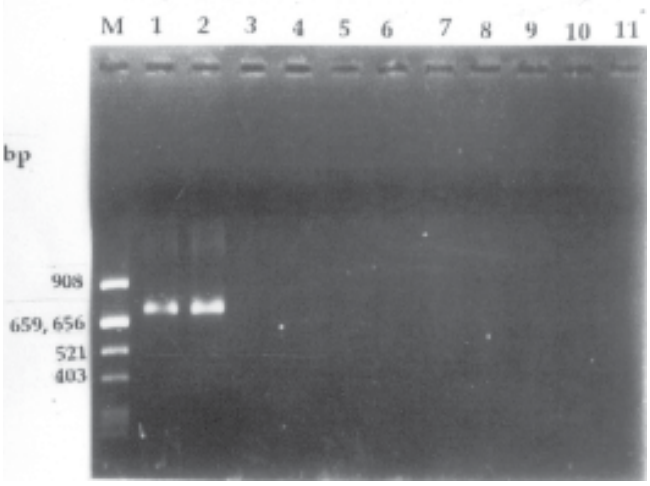

Fig. 1. ***M. bovis* specific PCR using primers Mbo F2 and Mbo R2 :** Lane M. : Marker (pBR 322 DNA digested with Alu I), Lane 1 : *M. bovis* (NC 317), Lane 2 : *M. bovis* (23K), Lane 3 : *M. bovirhinis* (NC 448), Lane 4 : *M. bovigenitalium* (NC 58) Lane 5 : *M. mycoides* subsp. *mycoides* SC (PG1), Lane 6 : *M. mycoides* subsp. *mycoides* SC (NCVP-1/86), Lane 7 : **M**. sp. bovine group 7 (PG 50), Lane 8 *: M. alkalescens* (NC 242), Lane 9 : *M. agalactiae* (RPNS 216), Lane 10 : *M. agalactiae* (RPNS 200), Lane 11 : *Acholeplasma laidlawii* (NC 313).

M. bovis- specific PCR assay have the potential for specific detection of *M. bovis* in clinical samples such as milk, meat, nasal swabs and even contaminated field materials and slaughter house samples in a very short period of time without any cross-amplification with other mycoplasmas as compared to lengthy and cumbersome procedures involved in serological and biochemical tests.

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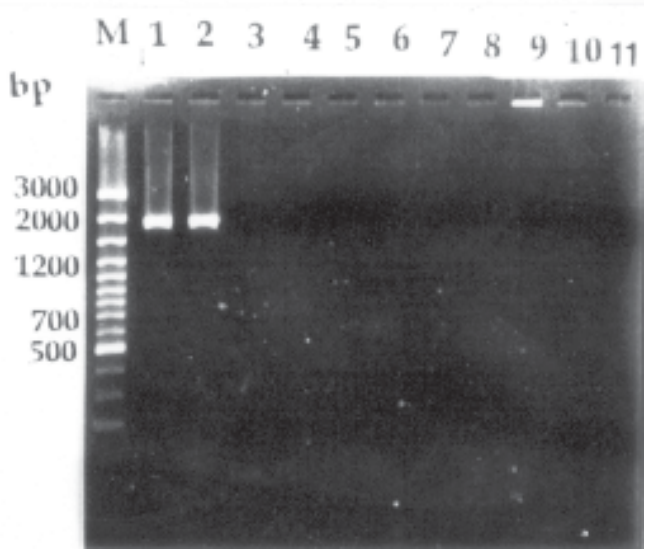

Fig. 2. ***M. bovis* specific PCR using primers PpMB 920-1 and PpMB 920-2:** Lane M. : Marker (Generuler), Lane 1 : *M. bovis* (NC 317), Lane 2 : *M. bovis* (23K), Lane 3 : *M. bovirhinis* (NC 448), Lane 4 : *M. bovigenitalium* (NC 58) Lane 5 *: M. mycoides* subsp. *mycoides* SC (PG1), Lane 6 : *M. mycoides* subsp. *mycoides* SC (NCVP-1/86), Lane 7 *: M.* sp. bovine group 7 (PG 50), Lane 8 *: M. alkalescens* (NC 242), Lane 9 : *M. agalactiae* (RPNS 216), Lane 10 : *M. agalactiae* (RPNS 200), Lane 11 : *Acholeplasma laidlawii* (NC 313).

REFERENCE

Bocklisch, H., Pfutzner, H., Martin, J., Templin, G. and Kreeusel, S. (1986). Arch. Exp. Veterinar. Med., **40** : 48.

Carmichael, L.E. St., Gearge, T.D., Sulliva, M.D., and Horsfall, N. (1972). Cornell Vet**. 62**: 653.

Ernϕ, H. and Stipkovits, L. (1973). Acta. Vet. Scand., **14**: 436.

Ghadersohi, A., Coelen, R.J. and Hirst, R.G. (1997). Vet. Microbiol. **56** : 87.

Ghadersohi, A., Hirst, R.G., Forbes-Faulkener, J. and Coelen, R.J. (1999). Vet. Microbiol. **65** : 185.

Gunning, R.F. and Shephered, P.A. (1996). Vet.Rec., **139** : 23.

Hotzel, H., Demuth, B., Sachse, K., Ptlitsch, A. and Pfutzener, H. (1993). Rev. Sci. Tech. Off. Int. Epiz., **12** : 581.

Hotzel, H., Sachse, K. and Pfutzner, H. (1996). J. Appl. Bacteriol., **80** : 505.

Howard, C.J., Thomas, L.H. and Parsons, K.R. (1987). Isr. J. Med. Sci., **23** : 621.

Jasper, D.E. (1977). J. Am. Vet. Med. Assoc., **170** : 1167.

Johansson, K.E., Berg, L.O., Bolske, G., Dniz, S., Mattsson, J., Persson, M. and Petterson, B. (1996). In cost 826, Agric. and Biotech, Luxembourg. Pp. 88.

Mattsson, J.G., Guss, B. and Johansson, K.E. (1994). FEMS Microbiol. Lett., **45** : 325.

Pinbow, C.C. Butler, J.A., Sachse, K., Hotzel, H., Timms, L.L. and Rosenbusch, R.F. (2001). J. Dairy Sci., **84** : 1640.

Simecka, J.W., Davis, J.K., Davidson, M.K., Ross, S.E., Stadtlander, C.T., and Cassel, G.H. (1992). In Maniloff, J., McElhancy, R., Finch, L. and Baseman, J. (Eds.) Mycoplasma : Molecular biology and Pathogenesis. Pp. 391.

Wilson, K. (1987). Preparation of genomic DNA from bacteria. Current protocols in Molecular biology, Unit 2: 4-1. New York, Wiley.