Isolation and molecular identification of *Mycoplasma equigenitalium* from equine genital tracts in northern India

Nehra, K.[1]; Rana, R.[2]; Viswas, K. N.[3]; Arun, T. R.[1]; Singh, V. P.[4]; Singh, A. P.[5*] and Prabhu, S. N.[6]

[1]Graduated from Indian Veterinary Research Institute, Izatnagar, 243122, Bareilly, Uttar Pradesh, India; [2]Referral Lab on Mycoplasma, Division Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar, 243122, Bareilly, Uttar Pradesh, India; [3]Division Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar, 243122, Bareilly, Uttar Pradesh, India; [4]Division of Bacteriology, Indian Veterinary Research Institute, Izatnagar, 243122, Bareilly, Uttar Pradesh, India; [5]Department of Microbiology, College of Veterinary & Animal Sciences (COVs&AH), Pt Deen Dayal Veterinary University (DUVASU), Mathura, 281001, Uttar Pradesh, India; [6]Ph.D. Scholar in Veterinary Pathology, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, 243122, Bareilly, Uttar Pradesh, India

[*]***Correspondence:*** *A. P. Singh, Department of Microbiology, College of Veterinary & Animal Sciences (COVs&AH), Pt Deen Dayal Veterinary University (DUVASU), Mathura, 281001, Uttar Pradesh, India. E-mail: drajay_vet@yahoo.co.in*

(Received 25 Dec 2013; revised version 1 Nov 2014; accepted 22 Nov 2014)

Summary

Although *Mycoplasma equigenitalium* has been implicated in equine reproductive problems, its prevalence is largely unexplored due to the lack of specific diagnostic tests. To address this limitation, the authors developed and optimized species-specific primer pairs that target *M. eguigenitalium rpo*B (RNA polymerase B subunit) gene sequences. The specificity of the PCR assay developed in this study was determined using 12 field isolates including the type strain of *M. equigenitalium* and other *Mycoplasma* species. In the field study, a total of 122 mare and stallion samples comprising of 50 clinical and 72 random samples were subjected to species-specific PCR assay to detect *M. equigenitalium* in equine genital tracts. *Mycoplasma equigenitalium* (MEG) species-specific PCR detected 22.13% positive samples; however, only 9.01% of the samples were found to be positive using the conventional culture technique. The PCR established in this study could be used for rapid, specific and accurate diagnosis of *M. equigenitalium* strains. To the authors' knowledge, this is the first report addressing the development and evaluation of species-specific PCR to detect *M. equigenitalium*.

Key words: Diagnosis, *Mycoplasma equigenitalium*, Polymerase chain reaction, *rpo*B gene

Introduction

Mycoplasma equigenitalium is associated with infertility, endometritis, vulvitis and abortions in mares, and reduced fertility and balanoposthitis in stallions (Moorthy *et al*., 1977; Heitmann *et al*., 1979; Kirchhoff *et al*., 1979; Spergser *et al*., 2002). The impact of *M. equigenitalium* on equine reproductive health is apparently inconsistent, largely because of strain differences in pathogenic potential, host factors and major roles played by other determinants (Bermúdez *et al*., 1992). Nevertheless, *in vitro* studies of experimental infections have reported *M. equigenitalium* to cause ciliostasis and cell degeneration in chicken-embryo tracheal explants (Bermúdez *et al*., 1988; Miller *et al*., 1994).

Identification of *M. equigenitalium* in clinical samples by standard bacterial culture is time consuming and requires complex media, special handling and further testing for species determination. Confirmed identification of *M. equigenitalium* at the species level is then achieved by carrying out digitonin sensitivity tests followed by metabolic inhibition tests which cause growth inhibition with species specific anti-sera, (Taylor-Robinson and Furr, 1997). Presently, only few diagnostic laboratories have specialized facilities and the required expertise to isolate and characterize Mycoplasmas. Therefore, the importance of *M. equigenitalium* infection in equine genital disorders may be underestimated.

PCR based detection of pathogens has recently gained momentum and emerged as an important tool for disease diagnosis. 16S rRNA based PCR targeting species-specific regions in the 16S rDNA and 16S/23S rDNA intergenic spacer (IGS) sequences were used for mollicute detection (Harasawa, 1999; Tang *et al*., 2000; Kong *et al*., 2001). Although 16S rDNA PCR is by far the most widely used technique, it is critically limited as a phylogenetic marker in *Mycoplasma*, because there are two or more 16S rRNA operons in many *Mycoplasma* species showing high interspecies variations (Woese *et al*., 1980; Pettersson *et al*., 1996; Konigsson *et al*., 2002). The *rpo*B gene, or the DNA-dependent RNA polymerase gene, has been proposed as a genome similarity predictor, and an alternative to 16S rDNA gene sequencing for biodiversity studies (Helal *et al*., 2008). A high degree of variation in terms of size and sequence was observed among species by the phylogenetic analysis of *rpo*B sequences of 26 *Mycoplasma* species. The *rpo*B gene was described as a useful target for bacterial identification and phylogenetic studies (Mollet *et al*., 1997). The extreme sequence diversity of *rpo*B gene sequence in the *Mycoplasma* species can be viewed

as a good alternative molecular marker and an interesting choice to differentiate or identify wild types of Mycoplasmas (Kim *et al*., 2003).

The present study aimed to develop a *M. equigenitalium* species-specific PCR for the identification of *M. equigenitalium* directly from clinical samples.

Materials and Methods

Sample collection

Samples were collected from different states in northern parts of India including Rajasthan, Punjab, Haryana, Jammu and Kashmir, Uttar Pradesh and Maharashtra (Fig. 1). Forty eight clinical samples comprising of preputial swabs and washings from stallion and fossa glandis swabs, vaginal discharge/swabs and clitoral fossa swabs were collected from mares subjected to various reproductive disorders, while 9 other samples were collected randomly. Another group of 63 samples (frozen and neat semen) was taken at random from stallions subjected to semen evaluation, while 2 clinical samples were from stallions with balanoposthitis. The swab samples were collected in duplicate, one kept for culture and the other for PCR.

Isolation of *M. equigenitalium*

All samples were cultured in modified PPLO broth media (Carmichael *et al*., 1972). The swabs were squeezed in 2 ml of modified PPLO broth (pH = 7.6-7.8) growth medium containing the following components per liter: 450 ml Hank's growth medium, 450 ml beef heart infusion, 100 ml horse serum, 2.5 ml (10% w/v) thallous acetate (Sigma) and 5 ml (2,00,000 IU/ml) penicillin (Glaxo Laboratories) and 4 ml 1% (w/v) phenol red. Hanks growth medium was prepared by mixing 225 ml Hank's buffered salt solution (HBSS), 5.0 g lactalbumin hydrolysate (LAH) and 1.0 g yeast extract (Difco) in a 1 L final volume.

After inoculating the suspected specimen in modified PPLO broth, 10 fold dilutions were made up to 10^{-4} with PPLO medium. The media were incubated at 37°C for about one week. In case of lack of growth, turbidity or any pH change in liquid media, it was further subcultured and observed for another week to 10 days. Likewise, frozen semen, neat semen and preputial washing samples were diluted and processed in modified PPLO broth (pH = 7.6-7.8). Cultures were observed daily for colour change. Suspected growth in the medium with a faint translucent turbidity was confirmed by transfering about 0.2 ml onto a solid PPLO agar medium and incubation in micro-aerophilic conditions at 37°C for one week to 10 days. The growth of *M. equigenitalium* isolates was confirmed using digitonin sensitivity and growth inhibition tests conducted with specific standard antiserum, glucose fermentation, phosphatase activity, arginine hydrolysis, liquification of coagulated serum, tetrazolium reduction and film and spot formation tests. Biochemical tests were performed according to the methods described by Aloutto *et al*. (1970).

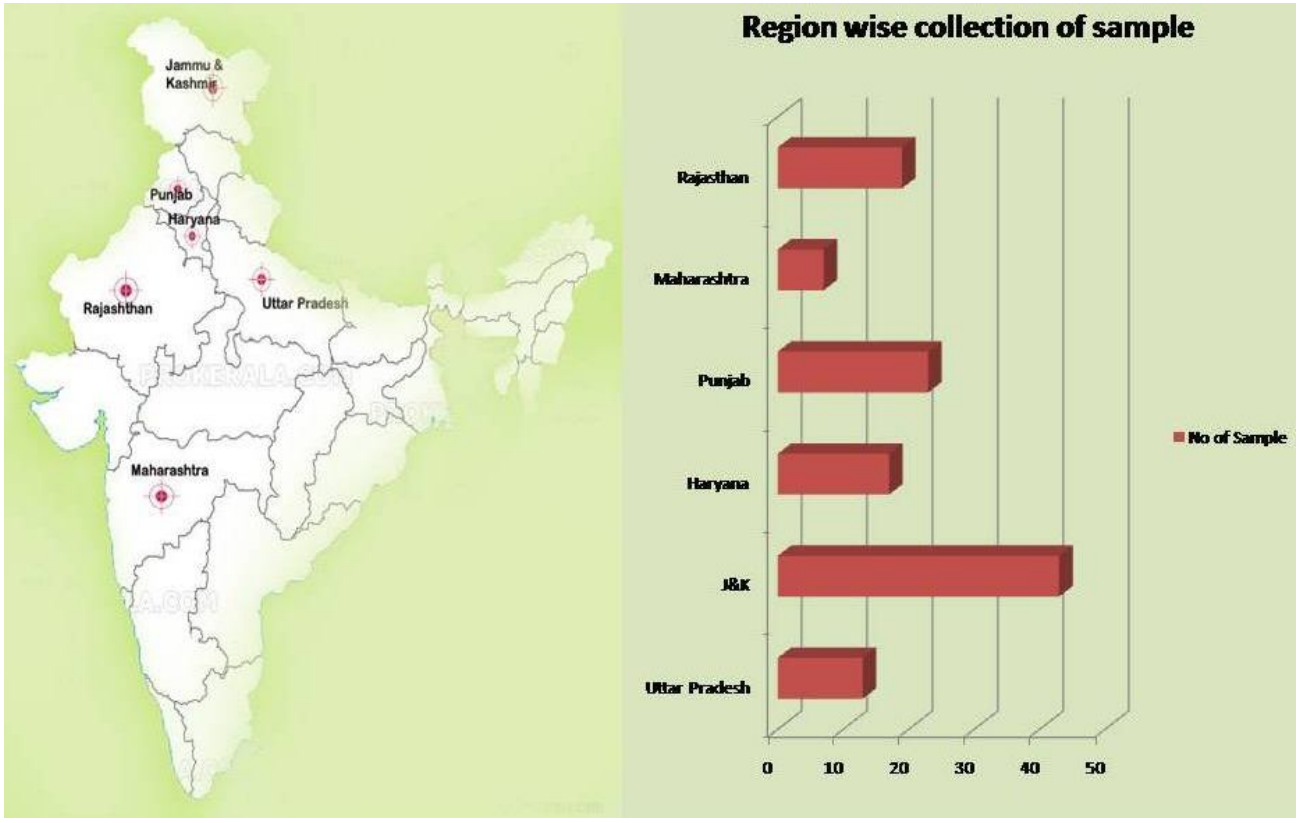

Fig. 1: Source and number of clinical and random samples collected from different states of India

Sample preparation for PCR analysis

Bacterial strains

Genomic DNAs from bacterial cultures were prepared as described by Garcia *et al*. (1988). Briefly, cells were suspended in 20 mM Tris-HCl (pH = 8.0) containing 10 mM EDTA, 50 mM glucose, and 2 mg lysozyme per ml. After the addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.5%, the DNA was incubated with 0.1 mg of proteinase-K per ml at 55°C overnight. The DNA was phenol extracted, ethanol precipitated, and suspended in distilled water. Concentrations of genomic DNA isolated from different bacterial strains were measured using a NanoDrop-8000 spectrophotometer (Thermo Fisher scientific, USA) and estimated to be in the range of 1.8-4.6 µg/ml, with the average of 2.56 µg/ml.

Genital swabs

For sample preparation, swabs were suspended in 200 µL sterile phosphate buffered saline (PBS). The 200 µL suspensions and 100 µL semen or pre-ejaculatory fluid samples were centrifuged at 14,000 g for 20 min. Pellets were re-suspended in 385 µL of a lysis buffer containing 20 mM Tris-HCl (pH = 8.0), 1 mM ethylene diamine tetra acetate (EDTA), 30 mM dithiothreitol, 0.5% sodium dodecyl sulphate (SDS) and 0.4 mg proteinase K/ml. The mixture was incubated with agitation overnight at 55°C. DNA was isolated by cetyl trimethyl ammonium bromide (CTAB) extraction as previously described by Kaltenböck *et al*. (1997). Phenol-chloroform extraction was followed by precipitation with 3 M sodium acetate and 96% ethanol. The DNA was pelleted, washed with 70% ethanol and re-suspended in 50 µL distilled water.

Primer design

Published *rpo*B gene sequences of Mollicutes were retrieved from GenBank (NIH, Bethesda, MD), and homology trees were generated using Lasergene® software (DNASTAR, Madison, Wis.). Multiple sequence alignments (MSA) were performed using the Clustal W algorithm. The alignments were visually scanned for unique non-homologous DNA sequences between clusters of highly conserved DNA sequences representing *Mycoplasma* species (Fig. 2). PCR primers were developed based on these sequences while maximizing established primer design criteria. Potential primer sequences were checked for lack of sequence homology with other prokaryotic and eukaryotic DNA sequences using a BLAST (NCBI, Bethesda, MD) search.

M. equigenitalium species-specific PCR

Specific primers directed against the *rpo*B gene of *M. equigenitalium* were designed and used to amplify a 922 bp product. The oligonucleotide sequence for forward primer MEG-(F) was 5′-AGT GAG GGT GAG CTT TCG CTT GG-3′ and the reverse primer was MEG-(R) 5′-ATG GCC CAA TGC TCC GTG CG-3′. The amplification reaction was performed in a 25 µL volume containing 0.2 mM dNTP's (Fermentas, GmBH, Germany), 15 pmol of each primer (GCC Biotech, India), 1 unit Taq polymerase (Fermentas) and 2 µl of the DNA template. The PCR program was performed for 30 cycles with denaturation (94°C for 1 min), annealing (55°C for 45 s) and extension (72°C for 1 min) in an automated DNA thermal cycler (Eppendorf Mastercycler®, USA). PCR products were analyzed by 1.5% agarose gel electrophoresis. A negative control (the PCR reagents excluding the DNA template) and a positive control, using DNA from *M. equigenitalium* (NCTC 10176), were also included.

Specificity and sensitivity of primers

To determine primer specificities, strains of *Mycoplama* species widely associated with equine reproductive tract including *M. equigenitalium* T37 (NCTC 10176), *Acholeplasma laidlawi* (NCTC 10171) *Acholeplasma equifetale* (NCTC 10116), *M. subdolum,* as well as field isolates of nine *M. equigenitalium*, six *A. laidlawi* and two *A. equifetale* were used in the study. *M. agalactiae*, *M. Capri* and *M. felis* isolates obtained from the Referral Laboratory on Mycoplasma, Division of Bacteriology, Indian Veterinary Research Institute, India, were also used.

Genomic DNA from 12 *M. equigenitalium* strains and other *Mycoplasma* species belonging to equine,

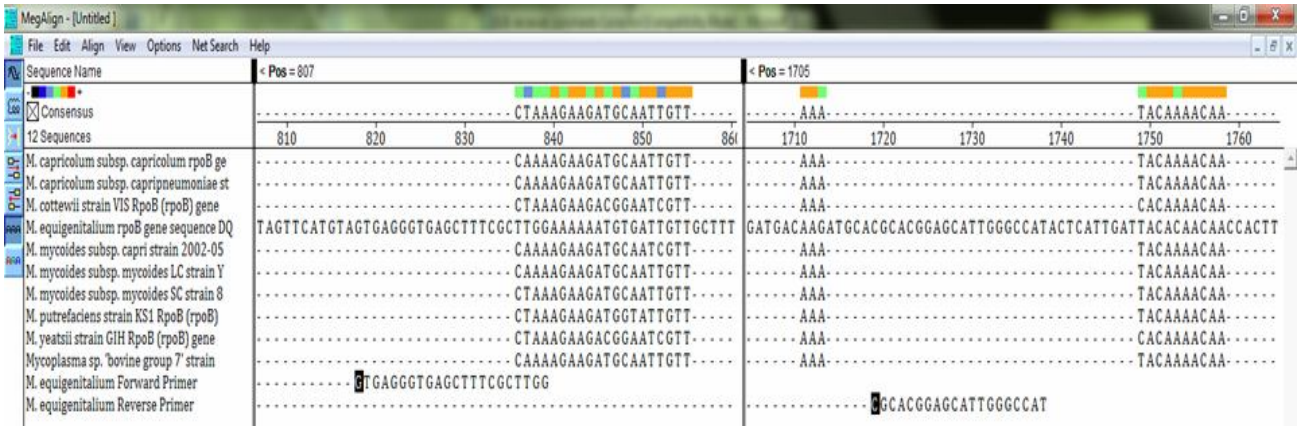

Fig. 2: Alignment report of *rpo*B genes in different *Mycoplasma* species

feline, and ruminant isolates were tested using the *M. equigenitalium* species specific primer (MESSP) pair. In addition, the specificity of the MESSP pair was tested with 122 clinical swabs. MESSP sensitivity was evaluated by qualitative PCR analysis. The genomic DNA of *M. equigenitalium* culture was isolated and quantified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Purified DNA was 10 fold serially diluted, each dilution being used as a template and tested by MESSP PCR to determine lower detection limits.

Results

Clinical evaluation

A total of 122 genital sample swabs were cultured to isolate the bacterium of 50 clinical and 72 random samples. The results are summarized in Table 1. *M. equigenitalium* was isolated from 11 clinical samples, whereas 27 others, including the culture-positive samples, were found to be positive using the direct PCR. The highest percentage of culture positive samples (16.66%) belonged to abortion cases, followed by 15.78%, 13.04%, and 5.55%, from metritis, repeat breeders and random samples respectively. Our results from the direct PCR showed the highest prevalence of *M. equigenitalium* (47.82%) in the repeat breeders, followed by, 36.84%, 16.66%, 11.11%, from, metritis, abortion and random samples respectively.

Primer specificity and sensitivity of the PCR assay

Specificities of the selected PCR primers were tested using DNA from one reference strain of *M. equigenitalium* and 11 *M. equigenitalium* field isolates. An amplified product of 922 base pairs was generated with all tested isolates (Fig. 3a). The 922 bp *rpo*B PCR product was sequenced by an automated DNA sequencer (Chromus Biotech, Bangalore), and the gene sequence was submitted to GenBank (GenBank accession No.: KC485074.1). DNA isolated from a variety of other *Mycoplasma* species (as explained in the previous section) was also subjected to PCR, but none amplified the specific target sequence (Fig. 3b). Lower limits required to detect the *M. equigenitalium* isolated DNA by PCR were examined. The results showed that 50 pg of purified DNA in the reaction mixture was the minimum needed to obtain a detectable PCR product (Fig. 4).

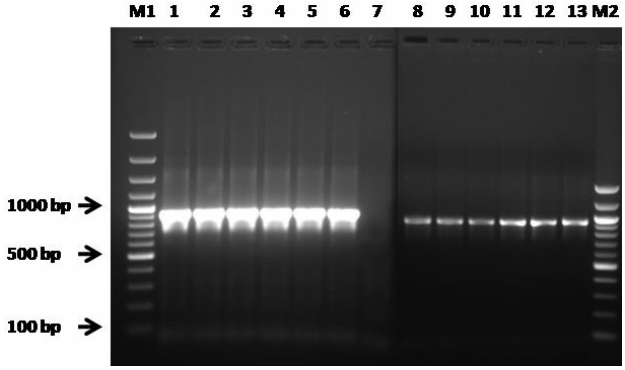

Fig. 3a: Species-specific PCR amplification for *Mycoplasma equigenitalium*. Lane M1 and M2: 100 bp DNA ladder marker, Lane 1-5 and 8-13: DNA of 11 *M. equigenitalium* isolates from clinical samples, Lane 6: Reference *M. equigenitalium* DNA, and Lane 7: Negative control

Discussion

Mycoplasmas have been isolated from uterus, cervix and vaginas of mares, the clitoral fossa being most likely the "ecological niche". In stallions, the preferred isolation site is the fossa glandis, apart from other common sources like urethra, prepuce, penis, and fresh or frozen semen (Lemcke and Kirchhoff, 1979; Bermúdez *et al*., 1987; Spergser *et al*., 2002). Hence, clitoral fossa and fossa glandis swabs from mares and stallions were chosen in this study as preferred clinical samples for obvious reasons. The impending pathogenic role of *M. equigenitalium* in reproductive dysfunctions is yet to be recognized clearly, and further studies are required to ascertain its pathogenic potential (Bermúdez *et al*., 1988; Miller *et al*., 1994). Information regarding the occurrence and significance of *M. equigenitalium* in equine reproductive disorders is scarce in scientific veterinary literature, largely because the isolation of the organisms is very tedious, laborious, and time consuming. At the same time, serological tests are not very sensitive and specific. PCR based diagnoses can thus be used as effective tools for detecting pathogens in clinical samples, either directly or in conjunction with culture techniques. The aim of this study was to develop

Table 1: Percentage of culture and PCR positive samples and distribution of *M. equigenitalium* among samples collected from clinically affected and random animals

Sample	Sex	Clinical condition	No of sample	Culture positive sample	PCR positive sample
Clinical	Female	Abortion	6	N=1 (% 16.66)	N=1 (% 16.66)
		Repeat breeder, infertility	23	N=3 (% 13.04)	N=11 (% 47.82)
		Metritis	19	N=3 (% 15.78)	N=7 (% 36.84)
	Male	Balanoposthitis	2	0	0
	Total		50	N=7 (% 14.00)	N=19 (% 38.00)
Random	Female	Apparently normal	9	0	N=4 (% 44.45)
	Male	Apparently normal	63	N=4 (% 6.35)	N=4 (% 6.35)
	Total		72	N=4 (% 5.56)	N=8 (% 11.11)
Grand total			122	N=11 (% 9.01)	N=27 (% 22.13)

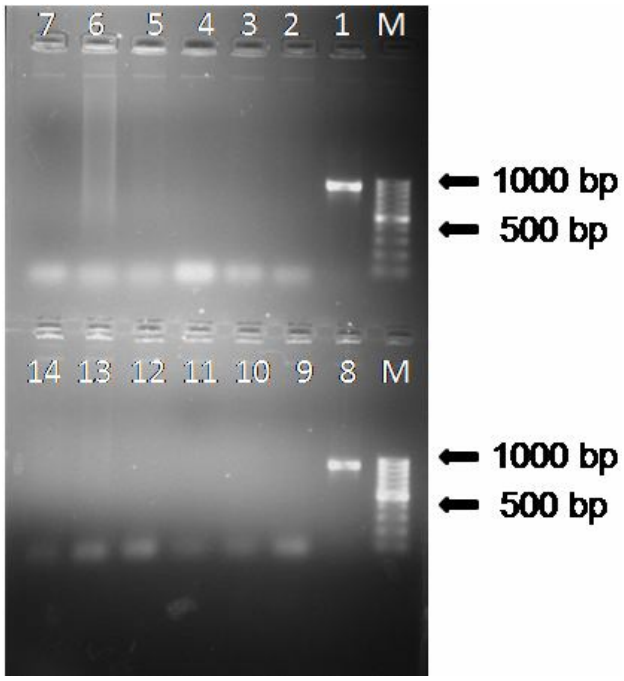

Fig. 3b: Specificity of *Mycoplasma equigenitalium* species specific PCR. Lane M: 100 bp ladder, Lane 1 and 8: *Mycoplasma equigenitalium*, Lane 2: *Acholeplasma equifetale*, Lane 3: *Acholepasma laidlawi*, Lane 4: *Mycoplasma bovis*, Lane 5: *Mycoplasma mycoides* subsp. capri, Lane 6: *Mycoplasma agalactiae*, Lane 7: *Mycoplasma bovirhinis*, Lane 9: *Brucella abortus*, Lane 10: *E. coli*, Lane 11: *Pasteurella multocida*, Lane 12: *Arcobacter butzleri*, Lane 13: *Listeria monocytogens*, and Lane 14: *Leptospira interrogans*

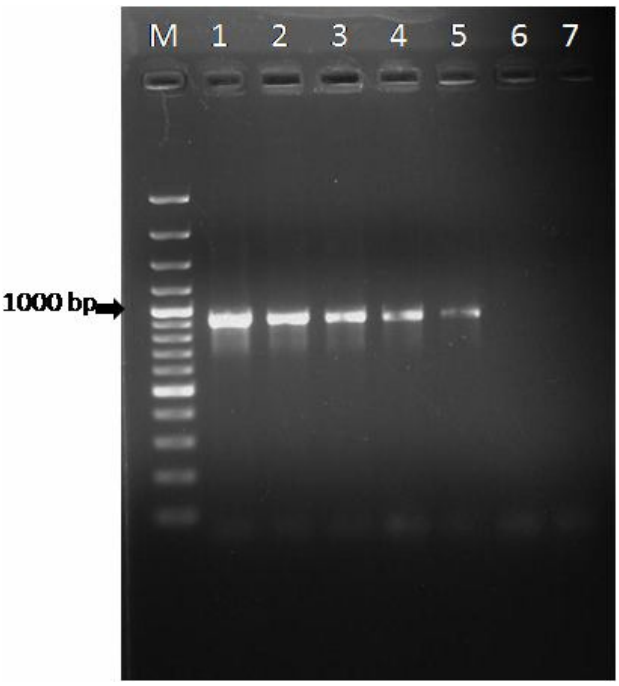

Fig. 4: Gel electrophoresis of PCR products amplified from serial dilutions of *Mycoplasma equigenitalium* DNA. Lane M: 100 bp ladder, Lane 1: 500 ng, Lane 2: 50 ng, Lane 3: 5 ng, Lane 4: 500 pg, Lane 5: 50 pg, Lane 6: 5 pg, and Lane 7: 500 fg

a novel PCR assay specific to *M. equigenitalium* and to apply it to detect *M. equigenitalium* DNA taken directly from clinical samples.

In an attempt to develop a species-specific PCR for *M. equigenitalium*, the *rpo*B gene was targeted for the construction of specific primers. The PCR reaction using the *rpo*B gene-based primer set described here was found to be very specific since a precise amplification of 922 bp fragments was obtained using the l2 *M. equigenitalium* isolates tested. The same test gave negative results with phylogenetically related *Mycoplasma* species other than *M. equigenitalium*, some of which are known to be present in the genital tracts of horses.

As evident from the results presented in Table 1, the sensitivity of the PCR assay is superior to that of the conventional *Mycoplasma* culture technique. However, a combination of culture and PCR is to date the most sensitive approach. Of the tested samples, 9.01% are positive as shown by the conventional culture technique, while 22.13% resulted positively using the *M. equigenitalium* species specific PCR. The results clearly indicate that a considerable number of horses carrying *M. equigenitalium* remain unidentified by current culturing techniques, meaning that *M. equigenitalium* infection is more prevalent and that its importance has probably been underestimated.

Both culture- and PCR-based studies show the maximum number of positive clinical samples to belong to cases of metritis and repeat breeders, highlighting the subclinical involvement of these pathogens. Khurana *et al*. (2004) report an occurrence of 15.9% seroprevalence of antibodies to *M. equigenitalium* amongst repeat breeder and 41.2% seroprevalence in cases of metritis by *M. equigenitalium* specific ELISA, which is in close agreement with the results of the present study.

A significant number (6.35%) of apparently healthy stallions without any sperm abnormalities were found to be positive for *M. equigenitalium*, hence its clinical relevance in semen should not be over-interpreted. This may be due to the colonisation of *M. equigenitalium* in non-testicular tissues, which serves as a source of contamination during ejaculation. This is in agreement with reports on the lack of correlation between sperm abnormalities and isolation of Mycoplasmas or other potentially pathogenic bacteria in stallions and bulls (Malmgren *et al*., 1998; Spergser *et al*., 2002).

The analysis of the findings from the present study implies that there is indeed a prevalence of *M. equigenitalium* infections in India. The use of the *rpo*B gene-based species-specific *M. equigenitalium* PCR described here can help increase the number of laboratories detecting *M. equigenitalium* in equine genital infections and enhance current knowledge on the importance and distribution of *M. equigenitalium*.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Acknowledgements

We sincerely acknowledge the help extended to us by Dr. R. A. J. Nicholas, VLA Weybridge, UK for providing the standard genomic DNA and/or equine origin culture. We also wish to acknowledge the help given to us by Dr. R. K. Singh, Director of the National Research Centre on Equines and VTCC, Hisar, India for his valuable guidance and suggestions. We also thank the Director of the Indian Veterinary Research Institute, Izatnagar, India for financial assistance to carry out this work.

References

Aloutto, BB; Wittler, RG; Williams, CO and Faber, JE (1970). Standardized bacteriological techniques for characterization of *Mycoplasma* species. Int. J. Syst. Bacteriol., 20: 35-38.

Bermúdez, VM; Miller, RB; Johnson, WH; Rosendal, S and Ruhnke, L (1987). The prevalence of *Mycoplasma* spp. and their relationship to reproductive performance in selected equine herds in southern Ontario. J. Reprod. Fertil. Suppl., 35: 671-673.

Bermúdez, VM; Miller, RB; Rosendal, S; Fernando, MA; Johnson, WH and O'Brien, PJ (1992). Measurement of the cytotoxic effects of different strains of *Mycoplasma equigenitalium* on the equine uterine tube using a calmodulin assay. Can. J. Vet. Res., 56: 331-338.

Bermúdez, VM; Miller, RB; Rosendal, S and Johnson, WH (1988). *In vitro* cytopathic effect of *Mycoplasma equigenitalium* on the equine uterine tube. In: Stanek, G; Cassell, GH; Tully, JH and Whitcomb, RF (Eds.), *Recent advances in mycoplasmology*. *Proceedings of the Seventh Congress of the International Organisation of Mycoplasmology*. Baden, near Vienna, 1988. Suppl. 20: 419-428.

Carmichael, LE; George, TD; Sulliva, MD and Horsfall, N (1972). Isolation and characterization studies on an ovine *Mycoplasma* responsible for proliferative interstitial pneumonia. Cornell. Vet., 62: 654-679.

Garcia-Arribas, ML; Plaza, CJ; de la Rosa, MC and Mosso, MA (1988). Characterization of *Bacillus cereus* strains isolated from drugs and evaluation of their toxins. J. Appl. Bacteriol., 64: 251-264.

Harasawa, R (1999). Genetic relationships among Mycoplasmas based on the 16S/23S rRNA spacer sequence. Microbiol. Immunol., 43: 127-132.

Heitmann, J; Kirchhoff, H; Petzoldt, K and Sonnenschein, B (1979). Isolation of Acholeplasmas and Mycoplasmas from aborted horse fetuses. Vet. Rec., 104: 350.

Helal, M; El-Gindy, H; Gaeta, G and Sintchenko, V (2008). High performance multiple sequence alignment algorithms for comparison of microbial genomes. In: *Proceedings of 19th International Conference on Genome Informatics (GIW 2008)*. Gold Coast, Australia, 2008.

Kaltenböck, B; Schmeer, N and Schneider, R (1997). Evidence for numerous omp1 alleles of porcine *Chlamydia trachomatis* and novel *Chlamydial* species obtained by PCR. J. Clin. Microbiol., 35: 1835-1841.

Khurana, SK; Garg, DN and Kumar, A (2004). Sero-prevalence of *Mycoplasma equigenitalium* antibodies in equines by indirect ELISA. J. Immunol. Immunopathol., 6: 77-78.

Kim, KS; Ko, KS; Chang, MW; Hahn, TW; Hong, SK and Kook, YH (2003). Use of *rpo*B sequences for phylogenetic study of *Mycoplasma* species. FEMS Microbiol. Lett., 226: 299-305.

Kirchhoff, H; Naglić, T and Heitmann, J (1979). Isolation of *Acholeplasma laidlawii* and *Mycoplasma equigenitalium* from stallion semen. Vet. Microbiol., 4: 177-179.

Kong, F; James, G; Gordon, S; Zelynski, A and Gilbert, GL (2001). Species-specific PCR for identification of common contaminant mollicutes in cell culture. Appl. Environ. Microbiol., 67: 3195-3200.

Konigsson, MH; Bolske, G and Johansson, KE (2002). Intraspecific variation in the 16S rRNA gene sequences of *Mycoplasma agalactiae* and *Mycoplasma bovis* strains. Vet. Microbiol., 85: 209-220.

Lemcke, MR and Kirchhoff, H (1979). *Mycoplasma subdolum*, a new species isolated from horses. Int. J. Syst. Bacteriol., 29: 42-50.

Malmgren, L; Engvall, EO; Engvall, A and Albihn, A (1998). Aerobic bacterial flora of semen and stallion reproductive tract and its relation to fertility under field conditions. Acta Vet. Scand., 39: 173-182.

Miller, R; Chelmonska-Soyta, A; Smits, B; Foster, R and Rosendal, S (1994). Ureaplasma diversum as a cause of reproductive disease in cattle. Vet. Clin. N. Am. Food A., 10: 479-490.

Mollet, C; Drancourt, M and Raoul, D (1997). *rpo*B sequence analysis as a novel basis for bacterial identification. Mol. Microbiol., 26: 1005-1011.

Moorthy, ARS; Spradbrow, PB and Eisler, MED (1977). Isolation of Mycoplasmas from the genital tract of horses. Aust. Vet. J., 53: 167-169.

Pettersson, B; Leitner, T; Ronaghi, M; Bolske, G; Uhlen, M and Johansson, KE (1996). Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J. Bacteriol., 178: 4131-4142.

Spergser, J; Aurich, C; Aurich, JE and Rosengarten, R (2002). High prevalence of Mycoplasmas in the genital tract of asymptomatic stallions in Austria. Vet. Microbiol., 87: 119-129.

Tang, J; Hu, M; Lee, S and Roblin, R (2000). A polymerase chain reaction based method for detecting *Mycoplasma/Acholeplasma* contaminants in cell culture. J. Microbiol. Methods. 39: 121-126.

Taylor-Robinson, D and Furr, PM (1997). Genital *Mycoplasma* infections. Mid. Eur. J. Med., 109: 578-583.

Woese, CR; Maniloff, J and Zablen, LB (1980). Phylogenetic analysis of the Mycoplasmas. P. Natl. Acad. Sci., 77: 494-498.