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Comparison of Serum Antibody ELISA with Antigen Detection Assay for Diagnosis of *Mycoplasma equigenitalium* Infection in Equines

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ABSTRACT

Mycoplasmas cause varied respiratory and genital problems in man and animals. They have great economic importance because of their ability to resist common antibiotics and to survive the cryopreservation of semen during artificial insemination. *Mycoplasma equigenitalium* is associated with reproductive problems of equines. Though several serological tests can be used for measurement of antibodies to equine-mollicutes, the valuable tests are complement fixation and ELISA. However, ELISA is serotest of choice to detect antibodies in equines against *Mycoplasma* because of its rapidity, sensitivity and specificity. Capture ELISA could be a useful method for detecting *M. equigenitalium* antigen in swabs, fluids and tissues where it may not be possible by cultural methods. Here, in the present study, we have done antibody detection by ELISA and antigen detection by capture ELISA. The seroprevalence of *M. equigenitalium* by indirect ELISA was found to be 20.2% among equines with reproductive problems and only 3.7% in apparently healthy equines. In comparison 4.8% equines with reproductive problems were culturally positive and 9.6% equines were detected positive by capture ELISA. No apparently healthy equid was found positive either culturally or by capture ELISA assay. It shows that indirect antibody ELISA provides a higher possibility of *M. equigenitalium* antibody detection for mass screening and is less cumbersome than cultural or antigen detection method by capture ELISA.

Key words: *Mycoplasma equigenitalium*, seroprevalence

INTRODUCTION

Mycoplasmas are the smallest free living organisms lacking cell wall. They are associated with mucosal surfaces and are responsible for varied respiratory and urogenital diseases of man and animals. They are reported to be isolated from vagina, cervix, uterus and clitoral fossa of mares and from prepuce, urethra and semen of stallions with reproductive problems as well as apparently healthy equines (Garg, 1999). *Mycoplasma equigenitalium* was first isolated from cervix of mares (Kirchhoff, 1978), later this organism was reported to be isolated from other sites of reproductive tract of equines also (Kirchhoff *et al*., 1979, 1980; Naglic *et al*., 1980; Mani *et al*., 1985; Bermudez *et al*., 1987; Copes *et al*., 1995; Khurana and Garg, 2001). Seroprevalence of *M. equigenitalium* has also been determined from India (Khurana and Malik, 2009).

Mycoplasmal infections are known to increase the level of serum antibodies which are detected by various serological tests. The valuable tests used for measurement of antibodies to

equine-mollicutes are complement fixation and enzyme linked immune sorbent assay (ELISA). However, ELISA is considered a serotest of choice because of its rapidity, sensitivity and specificity.

Ammar *et al*. (1980) tested sera samples from 58 aborting thoroughbred and other mares in Germany for antibodies against *M. equigenitalium*, *M. equirhinis*, *M. subdolum*, *M. pulmonis*, *M. felis*, *A. laidlawii*, *A. hippikon* and *A. equifetale* by ELISA. Kirchhoff *et al*. (1982) found high percentage for antibodies against *M. equigenitalium* due to the fact that *M. equigenitalium* has been found more frequently in genital tract of mares and commonly in aborted fetuses and horse semen.

Rosendal *et al*. (1986) found that indirect haemaggtination and metabolic-inhibition test were suitable for diagnosis of *M. felis* infection in horses.

Wood *et al*. (1997) observed *M. felis* by indirect haemagglutination assay in 22 of 25 horses in a respiratory disease outbreak.

Dieckmann *et al*. (2010) detected haemotropic mycoplasmas for the first time from the blood of two horses suffering from poor performance and anaemia and were found to be closely related to *M. haemofelis* and *Candidatus* Mycoplasma haemobos. Further, Dieckmann *et al*. (2012) correlated the occurrence of haemotropic mycoplasmas with haematogical findings.

Very little work has been done on seroprevalence of equine mycoplasmas, however, significant work has been done on human mycoplasmas including agglutination screening test (Sobieszczanska *et al*., 2014), IgM and IgG ELISA (Noorbakhsh *et al*., 2013).

Since, no systematic work was done in past to know the extent of *M. equigenitalium* prevalence, a study was undertaken to determine the prevalence of *M. equigenitalium* among apparently healthy as well as diseased equine population by ELISA.

MATERIALS AND METHODS

Determination of seroprevalence of *Mycoplasma equigenitalium* by indirect-ELISA: The serum samples collected from equines with various reproductive disorders (repeat breed, metritis, balanoposthitis, abortion) and apparently healthy were screened for *M. equigenitalium* antibodies titre using indirect-ELISA (Boothby *et al*., 1981) with some modifications.

Antigen: The freeze-dried culture of *M. equigenitalium* (NCTC 10176/T-37) was reconstituted in 0.5 mL of SLM, incubated at 37°C for 4-5 days and a uniform milky turbidity throughout the medium was observed. The culture was grown in increasing volumes of SLM with final volume of 1 L. The resultant growth was centrifuged at 10000×g at 4°C for 30 min. The pellet of organisms was washed with PBS three times (pH 7.2) and suspended in 10 mL PBS and kept at 4°C. This suspension was sonicated at 180-200 watts for 3 cycles of 4 min each and protein content was estimated (Lowry *et al*., 1951). It served as stock antigen for immunization.

Substrate: A solution of orthophenylene diamine dihydro-chloride (OPD, Sigma, USA) prepared as per manufacturer's recommendations.

Optimization of antigen and conjugate: Disposable microtitre plates (Linbro, typeIS-FB-96, Flow Lab, UK) were used as a vehicle for *M. equigenitalium* antigen. The optimum concentration of antigen was determined by chequer-board titration. Stock antigen of *M. equigenitalium* (protein conc 4.06 mg mL^{-1}) was diluted to 1:50, 1:100, 1:150, 1:200 in carbonate-bicarbonate buffer (pH 9.6). The conjugate was diluted to 1:1000, 1:2000, 1:3000, 1:4000 in 0.1 M PBST and known

positive and known negative sera were diluted two folds from 1:10-1:1280. The ELISA was performed using different dilutions of antigens, conjugate and known positive (Table 1) and negative sera. The optimum dilution of coating antigens was found to be 1:100. In the present study, the optimum dilution of conjugate was observed as 1:2000.

Procedure: The microtitre plates wells were coated at 0.05 mL (2.03 μg protein/well) of optimum dilution (1:100) of *M. equigenitalium* antigen for overnight at 37°C. The coated plates were washed with 0.1 M PBST (pH 7.4), air dried and stored at 4°C in polythene packs and used within a week without loss of activity.

The test serum diluted serially two folds from 1:10-1:1280 in 0.1 M PBS was added to each well (0.05 mL) and kept at 37°C for 1 h. Unbound serum was removed by washing the plates three times with PBST. Optimum dilution (1:2000) of anti-horse-IgG conjugate in PBST (pH 7.6) was added to each well (0.05 mL) and again incubated at 37°C for 1 h. Plates were washed three times with PBST and 0.1 mL freshly prepared OPD substrate was added. The plates were incubated for 5 min at 37°C and absorbance was measured at 492 nm in Oraganon Teknika Reader 530. The dilutions of serum ranged from 1:10-1:1280. The maximum absorbance value for known negative serum was considered as cut-off value for calculating end titre of the sera tested in that plate. The cut-off values in this study ranged from 0.400-0.823. The ELISA titre of 1:40 and above was considered as positive.

RESULTS AND DISCUSSION

Seroprevalence of *Mycoplasma equigenitalium* antibodies in equine by indirect-ELISA: Two hundred nineteen serum samples from diseased as well as from healthy equines were examined for *M. equigenitalium* ELISA antibodies by indirect-ELISA. Result of *M. equigenitalium* antibodies amongst 84 equines with various reproductive disorders (63 repeat breed, 17 metritis, 2 abortion, 2 balanoposthitis) and 135 apparently healthy equines is given in Table 2.

Table 1: Immunization schedule for raising hyperimmune serum/ known positive serum against *Mycoplasma equigenitalium* in donkey foal (equine)

Weeks	Inoculum*	Distribution and site
1/7	7.5 mL killed antigen+7.5 mL CFA	15 mL distributed subcutaneously at prescapular region
4/7	7.5 mL killed antigen	7.5 mL distributed subcutaneously at prescapular region
2	7.5 mL killed antigen+7.5 mL CFA	15 mL distributed subcutaneously at prescapular region
4	4.5 mL killed antigen	4.5 mL intravenously
8	7.5 mL live antigen (CFU 2X 10 mL)	7.5 mL distributed subcutaneously at prescapular region
9	7.5 mL live antigen (CFU 2X 10 mL)	7.5 mL distributed subcutaneously at prescapular region

*Stock antigen diluted to Brown's opacity tube no. 10 with PBS (pH 7.2), ** CFA: Complete Freunds's adjuvant (Difco, USA), Enzyme-antiglobulin conjugate: Horse-radish peroxidase labelled rabbit anti-horse-IgG (Prod cat no. A9292. Sigma, USA)

Table 2: Detection of *Mycoplasma equigenitalium* by culture and ELISA (capture, indirect)

Reproductive health status of equines with total numbers in parentheses	Equines positive for *Mycoplasma equigenitalium*			
	Culture	Capture-ELISA	Indirect-ELISA	Indirect-ELISA+capture+ELISA
(A) Diseased (84)				
Repeat breed (63)	2 (3.2)*	4 (6.4)	10 (15.8)	3 (4.7)
Metritis (17)	2 (11.8)	4 (23.6)	7 (41.1)	3 (17.7)
Aborted fetus (2)	0 (0)	0 (0)	0 (0)	0 (0)
Balanoposthitis (2)	0 (0)	0 (0)	0 (0)	0 (0)
Total	4 (4.8)	8 (9.6)	17 (20.2)	6 (7.1)
(B) Apparently Healthy (135)	0 (0)	0 (0)	5 (3.7)	0 (0)
Grand total (219)	4 (1.8)	8 (3.6)	22 (10)	6 (2.7)

*Values in parentheses indicate percentage

Twenty three out of 84 equines (27.4%) with reproductive disorders (63 repeat breed, 17 metritis, 2 abotred fetus, 2 balanoposthitis) had *M. equigenitalium* antibodies with ELISA titre ranging from 1:10-1:640, whereas eight out of 135 (5.9%) apparently healthy equines had *M. equigenitalium*-ELISA antibodies with a titre range from 1:10-1:160. Considering a titre of ≥1:40 as specific *M. equigenitalium*-ELISA as titre, 17 out of 84 (20.2%) diseased equines proved positive. Disease-wise specific seroprevalence of *M. equigenitalium* in equines ranged from 0- 41.2% (0% abortion for and balanoposthitis, 15.9% for repeat breed and 41.2% for metritis) (Table 2). Observations on apparently healthy equines revealed that specific ELISA *M. equigenitalium* antibodies were present in 5 of 135 (3.7%) equines, Table 2.

The results of indirect ELISA have suggested a higher seroprevalence of specific *M. equigenitalium* in diseased equines (20.2%) than those in healthy equines (3.7%) (Table 2). Table 3 shows seroprevalence of *M. equigenitalium* ELISA antibodies in equines with various reproductive strata.

Overall prevalence of *Mycoplasma equigenitalium* in equines: The prevalence of *M. equigenitalium* in equines population under study was investigated by employing three methods viz. cultural detection, capture-ELISA and indirect-ELISA, the results of which are summarized in Table 1.

Culturally, *M. equigenitalium* were isolated from four of 84 (4.8%) equines with reproductive disorders; two from repeat breed and two from metritis cases. However, apparently healthy equines population of 135 proved culturally negative for *M. equigenitalium*. Capture-ELISA detected *M. equigenitalium*-Ag in 8 of 84 (9.6%) diseased equines but not in 135 healthy equines. *Mycoplasma equigenitalium* ELISA-Ab were detected in 20.2% diseased and 3.7% healthy equine population.

The analysis of results indicated that all the four 4 cases of equine-reproductive disorders which were culturally positive for *M. equigenitalium* also proved positive by capture-ELISA and indirect-ELISA. *Mycoplasma equigenitalium* was detected only by indirect-ELISA in 3.7% of healthy equine population but neither by cultural nor by capture-ELISA. The prevalence of *M. equigenitalium* antibodies was much higher (10%) in comparison to detection by culture (1.8%) and capture ELISA (3.6%). However, there are hardly any previous studies of this kind for comparative analysis. Khurana and Malik (2009) found overall seroprevalence of *M. equigenitalium* among apparently healthy indigenous equine population in India to be 5.96% with a range of 0-19.0% in different States of the country (Haryana 9.6%, Rajasthan 7.2%,

Table 3: Seroprevalence of *Mycoplasma equigenitalium* ELISA antibodies in equines with various reproductive state

	Equines examined with numbers in parentheses					
ELISA titre	Apparently healthy (135)	Repeat breed (63)	Metritis (17)	Aborted foetus (2)	Balanoposthitis (2)	Total diseased (84)
0	127 (94.0)*	48	9	0	0	57 (67.0)
1:10	2 (1.5)	3	1	0	0	4 (4.8)
1:20	1 (0.74)	2	0	0	0	2 (2.4)
1:40	3 (2.25)	0	2	0	0	2 (2.4)
1:80	1 (0.74)	4	3	0	0	7 (8.3)
1:160	1 (0.74)	2	1	0	0	3 (3.6)
1:320	0 (0)	3	1	0	0	4 (4.8)
1:640	0 (0)	1	0	0	0	1 (1.2)
ELISA +**	5 (3.7)	10 (15.9)	7 (41.2)	0 (0)	0 (0)	17 (20.2)
ELISA -	130 (96.3)	53 (84.1)	10 (58.8)	2 (100)	2 (100)	67 (79.8)

*Values in parentheses indicate percentage, **A titre of 1:40 and above was considered ELISA positive for *Mycoplasma equigenitalium*

Uttaranchal 2.0%, Karnataka 2.3%, Punjab 10.4%; Uttar Pradesh 3.4%, Gujarat 0%, Andhra Pradesh 0%, Maharashtra 2.9%, West Bengal 0%, Tamil Nadu 0%, Meghalaya, 19.0%, Jammu and Kashmir 7.8%, Delhi 0%, Himachal Pradesh 5.5%, Bihar 3.3% and Madhya Pradesh 4.0%). Chang *et al*. (2014) have compared real-time polymerase chain reaction with serological tests. Respiratory samples from two hundred ninety children with *Mycoplasma pneumonia* clinically were tested for *M. pneumoniae* by RT-PCR. Fifty-four children (19%) were found positive. Meanwhile, of the 63% (182/290) of these children, 44 (24%) were found to be positive for immunoglobulin M (Chang *et al*., 2014). Bacterial loads of *M. pneumoniae* could not be correlated with clinical outcomes. However, detailed studies incorporating both serological methods and antigen detection methods including capture ELISA and PCR are required to increase the possibility of detection of *M. equigenitalium* from equines with a aim of timely treatment of disease and to prevent its spread.

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