Research Article

Detection of *Mycoplasma equigenitalium* from Equines by Capture-ELISA

Sandip Kumar Khurana[1*], Daya Nand Garg[2], Yarvendra Singh[2]

[1]National Research Centre on Equines, Hisar, 125 001, Haryana, India; [2]Department of Veterinary Public Health, College of Veterinary Sciences, LUVAS, Hisar, 125 004, Haryana, India.

Abstract | Mycoplasmas are smallest self-replicating prokaryotes and responsible for varied genital and respiratory disease conditions in animals as well as human beings. They assume greater importance due to their resistance for common antibiotics and ability to survive the temperature of cryopreservation during artificial insemination. *Mycoplasma equigenitalium* has been implicated in reproductive and genital problems of mares and stallions. The conventional cultural methods are quite cumbersome and time consuming. In this study detection rate by capture-ELISA (9.6%) was higher than cultural methods (4.8%) for diseased equines. Overall detection rate by capture-ELISA (3.6%) was also higher than cultural methods (1.8%). Therefore capture-ELISA becomes a simple, rapid and sensitive alternative tool for detection of *M. equigenitalium*.

Keywords | Mycoplasma equigenitalium, Capture-ELISA

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***Correspondence** | Sandip Kumar Khurana, National Research Centre on Equines, Hisar, India; **Email:** sandipkk2003@yahoo.co.in
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INTRODUCTION

Mycoplasmas are smallest wall-less prokaryotes capable of self replication and are common inhabitants of genital and respiratory tract of animals and human beings. They have been isolated from equines with reproductive problems as well as apparently healthy equines from vagina, cervix, uterus and clitoral fossa of mares, and from prepuce, urethra and semen of stallions (Garg, 1999). *Mycoplasma equigenitalium* was first isolated from cervix region of mares (Kirchhoff, 1978), since then this organism has been isolated from various sites from reproductive tract of equines (Kirchhoff et al., 1979; Kirchhoff et al., 1980; Naglic et al., 1980; Mani et al., 1985; Burmudez et al., 1987; Copes et al., 1995; Khurana and Garg, 2001). Seroprevalence of *M. equigenitalium* has also been reported (Khurana et al., 2004; Khurana and Malik, 2009). Mycoplasmas are important because of their resistance to conventional antibiotics and ability to survive the temperature of cryopreservation, thus may be transmitted during artificial insemination.

Cytadosorption localizes the mycoplasmas on membrane of infected cells, these can be demonstrated in fluid expressed from internal organs of equines including aborted fetus by light microscopy after intensified Giemsa staining. Advanced procedures like UV microscopy following DNA binding flurochrome staining, acridine orange staining, fluorescent antibody, immunoperoxidase, counter-immuno-electrophoresis, ELISA, immune adherence haemagglutination tests are all capable of detecting mycoplasma antigen in body tissues and fluids but their application and value to equine mycoplasmosis have not been explored.

Boothby et al. (1986) performed capture-ELISA using monoclonal antibodies to detect *M. bovis* in milk samples from dairy experiencing an epizootic of mastitis. This method was reported to be 100 per cent specific. Broth enrichment increased the sensitivity from 65 % to 86% compared with standard culture methods. Nielson et al. (1987) have also used this technique for detection of *M. bovis* antigen in semen and perpetual washings of bulls. The technique had excellent specificity and the sensitivity of assay was approximately 200 colour changing units/ml. Ball et al. (1994) have used an antigen capture ELISA for detection of *M. bovis* in pneumonic calf lungs and na-

sal swabs. Rodriguez et al. (1996) used capture- ELISA for detection of *M. mycoides* subsp *mycoides* using monoclonal using monoclonal antibodies. The test was found to be specific and the sensitivity increased by overnight or 48 hr enrichment. Spergser et al. (2002) have reported high prevalence of mycoplasma in genital tract of stallion by PCR assay and cultural methods. Dahiya et al. (2009) have found capture-ELISA more sensitive than cultural isolation for *M. mycoides* subsp *mycoides*. Brooks et al. (2009) have developed a simple and specific capture/ sandwich ELISA for detection of *M. mycoides* subsp *mycoides* (SC) for screening large number of samples for CBPP. Soderlund et al. (2011) have developed a real time PCR for detection of *M. felis*. Guimaraes et al. (2014) have developed a multiplex microbead immunoassay for detection of *M. suis* antigens, which was found to be more sensitive than qPCR. Meng et al. (2014) have developed a colloidal gel-based immunochromatographic assay for highly specific, sensitive and rapid detection of *M.suis*.

Capture ELISA has not been used for detection of equine mycoplasmas. But, it is useful in detection of mycoplasmal antigens from various species as evident from various refences cited here earlier. It could be a very easy and useful method for dectecting *M. equigenitalium* anigen in swabs, fluids where it might not be possible by cultural methods.

MATERIALS AND METHODS

A capture ELISA technique (Nielson et al., 1987) with some modifications using polyclonal antiserum raised in rabbit and donkey foal against *M. equigenitalium* (NCTC 10176/T-37) was adopted for detection of *M. equigenitalium*-anigen in vagival swab/ preputial swab samples and specimens (heart blood, stomach content and tissue from lungs, heart, liver, spleen, kidneys) from aborted foals. The brief methodology is given below.

Test-sample Preparation

The vaginal swabs/preputial swabs from equines and other specimen from aborted fetuses were processed as per Ball et al. (1994) with some modifications. Specimens were transferred in Standard Liquid Medium (SLM) containing PPLO broth, yeast extract, inactivated horse serum, calf thymus DNA, thallium acetate and benzyl penicillin, then incubated at 37°C for 4-5 days, the enriched samples were centrifuged at 10,000 g at 4°C for 30 min, washed thrice with PBS (7.2), followed by 3 cycles of freezing and thawing process and kept at -20°C until used in capture-ELISA as antigen. The known positive antigen sample was prepared by adding *M. equigenitalium* (NCTC 10176/T-37) organisms in PBS (10^5 CFU /ml) which was also processed for three cycles of freezing and thawing. The PBS without any mycoplasma organisms served as negative control.

M. equigenitalium Antisera

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. The culture was grown in gradually increasing volumes of SLM with final volume of 1 litre. The resultant growth in gradually in one litre SLM was centrifuged at 10000xg at 4°C for 30 min. pellet of organisms was washed thrice with PBS (pH 7.2) and suspended in 10 ml PBS which was stored at 4°C. It was sonicated at 180-200 watts (Braunsonic–1510) for 3 cycles of 4 min each and protein content was estimated (Lowry et al., 1951). It served as stock antigen for immunization of rabbit and donkey foal.

Healthy mycoplasma free rabbits (New Zealand white) and donkey foals (6 months) were immunized as per schedule (Table 1) and bled one week after the last injection to harvest *M. equigenitalium* hyperimmune serum. Pre-immune serum of rabbit and donkey foal were collected for their usage as known negative serum for conducting capture-and indirect-ELISA.

Enzyme-antiglobulin-conjugate and Substrate

Horse-radish-peroxidase labelleed rabbit anti-horse IgG (Prod. Cat. No. A9292, Sigma, USA) was used as enzyme-antiglobulin-conjugate. A solution of orthophenylene diamine dihydro-chloride (OPD, Sigma, USA) prepared fresh was used as substrate.

Optimization of Antisera and Conjugate

The optimum dilution of equine anti-*M. equigenitalium* (NCTC 10176/T-37) serum was determined by chequer-board titration with known positive and negative samples. Rabbit anti-*M. equigenitalium* serum was diluted 1:40,1:80, 1:160, and 1:320 in 0.1M carbonate-bicarbonate buffer (pH 9.6) before coating the wells of microplates. The equine anti-*M. equigenitalium* serum was diluted to 1:40,1:80, 1:160 and 1:320 in 0.1 M PBS with 0.05per cent tween-20 (PBST). The dilution of conjugate 1:1000, 1:2000, 1:3000 and 1:4000 were prepared in PBST. The capture-ELISA was performed using the above prepared dilutions of rabbit and equine *M. equigenitalium* sera and conjugate. Optimum dilution of conjugate was 1:2000 whereas dilution for both rabbit and equine *M. equigenitalium* serum was 1:160, which were the highest dilutions resulting in maximum colour change and contrast between known positive and known negative antigen samples. In each plate, positive control consisted of *M. equigenitalium* (NCTC 10176/T-37) organisms (10^7 CFU/ml) in PBS (pH 7.2) which were serially two-fold diluted; from 1:10 to 1:1280.

Procedure

Distilled water rinsed well of microtitre plate (Linbro, type IS-FB-96, Flow Lab, UK) were coated for 18 hrs. at 37°C with 0.05 ml of rabbit anti- *M. equigenitalium* serum diluted

Table 1: Immunization schedule for raising hyperimmune serum against *M. equigenitalium* in rabbit and donkey foal

Schedule for rabbit

Week	Inoculum*	Distribution and site
1/7	1.8 ml killed antigen+1.8 ml CFA **	Subcutaneously 0.4 ml in each rear foot-pad, 0.8 ml distributed at 4 site on the back and 2.0 ml intramuscularly in the thigh region
3	1.25 ml killed antigen	1.0 ml intramuscularly and 0.25 ml intravenously
4	0.25 ml killed antigen	0.25 ml intravenously
5	0.25 ml killed antigen	0.25 ml intravenously
8	0.25 ml killed antigen	0.25 ml intravenously

Schedule for donkey foal

Week	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 10^9ml)	7.5 ml distributed subcutaneously at prescapular region
9	7.5 ml live antigen (CFU 2X 10^9ml)	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).

Table 2: Appearance of precipitin, growth inhibiting and ELISA-antibodies in experimentally antigenized donkey foal with *M. equigenitalium* (NCTC 10176/T-37)

Day/week post immunization	Anti M. equigenitalium antibodies detected with		
	AGIPT	GI	ELISA titre
1 day	-	-	-
4 day	-	-	-
7 day	-	-	1:40
2 week*	-	-	1:80
3 week	-	-	1:640
4 week*	-	-	1:640
5 week	+	+	1:1280
6 week	+	+	1:1280
7 week	+	-	1:1280
8 week*	+	-	1:1280
9 week*	+	+	1:2560
10 week	+	+	1:5120

*= booster dose; AGIPT=agar gel immune precipitation test; GI= growth inhibition; + = positive; - = negative

to 1:160 in 0.1 M carbonate bicarbonate buffer (pH 9.6). The plate wells were washed with 0.1 M PBS (pH 7.4) having 0.05 per cent (v/v) tween-20 (PBST) and air-dried. The coated plates were stored at 4°C till use. Blocking of plates was done with addition of 0.05 ml bovine serum albumin (20%) in each well and incubated at 37°C. After 4 washings and air drying of plate wells, equine *M. equigenitalium* serum diluted to 1:160 was added to each well (0.05ml) and incubated at 37°C for 1 hr and followed by 4 washings with PBST. The prepared test antigen in PBS (0.05 ml) was added in duplicate to plate wells and incubated for 1 hrs. at 37°C for another hour. After another washing step, optimally diluted anti-equine immunoglobulin horse radish-peroxidase conjugate (1:2000) was added (0.05 ml) and again incubated at 37°Cfor 1 hr. followed by four washings. After air drying the plates, 0.1 ml of enzyme substrate (OPD) was added to each well and incubated at room temperature (25-30°C) for 5 min. Stopping reagent (1 M H_2SO_4 solution) was added (0.1 ml) to each well and absorbance was measured at 492 nm in a Organon-teknika Reader 530 (Organon, Germany). Known positive and negative antigen samples were included for comparison with each test plate.

Interpretation

The mean absorbance of known negative antigen (5 tests) and known positive antigen (5 tests) was calculated. ELISA- value (mean absorbance of test sample mean absorbance of known negative sample) for each sample was calculated. An ELISA-value below 1.5 was considered negative.

RESULTS AND DISCUSSION

Appearance of Precipitin, Growth Inhibiting and ELISA-antibodies in Experimentally Antigenized Donkey Foal

Appearance of precipitin, growth inhibiting and ELISA-antibodies in experimentally antigenized donkey foal with *M. equigenitalium* (NCTC 10176/T-37) is given in Table 2. The results indicated that ELISA antibodies gradually increased from nil (pre-immune titre) to 5-week

time and it remained at 1:1280 till 8th week. Subsequently, it increased to 1:2560 and 1:5120 by 9th and 10th week, respectively. The precipitin antibodies also appeared at 5-week post immunization with *M. equigenitalium* antigens and were observed till 10th week. The growth inhibiting antibodies appeared at 5th week but disappeared at 7th to 8th week. However, *M. equigenitalium* precipitins were again recorded at 9th and 10th week post- immunization.

Detection of *M. equigenitalium* Antigen in Equines

All the 219 equine samples, which included frozen semen, neat semen, prepucial swab, vaginal swab, cervical swab, aborted foetus samples, were subjected to detection of *M. equigenitalium* antigen by capture-ELISA. *M. equigenitalium* antigen was detected in samples from 4 of 63 (6.4%) repeat breed and 4 of 17 (23.6%) metritis mares. These detection rates were higher in comparison to detection by culture method, which were 3.2 and 11.8 per cent correspondingly. However, from 135 healthy equines *M. equigenitalium* antigen was not detected in any of samples. The results obtained are given in Table 3.

Table 3: Detection of *M. equigenitalium* by culture and ELISA (capture)

Reproductive health status of equines with total numbers in parentheses	Culture	Capture-ELISA
(A) Diseased (84)		
Repeat breed (63)	2 (3.2)*	4(6.4)
Metritis (17)	2 (11.8)	4 (23.6)
Aborted fetus (2)	0(0)	0 (0)
Balanoposthitis (2)	0(0)	0(0)
Total	4(4.8)	8(9.6)
(B) Apparently Healthy (135)	0(0)	0(0)
Grand total (219)	4(1.8)	8 (3.6)

*Figures in parentheses indicate percentage.

The overall prevalence of *M. equigenitalium* was also higher (3.6%) by detection of its antigen by capture ELISA than by cultural methods (1.8%) in equines with reproductive disorders (Table 3).

Spergser et al. (2002) have reported high prevalence of mycoplasmas in the genital tract of asymptomatic stallions in Austria, where *M. equigenitalium* was isolated from 55 out of 116 stallions. The dominance of *M. equigenitalium* in the genital tract of equines has been confirmed (Kirchhoff et al., 1979; Kirchhoff et al., 1980; Naglic et al., 1980; Zgorniak-Nowesielka et al., 1984; Mani et al., 1985; Bermudez et al., 1987; Copes et al., 1995; Khurana and Garg, 2001). In this study, *M. equigenitalium* antigen was detected in samples from 4 of 63 (6.4%) repeat breed and 4 of 17 (23.6%) metritis mares, which is significant prevalence and the detection was two-times higher than detection by cultural method. The overall prevalence of *M. equigenitalium* was higher also (3.6%) by detection of its antigen by capture-ELISA than by cultural methods (1.8%) in equines with reproductive disorders. Therefore, ELISA has been found a very easy, sensitive and rapid method for mass screening of *M. equigenitalium* anigen in equines especially when compared with cultural methods.

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