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BACTERIOLOGICAL STUDIES ON MYCOPLASMA INFECTION OF BOVINE GENITAL TRACT

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SUMMARY

A total of 70 vaginal swabs were collected from cows (forty five from cows suffering from congestion and purulent vaginal discharge, and 25 from apparently healthy cows). Also ten vaginal swabs were collected from buffaloes suffering from congestion and purulent vaginal discharge. The collected vaginal swabs were subjected to bacteriological examination for *Mycoplasma* pathogens and the recovered species were identified using PCR. Vaginal swabs from apparently healthy cows were negative for Mycoplasma isolation, while seven isolate were recovered from vaginal swabs of diseased cows with an incidence of 15.6 %. On the other hand, five isolates were recovered from the vaginal swabs of diseased buffaloes with an incidence of 50 %. All Mycoplasma isolates were identified by PCR using M. *bovis*, *M. bovigenitalium*, and *M. bovine* group 7 specific primers. No isolates reacted with *M. bovine* group 7, while 2 and 6 isolates had amplified fragments at 442 and 928 bp against *M.bovis*, *M.bovigenitalium*, respectively. The obtained data indicated that *M. bovis* was recovered from vaginal swabs of cows and buffaloes with an incidence of 2.2 % and 10 %, respectively, while the incidence of *M. bovigenitalium* was 11.1 % and 10 %, respectively. Ureaplasma could be identified from the collected samples of cows and buffaloes with an incidence of 2.2 % and 20 %, respectively. Furthermore, another Mycoplasma isolate (unidentified) was recovered from buffalo samples.

INTRODUCTION

DNA amplification techniques offer considerable promise for the identification of Mycoplasma. They avoid the antigenic cross-reactivity and variability that hinder serological methods, and they allow easier standardization between laboratories. As of today, arrays of primers specific to differ-

ent members of genus Mycoplasma have been proposed (Monnerat et al., 1999; Persson et al. 1999 and Schankster et al., 2002) Laboratory diagnosis of Mycoplasma species using conventional methods are usually difficult and time consuming Therefore, other techniques such as PCR are generally preferred by the majority of laboratories (Thomas et al 2004 and Bashiruddin, et al., 2005). PCR is rapid when compared to the traditional culture and serological techniques (typically taking less than 24 h. compared with up to 2 weeks for serological or culture based diagnosis) (Mcauliffe et al., 2003). This work was conducted to recover *Mycoplasma* pathogens responsible for genital tract of bovine and to elucidate the role of PCR in *Mycoplasma* species identification. In the present work both conventional bacteriological methods and PCR detection were used to detect bovine mycoplasmas.

MATERIALS AND METHODS

1. Samples:

A total of 70 vaginal swabs were collected from cows (forty five were collected from cows suffering from congestion of the vaginal mucous membrane and purulent vaginal discharge, and 25 from apparently healthy cows). Also ten vaginal swabs were collected from buffaloes suffering from congestion and purulent vaginal discharge. The collected swabs were directly inoculated into Stuart's transport medium (Difco) or HI Mycoplasma broth medium (Difco) and transported to the laboratory as soon as possible in an ice box for bacteriological examination.

2. Isolation of Mycoplasma (Sabry and Ahmed, 1979):

A. Direct method: The collected swabs were spread on Brain Heart Infusion (BHI) Agar and modified Hay flick's agar media, incubated at 37°C with 5-10% CO_2 in a humid chamber for 10-14 days and examined microscopically by stereomicroscope every 2-3 days for the appearance of typical colonies.

B. Indirect method: The examined samples were inoculated into *Mycoplasma* broth (B_0). After 2-3 days of incubation, B0 cultures were plated onto BHI Agar plates (P_1). At the same times they were transferred to broth (B_1). After another 3 days, B_1 cultures were spread onto BHI Agar plates (P_3). After 6 days, Mycoplasma broth cultures (B_0) were plated on agar (P_2). After other 3 days, B_1 cultures were plated on (P_4). Incubation was performed at 37°C with 5-10 % CO_2 for 2 weeks and examined by stereomicroscope every 2-3 days. Agar blocks from suspected Mycoplasma colonies (having characteristic fried egg appearance) were taken in BHI broth medium and incubated at 37°C for 2-3 days then subjected to purification and further identification.

3. Purification and maintenance of Mycoplasma isolates (Subcommittee on taxonomy of Mollicutes, 1979):

The broth culture was filtrated through 0.22um

millipore membrane filter. The filtrate was plated onto BHI and modified Hayflick's agar plates. Single colony was selected and transferred to broth culture. This technique was repeated for three times. *Mycoplasma* culture was maintained by freezing at -20°C in 2 ml aliquots of actively growing broth culture and in a form of agar blocks in sterile screw capped vials.

4. Bacteriological identification of the isolates by:

A. Microscopical examination: The isolates were examined under a stereomicroscope or inverted microscope for detection the characteristic "Fried egg appearancesî of *Mycoplasma* colonies".

B. Digitonin sensitivity (Freundt et al., 1973).

C. Glucose fermentation test (Enro and Stip kov its, 1973).

D. Arginine hydrolysis test (Frenske and Ken ny, 1976).

E. Phosphatase activity in broth culture (Bur ger]et al., 1967).

F. Urease activity (Shepared, 1976).

5. Identification of *Mycoplasma* species using Polymerase chain reaction (PCR) according to Sambrook et al. (1989) and Riffon et al., (2001):

5.1. Extraction of DNA from Mycoplasma iso lates according to Sritharan and Barker (1991):

The pellets of *Mycoplasma* isolates were re-suspended in 200 μl Tris-EDTA buffer (pH 8.0) and heated block at 105°C for 25 minutes. They were left to cool at room temperature and centrifuged at 14,000 xg for 10 minutes, then transferred to a fresh tube with addition of double volume absolute ethanol and 0.1 volume 3M sodium acetate (pH 5.2.). The test tubes were kept a t-20 °C for overnight. The DNA was pelleted by centrifugation at 14,000 xg / minute for 20 minutes, followed by washing with 70 % ethanol and re-centrifugation at 14,000 xg/ minute for 10 minutes. The pellet was dried and re-suspended in 20 μl sterile distilled water.

5.2. Estimation of purity and concentration of DNA:

The concentration and the purity of DNA that had been extracted were determined by estimation of the optical density at wave length of 260 and 280 nm using the spectrophotometer. The concentration was calculated as follows:

1 OD at 260 nm = 50 μg/ml. The purity of DNA = OD at 260/OD. at 280 nm.

The purity of DNA had a value of 1.8, where the optimum is between 1.8 - 2.

5.3. Running of PCR according to Riffon et al. (2001):

The amplified reaction was performed in 50 μl volumes in PCR tubes. The reaction mixture consisted of 5 μl (200ng) of extracted DNA template from bacterial culture, 5 μl 10x PCR buffer, 1 μl

dNTPs (40μM), 1 μl Ampli Taq DNA polymerase, 1 μl (50 pmol) from each primers pairs (Table 1) and the volume of the reaction mixture was completed to 50 μl using double distilled water. 40 μl paraffin oil was added and thermal cycler was adjusted as follows:

Initial denaturation: 95°C/1 minute

First cycle:

Denaturation- - - - - - - 94°C/1 minute

Annealing - - - - - - - - -as in Table (4).

Extension - - - - - - - - - 72°C/1 minute

The first cycle was repeated 35 times.

The final extension: 72°C/5 minute

The PCR products were stored in the thermal cycler at 4°C until they were collected.

5.4. Screening of PCR products by agarose gel electrophoresis according to Sambrook et al. (1989):

Two grams of agarose were added to 100ml Tris acetate EDTA (TAE) buffer. The agarose was autoclaved for 10 minutes and 0.5 μg/ml ethiduim bromide was added and then left to cool; to room temperature. The gel tray was tapped and the worm agarose was poured in. The comb was inserted in the agarose which was left to polymerize. After hardening, the tray was untapped, the comb was removed and the gel was applied to electrophoresis cell. The cell was filled with TAE buffer. 10 μl of each of the PCR product sample were applied to the gel along with 5μL molecular weight marker after mixing each with 1 μL load-

Table (1): Shows oligonucleotide primers used for amplification of DNA recovered from *Mycoplasma* isolates:

Primer designation	Specificity	Length	Sequence (5-3)	Amplified Product size (bp)	Annealing temperature	Reference
MBsf-MN	*M. bovis*	19	CCA GCT CAC CCT TAT ACA T	442	52 °C/1minute	**(Pinnow et al., 2001)**
MBsr-MN		19	TGATC ACC ATT TAG ACC G			
MBmf-MN-928	*M. bovigenitalium*	18	ACC ATG GGA GCT GGT AAT	928	56°C/1 minute	Gene Bank # AY 780797
MBmr-MN-927		18	TAG TTC ACT ATC GGT GTC			

ing buffer on a piece of parafilm . Each mixture was applied to a slot using 10μL micropipette. The electrophoresis cell was covered and the power supply was adjusted at 10 volt / cm. the gel was taken out from the cell and examined under short wave UV transilluminator. The gel was photographed in order to obtain permanent record using digital camera (Acer CR-5130, China).

RESULTS AND DISCUSSION

The aim of this study was to record the occurrence of mycoplasmal pathogen in the genital tracts of cows and buffaloes and to test PCR application for species identification of *Mycoplasma*. The effect of mycoplasmas on the reproductive function of cattle has been reviewed by a number of authors (Kirkbride, 1987 and Eaglesonme et al., 1992). As shown in Tables (2 and 3), 7 and 5 isolates could be identified from vaginal swabs collected from diseased cows and buffaloes with an incidence of 15.6 % and 50 %, respectively. The *Mycoplasma* positive animals may be infected via the artificial insemination with infected semen. In this concern Nicholas and Ayling (2003) concluded that *Mycoplasma* contaminated frozen semen can remain infectious for years and probably represents an overlooked infection source.

Identification of *Mycoplasma* as the causative agents of disease is often hindered by the lack of rapid diagnostic tests together with similarities in the clinical diseases that they cause. Conventional methods of diagnosis are based on culture and serological tests, e.g., the complement fixation test (Muthomi and Rurangirwa, 1983), enzyme-linked immunosorbent assay (Ball and Finaly, 1998), and immunoblotting (Nicholas et al., 1996). These techniques can be time consuming, insensitive, and non-specific.

In the present investigation conventional methods of diagnosis of *Mycoplasma* were conducted. Firstly, the characteristic fried egg colony of *Mycoplasma* was recorded by all isolates as shown in Photo (1). The characteristic colony appeared as tiny, smooth circular, translucent masses with a dense raised central area (Quinn et al., 2002). Digitonin sensitivity of the isolates was carried out to differentiate between members of *Mycoplasmataceae* and *Acholeplasmataceae* (Photo. 2). All detected isolates were sensitive to digitonin and belonged to the family *Mycoplasmataceae* (Freundt, 1983). The detected isolates were cultured onto *Ureaplasma* medium to differentiate between *Ureaplasma* and *Mycoplasma*. Three isolates were grown on *Ureaplasma* medium, which represented *Ureaplasma* species (5.5%), meanwhile 9 isolates were negative for urease activity on the medium so they were belonging to *Mycoplasma* species. The data recorded in Table (2) indicated that *Ureaplasma* could be identified from vaginal swabs of cows and buffaloes 2.2 % and 20 %, respectively.

PCR can be expected to provide higher efficiency for practical laboratory diagnosis because of its rapidity, high sensitivity and specificity. PCR assays were developed for a number of animal mycoplasmas, e.g., *M. mycoides* subsp. mycoides SC (Dedieu et al., 1994 and Hotzel et al., 1996) and *M. capricolum* subsp. *capripneumoniae* (Bascunana et al., 1994). While detection of *M. bovis* by PCR from broth culture or DNA extracts can be carried out on a routine basis (Hotzel et al., 1996). *M. bovis* and *M. agalactiae* are phenotypically and genotypically closely related. As both species share an unusually high number of related antigens and common epitopes (Flitman-Tene et al., 1997), therefore, differentiation between them present some difficulties, particularly, when serological methods are involved. The similarity of certain metabolic pathways (Abu-Amero et al., 2000) also puts limitation on biochemical differentiation testes.

The use of species-specific PCR provides an alternative approach to identify *Mycoplasma* species, which overcomes the potential problems associated with conventional test methods. *M. bovis* and *M. bovigenitalium* have been associated with infertility and reproductive failure in cattle. Both mycoplasmas have been isolated from semen and are transmitted by natural breeding and by artificial insemination. As a preventive measure against this type of microbial contamination, antibiotics have been added to the commercial cry preserved semen (Eaglesonme et al., 1992 and Bousseau et al., 1998).

In the present study species-specific primers \ used for detection of *M. bovis*, *M. bovigenita* and *M. bovine* group 7. Unfortunately no isol reacted with primers specific to M. bovine gr While, as shown in Photos. (3 & 4), ampl fragments at 442 and 928 bp were formed fo *bovis* and *M.bovigenitalium*, respectively. bovis-specific PCR developed by Ghadersol al. (1997) provided a more rapid and sens method for the detection of *M. bovis* comp to conventional culture methods. Moreove was the first PCR method described for the d tion of *M.bovis* from clinical material (Hay and Hirst, 2003). As shown in Table (3) M. b and *M.bovigenitalium* were detected in va swabs of diseased cows (2.2 % and 11.1 %) buffaloes (10 % each), respectively. Bielans al. (2000) recognized that *M. bovis* and *M. l genitalium* have the ability to colonize in th productive tract and to produce severe salpi oophoritis. Sprecher et al. (1999) recorded presence of *Hemophilus somnus*, *Mycopla bovigenitalium*, *Arcanobacterium pyogenes Ureaplasma diversum* in *seminal cultures*,.

The male genital tract can become infected *M. bovis* through contact with other anima possibly, via a heavily contaminated environ (Nicolas and Ayling, 2003). Infection of the puce or urethra by *M. bovis* leads to an ascer infection of the testes causing orchitis, vesic

and decrease of semen quality and ultimately shedding in the semen (Krausel et al., 1989).

Table (2) Distribution of members of F. *Mycoplasmataceae* in collected vaginal swabs among the examined cows and buffaloes

Sources of isolates	Apparently healthy			Diseased			Total		
	No. of examined samples	N0.	%	No. of examined samples	N0	%	No. of examined samples	No.	%
Buffaloes	-	-	-	10	5	50	10	5	0
Cows	25	0	0	45	7	15.6	70	7	0

No = Number of positive.
% = Percentage was calculated according to number of examined sample.

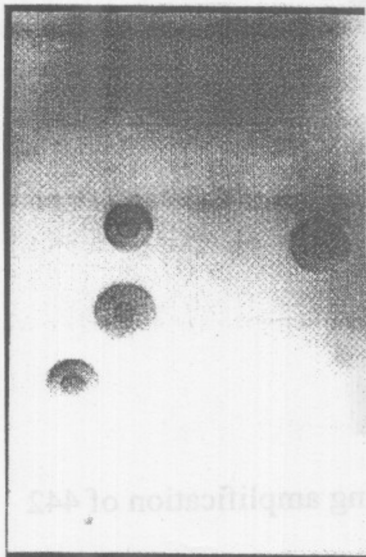

Photo. (1): Shows characteristic fried eggg colony of *Mycoplasma* species.

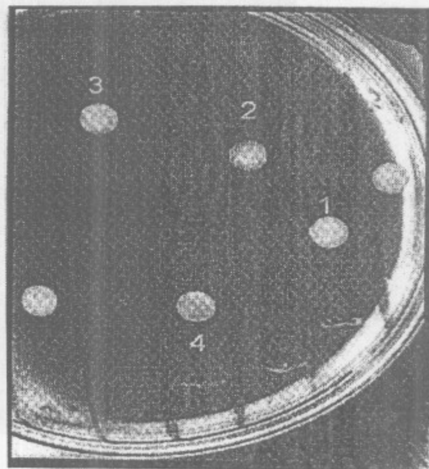

Photo. (2): Shows digitonin se... test .., 2 and 3 were se... to digitonin (*Mycplasma* ... *Ureaplasma*), while 4 resistant (*Acholeplasma* ... *trol*)

Table (3) Distribution of *Mycoplasma* and *Ureaplasma* species in vaginal swabs collected from the examined diseased cows and buffaloes

Source of isolates	No. of examind samples	No. of isolates	*M. bovis*		*M. Bovigenitalium*		*Ureaplasma*		Other *Mycoplasma* species	
			No.	%	No.	%	No.	%	No.	%
Cows	45	7	1	2.2	5	11.1	1	2.2	0	0
Buffaloes	10	5	1	10	1	10	2	20	1	10
total	55	12	2	3.6	6	10.9	3	5.5	1	1.8

No = Number of positive.

% = Percentage was calculated according to number of examined sample.

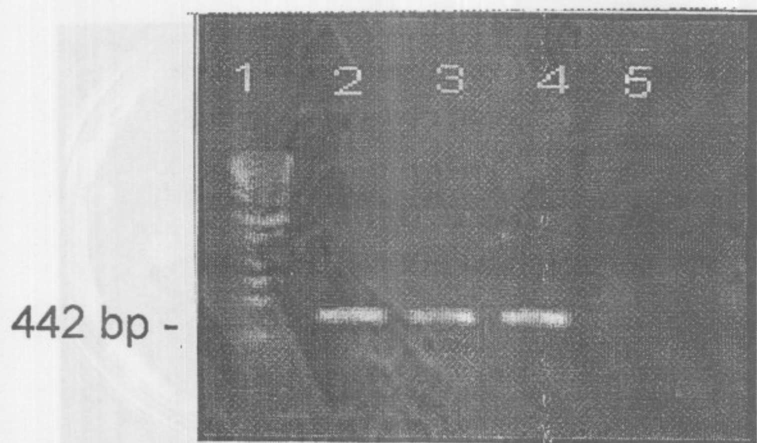

Photo. (3): Shows agarose gel electrophoresis showing amplification of 442 bp fragment of *M. bovis*

Lane (1): DNA ladder

Lane (2): Reference strain (control +ve)

Lanes (3&4): *M. bovis* (samples)

Lane (5): *M. bovigenitalium* (control –ve)

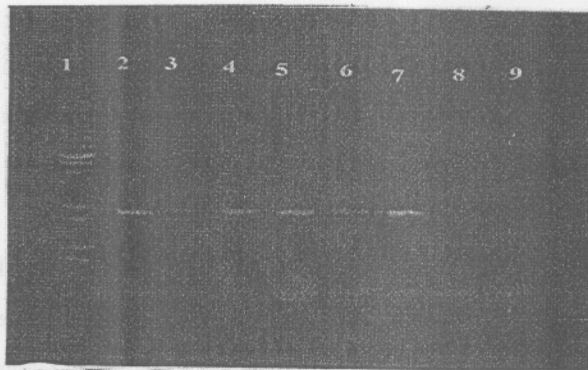

Photo. (4): Shows agarose gel electrophoresis showing amplification of 928 bp fragment of *M. bovigenitalium*

Lane (1): DNA ladder
Lane (2): Reference strain (control +ve)
Lanes (3-8): *M. bovigenitalium* (samples)
Lane (9): *M. bovis* (control -ve)

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دراسة بكتيريولوجية عن عدوي الميكوبلازما في الجهاز التناسلي للماشيه

تم جمع عدد ٧٠ عينة من الجهاز التناسلي للماشية (منهم ٤٥ عينة من ابقار بها التهابات وافرازات صديدية من الجهاز التناسلي و٢٥ عينة من ابقار سليمة ظاهريا). كذلك تم جمع عدد ١٠ عينات من جاموس به التهابات وافرازات صديدية. تم فحص العينات بكتروولوجيا لعزل ميكروب الميكوبلازما وتم تصنيف العترات المعزولة بأستخدام اختبار تفاعل البلمرة المتسلسل. العينات المأخوذة من الابقار السليمة كانت خالية من ميكروب الميكوبلازما بينما تم عزل سبع معزولات من الميكوبلازما من العينات المأخوذة من الابقار المريضة بنسبة ١٥.٦%. من ناحية اخري تم عزل ٥ معزولات ميكوبلازما بنسبة ٥٠% من العينات المأخوذة من الجاموس المصابة. تم تصنيف عترات الميكوبلازما المعزولة بأستخدام تفاعل البلمرة المتسلسل حيث صنفت عتراتان *M. bovis* وعدد ٦ عترات *M. bovigenitalium* وعترة من الميكوبلازما لم يتم تصنيفها. كذلك اوضحت النتائج ان *M. bovis* تم عزلها بنسبة ٢،٢ و ١٠% من الابقار والجاموس علي الترتيب. وتم عزل *M. bovigenitalium* بنسبة ١١،١% و ١٠% من عينات الابقار والجاموس علي الترتيب، وفي كل العترات المعزولة لم يتم عزل *M. bovis group 7*. كذلك تم عزل اليوروبلازما بنسبة ٢،٢ و٢٠% من عينات البقار والجاموس المفحوصة علي الترتيب.