Detection of *Mycoplasma bovis* and *Mycoplasma bovigenitalium* in Cattle and Buffalo in Egypt Using Dot ELISA and PCR with Anti -Microbial Trials

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Abstract: Mycoplasmae have the ability to colonize the respiratory and reproductive tracts of bovine. Consequently, these microorganisms can be associated with infertility and pneumonia. A total of 555 cows and 130 buffalo's samples (nasal swabs, lung tissues, tracheal swabs, bronchial lymph nodes and vaginal swabs) was collected from different Egyptian governorates and investigated. *Mycoplasma* isolates collected from the respiratory tract of cattle and buffaloes were typed as *M. bovis* (2.7 and 1.7%, respectively) and other *Mycoplasma* species (10.8 and 4.2%, respectively). *M. bovis* was typed from vaginal swabs of the examined cows and buffaloes with incidence of 2.2 and 10 %, respectively, while the incidence of *M. bovigenitalium* was 13.3 and 10 %, respectively. Treatment of *Mycoplasma* diseases is difficult since *Mycoplasma* species lack a cell wall and are thus resistant to some commonly used antibiotics. Exposure of *M. bovis* and *M. bovigenitalium* isolates to MIC against five antibiotics including erythromycin, gentamicin, tetracycline, streptomycin and lincomycin in comparison to reference strains revealed that lincomycin, erythromycin and streptomycin are more effective and able to get rid of *M. bovis* and *M. bovigenitalium*.

Key words: *Mycoplasma bovis* · *Mycoplasma bovigenitalium* · Cattle · Buffalo · Egypt · Dot ELISA · PCR

INTRODUCTION

Mycoplasmas cause some of the most serious and, economically, most costly diseases of cattle. *M. bovis* is a major and often overlooked, cause of calf pneumonia, mastitis, arthritis and other conditions. In the U K, it was estimated that up to 1.9 million cattle are affected annually by respiratory diseases which cost the cattle industry £54 million [1]. Furthermore, approximately 157,000 calves die annually as a result of pneumonia and related illnesses which would have a potential market value of £99 million [2]. It is likely that *M. bovis* is responsible for at least a quarter to a third of these losses although this is likely to be an underestimate [3]. In the USA, the cost of *M. bovis* infections as a result of loss of weight and carcass value has been estimated at $32 million per year [4]. The losses due to bovine mastitis caused by *M. bovis* may be higher than that due to respiratory diseases with estimates from the USA of up to $108 million per year with infection rates up to 70% of a herd [4]. *M. bovis* can act as a primary pathogen, yet many cases are coinfected with other bacteria or viruses and evidence suggests that *M. bovis*

colonizes and perpetuates lung lesions that were initiated by other bacteria, such as *M. haemolytica* [5].

Treatment of *Mycoplasma* diseases is difficult since *Mycoplasma* species lack a cell wall, which differentiates them from bacteria and is thus resistant to some commonly used antibiotics. Many *in vitro* studies have compared the susceptibility of *M. bovis* to a range of antibiotics. Also, Nigerian medicinal plants were analyzed by Muraina *et al.* [6] for antimycoplasmal activity. The prophylactic use of antibiotics is generally undesirable, but their use may be justified when calves are introduced to a site with a history of *M. bovis* infection in which high levels of mortality are being sustained. *M. bovis* strains in Europe are becoming resistant to antibiotics traditionally used for treatment of *Mycoplasma* infections in particular oxytetracyclines, tilmicosin and spectinomycin [7], while the fluoroquinolones are still effective. but their use in animals is controversial [3].

The aim of this study was to identify *M. bovis* and *M. bovigenitalium* from bovine respiratory and genital tracts and to determine the effect of different antimicrobial agents against *M. bovis* and *M. bovigenitalium*.

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MATERIALS AND METHODS

Samples: A total of 685 samples (555 cows and 130 buffaloes) was collected from different Egyptian governorates and examined to study the incidence of mycoplasmae in the respiratory and genital tracts. Samples from the nose, lung, trachea, bronchial lymph node and vagina were collected from the examined animals.

Isolation and Identification: The specimens were inoculated onto Modified Hay flick's medium [8] to isolate *M. bovis* and *M. bovigenitalium.* All media were incubated at 37°C in a moist 10% CO_2 incubator for three to five days [9]. The isolates were examined under inverted microscope to detect the characteristic fried egg colonies. All isolates were identified using a combination of conventional identification methods {digitonin sensitivity [10], glucose fermentation [11], arginine hydrolysis [12], phosphatase activity [13] and growth inhibition test [14]} and dot ELISA [15], immunoblotting [16] and PCR[17] using the following oligonucleotide primers:

Mbsf-MN (5' CCAGCTCACCCTTATACAT) and MBsr-MN (5' TGAATCACCATT TAGACC G) for detection of *M. bovis* at 442 [18]; MBsr-MN (5' ACC ATG GGA GCT GGT AAT) and MBmr-MN-927 (5' TTC TTA CTT CTA AAG TAT) for detection of *M. bovigenitalium* at 928bp (Gene Bank # AY 780797).

Minimum Inhibitory Concentration (MIC) According to Hannan [19]: IC for *M. bovis* and *M. bovigenitalium* against five antibiotics including streptomycin sulphate (Bio Basic, RD0115), erythromycin (Kahira Pharm. and Chem. Ind. Co., No. 6369), gentamicin (Bio Basic, GB0217), tetracycline HCL (ROTH) and lincomycin (PHARCO) was investigated. Both liquid and solid MIC methods were used to determine MIC among *M. bovis* and *M. bovigenitalium* using modified Hay flick's medium supplemented with 0.5% (w/v) sodium pyruvate and 0.005% (w/v) phenol red.

Liquid MIC Method:
Preparation of *Mycoplasma* Inoculum for MIC Testing (Viable Counting Method for Liquid MIC Assays): One ml of *Mycoplasma* culture was added to 9 ml of sterile diluent (*Mycoplasma* broth or broth base) and the suspension was vortexed for 5 seconds to reduce possible clumping of the mycoplasmas. Ten-fold dilutions of the vortex suspension from 10^{-2}-10^{-9} were prepared in

0.9 ml volumes. Only 0.1 ml of sterile *Mycoplasma* broth at the appropriate pH was added to 8 consecutive wells in a horizontal row of a round bottomed microdilution, wells 1-8. Only 0.2 ml of sterile *Mycoplasma* broth was added to well number 12 (sterility control). Only 0.1 ml of each *Mycoplasma* dilution was transferred to the 0.1 ml volumes of broth in a microdilution plate beginning at well number 8 and finishing at well number 1. The microdilution plate was sealed using an adhesive sealing film, firstly by puncturing the seal on all unused wells to prevent lifting of the film due to air expansion during incubation. The sealing was completed by rubbing the surface of the film around each well with a smooth object such as the back of a spoon (manufactured from plastic or horn). The plate was incubated at 36 ± 1°C until color changes are complete. The result was recorded; the lowest dilution to show a color change denotes the reciprocal of the number of color changing units (ccu) in 0.1 ml of undiluted *Mycoplasma* culture. The culture in *Mycoplasma* broth was diluted to give between 10^3 ccu and 10^5 ccu per ml.

Preparation of Antimicrobial Solutions for MIC Testing: Antimicrobial agent concentrations ranged from 0.016 to 10.6 µg/ml for erythromycin, gentamicin and tetracycline and 128 to 312 µg/ml for streptomycin and lincomycin were used.

Modified MIC Assay Method [20]: Only 0.1 ml of each compound dilution was transferred to separate consecutive wells running horizontally on a microdilution plate (wells 1 to 9). Only 0.2 ml of sterile *Mycoplasma* broth at the appropriate pH (e.g. pH 7.6) was added to well number 10 as a sterility control. Only 0.2 ml of sterile *Mycoplasma* broth was added to well number 11 and adjusted to the required end-point (pH 6.8). Then 0.1 ml of sterile *Mycoplasma* broth at the appropriate pH was added to row number 12. In a separate row, 0.1 ml of antibiotic- free *Mycoplasma* broth was added at the appropriate pH containing the antibiotic solvent at the same concentration used to dissolve the antimicrobial agent (solvent control). Only 0.1 m of *Mycoplasma* inoculum was added to each well containing the antibiotic dilutions (wells 1 to 9), to the growth control well (well 12) and to the solvent control well. The microdilution plate was sealed with adhesive film and incubated at 36 ± 1°C until the color in the growth control well first matched that of the end-point control. The plates must be examined at frequent intervals in early morning and late afternoon. The initial MICs were recorded; the lowest antibiotic

concentration to show no change in color when the color of the growth control well matches that of the pH end-point control. By definition this is the MIC value, however if plates are re-incubated the end-point in liquid assay frequently shifts. For this reason, a second reading is often taken and is referred to as the final MIC. Final readings can be recorded 7 days later or 14 days. The plate was incubated until color changes are complete in the wells containing the inoculum challenge control viable count.

Solid agar MIC Method

Viable Counting Method for Solid MIC Assays: One ml of *Mycoplasma* culture was added to 9 ml of sterile diluent (*Mycoplasma* broth or broth base) and the suspension was vortexed for 5 seconds to reduce possible clumping of the mycoplasmas. Ten-fold dilutions of the vortex suspension from $10^{-2}–10^{-9}$ were prepared in 0.9 ml volumes. Two ml of each *Mycoplasma* dilution (in triplicate or more for greater accuracy) were transferred to the surface of freshly prepared and dried *Mycoplasma* agar plates using a micropipettes and filter tip. When the droplets of each suspension have been absorbed into the agar, the plate was incubated at $36 \pm 1°C$ under the appropriate atmospheric conditions (e.g. aerobic, 95% N2 + 5% CO2 or anaerobic) in sealed containers containing moistened paper toweling. The seeded plates were incubated until the *Mycoplasma* colonies were well developed on the plates inoculated with the lower dilutions of the *Mycoplasma* suspension. Plates with a moderate growth of well separated colonies were selected for counting. Avoid plates with low numbers of colonies. The number of colonies produced by each 2 ml droplet was counted using inverted microscope. The number of cfu per 2 ml of inoculum was the mean count from the three droplets.

Modified MIC Solid Method: Agar plates were prepared containing a range of antimicrobial concentrations in 9 cm plastic Petri dishes. Control drug free plates were also prepared. The plates were dried. Each plate was inoculated with 2 ml containing $10^3 - 10^5$ ccu of the *Mycoplasma* culture to be tested using micro-pipettes with filter tips. Growth control plates without antibiotics must be included in each test. The following controls were included: reference strain (the type strain of each *Mycoplasma* species being tested), plates incorporating the solvents used for each antimicrobial at the same concentration as that used in the highest concentration of the drug containing plates and a challenge inoculum

growth control. When the inoculum droplets have been absorbed, the plates were incubated at $36 \pm 1°C$ aerobically or in an atmosphere of 95% N2 + 5% CO_2. Incubation should be maintained in moist conditions, in sealed plastic bags or gas jars, for 7 days. MICs were recorded by comparing the amount of mycoplasmal growth on each plate with that on the antibiotic-free growth control plate using inverted microscope. The end-point (MIC) was the lowest concentration of antimicrobial to cause 50% inhibition of growth compared with that on the control plate. A 50% end-point has been taken to eliminate plates in which antibiotics show a "tailing effect" (very small numbers of mycoplasmas persisting beyond, what appeared to be, the end-point). Very often readings could be carried out macroscopically, but end-points should always be checked microscopically.

RESULTS AND DISCUSSION

Among respiratory mycoplasmas, *M. bovis* is the species most commonly associated with calf pneumonia and bovine respiratory disease (BRD) complex in Europe, North America and Israel, causing huge losses for the cattle industry worldwide [21-24]. As demonstrated in Table 1 *M. bovis* was isolated from the examined respiratory tract of cattle and buffaloes with incidence of 2.7 and 1.7%, respectively. Several studies have shown that *M. bovis* is the most common bacterium in feedlot cattle affected by chronic unresponsive pneumonia [25] and in veal calves with fatal bronchopneumonia [26]. In Israel, *M. bovis* was isolated from 26 to 54% of case submissions of cattle with pneumonia during 2004-2007, with the highest incidence (54%) in 2006 [27]. In Netherlands, Ter Laak *et al.* [28] found *M. bovis* in 20% of pneumonic lungs from fattening herds, but only in a small number of apparently healthy calves. In France, *M. bovis* was isolated from 30% of calf herds with pneumonia [29].

M. bovis was typed from vaginal swabs of the examined cows and buffaloes with incidence of 2.2 and 10, % respectively, while the incidence of *M. bovigenitalium* was 13.3 and 10 %, respectively (Table 1). Animals have become infected via the respiratory tract, the teat canal or genital tract; artificial insemination with infected semen is another common route [30]. The male genital tract can become infected with *M. bovis* through contact with other animals or, possibly, via a heavily contaminated environment. Infection of the prepuce or

Table 1: Types of *Mycoplasma* species isolated from bovine samples

				M. bovis		M. bovigenitalium		Other *Mycoplasma* species	
Sources of isolates		No. of examined samples	No. of isolates.	No.	%	No.	%	No.	%
Nasal swabs	Cattle	180	31	6	3.3	0	0.0	25	13.9
	Buffaloes	30	3	1	3.3	0	0.0	2	6.7
Trachea	Cattle	120	17	2	1.7	0	0.0	15	12.5
	Buffaloes	30	2	0	0.0	0	0.0	2	6.7
Lungs	Cattle	190	19	5	2.6	0	0.0	14	7.4
	Buffaloes	60	2	1	1.7	0	0.0	1	1.7
Bronchial lymph- nodes	Cattle	20	2	1	5.0	0	0.0	1	5.0
Vaginal swabs	Cattle	45	7	1	2.2	6	13.3	0	0.0
	Buffaloes	10	3	1	10.0	1	10.0	1	10.0
	Total	685	86	18	2.6	7	1.0	61	8.9

No = Number of positive

% = Percentage was calculated according to number of examined sample

Table 2: Minimum inhibitory concentration (MIC) determined by Microdilution method

	Antimicrobial agents tested								
	Streptomycin sulphate (µg/ml)		Erythromycin (µg/ml)		Gentamicin (µg/ml)		Tetracycline HCl (µg/ml)		Lincomycin (µg/ml)
Strains	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final
M. bovis (RS)	150	190	3.6	3.8	4.5	5.0	3.8	4.0	130	140
M. bovis (R)	230	250	4.0	4.6	6.0	6.4	4.2	4.6	140	160
M. bovis (G)	240	270	4.0	4.8	6.2	6.8	4.4	4.8	140	160
M. bovigenitalium (RS)	190	220	4.0	4.6	4.0	5.2	5.6	5.8	140	150
M. bovigenitalium (G)	270	300	5.4	6.0	5.4	7.0	6.8	7.2	150	160
M. bovigenitalium (G)	280	300	5.5	5.9	6.2	8.2	7.2	7.6	150	160

RS = Reference strain

R = *M. bovis* isolated from respiratory infection

G = *M. bovis* or *M. bovigenitalium* isolated from genital infection

urethra by *M. bovis* leads to an ascending infection of the testes causing orchitis, vesiculitis and decrease of semen quality and ultimately shedding in the semen [31]. *M. bovis* and *M. bovigenitalium* isolates were conducted the characteristic fried egg colony as shown in photo 1. The characteristic colony appeared as tiny, smooth circular, translucent masses with a dense raised central area [32]. Digitonin sensitivity of the isolates was carried out to differentiate between *Mycoplasmataceae* and *Acholeplasmataceae*. All detected isolates were sensitive to digitonin (Photo 2) and belonged to the family *Mycoplasmataceae*. Biochemical identification of *Mycoplasma* isolates was carried out according to Freundt [10]. The isolates were negative for glucose fermentation, arginine utilization and phosphatase activity. The isolates were serologically identified using growth inhibition test [14], dot ELISA, immunoblotting [33] as well as PCR (Photos 3-7). It was clear that the isolates were identified as *M. bovis* and *M. bovigenitalium*.

In this study, the antibiotic sensitivity test was successfully used to determine the MIC of 5 antibiotics (streptomycin, erythromycin, gentamicin, tetracycline and lincomycin) against *M. bovis* and *M. bovigenitalium* strains (*M. bovigenitalium* reference strain PG11 and *M. bovis* reference strain PG45) and isolates (2 *M. bovis* and 2 *M. bovigenitalium*) using microdilution and agar methods. Conventional techniques cannot be used for the determination of *Mycoplasma* antimicrobial drug sensitivity since *Mycoplasma* requires particular media to grow. No MIC breakpoints are widely accepted in veterinary medicine for the determination of *M. bovis* and *M. bovigenitalium* susceptibilities. Hannan [19] gave the guidelines and recommendations for determination of MIC testing of antimicrobial agents against veterinary *Mycoplasma* species. As shown in Tables 2 and 3 the MIC of streptomycin (>150 µg/ml), erythromycin (>3.6 µg/ml), gentamicin (>4.5 µg/ml), tetracycline (>3.8 µg/ml) and lincomycin (>130 µg/ml) against *M. bovis* were similar to those of *M. bovigenitalium* (190, 4, 4, 5.6 and 140 µg/ml, respectively).

Table 3: Minimum inhibitory concentration (MIC) determined by (Agar method)

	Antimicrobial agents tested									
	Streptomycin sulphate (µg/ml)		Erythromycin (µg/ml)		Gentamicin (µg/ml)		Tetracycline HCl (µg/ml)		Lincomycin (µg/ml)	
Strains	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final	MIC initial	MIC final
M. bovis (RS)	170	210	4.0	4.6	4.8	5.2	4.0	4.4	140	160
M. bovis (R)	260	290	4.4	4.8	6.4	6.6	4.4	4.8	160	170
M. bovis (G)	260	280	5.0	5.2	6.6	6.8	4.4	5.0	140	170
M. bovigenitalium (RS)	190	220	4.4	4.8	5.0	5.2	5.8	6.0	160	180
M. bovigenitalium (G)	290	310	5.8	6.2	5.8	7.2	7.2	7.6	180	200
M. bovigenitalium (G)	300	320	5.5	6.2	6.5	8.4	7.2	7.8	180	200

RS = Reference strain

R = *M. bovis* isolated from respiratory infection

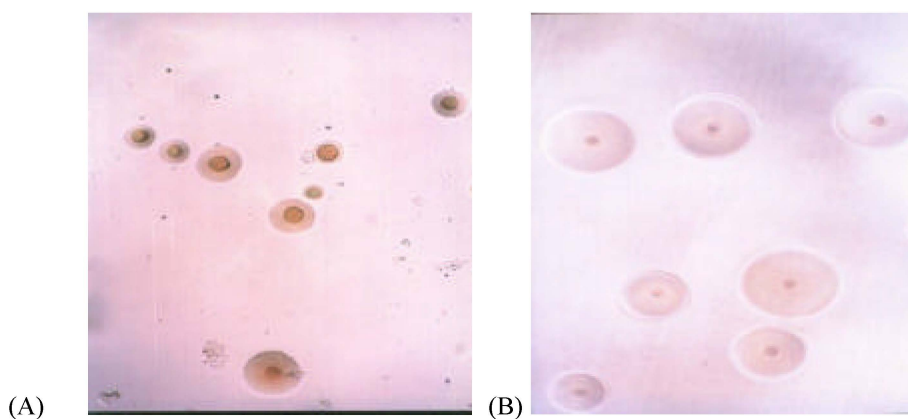G = *M. bovis* or *M. bovigenitalium* isolated from genital infection

(A)   (B)

Photo 1: Shows characteristic fried egg colony of *Mycoplasma* species.
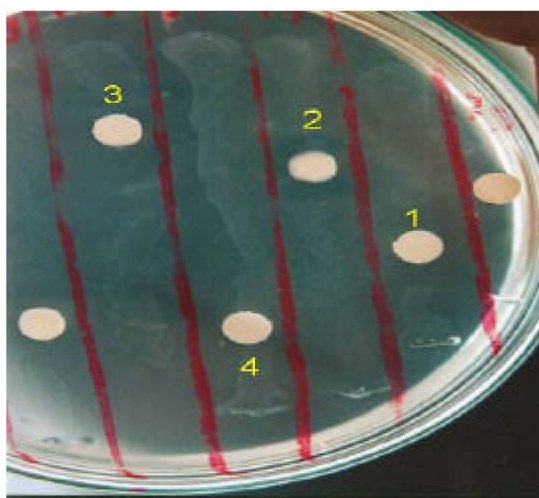(A):24 h *M. bovis* culture. (B): 72 h *M. bovigenitalium* culture.

Photo 2: Shows digitonin sensitivity test.
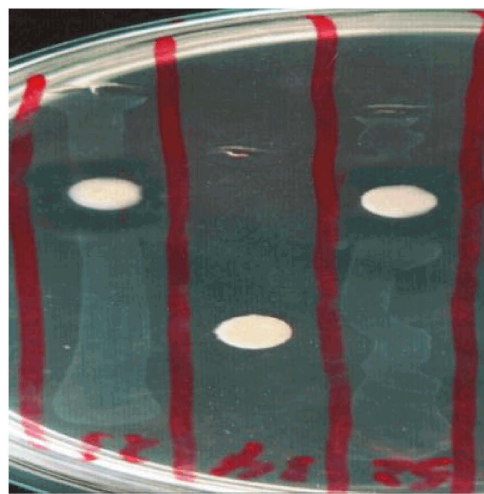1, 2 and 3 were sensitive to digitonin, while 4 was resistant (*Acholeplasma* control).

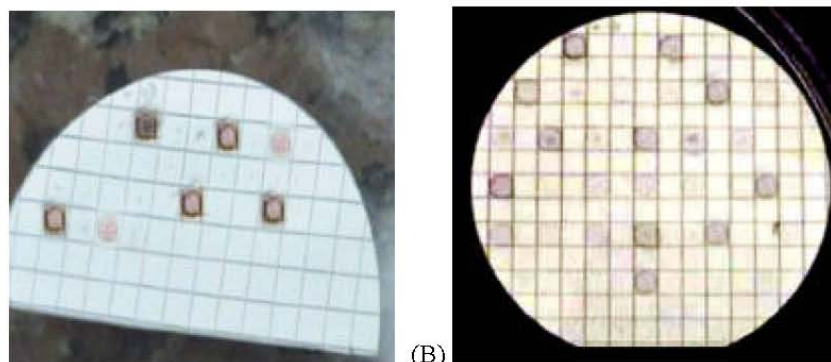Photo 3: Shows growth inhibition test.

(A)   (B)

Photo 4: Dot ELISA. A: Shows dot ELISA test against *M. bovigenitalium*, 5 strong positive and 2 weak positive
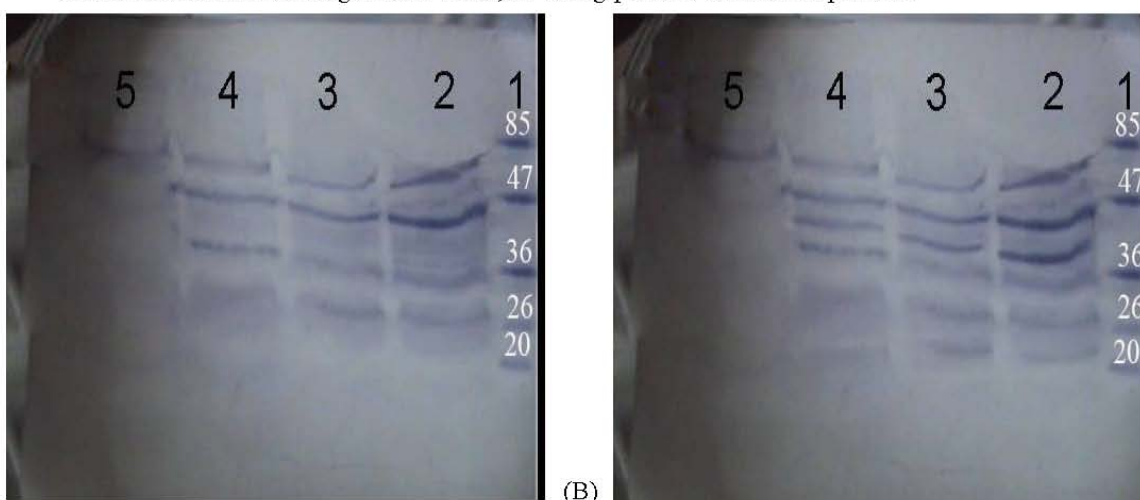B: Shows dot ELISA test against *M. bovis*, 11 strong positive and 8 weak positive.

(A)   (B)

Photo 5: Shows Immunoblotting against *M. bovis* and *M. bovigenitalium* isolates.
(A): Lane 1: protein marker, Lane 2: *M. bovis* reference strain PG45 (positive control), Lanes 3 & 4: *M. bovis* isolates and Lane 5: *M. bovigenitalium* reference strain PG11 (negative control). (B): Lane 1: protein marker, Lane 2: *M. bovigenitalium* reference strain PG11 (positive control), Lanes 3 & 4: *M. bovigenitalium* isolates and Lane 5: *M. bovis* reference strain PG45 (negative control).

M. bovis, like all mycoplasmas, is naturally resistant to many antibiotics because of its lack of a cell wall. Furthermore, over the last decade evidence has accumulated that *M. bovis* is becoming resistant to antibiotics which have traditionally been effective including the tetracyclines, tilmicosin and spectinomycin [7].

As shown in the same tables, MIC of tetracycline ranged from 3.8-7.8 µg/ml among the examined isolates and strains. Francoz *et al.* [34] recorded that *M. bovis* susceptibility against tetracycline was heterogeneous. These results were also suggestive of acquired resistance of *M. bovis* to tetracycline which has already been reported by other authors [7, 28]. MICs of erythromycin ranged from 3.6-6.2 µg/ml among the examined isolates and strains (Tables 9 and 10). All *M. bovis* strains were

resistant to erythromycin as recorded by Francoz *et al.* [34]. *M. bovis* had been previously reported to have MICs for erythromycin that ranged between 4 and 256 mg/ml [35] and MIC90 of 0.5 mg/ml [36]. It is clear that MIC determined by liquid medium assay was lower than those obtained by solid medium assay as previously recorded by Hannan [19]. Local *M. bovis* and *M. bovigenitalium* isolates have higher MIC values than standard strains (Tables 2 and 3), suggesting acquired resistance against streptomycin, erythromycin, gentamicin, tetracycline and lincomycin. *M. bovis* infections can have serious effects in both calves and adult cattle, but because of the costs and time traditionally taken for diagnosis, the true extent of the disease is often overlooked. Chemotherapy is a major and effective weapon against animal mycoplamosis but
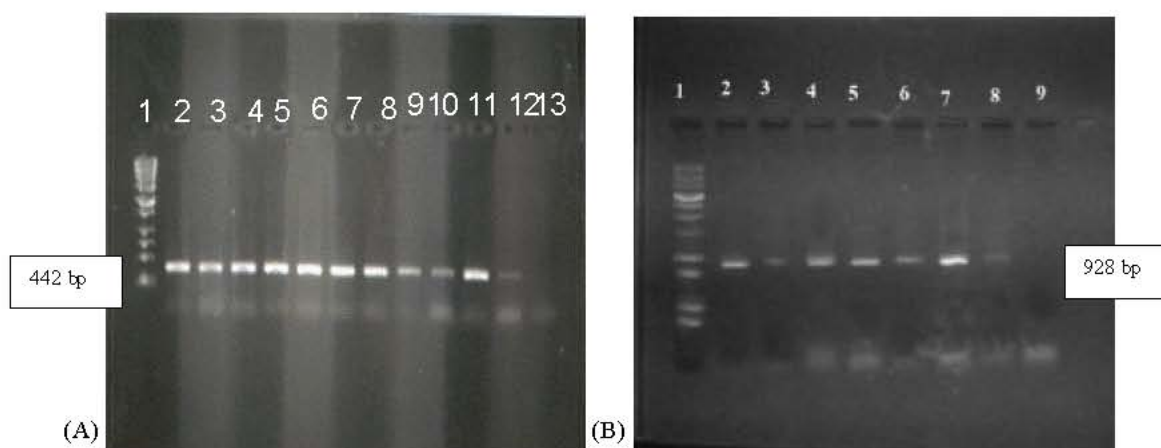
Photo 6:   Shows agarose gel electrophoresis showing amplification of 442 and 928bp fragments of *M. bovis* and of *M. bovigenitalium* respectively. (A): Lane 1: DNA ladder, Lane 2: *M. bovis* reference strain PG45 (control positive), Lanes 3 to 12: *M. bovis* isolates and Lane 13: *M. bovigenitalium* reference strain PG11 (negative control). (B): Lane 1: DNA ladder, Lane 2: *M. bovigenitalium* reference strain PG11 (control positive), Lanes 3 to 8: *M. bovigenitalium* isolates and Lane 9: *M. bovis* reference strain PG45 (negative control).

cannot offer a long term or environmentally sound solution. A vaccine is required urgently-ideally as a component of a multivalent bovine respiratory disease product.

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