j.Egypt.net.med.Assoc 77, no 4. 785 – 795 (2017)

# MICROBIOLOGICAL STUDIES ON *MYCOPLASMA* ISOLATED FROM EPIDIDYMIS OF CAMELS *(CAMELUS DROMEDARIES)*

By

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## ABSTRACT

In the present study, 48 testicles and sera of apparently healthy male camels were collected separately from El-Basateen abattoir, Cairo, Egypt. The testicles of different camels were aseptically separated and examined microbiologically for the presence of *Mycoplasma. Mycoplasma* species was isolated and identified biochemically from 10.41% (5/48) of the total collected samples. Further confirmation of the isolates with molecular characterization using PCR revealed 8.3% (4/48) of the collected samples were confirmed as *Mycoplasma bovis*. Serological investigation using indirect ELISA against *Mycoplasma bovis* antigen revealed a prevalence rate of 41.66% (20/48). ELISA found to be a rapid and applicable technique while PCR was confirmatory and more specific for the detection of *Mycoplasma bovis* infection in male camel genitalia.The minimum inhibitory concentration of antibacterial agents revealed that *Mycoplasma bovis* isolates were most sensitive to Enrofloxacin and Tylosin. The study focused the attention on the impact of *Mycoplasma bovis* infection as one of the suspected causes of infertility and or reproductive disorders in male dromedary camels.

## INTRODUCTION

Camels were formerly considered resistant to most of the diseases commonly affecting livestock, but as more research was conducted, camels were found to be susceptible to a large number of pathogenic agents. Increased interest in the camel as a multipurpose animal has been met with increased research into the etiology and pathology of camel diseases **(Abbas and Omer, 2005)**. But there is a general lack of epidemiological investigations explaining their diseases patterns **(Baumann and Zessin, 1992)**, this may be due to the fact that camel production is usually practiced on a migratory system with harsh living conditions that make such studies difficult and expensive to execute **(Khalafalla, 1998)**. *Mycoplasma* species are eubacteria that belonged to the class Mollicutes and are the smallest free-living and

self-multiplying microorganisms that lack cell wall **(Razin *et al.,* 1998)**. *Mycoplasma bovis* has been reported from cases of genital disorders and abortions **(Kaur *et al.,* 1987, Diemer *et al.,* 1996 and Abo-Elnaga *et al.,* 2012)**. The genital tract of male and female animals can harbor *M. bovis* and can be a source of infection **(Kreusel *et al.,* 1989)**.**Thanaa *et al.,* (2013)** reported the significance of *Mycoplasma arginini* in male dromedary camel. Male infertility has been reported following severe systemic or local infections in camelids **(Tibary *et al.,* 2006)**. Conventional culture method for identification of *M. bovis* is time-consuming and often gives false-negative results caused by overgrowth with another contaminating bacteria or fast-growing commensal mycoplasmas **(Nicholas *et al.,* 2008)**. Considering the limitations of individual tests, the use of at least two different laboratory tests (serological and molecular methods) may improve the diagnosis of *M. bovis* **(Szacawa *et al.,* 2016)**. Many investigators **(Ayling *et al.,* 1997, Konigsson *et al.,* 2002 and Moshkelani *et al.,* 2011)** concluded that, PCR represents a significant improvement on current tests as diagnosis of *Mycoplasma* infection can be made directly from clinical samples in less than 24 hours and it is a powerful and valuable tool for the identification of *Mycoplasma* isolates and the problems associated with some serological tests may be avoided.

## MATERIAL AND METHODS

**Samples:**

Testicles and sera of 48 adult (8-12 years) apparently healthy male dromedary camels were collected under sterile condition from EL- Basateen Abattoir, Cairo and transported directly to the lab. In ice box.

**Isolation and Identification of Mycoplasma species:**

The isolation of *Mycoplasma* was done according to **(Ruhnke and Rosendal, 1989)**. A testicle of each camel was sterilized externally and bacteriological swabs were taken from the tail of epididymis and transported into PPLO broth **(Difco)** and incubated at 37 $^{o}$C for 24 hours then platted on PPLO agar **(Difco)** plates under reduced oxygen tension in humidified candle jar for colonial characterization. Examination for *Mycoplasma* colonies was done after 48 hours then daily up to 7-10 days for fried egg colonial appearance. Followed by biochemical identification including:

- Digitonin sensitivity test **(Freundt, *et al.,* 1973)** to differentiate between family *Mycoplasmataceae* which is digitonin positive showing marked inhibition zone and family *Acheloplasmataceae.*

- Glucose fermentation test and arginine deamination test according to **(Erno and stipkovits, 1973)** were carried out**.** The molecular identification was performed by using PCR**;** extraction of DNA of isolates was done according to **(Ausubel *et al.,* 2003)**. Followed by running of PCR **(Riffon *et al.,* 2001);** the amplified reactions were performed in 50 µl volumes in micro amplification tubes. The reaction mixture consisted of 2 µl (200 ng) of extracted DNA template from bacterial cultures, 5 µl 10x PCR buffer, 1 µl dNTPs (40 µM), 1 µl Taq DNA polymerase, 1 µl (50 pmol) from primer pairs **(Yassi*n et al.,* 2004)**. The primer sequences are as follows: P1: 5 `GCA ATA TCA TAG CGG CGA AT 3`and P2: 5 ` TCT CAA CCC CGC TAA ACA TC 3`. The volume of the reaction mixture was completed to 50 µl using double distilled water. 40 µl paraffin oil was added and the thermos cycler was adjusted as follow; Initial denaturation: 94 ºC /2 minute. First cycle: denaturation 94 ºC /30 second, annealing: 48˚C /60 second, extension: 72˚C /150 second. The first cycle was repeated 35 times and final extension was at 72 ºC /5minutes. The amplified product size equals to 227 bp for *M. bovis* and loads 10 µl from PCR products.

**Serological examination:**

Indirect ELISA was done according to **(Cassell and Brown, 1983**) using a previously prepared antigen from identified *Mycoplasma bovis* isolate and anti-camel conjugate **(Abclonal, USA)**.

**Minimum Inhibitory Concentration (MIC):**

The test was performed as described by **(Hannan, 2000).** The antimicrobials (commercial products of Erythromycin, Tylosin, Oxytetracycline, Enrofloxacin and Lincomycin) were tested in serial two fold dilutions at concentrations ranging from 10.0 to 0.039 µg/ml. The M.I.C. was recorded as the lowest concentration of the antimicrobial that completely prevented a colour change (after adding pyrovate to broth). This typically occurred after 1 to 2 days. For comparison, a final reading was taken after 7-14 days incubation. The result was expressed in ug/ ml of active compound. The breakpoints of this test are as follow; value of 1 µg / ml as a guide to *Mycoplasma* susceptibility, with 2 - 4 µg / ml being intermediately susceptible and above 8 µg / ml being resistant as described by **(Ter Laak *et al.,* 1993).**

## RESULTS AND DISCUSSION

Reproductive disorders considered a major complaint in camel veterinary practice as camel herds suffer from low reproductive efficacy and birthing rates (rarely exceed 40% in nomadic herds and 70% in more intensive herds) and high neonatal loss sometimes reaching epizootic

proportions **(Kaufmann, 2005 and Tibary *et al.,* 2005)**. Different approaches to the diagnosis of *Mycoplasma* infection are used. For disease surveillance and herd screening, serological approaches are often used because of the low cost and rapid diagnosis but with some limitations in detection of the previous exposure and early infection stages **(Sachse *et al.,* 1993, Sachse and Frey 2003, Dudek *et al.,* 2013). Tibary *et al.,* (2005 and 2006)** reported that, *Mycoplasmas* is normal inhabitant in the respiratory, digestive and urogenital tracts of camels and there are little information about its role in the diseases of dromedary camels, this is partially due to the lack of investigations on the occurrence of *Mycoplasma* in camels, also, little data are available on the *Mycoplasma* flora of clinically healthy camels. **Elghazali *et al.,* (2011)** concluded that, the dromedary camel may act as carrier for *Mycoplasma* infection. *Mycoplasma* spp. infection was identified in 66 % of the single infected semen samples collected from infertile dromedary camels in Saudi Arabia **(Khaled *et al*., 2017)** which represents 30.9 % of the total samples. The obtained results (Table 1) revealed that, *Mycoplasma* species was recovered from 10.41% (5/48) out of all collected samples based on isolation, colonial characterization and the biochemical profile of the isolates which were sensitive to digitonin, negative for glucose fermentation and arginine hydrolysis. Confirmation was done by molecular characterization using PCR which revealed the characteristic electrophoretic pattern of *Mycoplasma bovis* at 227 bp for 4 out of 5 isolates with a rate of 8.3% (4/48) of the total collected samples. Other studies **(Ali *et al*., 1997, Nikolas and Ayling 2003 and Tibary *et al.,* 2006)** found that, *M.bovis* cause infections of the genital tract and may lead to infertility in male and female camels. Other *Mycoplasma* spp. were previously isolated as *Mycoplasma arginini* which isolated from male camel genitalia with a prevalence of 9% by **(Thanaa *et al.,* 2013)** who found that infection with *Mycoplasma arginini* resulted in increase in sperm abnormalities and abnormal acrosomes as well as decreased sperm motility and viability and concluded that the infection was occurred via the descending rout from the pneumonic lung and the ascending rout from the prepuce. Also **Gad *et al.,* (1989**) isolated *M. arginini* from the genital tract of camels and **Pfutzner and Sachse (1996)** reported that, the Infection of the male genital tract probably progresses upwards through the prepuce with *Mycoplasma* transmitted from a contaminated environment or descend to the testes producing orchitis, vasculitis and decreased of semen quality or shedding of the agent in the semen. The infection of male camel genitalia was occurred through two ways as previously described by **(El - Jakee *et al.,* 2008)** who concluded that, the

infection may be ascending manner from contaminated environment or from the she camel itself as bacterial infection of the epididymis usually occurs via two main routes, ascending infection from the urethra via the deference duct and accessory genital glands or hematogenous by descending infection from the pneumonic lung, or direct penetrating injury.

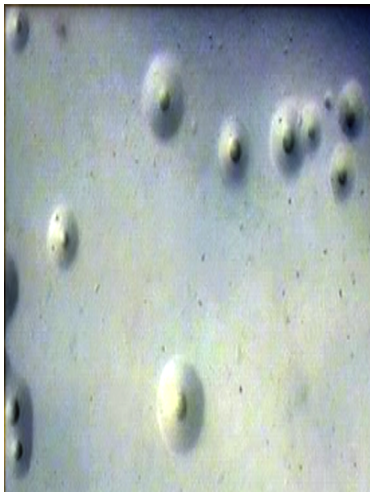

**Photo (1):** Fried egg appearance the characteristic colonial character of *Mycoplasma species.*

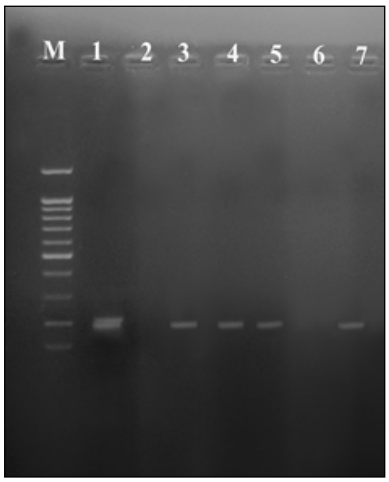


**Photo (2):** 1,cont. + ve. 2, cont. - ve.Characteristic elecrrophoretic pattern of *Mycoplasma bovis* at 227 bp**.** 6, -ve sample.

Regarding to the serological investigation (Table 1**)** it has revealed that, 41.66% (20 out of 48 serum samples) were seropositive to *Mycoplasma bovis*. **Egwu and Aliyu (1997)** conducted a sero-surveillance for *Mycoplasma* infection in clinical cases of pneumonia in the dromedary camel in Nigeria and found antibodies to *Mycoplasma mycoides* subspecies *mycoides* small colony by dot enzyme immunoassay and western blots at the rates of 12.1% and 6.8%, respectively.However,inKenya,to *Mycoplasma capricolum* subspecies *capripneumoniae* were detected in clinically normal cases by complement fixation test at the rates of 6.8% and 62.7%, respectively **(Paling *et.al.,* 1988)**. The difference between the bacteriological isolation (10.41%) and serology (41.66%) can be explained as false positive cases resulting from previous exposure and or non-genital *Mycoplasma* infection as under certain conditions *Mycoplasma* can cause alone or together with viruses and or bacteria some disorders **(Freundt, 1985)**. These results also go in hand with **(Razin *et al.,* 1998)** who found that**,** the highest number of seropositive cases without confirmation by antigen detection methods may be due to lack of specificity of the serological test, or that the antigen detection tests lacked

sensitivity or were limited by the sampling method, or that *M. bovis* is known to shed intermittently. Concerning the identification of *Mycoplasma* from apparently healthy animals may reveal the chronicity of the disease in agreement with **(Szacawa *et al.,* 2016)** who concluded that, the correlation between clinical signs indicating possible *M.bovis* infection and test positivity confirmed that clinical signs are not pathognomonic. In addition, PCR analysis of asymptomatic animals, have shown that many infected animals are unapparent, probably chronic carriers of these organisms **(El-Gmaal, 2007)**.

**Table (1):** Result of culture isolation and serology (ELISA) in the diagnosis of *Mycoplasma* infection.

| | | ELISA | | Total |
|---|---|---|---|---|
| | | Positive | negative | |
| Culture isolation | Positive | 3 | 2 | 5 (10.41%) |
| | Negative | 17 | 26 | 43 (89.58%) |
| Total | | 20 (41.66%) | 28 (58.33%) | 48 |

For the determination of the most suitable antibacterial agents to control the infection of *Mycoplasma bovis,* the minimum inhibitory concentration test was performed as described by **(Hannan, 2000)** using commercial products of Erythromycin, Tylosin, Oxytetracycline, Enrofloxacin and Lincomycin. *Mycoplasma bovis* isolates were found to be most sensitive to Enrofloxacin and Tylosin (Table 2). These results were in agreement with **(Zimmermann and Ross, 1975, Egwu, 1992 and Loria *et al.,* 2003)**. The high MIC value of erythromycin was also observed by **(Rosenbusch *et al.,* 2005)** who mentioned that erythromycin could not be recommended for treatment of *M. bovis* infections

**Table (2):** In vitro activities of anti-microbial agents (ug/ml) against *M. bovis* isolates.

| | Mean Values of MIC | | | | |
|---|---|---|---|---|---|
| anti-microbial agents / *Mycoplasma* isolates | Erythromycin | Tylosin | Oxytetracyclin | Enrofloxacin | Lincomycin |
| *M. bovis* | 0.625 | 0.078 | 0.312 | 0.078 | 0.156 |

Sensitive: 0-1 ug / ml. Intermediate susceptible: 2-4 ug / ml. Resistant: above 8 ug / ml. **(Ter Laak *et al.* 1993).** Camel infertility in males is considered a multi causes disorder and

different factors may adversely affect sperm quality and reduce fertility **(Waheed *et al.*, 2014)**. Male infertility has been reported following severe systemic or local infections in camelids **(Tibary *et al.*, 2006)**. Epididymis mycoplasmosis and its epidemiology in male camels is discussed in this study and could be a cause from different causes of infertility in agreement with **(Khaled *et al.*, 2017)** who characterized *Mycoplasma* spp. infection in 66% of the single infected semen samples collected from infertile dromedary camels in Saudi Arabia which represents 30.9% of total samples. In addition to **(El-Gamaal, 2007)** who isolated *Mycoplasma* spp. from camels in a total incidence rate of (18.7%) out of them (29.41%) were from the genital system, the highest recovery was from the preputial swabs (34%) with 34 isolates out of them 7 (20.60%) were *Mycoplasma bovis*. On the other hand **(Abo-Elnaga *et al.*, 2012)** isolated *Mycoplasma* spp. by 18 (33.9 %) and 23 (43.4%) from different samples collected from aborted, pregnant and infertile she camels and from aborted faeti and placenta, they identified *Mycoplasma bovis* in 12 (22.6%) and 18 (34%) by culture and PCR respectively. Therefore the role of genital mycoplasmosis in male and female camels is recorded and so, further studies are needed for its confirmation. As *Mycoplasma* spp. including *M. bovis* could be a cause of reproductive disorders and infertility in camels we recommend the previous bacteriological and molecular examination of male camel semen for *Mycoplasma* infection before the process of fertilization especially in those areas where reproductive disorders and or *Mycoplasma* infection were recorded.

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