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Application of the Polymerase Chain Reaction for Identification of *Mycoplasma* Isolated from Aborted She Camel

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Abstract: Infectious abortion is a significant cause of reproductive failure and economic losses in camels in Marriott Research Station-Desert Research Center, (Alexandria, Egypt) with previous history of *Mycoplasma* infections in this station. Thus the goal of this study was to detect *Mycoplasma* spp. A total of 53 samples were collected, out of them 47 were vaginal swabs (24 from pregnant, 20 from infertile and 3 from aborted she camels) and 3 samples were taken from each of stomach content and placenta of 3 aborted fetus. These samples were subjected to traditional culture methods and PCR specific for *Mycoplasma* spp. and *Mycoplasma bovis*. The results revealed that, *Mycoplasma spp*. was recovered from 18 (33.9 %) and 23 (43.4%) by culture and PCR respectively. At the same time, *Mycoplasma bovis* were recorded in 12 (66.7%) and 18 (78.2%) to the total no. of isolates by culture and PCR respectively. Results confirmed our assumption that, *Mycoplasma bovis* is the mainly cause of abortion which *was* recovered from each of aborted she camel, their placenta and their aborted fetus, In addition, PCR was faster, highly sensitive and specific for accurate detection of *Mycoplasma* spp. and *Mycoplasma bovis.*

Key words: Abortion · *Mycoplasma* · She · Camel · PCR.

INTRODUCTION

Mycoplasmas belong to the class Mollicutes and are among the smallest free living micro-organisms capable of auto- replication and are highly fastidious bacteria, difficult to culture and slow growing [1]. Many species are important veterinary pathogens causing many different diseases and occasionally abortion [1-3]. It is considered as highly contagious organism [4]. *Mycoplasma bovis* (*M. bovis*) is a very versatile pathogen and has been reported from cases of genital disorders and abortions [5, 6] and infertility [7] and reduction of semen fertility [8]. It is thought that they cause abortions by infecting the placenta or by causing fetal pneumonia. The genital tract of male and female animals can harbor *M. bovis* and can be a source of infection throw coitus and natural service [9] or via artificial insemination with deep frozen semen [10]. It can survive in frozen semen for several years [11]. *M. bovis* may be asymptomatically present as commensal organisms in the upper respiratory tract of older animals, where the *Mycoplasma* forms a constant source of infection for young animals that are more susceptible to developing clinical symptoms [12]. Consequently the pathogen cannot be detected during the incubation period. Moreover, the serological cross reactions among the *Mycoplasma spp*. (*M. spp*.) are a critical problem [13, 14]. In the absence of effective antibiotic or vaccination the only strategy currently available to control infection is the strict segregation of *M. bovis* infected animals from healthy herd.

The classical methods for detecting and identifying *M. bovis* are time consuming and complicated by serological cross-reactions between related organisms [15]. Additional problems can be caused by bacterial contamination of samples, as this usually prevents *Mycoplasma* growth [16]. In view of these difficulties, simpler, faster, and less hazardous and usually more sensitive and specific diagnostic methods are needed for detection of this organism [17]. Polymerase chain reaction (PCR) can yield rapid and specific diagnosis of infections caused by *M. bovis* [17, 18]. PCR with specific primers have better chance for the detection of *M. bovis* in both early and chronic infections [11]. In camels, birthing rate rarely exceed 40% in nomadic herds and 70% in more intensive herds [19].

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In addition to low birthing rates, camel herds suffer from high neonatal loss some times reaching epizootic proportions [19, 20]. The studies on the incidence and etiology of abortion in camelidae are scarce [19, 21] and little is known about the role of *Mycoplasma* in the etiology of diseases in camel. This is partially due to the lack of investigation on the occurrence of mollicutes such as *Mycoplasma, Uroplasma* or *Acholeplasma* in camels**.** Moreover, little data are available on the *Mycoplasma* flora of clinically healthy camels [22]. So the goal of this study was to throw out the light on *M. bovis* as a probable cause of abortion in she-camel in Marriott research station using PCR as a specific, sensitive and rabid technique for early diagnosis with comparison of culture method.

MATERIALS AND METHODS

Animals: Forty seven adult she-camels (24 pregnant, 20 infertile and 3 aborted) were subjected to examination in this study. The samples were collected from Marriott Research Station- Desert Research Center, Alexandria Egypt, for detection and identification of *Mycoplasma spp*. and *Mycoplasma bovis*, where this station had a previous history of *Mycoplasma* infection.

Samples: Fifty three samples were collected, out of them 47 vaginal swabs (24 pregnant, 20 infertile and 3 from aborted she camel) and 3 samples taken from each stomach content and placenta of 3 aborted fetus. Blood samples were also taken from examined animals for serological examination.

Serological Examination: Sera of examined animals were tested for presence of specific antibodies against abortifacient pathogens: *Brucella* spp by using Rose Bengal plate agglutination test [23]; *Chlamydophila abortus* by using Complement fixation test [24]; *Listeria monocytogenes* by agglutination test [25]; *Toxoplasma gondii* by using Toxolatex test kit [26] and Trypanosomosis using card agglutination test (CATT/T) according to the instructions of the manufacturer (Laboratory of Serology, Institute of Tropical Medicine, Antwerp, Belgium).

Organism: *Mycoplasma bovis* reference strain was provided from Prof. Dr. Ruhnke, Vet. Microbiol. Dept., Ontario Vet. Collage, Guelph, Ontario, Canada and used as a positive control.

Isolation and Identification of Mycoplasmas: Isolation of *Mycoplasmas spp.* was performed according to Ruhnke and Rosendal [27]. Biochemical and serological examination of the isolates were carried out according to Sabry [28] and Clyde [29]. Propagation of *Mycoplasma bovis* reference strain was preformed as described by Ruhnke and Rosendal [27].

Nucleic Acid Extraction: Genomic DNA was extracted from *Mycoplasma bovis* reference strain as described by Ghadersohi *et al*. [30]. From each swab of vaginal, placental and stomach content of aborted fetus samples, *Mycoplasma*-DNA was extracted according to Wit *et al.* [31] with some modifications and 5 µl of the final DNA solution were used per PCR.

Oligonucleotides Primers: The nucleotide sequences of primers used in the *Mycoplasma* group specific PCR assay were as follows: P1: upstream primer GPO-3, 5 `GGG AGC AAA CAG GAT TAG ATA CCC T 3`and P2: downstream primer MGSO, 5 `TGC ACC ATC TGT CAC TCT GTT AAC CTC 3` [32]. Primers were designed to detect *Mycoplasma bovis* by using primer 3 output software which is an internet program. The selected primers were tested by using Blast software which estimates the specificity of selected primers against all published sequences in the gene bank. The sequences are as follows: P3: 5 `GCA ATA TCA TAG CGG CGA AT 3` and P4: 5 ` TCT CAA CCC CGC TAA ACA TC 3`.

Polymerase Chain Reaction (PCR): The PCR assay conditions were performed as described by Hotzel *et al*. [33]. Each PCR mix contained 5 µl DNA sample, 5 µl of 10X Taq buffer (Gibco), 5 µl of deoxynucleotide triphosphates mix (2 mmol, promega), 1 µl (20 pmol/µl) each primer (P1and P2 primers in detection of *Mycoplasma spp*.; P3 and P4 in detection of *Mycoplasma bovis*), 0.5 µl of Taq polymerase (2 U, Gibco), 1.5 µl of 50 mmol MgCl2 and 31 µl of water. Each PCR mixture was overlaid with 40 µl of paraffin oil (Sigma) and amplified in a DNA thermal cycler (model 2400; Parkin-Elmer Cetus). After initial denaturation at 94°C for 2 min, 30 cycles of denaturation (94°C for 30 s), primer annealing (52°C for 60 s) and extension (72°C for 150 s) were run. Finally, all samples were incubated for an additional 5 min at 72°C to ensure that the final extension step was completed. PCR product from each reaction (10 µl) was separated on 1% agarose gel, stained with ethidium bromide and visualized using an ultraviolet transilluminator and photographed [34].

A visible band of appropriate size (270 bp in case of detection of *Mycoplasma spp*. and 227 bp in case of detection of *Mycoplasma bovis*) was considered as a positive reaction. To avoid false – positive PCR results the precautions for PCR described by Kwok and Higuch [35] were strictly followed. Positive control (all the components for the PCR with *M. bovis* reference strain DNA) and negative control (all the necessary components for the PCR except template DNA) were included in each set of amplifications.

Statistical Analysis: The sensitivity and specificity of the PCR assays were calculated according to Timmreck [36], taken the bacteriological isolation and identification as a gold standard.

RESULTS AND DISCUSSION

Camel is an important animal production resource in many areas of the world. The reproductive efficiency of camel is generally considerably low [20]. Uterine infection and abortion represent the major complaint in camel veterinary practice [37]. Regarding clinical symptoms: examined she-camels showed abortion with incidence (11.1%), infertility (42.5%). The aborted animals showed dyspnea, unsteady gait, uterine discharge, mild nervous manifestation and anorexia. The infected animals were treated with a combination of systemic broad spectrum antibiotics and uterine lavage [38].

Results regarding, causes of abortion in pregnant she-camel are shown in Tables (1and 3) and Photos (1 and 2). Out of 53 samples examined, 18 (33.9%) and 23 (43.4%) were positive for *M. spp.* by culture and PCR respectively. While 35 (66%) and 30 (56.6%) were found to be negative by culture and PCR respectively. Out of 18 (33.9%) and 23 (43.4%) isolates of *M. spp*. 12 (22.6 and 66.7%) and 18 (34 and 78.2%) (To the total no. of samples and isolates respectively), were found to be positive to *M. bovis* by culture and PCR respectively. Other performed serology tests for *Brucella, Leptospira, Chlamidophela, Listeria, Trypanosoma* and *Toxoplasma* showed negative results. Furthermore, another *Mycoplasma* isolate (unidentified) was recovered from the investigated samples. In Marriot Research station, there is a history of *Mycoplasma* infection in small ruminants [39] and cattle raised for meat production. These two factors contribute a constant source of infection to the adjacent camels and probably explain the high rates of *Mycoplasma* infection recorded in this study. This also represents a serious impact on the

Table 1: Comparison between culture and PCR for detection of *M. spp.* in different samples

		Result obtained			
		Culture		PCR	
Samples	No. of samples	No.	%	No.	%
Vaginal swabs from pregnant she camel	24	5	20.8	8	33.3
Vaginal swabs from infertile she camel	20	4	20	6	30
Vaginal swabs from aborted she camel	3	3	100	3	100
Stomach content from aborted fetus	3	3	100	3	100
Placental swabs	3	3	100	3	100
Total	53	18	33.9	23	43.4

Table 2: Comparison between culture and PCR for detection of *M. spp.* in different samples (Related to the total no. of samples)

	Bacteriological examination	
	Positive	Negative
PCR Positive	18	5
Negative PCR	0	30

Table 3: Comparison between culture and PCR for detection of *M. bovis* in different samples

		Result obtained			
		Culture		PCR	
Samples	No. of samples	No.	%	No.	%
Vaginal swabs from pregnant she camel	24	3	12.5	5	20.8
Vaginal swabs from infertile she camel	20	3	15	4	20
Vaginal swabs from aborted she camel	3	2	66.7	3	100
Stomach content from aborted fetus	3	2	66.7	3	100
Placental swabs	3	2	66.7	3	100
Total	53	12	22.6	18	34

Table 4: Comparison between culture and PCR for detection of *M. bovis* in different samples (Related to the total no. of samples)

	Bacteriological examination	
	Positive	Negative
PCR Positive	12	6
Negative PCR	0	35

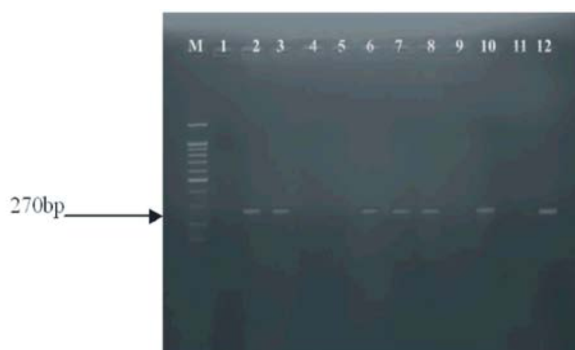

Photo 1: PCR amplification of *Mycoplasma spp.* DNA from different samples; M ,Molecular weight marker showed 100bp-1000bp DNA ladder (Hae 111 digest); lane (1) negative control; lane (2) positive control; lanes, 3,6,7,8,10, and 12 positive samples and lanes,4 ,5 ,9 , and 11 , negative samples.

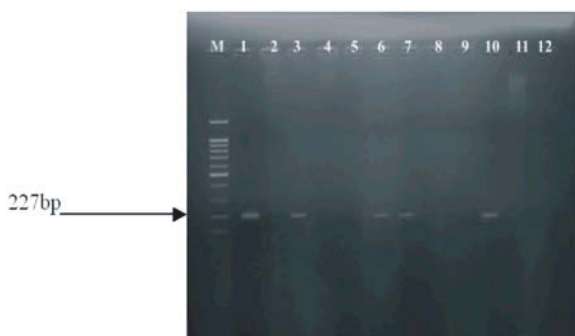

Photo 2: PCR amplification of *Mycoplasma bovis* DNA from different samples; M ,Molecular weight marker showed 100bp-1000bp DNA ladder (Hae 111 digest); lane 1 positive control; lane 2 negative control; lanes 3, 6, 7 and10, positive samples and lanes 4, 5, 8, 9, 11 and 12, negative samples.

fertility and overall production of the involved animals. The lower percentage of the isolation than that from PCR might be attributed to the fact that, isolation from chronically affected animals is some times difficult, because of the over growing of bacteria of secondary infections or the inhibitory effect of the administrated antibiotics [7]. The obtained percentage of *M. spp.* is lower compared with that recorded by Buzinhani *et al*.[40] who reported that, the percent of positive *Mycoplasma* is 63.4% isolated from 112 samples of vulvo-vaginal mucus of cows bearing reproductive disturbance. Also, Tambuwal *et al.* [41] detected *M. bovis* antibody in 41(66%) of cattle. On the other hand, the obtained percentage of *M. bovis* is higher than that detected by Buzinhani *et al.* [40] and El-Jakee *et al.* [42], who detected the same pathogen in 4 (9.8%) of vulvo-vaginal mucus of diseased cow and 7 (15.6 %) and 5 (50 %) from vaginal swabs of diseased cows and buffaloes respectively. This difference might be refereed to, species difference, immunostatus of the animals, methods of isolation and identification and consistent source of infection in Marriot station.

In the current work, there were 3 (11.1%) aborted cases from 27 pregnant she-camel. The cause of this abortion would mainly due to *M. bovis* which was isolated from each of, aborted one, their aborted fetus and their placenta. This finding is consistent with previous reports of Nicolas and Ayling [7], who confirmed that, from the infectious organisms causing infertility and abortion in animals is *M. bovis.* Also Futzner and Sachse [43] recorded that, *M. bovis* has been associated with genital infections and abortion in cows. Besides, it is responsible for several production diseases in cattle, including infertility [44].

Our result reveled that, *M. spp.* and *M. bovis* was recovered from the (30%) and (15%) respectively of infertile she camel, so it is mainly the cause of infertility. This go in hand with several investigators [7, 44, 45] who proved that, *M. bovis* cause infections of the genital tract which may lead to temporary or permanent infertility in the male and female camel. According to the available literature these results proved that*, M. bovis* is the main cause of abortion and infertility in she- camels and this go in hand with Kumar *et al*. [11] who recorded that, there is as such no survey available for the economic losses due to *M. bovis* infection. Tibary *et al*, Tibary. and Anouassi [19, 21] recorded that, the studies on the incidence and etiologies of abortion in camelidae are scarce.

Polymerase chain reaction (PCR) with specific primers has better chance for the detection of *M. bovis* in both early and chronic infections [11]. This came in agreement with our result in that, as shown in tables (1 and 3) and Photo (1 and 2), all bacteriologically positive samples were positive with PCR, whereas PCR detected 5 and 6 bacteriologically negative samples of *M. spp*. and *M. bovis* respectively and the 270bp and 227bp PCR product was observed on agarose gel electrophoresis for each of *M. spp*. and *M. bovis* respectively. On the other hand, the sensitivity and specificity of PCR were found as100% and 85.7% for *M. spp.* and 100% and 85.3% for *M. bovis* respectively as shown in tables (2 and 4). So, PCR was found to be rapid, highly sensitive and specific for accurate detection of *M. spp*. and *M. bovis* from the

investigated cases. This was confirmed by many investigators [17, 46- 50] who 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 h. and it is a powerful and valuable tool for the correct identification of *Mycoplasma* isolates. Since only small numbers of organisms are required for rapid identification, problems associated with some serological tests may be avoided. In conclusion, *Mycoplasma bovis* is of great importance as abortifacient agent in camels and should be followed in diagnosis and control. Also, this study revealed that, PCR is of major importance in diagnosis of camelidae mycoplasmic abortion.

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