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THE RELATION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS SHEDDING TO THE SEROCONVERSION IN THE CAMELS

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ABSTRACT

This study is aimed to examine the extent and nature of the *Mycobacterium avium* subspecies *paratuberculosis* (MAP) shedding among young camels. Attempt was also sought to fully genotype the camel MAP isolate. 450 faecal and serum samples were collected from young camel of age 1-5 years old. The PCR, ELISA, and faecal culture (Herrold's medium tubes with Mycobactin J) were used to detect MAP. The number of the ELISA positive samples was only 39 (8.7%) out of 450 while the PCR results were 188 (41.8%), but the samples that were positive to both tests were only 29 (6.44%). Unfortunately, the genotyping of the faecal culture isolates were shown that majority of them are environmental mycobacteria. In general, the current results and the previous observations indicated that young camels shed MAP continuously with low tendency to develop infection. High level of positive PCR results probably reflects the capability of the young camels to recirculate MAP in their environment rather than succumb to its infection. However, although camels recirculate MAP in their environment, it could propose that wildlife animals could be the prime source of MAP contamination to the camel environment. Hence, MAP shedding in young camels is not necessary means that the animals are infected or they will develop infection.

Key words: Camel, *Mycobacterium avium*, paratuberculosis

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease in domestic and wild ruminant like, cattle, sheep, goats, deer, antelope and bison worldwide (Stabel, 1997). In Saudi Arabia, Johne's disease was reported in sheep, goat, dairy cattle, and camel (Ahmed and Towfik, 1999; Alluwaimi *et al,* 1999; Al Hajri and Alluwaimi, 2007; Alluwaimi, 2008; Alhebabi and Alluwaimi, 2010).

Long incubation period is the main characteristic feature of MAP infection. Ingestion of faecal material, milk, or colostrum is the main route of infection. Infected cattle shed low amount of bacteria during the subclinical stage. However, during the clinical stage the shaded organisms in faeces increase dramatically. The major symptoms of infection are chronic diarrhoea, emaciation, decrease milk production, and infertility (Stabel, 1997).

Recent studies clearly demonstrated the influence of the stage of the disease and the nature of the shedding on the early detection of the disease with the ELISA (Nielsen, 2008; Dieguez *et al,* 2009). Using four different ELISA tests, faecal culture and PCR for the early diagnosis of the disease, it was demonstrated that the stage of the incubation period at which the test was performed has great influence on the early diagnosis of the disease. The MAP faecal culture incubation period lasts 12-16 weeks in which most of the positive animals are probably detected afterwards (Dieguez *et al,* 2009).

The ambiguity of factors that regulate the pattern of MAP shedding and the immunopathological mechanism involved in the initiating the humoral immune responses were seen the major factors that render ELISA and PCR sensitivity variable at the early stage of the incubation period (Nielsen, 2008).

The main objective of this study was to reveal the nature of the shedding of MAP infection in camel (*Camelus dromedarius*) in relation to the ELISA sensitivity in detection of the anti-MAP antibodies in the infected camels. The study was also aimed to isolate the camel MAP and carry out complete genotyping to reveal the nature of the MAP strain involved in the camel infection.

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Materials and Methods

Samples and animal

A total of 450 blood and faecal samples were collected from of 1-5 years old camels at Dammam abattoir, Saudi Arabia.

Detection of Anti MAP Antibodies using ELISA Tests

All serum samples were tested by commercial ELISA kit (ID VET Screen Paratuberculosis Indirect ELISA kits-France) for detection of the camel anti-MAP antibodies (Alhebabi and Alluwaimi, 2010). The test was applied according to the manufacturer's directions. Briefly, Samples were pre incubated in a neutralising buffer containing *Mycobacterium pheli,* after which transferred to the coated plates. An anti-ruminant IgG-peroxidase conjugate was then added to the micro wells and the substrate solution was added. Then the micro plate was read at 450 nm with plates were read by ELISA plate reader (Start Fax 2100, Awarness Technology Inc. USA).

Detection of MAP-DNA by Real Time PCR

Screening of the MAP DNA in the faecal samples was carried out with the real-time PCR kit (VetAlert, Tetracore, USA). The kit is made of two parts, the extraction kit and the DNA detection kit. This kit has primers and probe target the hspX gene of MAP. The extraction of the MAP DNA and its detection was performed according to the manufacturer's directions. Briefly, 1 gram of faecal material was transferred into a 5ml Sarstedt tube. 2ml of 1 x TE (10mM Tris-HCl+1mM EDITA) were added to each faecal sample. The faecal samples were placed into a disruption tube (containing glass beads). The tubes filled within the grooves of the disruption tubes and briefly vortexed with Mini Vortexer (VWR scientific product- USA) for a couple of seconds and then bead beated by using Biospec Mini-BeadBeater-16™ for 5min (Biospec Product, USA).

The samples then purified by centrifugation (Centrion K2202- Scientific Ltd-UK) in the disruption tubes for 10 minutes at 16,000g. The supernatant was placed in 2ml microcentrifuge tubes. 100µl of NABTM Buffer was added and the tubes were inverted for 5 times for the proper mixing. The samples were then centrifuged at 1200g for 3 minutes. The large brown coloured pellet was recovered and 560µl of binding buffer was added. The samples were vortexed for 5 sec and incubated at room temperature for 10 min. 560µl of 100% ethanol was added and mixed by vortexing for a few seconds. 630µl of each of the sample was added to the spin columns and centrifuged at 5,200x for 1 min and then 500µl of wash buffer A was added to the spin columns and recentrifuged for 1 minute and repeated with wash buffer B and further centrifugation without any buffers was also performed. The spin columns were placed into 1.5 ml eppendorf microcentrifuge tubes and 50µl of DNase free water was added to each of the spin columns to and incubated at room temperature for 1 minute. Then the columns were centrifuged at 5,200x for 1 minute to elute the DNA.

The targeted DNA was detected by adding 22.5µL of the mastermix to each thermocycler reaction tube or well. 2.5µL of each extracted sample, no template control, and positive control were added to the appropriate reaction tube or well. Reaction vessel was briefly centrifuge and loaded in the real-time thermocycler (Applied Biosystem 7500-USA). Cycling conditions were two steps PCR (95°C × 15 seconds, 62°C × 60 seconds) for 45 cycles. The reaction condition was set as outlined in manufactures manual as following: enzyme activation step: 10 minutes at 95°C, two step PCR: (95°C x 15 seconds, 62°C x 60 seconds) for 45 cycles, quencher to none, the reference dye as ROX and FAM as dye layer.

The results were interpreted according to the manufacturer's direction. Briefly, MAP DNA positive control on ABI PRISM® 7500; positive control was diluted from 25,000 gene copies to 1 gene copy. A run is considered valid if:

(a) The amplification curve of the positive control provided (25,000 gene copies / 2.5 µl) must have a cycle threshold value threshold cycle (Ct) that is between 20-26 cycles.

(b) The no-template control (NTC) must not cross the threshold prior to the endpoint of the test.

Faecal culture

The faecal samples were first decontaminated with MycoPrep reagents (Becton, Dickinson and Company, USA). Equal amount of faecal samples and MycoPrep were mixed in 50ml tube after, brief vortexing, samples were left to stand for 30 minutes. Sterile PBS up to 50 ml was added. The tubes were inverted for several times, and then they centrifuged at 3000g for 20 minutes. The supernatant was decanted and few drops of sediments were transferred to the Herrold's medium tubes with Mycobactin J. (BD Becton, Dickson And Company, USA). The inoculated tubes were incubated at 35°C in 5% CO_2 incubator in slant position with the caps loose. After one week, the tubes caps were tightened, and they placed in upright position in the incubator. The tubes were evaluated for the specific growth weekly.

Results

The animal group

The camels of age 1-5 years old was a group of animals which were mainly ELISA negative but PCR positives (Alhebabi and Alluwaimi, 2010). This group portraits the extent of the shedding and its pattern in relation to seroconversion.

The results of ELISA and PCR

There were only 39 (8.7%) out of 450 samples scored ELISA positive. The PCR positives, however were 188 samples (41.8%). The samples that were positive for both of the tests were only 29 samples (6.4%) (Table-1).

Table 1. The results of the ELISA and PCR of the total samples (450 faecal and serum samples).

	No.	Percentage
Elisa Positive	39	8.7%
PCR Positive	188	41.8%
Elisa & PCR Positive	29	6.44%

The results of the faecal cultures

The growth of some of the cultures was started as early as 2 weeks post incubation, while majority of the cultures were detectable after 5 weeks post incubation. The colonies were 0.5 cm in size, creamy colour, and they change the colour of the media from pale yellow to pale green.

The genotyping of the isolated colonies

The identity of the cultures was analysed by Dr. Beatriz R. Martínez at the Mycobacteria Unit, Centro de Vigilancia Sanitaria Veterinaria (VISAVET) (Centre for Veterinary Health Surveillance), Health Surveillance Centre, Madrid, Spain. Colonies from three cultures were selected according to their length of the incubation, 1 week, 3 weeks and 45 days. Initial analysis of the isolates indicated that they are PCR negative. To indicate whether these isolates are MAP the Hsp65 and the 16s rRNA genes were amplified and sequenced.

The comparison to the data in the genbank database was not possible to reveal precisely which mycobacteria they are because the sequenced DNA products were the same among several mycobacteria. However, the comparison of the three sequences among them showed that the isolates seem to be the same according to the 16s rRNA (the sequence of the reference 334 is slightly shorter than the others) (Table-2). On the other hand, the results obtained with the hsp65 gene are almost the same as those of the 16s RNA. Hence, it was revealed that all these Mycobacterium species are atypical and environmental mycobacteria (Table-2).

The isolates were identified as *Mycobacterium senegalense, Mycobacterium conceptionense, Mycobacterium fortuitum, Mycobacterium farcinogenes, Mycobacterium houstonense, Mycobacterium neoaurum, Mycobacterium smegmatis, Mycobacterium septicum* (Table-2).

Discussion

Mycobacterium avium subspecies *paratuberculosis* is widely detected in camels in Saudi Arabia (Alluwaimi, 2008; Alhebabi and Alluwaimi, 2010; Alharbi *et al,* 2012). Previous studies clearly demonstrated the feasibility of the commercially available bovine or ruminant ELISA and PCR techniques in the detection of MAP in camel (Alluwaimi, 2008; Alhebabi and Alluwaimi, 2010).

The group of camels (1-5 years old) was seen suitable for studying the nature of MAP shedding behaviour in the infected camels particularly to reveal if any of the known shedding patterns (transient, intermittent, low and high) is predominant. However, the outcome of the faecal culture genotyping has made this approach not completely practical. Nevertheless, the detection of high number of PCR positive animals in comparison to the ELISA positives and animals reacted positive to both of the tests, indicate clearly that camel has tendency of shedding high levels of MAP. The shedding of high levels of bacteria in the environment by this young group of camels without signs of infection, clearly demonstrate their heavily contaminated environment. Therefore, camels could have a tendency to transmit MAP to the environment continuously. This notion can be substantiated by our previous observations (Alhebabi and Alluwaimi, 2010). The positive PCR results of the 1-4 years-old camels were higher than the number of the ELISA positive camels of this group. Furthermore, the number of the camels that manifest the disease or seroconvert remains marginal in comparison to the PCR results (Alhebabi and Alluwaimi, 2010).

Studies that could incriminate the desert wildlife animals in Saudi Arabia to the transmitting MAP to farm animals were not addressed. However, extensive studies on rodents, rabbits, foxes, and birds in Scotland revealed that they are incriminated in transmitting MAP to the farm animals (Daniels *et al,* 2003; Beard *et al,* 2001).

In conclusion, young camels shed MAP continuously with low tendency to develop

Table 2. The results of the comparison of 16S rRNA and Hsp65 sequences of three isolates to each revealed that the isolates are not MAP but all are atypical environmental mycobacteria.

Reference	Growth time of culture	16S rRNA		Hsp65	
		Identification	Identity	Identification	Identity
MI1201822	45 days	*Mycobacterium senegalense*	100% identity	Not amplified	–
		Mycobacterium conceptionense	100% identity		–
		Mycobacterium fortuitum	100% identity		–
		Mycobacterium farcinogenes	100% identity		–
		Mycobacterium houstonense	100% identity		–
MI1201828	1 week	*Mycobacterium neoaurum*	99% identity	*Mycobacterium fortuitum*	99%
		Mycobacterium smegmatis	99% identity		
		Mycobacterium septicum	99% identity		
		Mycobacterium senegalense	99% identity		
		Mycobacterium conceptionense	99% identity		
		Mycobacterium fortuitum	99% identity		
MI12011830	3 weeks	*Mycobacterium senegalense*	99% identity	*Mycobacterium senegalense*	99% identity
		Mycobacterium conceptionense	99% identity	*Mycobacterium conceptionense*	98% identity
		Mycobacterium fortuitum	99% identity	*Mycobacterium fortuitum*	99% identity
		Mycobacterium farcinogenes	99% identity	*Mycobacterium farcinogenes*	99% identity
		Mycobacterium houstonense	99% identity		

infection. Majority of the tested animals behave as source of infection rather than succumb to it. However, although camels recirculate MAP in their environment, wildlife animals could be the prime source of MAP contamination. Hence, MAP shedding in young camels is not necessary means that the animals are infected or they will develop infection. Hence, the seroconversion most probably is the most trusted indication that could refer to the MAP infection in camels.

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