

PATHOLOGICAL AND MOLECULAR DIAGNOSIS OF PARATUBERCULOSIS AMONG DROMEDARY CAMELS IN SAUDI ARABIA

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ABSTRACT

This study was conducted on dromedary camels suffering from disease suspected Johne's disease. The clinical signs were chronic watery diarrhea, emaciation and decreased milk production. Inter-mandibular edema was noticed in two cases. Several investigations were performed, staining of intestinal smears with Ziehl-Neelsen stain, histopathology and PCR. Gross pathological examination revealed thickening of the intestinal wall with corrugation of the mucosa which extend in some cases to the colon and rectum along with granulomatous reaction in the liver. Mesenteric and ileocecal lymph nodes were moderately large and edematous. Histopathological lesions were diffuse granulomas characterized by extensive macrophages and epithelial cell infiltration into the mucosa and submucosa of the small intestine, and colon, with numerous acid-fast organisms. The ileocecal lymph node as well as the other mesenteric lymph nodes showed sinus histiocytosis, infiltration of macrophages and epithelial cells containing acid-fast bacilli. The different tissue, organs and fecal sample of infected camels with *Mycobacterium avium subspecies paratuberculosis* were strongly positive by IS900 PCR. In conclusion, the present study suggests more than one tool for the diagnosis of Johne's disease in dromedary camel.

Key words: Dromedary camel, paratuberculosis, PCR, histopathology.

INTRODUCTION

Camels in Saudi Arabia are farmed for their meat and milk. In recent times, the blooming of camel racing in the Gulf countries made camel as highly attractive. *Mycobacterium avium subspecies paratuberculosis* (MAP) causes Johne's disease in domestic and wild ruminant, cattle, sheep, goats, deer, antelope and bison worldwide (Stabel, 1997; Sharif *et al.*, 2013)). In Saudi Arabia, Johne's disease was reported in sheep, goat, dairy cattle, and camel (Al Hajri and Alluwaimi, 2007; Ghanem, 2013). The disease is accompanied by economic losses due to severe loss of weight and reduced milk production (Beauveau *et al.*, 2007; Kudahl and Nielsen, 2009).

The disease is characterized by the excretion of MAP in feces for months and years before the development of the clinical symptoms (Whittington and Sergeant, 2001). The exposure to contaminated feces constitutes one of the main risk factors for MAP transmission within the herd. There are many risk factors associated with the spread and persistence on MAP in livestock herds (Lombard, 2011). Till now, the disease is regarded as incurable (Singh *et al.*, 2011a).

The gross lesions of the disease are the thickness and corrugation of the intestinal mucosa, enlargement of mesenteric lymph nodes and hepatic granulomas (Mahmoud *et al.*, 2002). Extensive epithelioid cells infiltration in the intestinal mucosa, mesenteric lymph

nodes and liver are the main microscopic lesions of the disease (Hananeh *et al.*, 2013). Diagnosis of MAP is difficult because of the slow growth and the lack of sensitive tests to identify sub-clinically infected cattle. A wide array of procedures and laboratory tests ranging from conventional methods like ELISA test, Ziehl-Neelsen (ZN) stained smears, polymerase chain reaction (PCR) and histopathology of the ileum and mesenteric lymph nodes have been employed for its diagnosis (Buergelt and Ginn, 2000; Sikandar *et al.*, 2013). The technique right now that is the most focus of attention is the polymerase chain reaction (PCR). The potential value of the PCR in diagnosing *Mycobacterium avium subsp. paratuberculosis* infections have been recognized for some time and this technique has been applied in a variety of clinical samples (Salem *et al.*, 2012). Histological examination is still a reliable indicator (Kurade *et al.*, 2004; Sikandar *et al.*, 2012) and culturing technique is deemed as a gold standard for the diagnosis of paratuberculosis (Huntley *et al.*, 2005). Specific and sensitive diagnostic tools as well as a better understanding of the pathogenesis are needed to develop control programs to eradicate the disease. Therefore, the present study was carried out to investigate the clinical, pathological and molecular diagnosis of Johne's disease in dromedary camel.

MATERIALS AND METHODS

Animals: A total of 8 dromedary camels (five females and three males) aged between 4 to 6 years were suffering from disease suspected to be Johne's disease and admitted to the Veterinary Teaching Hospital, King Faisal University, Saudi Arabia for treatment. The camels were brought at separate time intervals within one month from the different location in the Saudi Arabia (Eastern Province). The animals were kept in separate pens to investigate the clinical disease, its pathology and etiology.

Necropsy and histopathological samples: Five camels with bad physical condition were submitted to necropsy. The camels were euthanized via intravenous route with sodium pentobarbital (60-80 mg/kg) according to the recommendations of the code of Practice for camels in Saudi Arabia. Animals were bled by severing the major vessels of the neck. The tissue samples were processed using sterile instruments, the excess fat and mesenteric attachments were trimmed off and intestinal contents were flushed out for the examination of intestinal mucosa. Tissue samples from organs displayed gross lesions such as the ileum, colon, mesenteric and ileocecal lymph nodes, and liver were collected. The tissues were trimmed and fixed at 10 % buffered formalin, embedded in paraffin wax, sectioned at 4-5µm thick and stained with haematoxylin and eosin (HE) as well as Ziehl-Neelsen (ZN).

DNA extraction and polymerase chain reaction:

Samples: Tissue samples, including ileum, colon, mesenteric and ileocecal lymph nodes in addition to fecal samples were collected and stored at -80°C till used for DNA extraction.

Fecal samples: Total DNA was isolated from fecal samples using the QIAamp DNA stool Mini Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's recommendation. Briefly, 180-220 mg of the samples were diluted by adding 1.4ml of ASL buffer, mixed by pulse vortexing and incubated at 70°C for 5 minutes. 1 inhibited tablet was added to 1.2ml of the supernatant samples and vortexing then centrifuged at full speed for 3 minutes. 15µl proteinase K was added to 200µl sample supernatant then 200µl buffer AL were added and vortexing for 15 seconds and incubated at 70°C for 10 minutes. 200 µl of absolute ethanol were added and mixed for 15 seconds by pulse vortexing. Complete lysate was transferred to a QIAamp spin column followed by centrifugation at full speed for 1 min. The binding DNA was washed by the addition of 500 µl AW1 buffer, then centrifuged at full speed for 1 min followed by the addition of 500 µl AW2 buffer then centrifuged at full speed for 3 min. The DNA was eluted from a column by adding 200 µl of buffer AVE,

incubation at room temperature for 1 min and centrifugation.

Animal tissues: Total DNA was isolated from the ileum, colon, mesenteric and ileocecal lymph nodes using the DNeasy Blood and Tissue Mini Kit (QIAGEN, Inc., Valencia, CA, USA) according to the manufacturer's recommendation. Briefly, 25 mg of the samples were cut up into small pieces and placed in a 1.5ml microcentrifuge tube, then added 180µl buffer ATL. 20µl proteinase K were added and mixed thoroughly by vortexing and incubated at 56°C until the tissue is completely lysed. 200 µl buffer AL was added and vortexing for 15 seconds. 200µl of absolute ethanol were added and mixed for 15 seconds by pulse vortexing. Complete lysate was transferred to a QIAamp spin column followed by centrifugation at 8000 rpm for 1 min, then the flow-through and collection tube were discarded. The binding DNA was washed by the addition of the 500µl AW1 buffer, then centrifuged at 8000 rpm for 1 min followed by the addition of the 500µl AW2 buffer then centrifuged at full speed for 3 min. The DNA was eluted from column by adding 200µl of buffer AE, incubation at room temperature for 1 min and centrifugation at 8000 rpm for 1 min.

PCR amplification: *M. Avium subsp. paratuberculosis* DNAs isolated from organs and fecal samples were amplified by PCR using primers P90⁺(5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3') and P91⁺(5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3') (Millar *et al.*, 1996). These primers are specific to IS900 that is an unusual DNA insertion element in the genome of *Mycobacterium avium subspecies paratuberculosis* (MAP). IS900 has been used extensively as a specific and sensitive DNA marker for identification and characterization of MAP from other mycobacterial species. PCR amplification was performed in a total volume of 20µl in a total volume 20µl of HotStartTaq® Plus Master Mix Kit (2x) "QIAGEN, Inc., Valencia, CA, USA". The PCR cycling profile consisted of one cycle for initial heat activation of 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minutes and final cycle for 10 minutes at 72°C. 10µl of each amplified product were analyzed by agarose gel electrophoresis on 1.5% agarose containing 0.5µg /ml ethidium bromide and visualized using gel documentation system.

RESULTS

The attached clinical signs were chronic and intermittent watery diarrhea for 1-4 weeks, emaciation and decreased milk production. Intermandibular edema (bottle jaw) was noticed in two cases.

Gross findings: The all necropsied camels were emaciated and showed gelatinous atrophy of subcutaneous and visceral fats. The lesions of the disease were confined to the ileum, but extend to involve the colon and rectum. The most prominent gross lesions were thickening and corrugation of the small intestine (ileum) up to three or four times normal thickness (figure 1a). The mucosa of

colon was folded into transverse ridges, similar to cerebral convolutions that could not be reduced by stretching (figure 1b). The ileocecal and mesenteric lymph nodes were congested and edematous, granular in some cases (figure 1c). Whitish granulomas of variable size and shapes were seen dispersed all over the hepatic tissue (3 cases) (figure 1d).

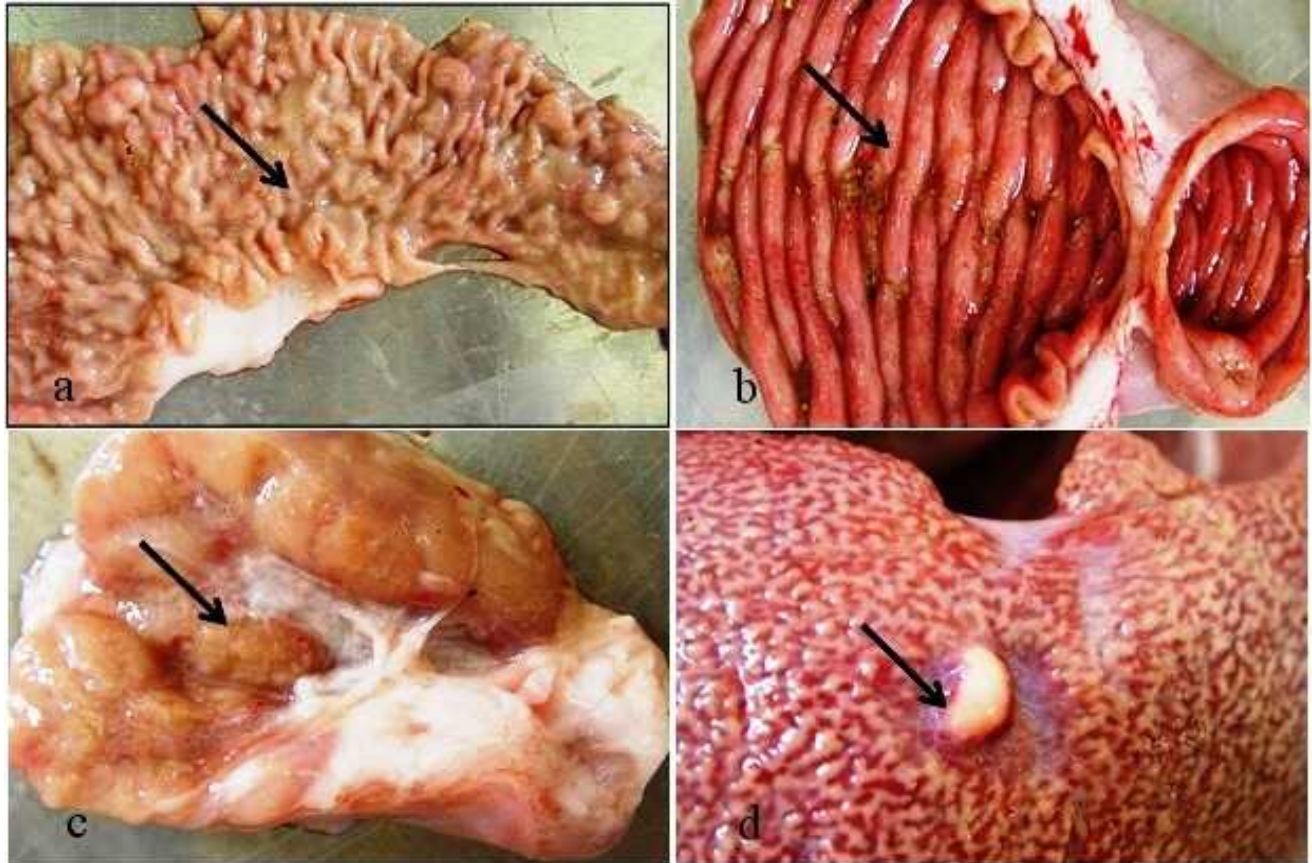


Figure 1. a- Ileum mucosa showing thickening and corrugations (arrow). b- Colon mucosa showing transverse ridges (arrow). c- Mesenteric lymph node with granular surface (arrow). d- Liver showing whitish nodule (arrow).

Microscopic findings: The consistent histologic findings in all cases were diffuse granulomas characterized by extensive foamy poorly basophilic macrophages, epithelioid cells and lymphocytes infiltration into the mucosa and submucosa of the small intestine with numerous acid-fast organisms (figure 2a). Similar findings were observed in the mucosa and submucosa of the colon, but the cellular infiltrations are less extensive (figure 2b). The ileocecal lymph node as well as the other mesenteric lymph nodes showed hyperplastic activation mostly in lymphoid follicles and medullary cords (figure 2c). This was associated with sinus histiocytosis,

infiltration of macrophages and epithelioid cells containing acid-fast bacilli. The Liver showed lepromatous type of granulomas, characterized by aggregates of macrophages, epithelioid cells and lymphocytes intermingled with a fibrous connective capsule (figure 2d).

Detection of the MAP DNA by PCR: The total DNA samples extracted from the different tissue and fecal sample of animal infected with *Mycobacterium avium subspecies paratuberculosis* were strongly positive by IS900 PCR (Figure 3).

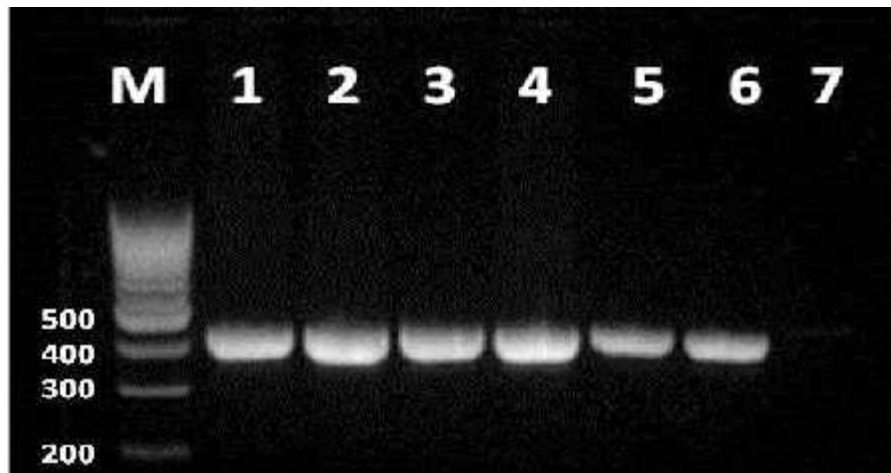


Figure 2. a- Ileum villi showing extensive aggregations of macrophage and epithelioid cells (arrow) HE bar 40 μ m. b- Colon submucosa, note aggregations of macrophage and epithelioid cells between and around the glands (arrows) HE bar 20 μ m. c- Mesenteric lymph node showing hyperplastic medullary cords (arrow) HE bar 20 μ m. d- Liver showing lepromatous granuloma, note macrophage and epithelioid cells intermingle with connective tissue capsule (arrow) HE bar 20 μ m.

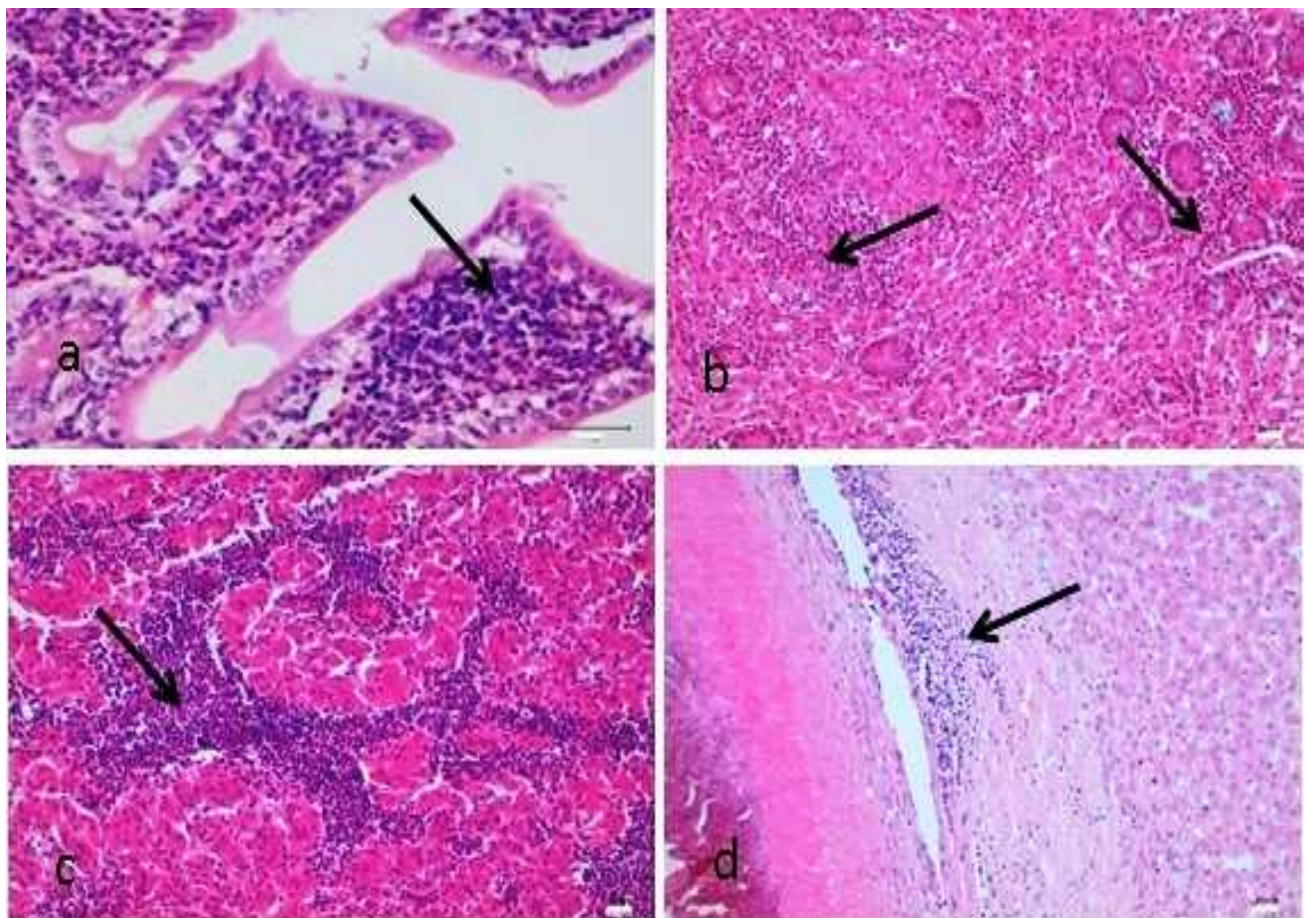


Figure 3. Ethidium bromide-stained agarose gel showing the approximately 400bp *Mycobacterium avium subspecies paratuberculosis* band products. Lane M: 100bp of PCR molecular weight markers; Lane 1: mesenteric lymph nodes; Lane 2: ileum; Lane 3: ileocecal lymph nodes; Lane 4: colon; Lane 5: faecal sample; Lane 6; DNA positive control of *Mycobacterium avium subspecies paratuberculosis* and Lane 7: Negative control DNA.

DISCUSSION

Mycobacterium avium subsp. *Paratuberculosis*(MAP) is the etiological agent of a severe gastroenteritis in ruminants, known as Johne's disease. It has a significant impact on the global economy (Salem *et al.*, 2005), and the isolation of MAP from intestinal tissue of Crohn's disease patients has led to concerns that it may be pathogenic for humans (Alluwaimi, 2007; Behr and Kapur, 2008; Singh and Gopinath, 2011). Thus, the pathogenic role of *M. paratuberculosis* in camel population and its efficient control are topics of intense debate (Almujalli and Al Ghamdi, 2013).

The present study confirmed that the clinical signs and pathology of Johne's disease in the camels are quite similar to those described for the disease in other farm animals (Sivakumar, 2006). When the ileum became thickened, reduce the ability to absorb fluids, diarrhea and a condition known as a protein-losing enteropathy occurred (Yayo Ayele *et al.*, 2001). In advanced Johne's disease, animals had lost a marked amount of weight. The fat layers surrounding kidney, heart and intestine were also missed and replaced by gelatinous material. Histopathological lesions were confined to small intestine, colon, mesenteric and ileocecal lymph nodes, and liver. The reaction was granulomatous in nature (Hananeh *et al.*, 2013). The granulomas in all cases seen so far in camels are of the lepromatous type and characterized by the presence of foamy poorly basophilic macrophages and epithelioid cells that contain large numbers of acid fast bacilli in liver surrounded by a fibrous capsule. Involvement of the liver was probably due to spread of infection by infected macrophages from the intestine through the blood stream. Paratuberculosis has been described in the liver and other vital organs such as lungs and kidneys in animals other than the camel (Mahmoud *et al.*, 2002). Diagnosis of paratuberculosis is difficult because of the fastidious growth pattern of the microorganism and the different host immune responses invoked during subclinical and clinical stages of infection (Al-Hajr and Alluwaimi, 2007). Methods of detection of *M. paratuberculosis* infection has also been developed using nucleic acid probes combined with the PCR (Singh *et al.*, 2011b). PCR tests for detection of *M. paratuberculosis* in fecal samples have vastly improved in recent years, leading to an increased sensitivity of detection of low shedders, including a detection level of one colony-forming unit(s) per gram of feces. This is done by amplifying the IS900 gene sequence which is the most reliable way for detecting cows shedding low levels of *M. paratuberculosis* in their feces (Stabel *et al.*, 2004).

In conclusion, the clinical signs and pathology of Johne's disease in camels are consistent with those of paratuberculosis in other farm animals. The camel has a high incidence of liver involvement of paratuberculosis

compared to other farm animals. PCR on camel fecal samples is a fast, reliable test and should be applied routinely when screening for MAP within herds suspected of paratuberculosis.

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