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# CLINICAL AND MOLECULAR INVESTIGATIONS OF JOHNE'S DISEASE AMONG SMALL RUMINANTS IN MAKKAH, SAUDI ARABIA

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**Abstract:** Paratuberculosis (PTB) or Johne's disease (JD) is a chronic debilitating disease caused by the *Mycobacterium avium* subsp. *paratuberculosis* (MAP). JD affects a wide range of animals including ruminants and characterized by enteritis and progressive diarrhea. The current study aimed to implement molecular tools for detection and identification of MAP among clinically suspected small ruminants in Makkah region, Kingdom of Saudi Arabia (KSA). A total of 2660 small ruminants from five different farms around Makkah were clinically investigated for characteristic signs of JD during the period of November 2013 to February of 2014. Out of investigated animals, 16 cases were selected as being suspected of JD infection based on the associated clinical symptoms (emaciation and unthriftiness with or without persistent diarrhea). Rectal scrapings were collected from all suspected animals and were subjected for molecular examination. Three different genetic targets were evaluated including 16S rDNA, insertion sequence 900 (IS900) and intergenic spacer (IGS). Initial examination of suspected specimens by amplification of universal bacterial primers for 16S rDNA revealed positive results in 6 out of 16 cases (37.5%). Further amplification of the *Mycobacterium*-specific IGS target from 16s rDNA-positive samples revealed the detection of 4 *Mycobacterium* species. Sequence analysis of the IGS sequence of these 4 *Mycobacterium* species revealed 2 cases only as *Mycobacterium avium paratuberculosis*. This finding was confirmed by the detection of MAP-specific IS900 target only from the same 2 cases. In conclusion, the current study genetically documented the first report of MAP (the causative agent of JD) among small ruminants of Makkah region.

**Key Words:** Johne's disease; microscopic examination; molecular investigation; small ruminants; Saudi Arabia.

## INTRODUCTION

Johne's diseases (JD) or paratuberculosis (PTB) is a chronic, progressive enteric disease of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis is a chronic infectious disease of cattle, small ruminants and wild ruminants, characterized by therapy-resistant diarrhea and weight loss. The spread of MAP could significantly hamper the livestock industry via its detrimental impact on animal health and the consequential loss in productivity [1, 2]. Identification of infected animals and their eradication form the basis of treatment and control of JD. Diagnosis of the disease depends on characteristic clinical signs confirmed by a wide range of diagnostic tests that categorized into those that identify the organism and those that identify an immunological reaction to the organism [3, 4].

Paratuberculosis usually pass through 3 main stages depending on the severity of clinical signs and the potential for shedding organisms into the environment with the subsequent detectability by different diagnostic assays [5, 6]. Silent infection is the first stage of infection that characterized by absence of clinical signs of infection. Infected animals in this stage may shed infectious organisms into the farm environment at levels below the threshold of detection by conventional methods [7]. The second stage is the subclinical infection, where animals at this stage do not yet have clinical signs of infection, but may be detected as infected by using cost-effective diagnostic tests [8, 9]. Some of these infected animals may be detected by fecal culture. However, focal lesions, variable rates of disease progression and shedding, and dilution of organisms in large volumes of intestinal content result in intermittent detection of fecal shedding [10]. Therefore, other infected animals may test negative by using current fecal culture techniques. Some animals may have detectable antibodies to MAP, particularly if they are getting close to entering the next stage of the disease (clinical phase) [11]. However, MAP fecal shedding usually occurs before a detectable antibody response [12]. The third stage is the clinical infection stage during which initial clinical signs following a prolonged incubation period start to appear. The first apparent sign is gradual weight loss, despite a normal appetite. Concurrent with the weight loss, the manure consistency becomes more fluid. The diarrhea may be persistent or intermittent, at first, with periods of normal manure consistency [5]. Most animals at this stage have a positive fecal culture and have increased serum antibody to detectable levels. Several

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commercial enzyme-linked immunosorbant assay (ELISA) and agar gel immunodiffusion (AGID) test were reported for detection of JD infection with variable sensitivities and specificities based on the stage of the infection and the prevalence of the disease [3, 4 and 13].

Due to the delays between time of infection and development of measurable immune system reactions and shedding, various "gold standards" have been utilized for MAP-infection status in the past. Tissue culture of MAP is considered the ideal gold standard test, because, even before fecal shedding or an immune response is present, it can detect growth of MAP in multiple organs, including the intestinal mucosa and submucosa, and regional lymph nodes [14]. However, fecal culture is not usually used as a routine method for diagnosis due to the high cost and logistical difficulties of sampling for tissue culture [4, 15, 16]. Alternatively, fecal culture can be applied to various species and sample types (feces, tissue, water, soil) during the clinical stage of the disease to confirm infection. However, culture is unreliable in sheep and some other ruminant species when using standard laboratory techniques. It is labor intensive and may require 8-24 weeks of incubation for colonies to be observed based on the type of media used and its sensitivity is low [3, 5 and 17] . In term of sensitivity, recent studies have shown the superiority of PCR-based molecular assays over conventional culturing methods for sensitive and accurate detection of MAP infection among subclinical and clinical cases [18]. Several genetic targets were evaluated for sensitive and accurate detection and identification of different *Mycobacterium* species including *Mycobacterium* species-specifc 16S rDNA sequence [19] and intergenic spacer (IGS) [20] as well as MAP-specific IS900 target [21].

In Saudi Arabia, sheep and goats constitute an integral part of the animal population and are raised principally by private breeders mainly for meat production due to their manageable size and feed requirements plus their ability to utilize low-grade food and limited pasture areas [22]. In Makkah hundreds of thousands to millions of sheep and goats are utilized annually during pilgrimage season. This necessitates the importation of large quantities of small ruminants from various parts of the world, which could represent a source of infection to the Saudi herds [23]. JD was reported in certain regions of Saudi Arabia among different animals species including small ruminant [24-27]. However, no reports had documented the presence of the disease in Makkah region. As a probing investigation, the current study aimed to document the presence of JD in Makkah region among clinically suspected small ruminants. For this purpose, 3 genetic targets (16S rDNA, IS900 and IGS) were used for molecular detection and identification of MAP- the causative agent of the disease.

## MATERIALS AND METHODS

### *Clinical investigation and sampling*

Five small ruminant farms around Makkah with a total number of 2660 animals (goats and sheep) were visited during the period of November 2013 to February of 2014. Clinically suspected animals were selected and isolated based on history and clinical examination. Inclusion criteria included animals suffering from debilitation and emaciation that is unresponsive to de-wormers and antibiotics, intermittent diarrhea or softened, pasty stools, low-grade fever, lethargy, and depression [28]. Fecal samples (including rectal swabs) were collected from clinically suspected sheep and goats. Preparation of the samples, faecal direct smears and molecular examination were carried out in the microbiology and molecular biology laboratories at the environmental and health research department, The Custodian of the Two Holy Mosques Institute for Hajj & Umrah Research, Umm Al-Qura University, Makkah Al-Mukaramah.

### *Conventional Microscopic examination*

Collected samples from clinically suspected cases were initially evaluated microscopic examination for expected acid fast bacilli. For this purpose direct smears were prepared from fecal specimens and rectal scrapings that were stained with standard ZN staining procedure according to Quinn et al. (1994) [29].

### *Molecular detection and identification*

***DNA extraction from fecal specimens:*** DNA was extracted from all collected specimens as previously described [30] using Promega Genomic Wizard DNA Purification Kit (Promega, Madison, WI, USA), with some modification. Briefly, the fecal material was diluted in PBS and homogenized with easyMIX® Lab Blender (AES Chemunex, Bruz Cedex, France) in filter bags (Seward, Thetford, Norfolk, UK). Pellets were washed with 1 ml of TE (10 mM Tris, pH 8, 10 mM EDTA) and were re-suspended in 100 µl of TE. After addition of 50 µl of 10% SDS the mixture was incubated for 30 min at 65°C. The lysates were centrifuged and supernatants were removed. The microtubes were then placed in a microwave oven (with specifications; LG grill, model No. MG-604AZ, input 220v-50/bHz, microwave 1350 w, RF output 900 w,2,4500 MH) and heated twice for 1 min at 900 W or three times for 1 min at 750 W. The pellets were dissolved in 200µl of TE and were extracted with an equal volume of phenol/chloroform/ isoamyl alcohol (25:24:1) for 15 min. The aqueous phase was recovered by centrifugation for 20 min and precipitated with ethanol

***Amplification of selected genomic targets:*** Three genomic targets were amplified from DNA obtained from all suspected animals' specimens. This included 16S rDNA, IGS and IS900. The hyper variable 500 bp. segment of the 16S rDNA was amplified using the previously described universal bacterial primer set [31]. In addition, the hyper variable region of the intergenic spacer (IGS) target was amplified from suspected samples using the previously described pan-*Mycobacterium* primer set ITS-A1 and ITS-A6 [32]. Finally, the MAP-specific IS900 target was amplified using primer set P90 (5_GAAGGGTGTTCGGGGCCGTC) and P91 (5_ GAGGTCGATCGCCCACGTGAC) as previously described by Khare *et al.,* (2004) [33]. All primer sets were obtained from IDT Integrated DNA technologies (IDT, Belgium).

In the PCR assay, 5 µl of template DNA (10 ng/µl) in a total reaction volume of 50µl to include PCR buffer [20 mmol Tris-HCL (pH 8.4) and 50 mmol KCl], 0.1mmol each of dNTP (deoxyribo nucleotide triphosphate), 1.5 mmol of $MgCl_2$, 0.3µmol of each primer, and 1.5 U of RED Taq DNA polymerase were used. The PCR amplification was performed in a Techne thermocycler model TC-312 starting with an initial denaturation step at 95°C for 10 min, followed by 35 cycles where each cycle consisted of a denaturation at 95°C for 1 min, an annealing at 64°C for 30 sec, and an extension step at 72°C for 1 min. After PCR, 10 µl of the PCR product was mixed with 5 µl dye mixture (0.25% bromophenol blue and 0.25% xylene cyanol in 15% Ficoll type 400) and electrophoresed in 1 µl Tris-acetate-EDTA buffer through a 1% agarose gel containing ethidium bromide (0.5µg/mL). Bands of the appropriate size were visualized using a UVP gel documentation system according to the manufacturer's instructions and identified by comparison with a 100-bp DNA ladder (DNA molecular weight marker Promega).

**IGS sequence analysis:** Purified IGS products from all IGS-positive samples were sequenced using the same IGS-amplification primer set. Obtained sequences were analyzed using both the custom MycoAlign database [32] and BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/).

## RESULTS

Sixteen clinically suspected cases were selected upon clinical examination of the total 2660 investigated small ruminants. These included 12 sheep and 4 goat distributed between different investigated farms (Table 1). Initial microscopic screening of ZN-stained direct faecal smears from all suspected cases, showed that five out of the suspected 16 cases (31.3%) were positive for acid fast bacilli (Figure 1).

**Table 1:** Numbers of clinically suspected small ruminants after clinical examination of investigated animals.

| Investigated farms | Clinically suspected / Total investigated cases | | |
|---|---|---|---|
| | Sheep | Goat | Total |
| Farm 1 | 4 / 800 | 0 / 50 | 4 / 850 |
| Farm 2 | 4 / 1000 | 0 / 80 | 4 / 1080 |
| Farm 3 | 4 / 400 | - / - | 4 / 400 |
| Farm 4 | - /- | 2 / 100 | 2 / 100 |
| Farm 5 | 0 / 90 | 2 / 140 | 2 / 230 |
| Total | 12 / 2290 | 4 / 370 | 16 / 2660 |

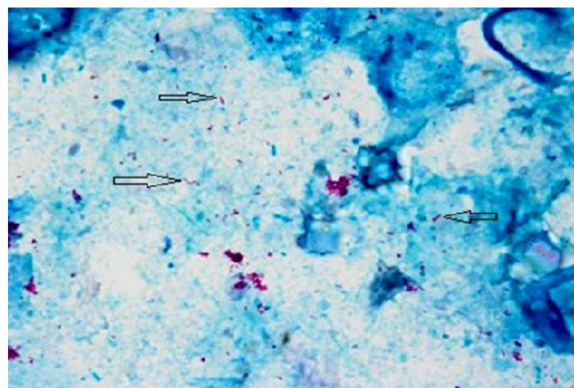

**Figure 1:** Pairs and clumps of the red stained short bacilli on blue background in ZN-stained direct fecal smears.

Molecular-based detection and identification assays revealed that 6 samples were initially positive for the bacterial-specific 16S rDNA target (Figure 2). Of the 6 16S rDNA-positive cases, 4 isolates were positive for the IGS target and typed as *Mycobacterium* species, and 2 isolates were negative for IGS target and proposed as non-*Mycobacterium* species (Figure 3). On the other hand, examination of all suspected samples with the MAP-specific IS900 targets revealed only 2 positive cases (Figure 4). Sequence analysis of the obtained *Mycobacterium* species-specific IGS targets from suspected mycobacterial isolates identified 2 isolates as being MAP, which confirmed the IS900 target findings, while the other 2 isolated were identified as being *M. tuberculosis* complex and *M. fortuitum sequevar Mfo-B*. The genetically confirmed 2 MAP cases were one sheep from farm 1 and one goat form farm 4.

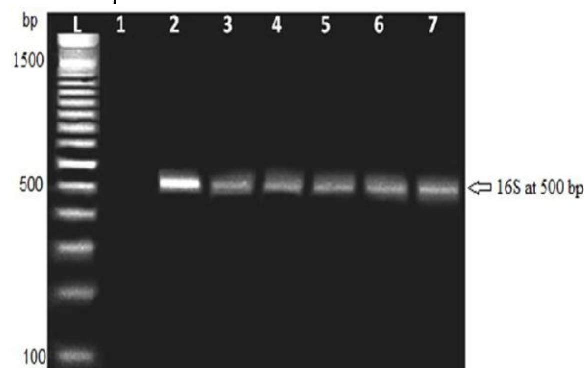


**Figure 2:** Amplification of 16S rDNA target using universal primers. Lane (L) 100 pb. DNA ladder. Lane (1) negative control showing no 16S product. Lanes (2-7) showing 500 bp product of 16S target from all suspected DNA samples.

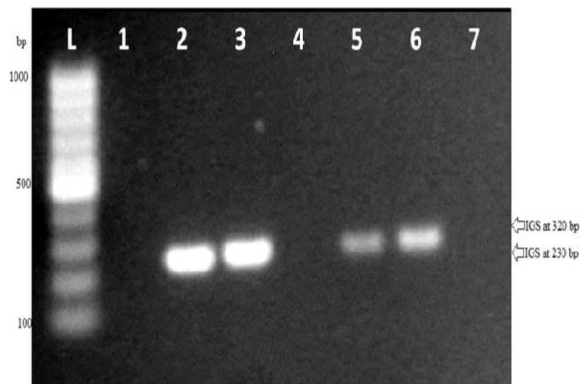


**Figure 3:** Amplification of IGS target using pan-*Mycobacterium* primers. Lane (L) 100 pb DNA ladder. Lane (1) negative control showing no IGS product (230, 320). Lanes (2, 3, 5 and 6) showing positive product of *Mycobacterium*-specific IGS target. Lanes (4 and 7) showing negative product of *Mycobacterium*-specific IGS target.

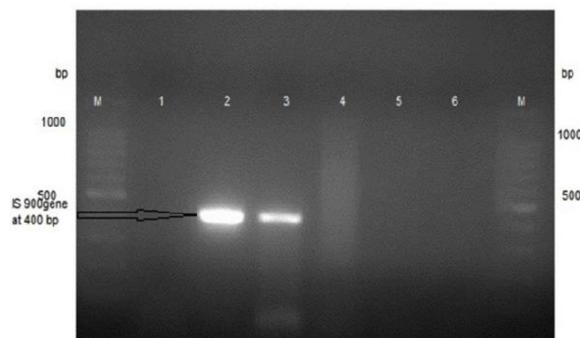


**Figure 4:** Detection of MAP-specific IS900 target. Lane: M, 100-bp DNA ladder. Lane (1) control negative showing no IS900 product. Lane (2 and 3) showing positive IS900 gene at (400bp). Lane (4, 5, 6) showing negative product IS900 genes.

## DISCUSSION

Isolation of MAP from intestinal tissue of Crohn's diseased patient [34-37] has intensified the interest towards the epidemiology and spread of John's disease among farm animals. The increasing evidences that links JD in animals to crohn's disease [38] has led the concern that JD-infected farm animals could act as reservoir for Crohn's disease infection among contact humans. In Saudi Arabia, sheep and goats constitute an integral part of the animal population and are raised principally by private breeders mainly for meat production [22]. JD was reported in Saudi Arabia among different farm animals including dairy cattle [25] and camels [24]. In addition, the disease was also reported among sheep and goat in different regions of Saudi Arabia as Al-Qassim [26] and Al-Ahsaa [27]. However, no reports had documented the presence of the disease in Makkah region. In Makkah, in addition to the internal risk factors, the importation of large quantities of small ruminants from various parts of the world during pilgrimage season represent another external risk factor for spreading of JD among animal herds with the subsequent potential risk for Crohn's infection in human [23, 38]. With regard to the pathogenesis of JD in small ruminants, young animals, less than six months of age, are thought to be the most susceptible to infection [39]. However, the long period between time of infection and detection of apparent clinical symptoms [5] could have make these imported young small ruminants (1-2 years old), a potential source for dissemination of JD among local herds in Makkah as they might be imported during the subclinical stage of the disease. The research team, driven by the ambiguity status of JD in Makkah region, carried out a probing investigation to document the presence of JD among small ruminants in Makkah. For this purpose, 3 genetic targets (16S rDNA, IS900 and IGS) were used for molecular detection and identification of MAP-the causative agent of the disease among clinically suspected cases. The study started by clinical investigation of a total of 2660 small ruminants from five different sheep and goat farms. Selection of suspected animals based on specified inclusion criteria [28] and resulted in selection of 16 (0.6%) suspected cases (0.5% of investigated sheep and 1.08% of investigated goats). Clinical signs of JD in small ruminants are not specific and could be confused with other diseases as intestinal parasitism, chronic malnutrition, caseous lymphadenitis (especially animals with internal abscesses), ovine progressive pneumonia (OPP), environmental toxins, and cancer [28, 40]. Initial microscopic screening of ZN-stained smears of collected fecal specimens and rectal swaps revealed suspected *Mycobacterium* species (positive acid fast bacilli) in 5 cases (31.3%) out of the investigated 16 clinically suspected ones. Although ZN staining is the most rapid and cost-effective screening method, it lacks the required sensitivity [19]. Moreover, acid-fast bacilli other than MAP could be detected in fecal specimens including other pathogenic and environmental *Mycobacterium* species [41]. Therefore, an additional specific and confirmatory test required to evaluate MAP infection among suspected cases. In this regard, fecal culture is considered the ideal gold standard test for detection and identification of MAP [14]. Fecal culture can be applied to various species and sample types (feces, tissue, water, soil) during the clinical stage of the disease to confirm infection. However, culture is unreliable in sheep and some other ruminant species when using standard laboratory techniques as it lacks the required sensitivity. In addition it is labor intensive and may require 8-24 weeks of incubation for colonies to be observed based on the type of media used [3, 5 and 17]. Alternatively, recent studies have shown the superiority of PCR-based molecular assays over conventional culturing methods for sensitive and accurate detection of MAP

infection among subclinical and clinical cases [18]. Using 3 different genetic targets, implemented molecular assays revealed that 6 samples were initially positive for the bacterial-specific 16S rDNA target. Of which, 4 samples were confirmed as being *Mycobacterium* species while the other 2 samples were regarded as non-*Mycobacterium* species based on the amplification of the IGS target using pan-*Mycobacterium* specific primers. Sequence- dependent identification has been shown to be an especially effective molecular tool that provides rapid and accurate differential identification of *Mycobacterium* species [32, 42]. Most molecular approaches have focused on the conserved 16S small subunit ribosomal DNA (rDNA) sequence [31, 43]. However, the fact that the product of the 16S rDNA gene serves a vital function in the bacteria makes the frequency of permissible mutations in this gene inherently limited. This limitation results in the presence of identical or highly homogenous 16S rDNA sequences among some of the *Mycobacterium* species, making the differentiation of some closely related species difficult [20, 32 and 44]. Alternatively, sequence analysis of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes has been successfully used to differentiate among closely related *Mycobacterium* species [32, 45 and 46]. Which make it a good target for molecular-based detection and identification of MAP among sub-clinically and clinically affected animals. Further investigation of the obtained IGS amplicons by sequence analysis identified only 2 samples as being MAP, while the other 2 isolated were identified as being *M. tuberculosis* complex and *M. fortuitum sequevar Mfo-B*. This finding confirmed the findings of IS900 target, which were detected only from the same 2 samples identified by IGS-sequence analysis. Sequences of IS*900* proved to be highly sensitive and specific markers of *M. paratuberculosis* among other slowly growing, acid-fast bacteria. IS*900* has a unique nucleotide sequence which can be specifically detected by hybridization or PCR techniques. Previous studies showed that IS*900* has been detected in all reference and vaccine strains as well as in field isolates of *M. paratuberculosis* from several hosts but never in other bacterial species [21, 34 and 47]. The genetically confirmed 2 MAP cases were one from sheep and one from goat.

## CONCLUSION

In conclusion, clinical screening of small ruminants for suspected cases of para tuberculosis, confirmed by both conventional and molecular identification were able to document John's disease for the first time among small ruminants in Makkah region using both clinical and molecular identification. Based on the current finding, a wide range screening study among small ruminants is recommended for detection of the disease among sub-clinical cases, which could represent a potential zoonotic hazard for human being as well as being a source for dissemination of infection among other animals in the region.

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