Research Article

Phenotypic and Molecular Detection of Mycobacterium avium subsp. paratuberculosis in Small Ruminants Clinically Suspected with Johne’s Disease

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ABSTRACT

Johne’s disease (JD) or Paratuberculosis (PTB) has gained great attention from many industrial countries for its severing economic losses and possibly zoonotic concerns. In the current study conventional clinical and direct microscopic examination compared to real time polymerase chain reaction (RT-PCR) were used to diagnose JD in clinically suspected small ruminants. Clinical examination revealed 130 (8.7%) suspected cases that showed history of emaciation and diarrhea out of the total examined (1500) animals. Direct microscopy of Ziehl-Neelsen (ZN) stained smears (130) revealed 62 (47.7%) acid fast bacteria resembled Mycobacterium avium subsp. paratuberculosis (MAP). RT-PCR insertion sequence gene (IS900) detected MAP in 25 (65.8%) out of 38 fecal samples harbored acid fast bacilli. We were concluded and recommended that RT-PCR considers the most rapid confirmatory method for screening and diagnosis of the MAP in comparison to low specific conventional phenotypic methods, which still remained valuable techniques in the diagnosis of JD in developing countries.

Key-words: Acid fast bacteria, IS900 gene, Johne’s disease, Paratuberculosis, Ziehl-Neelsen stain

INTRODUCTION

Johne's disease or Paratuberculosis is nowadays viewed as one of the most serious chronic bacterial diseases of ruminant which limits animal industry worldwide [1]. The disease is caused by Mycobacterium avium subsp. paratuberculosis (MAP) of the genus Mycobacterium. MAP is an aerobic, non-motile acid fast bacterium. Members of the genus Mycobacterium have a lipid-rich, hydrophobic cell wall, which is substantially thicker than most other bacteria [2]. The thickness and fatty composition of the cell wall render mycobacteria impermeable to hydrophilic nutrients and resist heavy metals, disinfectants and antibiotics interaction [3]. Recently MAP thought to be incriminated in Crohn’s disease of humans. Clinical signs of Paratuberculosis in small ruminants are not specific and could be confused with other diseases as intestinal parasitism, chronic malnutrition, ovine progressive pneumonia (OPP), caseous lymphadenitis, environmental toxins, and cancer [4]. Epidemiological data indicated the distribution of the disease worldwide in both developed and developing countries in Europe, North America, South America, Asia, Australia and Africa [5]. In Saudi Arabia, in Grenada [6], West Indies [7] and in Cyprian [8] many diagnostic tests were used for diagnosis of paratuberculosis in ruminants.

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Microscopic examination of Ziehl-Neelsen stain method usually used, however, it has variable sensitivity and specificity depending upon the stage of disease [4]. Fecal culture demonstrates low sensitivity, require long incubation period for growth as well as its labor intensive so it cannot be recommended as a screening test to detect shedding of MAP in either goats or sheep [9]. Recently, IS900 and F57 insertion sequence genes have enabled the specific identification of minimum amounts of bacterial DNA by different polymerase chain reaction (PCR) techniques [10,11]. Therefore, the aim of this study was to identify and confirm M. avium subsp.

MATERIALS AND METHODS
Clinical examination and sampling- A total number of 1500 small ruminants were investigated in El Qassim region KSA. Based on history and clinical examination animals showed signs of emaciation, diarrhea or softened feces, pasty stools, low-grade fever, lethargy, and depression and chronic history of weight loss were supposed to be infected [4]. One hundred and thirty 130 clinical PTB suspected animals were selected and subjected for microscopic screening test using fecal ZN stained smears. Fecal samples were collected in sterile containers from clinically suspected sheep and goats and preserved for further examination. The study proceeds from February to October 2015 in Department of Clinical laboratory, Teaching Hospital, AL Qassim region, Saudi Arabia.

Traditional microscopic examination- From the collected fecal samples direct smears were prepared, stained and microscopically examined for expected acid fast bacilli using ZN stain method [12].

Molecular methods
DNA extraction from fecal samples
Preparation of the samples- Fresh, moist 38 fecal samples of clinical JD suspected animals were aliquoted in 2 ml cryo tubes and immediately frozen and stored at -80°C until used. The fecal samples were prepared for extraction by using two grams protocol of MAP Extraction System (Tetracore, USA) according to the manufacturer’s instructions.

Pre-treatment of the samples- In this step, 200 µl resuspension of the sample was mixed with 180 µl of magna pure bacterial lysis buffer (Roche Diagnostics GmbH, Mannheim-Germany) and 20 µl of proteinase k. The mixture was mixed thoroughly and incubated for 10 minutes at 65°C then after boiled for 10 minutes at 95°C, centrifuged at low speed, then chilled in ice from which 400 µl were transferred to magna pure compact sample tube.

DNA Purification- The extraction of DNA was carried out in a fully automated Magna Pure Compact system (Roche Diagnostics GmbH, Mannheim-Germany) according to the manufacturer's instructions.

Real Time Quantitative PCR- A real time qPCR assay, that was applied for detection of presence of MAP bacteria, based on amplification of a 177 bp fragment of MAP insertion element IS900 with set of specific primers and probe labeled with light cycler red 640 dye as described by Beumer et. al. [13] and Rajeev et. al. [14]. In this assay, the Light cycler Fast Start DNA master hybprobe kit (Roche Diagnostics GmbH, Mannheim-Germany) and the light mix M. avium sp. paratuberculosis (MAP) kit (TIB MOLBIOL GmbH-Berlin -Germany) was used in an amplification reaction mixture. The mixture consisted of 2 µl 10x master mix, 2.4 µl 25 mM Mg²⁺, 2 µl of specific primers and probe sets solution and 5 µl DNA templates and completed to 20 µl with 8.4 µl PCR grade water. The PCR experiment was carried out in the Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim-Germany) with a protocol consisted of four thermal program steps: initial denaturation one cycle at 95°C for 10 min, amplification in 50 cycles, each cycle segmented to 95°C for 5sec, 62°C for 5sec, and 72°C for 15 sec and finally melting in one cycle with 3 thermal steps (95°C for 20 sec, 40°C for 20 sec and 85°C). The amplification crossing (CP) and melting (Tm) points were detected in 640 channels.

Statistical Analysis- The analysis was performed as described in light cycler instrument operator’s manual, using the second derivative maximum method. The obtained data were analyzed with quantification analysis mode and the amplification signals were reported as crossing points (cycle’s threshold) in channel 640. For further identification, the melting curve analysis mode was performed and specific melting points were detected by the same channel.
RESULTS
Clinical examination of targeted animals showed 130 (75 goats and 55 sheep) JD suspected animals out of 1500 total examined. Suspected animals showed clinical signs include chronic weight loss, non-curable diarrhea, emaciation terminated by death. Microscopic screening of Ziehl-Neelsen’s stain for rectal scraping smears from suspected cases show that 62 (47.7%) out of 130 were harboring acid fast bacteria (Fig. 1 & Fig. 2) of the positive cases 41 (54.7%) out of the 75 were goats and 21 (38.2%) out of 55 were sheep (Table 1). RT-PCR examined fecal samples (n=38) using IS900 gene revealed positive results of 25 (62.5%) samples. These results indicated high sensitivity of the molecular test in contrast to the conventional clinical and microscopic methods. Twenty five (25) fecal samples tested were consistently positive for MAP insertion element IS900 by real time quantitative PCR (62.5%) (Table 2) and (Fig. 3 & Fig. 4). The resulted cycle thresholds (Ct) range from 15.99 to 36.01, with a mean of 28.2712 and melting points (Tm) range from 66.03 to 68.85, with a mean of 67.9556 and 0.47178 standard deviations.

Table 1: Numbers of clinically examined, suspected and acid fast positive cases

| Animal species | Total examined animals | Clinical suspected cases | Positive fecal Zn stain test | Percentage |
|----------------|------------------------|--------------------------|----------------------------|------------|
| goat           | 900                    | 75                       | 41                         | 54.7       |
| sheep          | 600                    | 55                       | 21                         | 38.2       |
| Small ruminants | 1500                  | 130                      | 62                         | 47.7       |

Fig. 1: Direct rectal scrapings smear from sheep shows acid fast clumps, Ziehl-Neelsen Stain X100

Fig. 2: Direct rectal scrapings smear from goat shows acid fast clumps, Ziehl-Neelsen Stain X100

Table 2: Quantitative RT-PCR, Ct and Tm results of PAM in the fecal samples in light cycler 2.0 and analyzed with absolute and melting curve modes

| No. of Samples | CT * | Tm** |
|----------------|------|------|
| 1              | 29.11| 68.32|
| 2              | 27.98| 68.18|
| 3              | 34.56| 67.91|
| 4              | 26.89| 68.05|
|   | Ct-Cycle's threshold | Tm-Melting point temperature |
|---|------------------|-----------------------------|
| 5. | 26.17            | 68.22                       |
| 6. | 25.02            | 68.31                       |
| 7. | 33.98            | 68.29                       |
| 8. | 28.96            | 68.09                       |
| 9. | 28.81            | 66.03                       |
| 10. | 28.25           | 67.92                       |
| 11. | 35.85            | 67.89                       |
| 12. | 30.81            | 67.95                       |
| 13. | 27.04            | 67.91                       |
| 14. | 33.56            | 67.86                       |
| 15. | 31.12            | 67.77                       |
| 16. | 28.48            | 67.98                       |
| 17. | 24.77            | 68.41                       |
| 18. | 28.69            | 67.85                       |
| 19. | 20.87            | 68.28                       |
| 20. | 25.25            | 68.00                       |
| 21. | 15.99            | 68.29                       |
| 22. | 27.03            | 68.08                       |
| 23. | 36.01            | 67.72                       |
| 24. | 15.99            | 68.29                       |
| 25. | 35.59            | 67.29                       |

* Ct- Cycle's threshold, ** Tm- Melting point temperature

Fig. 3: Displays the amplification curves and crossing points as analyzed by absolute analysis mode in light cycler 2.0
DISCUSSION
Detection of Paratuberculosis is important for control and eradication program. In the current study, the clinical examination of targeted animals showed 130 JD suspected small ruminants out of 1500 total examined. Initial microscopic screening of Ziehl-Neelsen’s stain for rectal scraping smears from suspected cases shown that 62 (47.7%) out of 130 were harboring acid fast bacteria of the positive cases. These results were similar to studies conducted by Liapi, et al. [8] in Cyprian dairy goat herds and it is higher than results obtained by Atif et al. [6] and Kumthekar [7]. Although ZN staining is the most rapid screening method, it lacks the required sensitivity in the diagnosis of PTB; therefore, molecular methods were alternatively used for rapid diagnosis of MAP in farm animals [15]. Samples culture is labor intensive and may require 8–24 weeks of incubation for colonies to be observed based on the type of media used [16]. RT-PCR examined fecal samples (n=38) using IS900 gene revealed positive results of 25 (62.5%) samples. These results indicated high sensitivity of the molecular test in contrast to the conventional clinical and microscopic methods. Twenty five (25) fecal samples tested were consistently positive for MAP insertion element IS900 by real time quantitative PCR (62.5%). These results evaluated traditional methods (Zn stain) is a lower specificity in compare to molecular methods (RT-PCR) and the findings agreed with Kawaij et al. [16]; Sonawane and Tripathi [17]. This study indicated that the RT-PCR is more rapid, specific and sensitive test for screening and diagnosis of M. avium sub sp. paratuberculosis in fecal samples of small ruminants.

CONCLUSIONS
This study included the clinical screening of small ruminants for suspected cases of paratuberculosis, confirmed by both phenotypical and molecular identification were able to document paratuberculosis among small ruminants in Qassim region. Based on the current finding, RT-PCR was diagnosed and confirmed test of paratuberculosis among small ruminants and a wide range of study among small ruminants were recommended for detection of the disease, which could represent a potential zoonotic hazard of infection among other animals in the region.

According to our current finding, we recommend the application of RT-PCR as a confirmatory diagnostic test as well as epidemiological screening test in combating and eradication programs of paratuberculosis in farm animals.

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