eISSN: 2455-1716
Hamid *et al.*, 2018
DOI:10.21276/ijlssr.2018.4.5.8

Research Article

open access

Histopathological and RT-PCR Detection of *Mycobacterium paratuberculosis* in Tissues of Clinically Suspected Small Ruminants

Mohamed A. I. Hamid[1*], Galal Eldin E. Mohammed[2], Amel O. Bakhiet[3], Elhassan M. A. Saeed[4]

[1]Pathologist, Qassim University Teaching Veterinary Hospital, Saudi Arabia
[2]Professor, Department of Microbiology, Pathology and Parasitology, Faculty of Veterinary Medicine, Sudan University of Science and Technology, Sudan
[3]Professor, Deanship of Scientific Research, Sudan University of Science and Technology, Sudan
[4]Associate Professor, Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Saudi Arabia

***Address for Correspondence:** Mr. Mohamed Alamin Ibrahim Hamid, Pathologist, Qassim University Teaching Veterinary Hospital, Saudi Arabia

Received: 11 Apr 2018/ Revised: 09 Jun 2018/ Accepted: 16 Aug 2018

ABSTRACT

Paratuberculosis (PTB) remains one of the most obstacles limit animal breeding sector all over the world. The current study aimed to detect the etiology of PTB in tissues of clinically suspected small ruminants using histopathological and real-time polymerase chain reaction (RT-PCR) methods. Clinical examination shown 10 (26.4%) PTB suspected cases out of the total (38) examined the animals. The suspected cases were euthanized, necropsied, gross lesions were recorded and tissue samples were collected for histopathological and molecular procedures. Grossly intestinal and mesenteric lymph nodes thickening, corrugations and edematous swellings were recorded. Semi-thin sections of the intestine and mesenteric lymph nodes stained with toluidine blue demonstrated MAP organism inside epithelial cells and macrophages. RT-PCR detected MAP IS900 gene in all suspected cases (100%), thus we recommend using RT-PCR as a rapid sensitive method in the diagnosis of PTB.

Key-words: IS900 gene, *Mycobacterium*, Paratuberculosis Semi thin sections, Toluidine blue

INTRODUCTION

Paratuberculosis (PTB) is a chronic debilitating incurable granulomatous disease affects cattle, sheep, goats, deer, camelids and wild ruminants worldwide. The disease has also reported in other animals like wild rabbits, pigs, horses, birds, and carnivores [1-3]. PTB leads to severe economic losses due to productivity reduction, deaths and cost of control programs. The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the suspected causative agent of crohn's disease of man, so the disease is ranked as one of zoonoses and classed in risk group-2 for human infection [4,5].

How to cite this article
Hamid MAI, Mohammed GEE, Bakhiet AO, Ali Saeed EM. Histopathological and RT-PCR Detection of *Mycobacterium paratuberculosis* in Tissues of Clinically Suspected Small Ruminants. Int. J. Life Sci. Scienti. Res., 2018; 4(5): 2012-2018.

Access this article online
www.ijlssr.com

The disease has unusual long-term incubation period with developing of proliferative granulomatous/ lepromatous enteritis which results in subclinical digestive symptoms later may progress to severe clinical signs according to the affected host. Clinical JD is observed mostly in adult animals with characteristic signs including intermitted or progressive projectile watery diarrhea leading to emaciation, wasting and loss of production. Pathological lesions related to the chronic enteritis are found at necropsy [6,7]. Over a period in the infected host, MAP organisms proliferate extensively in tissues of an infected host which could be easily demonstrable in tissues and faeces by genetic tests. Regular screening of the farm animals by a number of tests including bacterial culture, ELISA, PCR and necropsy of the dead animals provide evidence of MAP infection. At necropsy and histopathology, ruminant showing granulomatous enteritis with demonstrable an abundant Acid Fast Bacteria are tentatively diagnosed as cases of

paratuberculosis [8]. Molecular tests since the discovery of IS900 gene [9], offer a great promising, sensitive and specific diagnostic assays for detection of MAP infection. Real-time PCR assays are endowed with higher sensitivity and help in determining the load of infection in environmental samples, faeces, and milk [10]. The application of PCR to detect genome directly from tissues is a practical and valuable approach for laboratory confirmation of infectious diseases. Therefore this study aimed to an application of real-time polymerase chain reaction and histopathology section in tissues samples of small ruminants in the investigated area.

MATERIAL AND METHODS

Clinical examination and sampling- A total number of 38 small ruminants were examined for suspicion of PTB infection in AL Qassim region. The selection based on history and clinical examination. The animals showed signs of emaciation, not curable diarrhea or softened faeces, pasty stool and chronic history of weight loss were suspected to be PTB infected (n=10) 5 sheep and 5 goats. The suspected cases were euthanized and examined for gross pathology. The intestine tissues and mesenteric lymph nodes were taken and prepared for histopathological examination and extraction of DNA for the RT-PCR method. The study proceeds from February to October 2015 in the Department of Clinical Laboratory, Teaching Hospital, AL Qassim region, Saudi Arabia.

Histopathologically procedure- Intestinal and mesenteric lymph node lesions were collected and immediately preserved in 10% formal saline for histopathology. Semi thin tissue sections for histopathological examination was carried out according to the method described by Hoffman *et al.* [11].

Molecular detection and identification

DNA Extraction from tissues samples- DNA was extracted from intestine tissues (n=10) suspected samples using DNeasy® Blood & Tissue Kit (Qiagen). Briefly, 25 mg from intestine tissues were taken and cut into small pieces, placed in a 1.5 ml microcentrifuge tube, 180 µl Buffer ATL and 20 µl proteinase K were added. The mixture vortexes incubated at 56°C until completely lysed and continuous the procedure according to the kit manufacturer's protocol. The eluted DNA was stored at -20 C until used in the PCR downstream reaction.

Real time Quantitative PCR- A real-time qPCR assay was applied for detection of MAP bacteria, based on amplification of a 177bp 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.* [12]; Rajeev *et al.*[13]. In this assay, the Light cycler Fast Start DNA master hybprobe kit (Roche Diagnostics GmbH, Mannheim-Germany) and the light mix 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 Mg^{2+}, 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 5 sec, 62°C for 5 sec and 72°C for 15 sec, and finally melting in one cycle with 3 thermal steps (95°C for 20 sec, 40°C for 20sec 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 investigated animals showed 10 (5 goats and 5 sheep) JD suspected animals out of 38 total examined. Suspected animals showed clinical signs included chronic weight loss, non-curable diarrhea, emaciation terminated by death. Gross pathology of suspected cases revealed thickening of the intestinal wall, corrugation and edematous of the mucosa and mesenteric lymph nodes hypertrophy and caseation (Fig. 1 and 2). Other pathological lesions; muscular atrophy emaciation, fatty and mucoid degeneration, alopecia,

eISSN: 2455-1716
Hamid *et al*., 2018
DOI:10.21276/ijlssr.2018.4.5.8

edema, serous exudates in body cavities and anemia Histopathological examination of intestine tissues of suspected cases revealed cellular infiltration of epithelioid, lymphocyte, macrophage and giant cells and demonstration of free and phagocytosed acid fast bacilli (Fig. 3 & Fig. 4). The molecular examination of tissues from suspected animals using real time polymerase chain reaction confirmed MAP infection in all 10 suspected tissue samples (Table 1) and (Fig. 5 & Fig. 6). The resulted cycle thresholds (Ct) range from 14.5 to 32.72, with a mean of 25.587 and melting points (Tm) range from 68.41 to 68.85, with a mean of 68.53 and 0.339 standard deviations.

were seen.

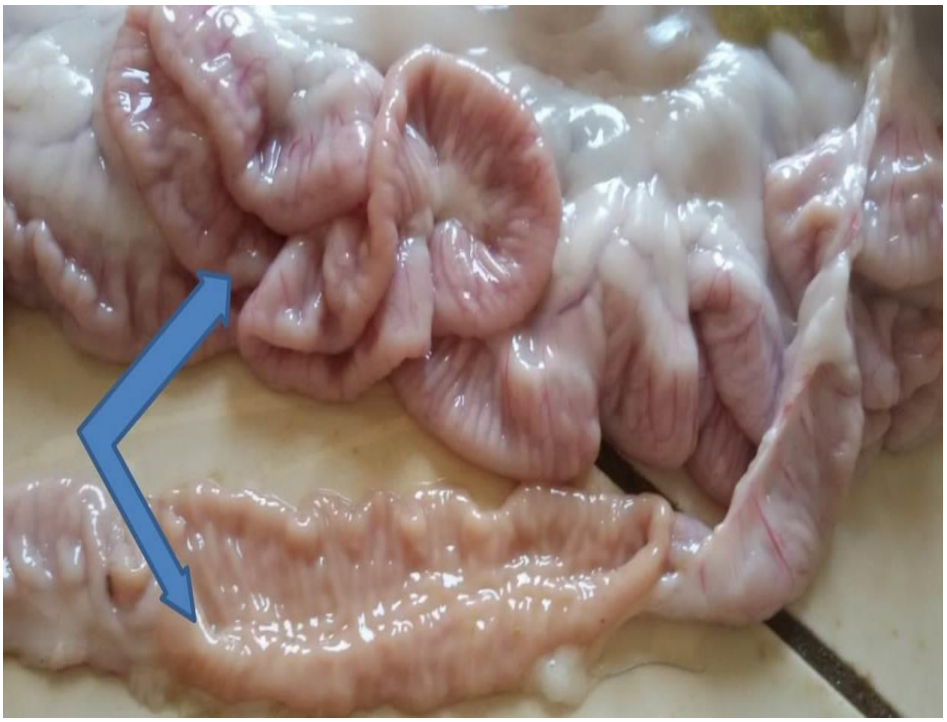

Fig. 1: Intestine of infected sheep shows corrugations, thickening and edematous of the mucosal wall

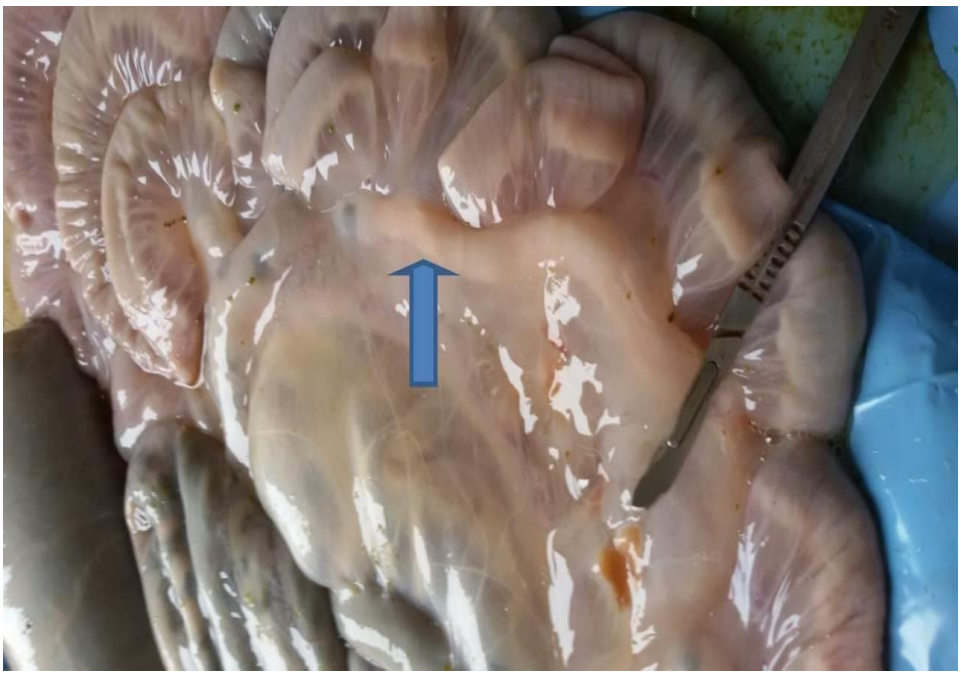

Fig. 2: Mesenteric lymph nodes of infected goat show thickening, cording and edematous swelling

eISSN: 2455-1716
Hamid *et al*., 2018
DOI:10.21276/ijlssr.2018.4.5.8

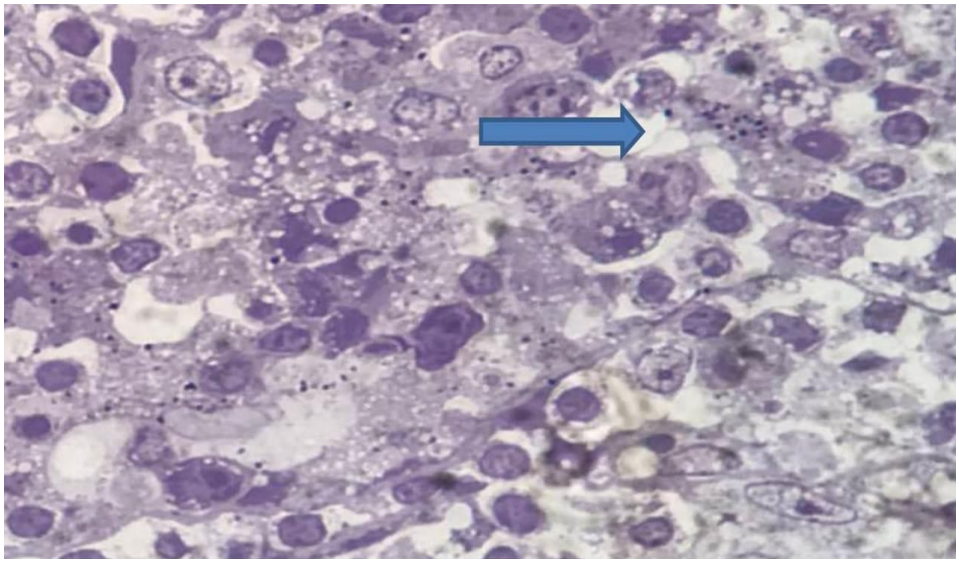

Fig. 3: Semi thin section of mesenteric lymph node shows presence of coccobacilli organisms of MAP and infiltration of mononuclear cells, toluidine blue stain, 100X

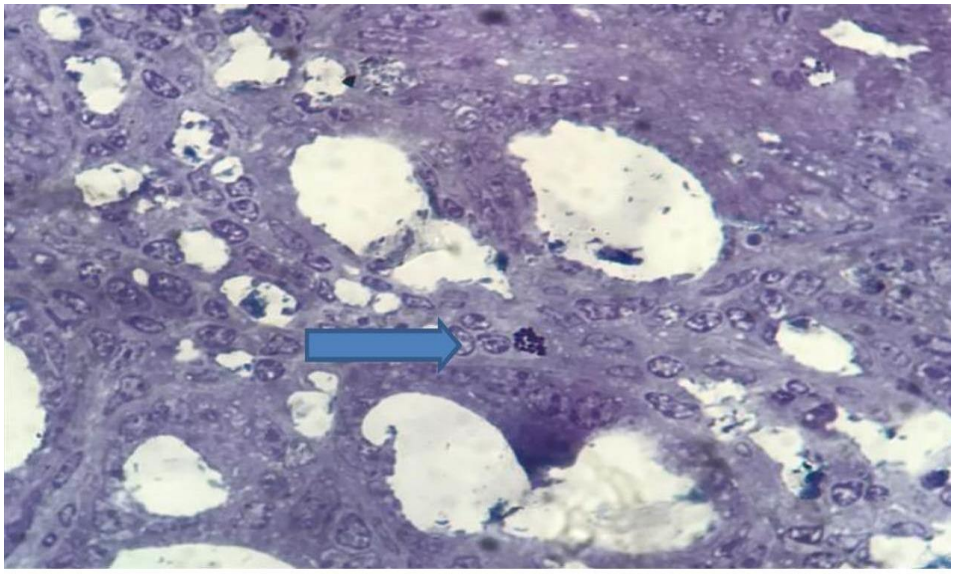

Fig. 4: Semi thin section of intestine section shows presence of coccobacilli organisms of MAP and infiltration of mononuclear cells, toluidine blue stain, 100X

Table 1: Contains cycle's threshold and melting point temperature of PAM tested by real time qPCR in light cycler 2.0 and analyzed with absolute and melting curve modes

No. of Samples	CT*	Tm**
1.	32.72	68.75
2.	14.50	68.75
3.	29.73	68.85
4.	19.76	68.44
5.	28.74	68.70
6.	24.77	68.41

DOI:10.21276/ijlssr.2018.4.5.8

7.	28.07	68.74
8.	17.98	68.59
9.	31.67	68.81
10.	27.93	68.26

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

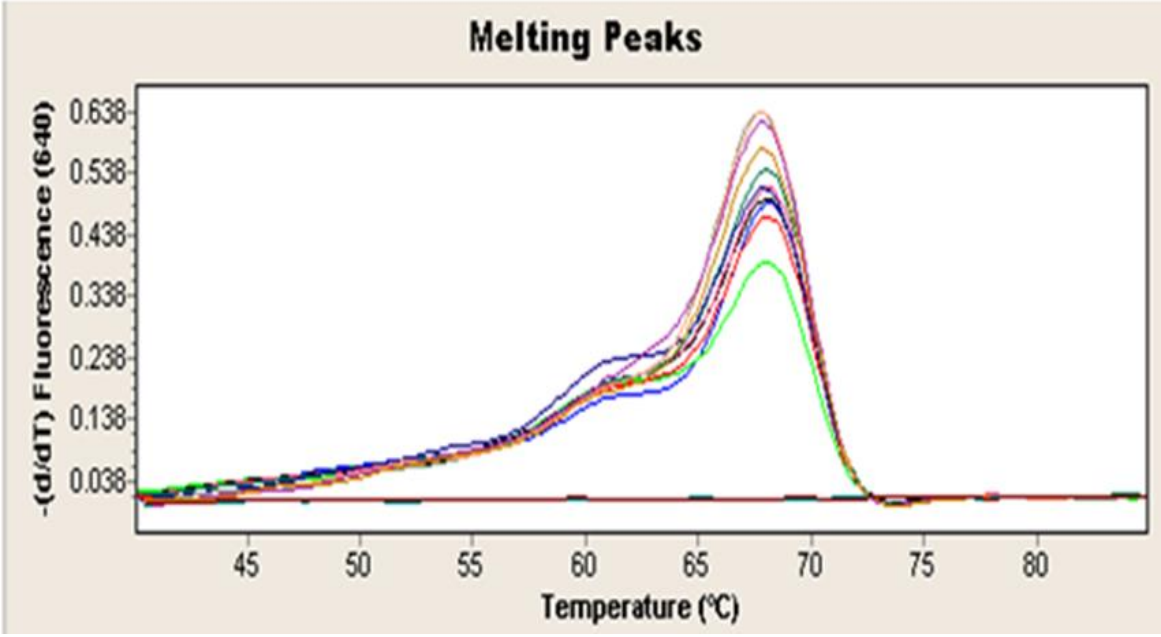

Fig. 5: Displays the melting curves and melting temperatures point as analyzed by melting analysis mode in light cycler 2.0

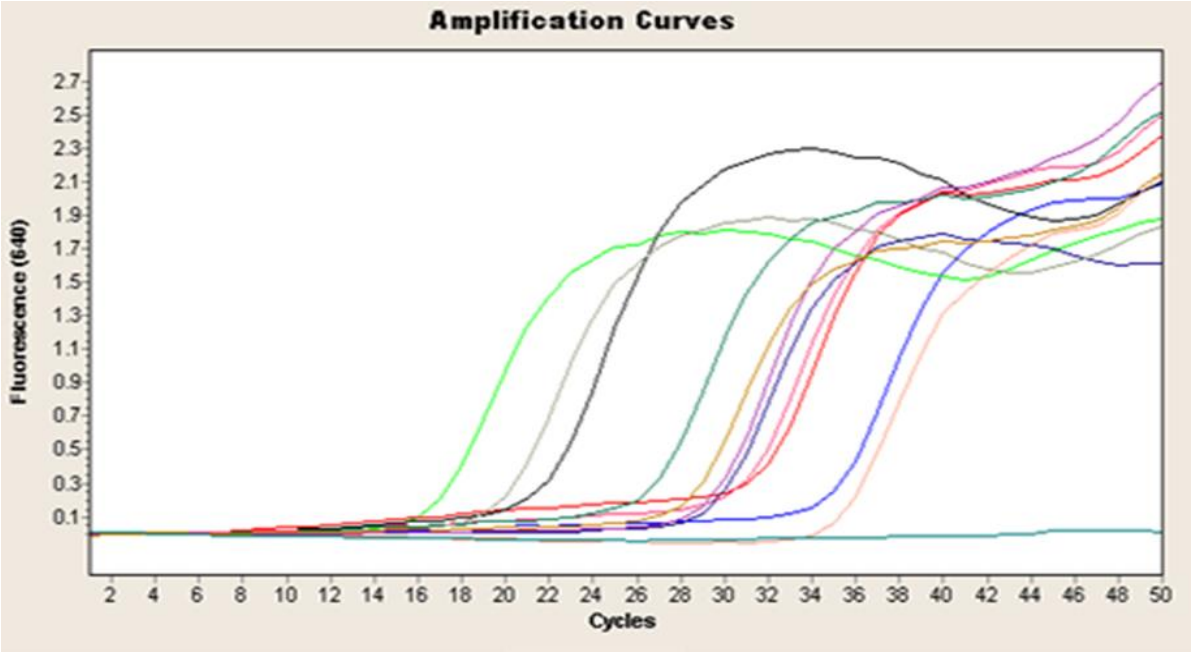

Fig. 6: Displays the amplification curves and crossing points as analysed by absolute analysis mode in light cycler2.0

DISCUSSION

Johne's disease is serious economic and animal health consequences in domesticated ruminants (including sheep and goats) throughout the world [14,15]. In the current study, Clinical examination of investigated small ruminant showed 10 (5 goats and 5 sheep) JD suspected animals out of 38 total examined. Suspected animals shown clinical signs included chronic weight loss, non-curable diarrhea, emaciation terminated by death. Clinical signs of JD in small ruminants are not specific and could be confused with other diseases as intestinal parasitism, chronic malnutrition, caseous lymphadenitis, ovine progressive pneumonia (OPP), environmental toxins, and cancer [16,17]. Gross pathology of suspected cases revealed thickening of the intestinal wall, corrugation and edematous of the mucosa and mesenteric lymph nodes hypertrophy and caseation. These results came in line obtained by Atif *et al.* [18]. Histopathological examination of intestine tissues of suspected cases revealed cellular infiltration of epithelioid, lymphocyte, macrophage and giant cells and demonstration of free and phagocytosed acid fast bacilli. These findings agreed with Clarke and Little [8]. Real time polymerase chain reaction confirmed MAP infection in all 10 suspected tissue samples. These results agreed with Green *et al.* [9] and Kawaji *et al.* [10]. This study confirmed the presence of MAP in tissues of suspected small ruminants when histopathological and RT-PCR methods were adopted.

CONCLUSIONS

Clinical screening of small ruminants for suspected cases of para tuberculosis was confirmed by using histopathological and real-time polymerase chain reaction (RT-PCR) methods. Grossly, intestinal and mesenteric lymph nodes of suspected cases revealed thickening, corrugation and edematous swelling. Histopathologically, semi thin sections from the intestine and mesenteric lymph nodes stained with toluidine blue demonstrated MAP organism inside epithelial cells and macrophages. RT-PCR detected MAP IS900 gene in all suspected cases (100%) so we recommended using RT-PCR as a rapid sensitive method in diagnosis of PTB.

Based on the current study, early diagnosis among small ruminants is recommended for detection and control of the disease among sub-clinical animals, which could represent a source for dissemination of infection among other animals.

ACKNOWLEDGMENTS

The authors would like to thank Dr. El Tigani, Asil A. El Tigani for his kind participation in planning for the research and collection of research samples.

CONTRIBUTION OF AUTHORS

Research concept- Elhassan MA Saeed, Mohamed AI Hamid

Research design- Mohamed AI Hamid, Amel O Bakhiet

Materials- Elhassan MA Saeed, Mohamed AI Hamid

Data collection - Mohamed AI Hamid

Data analysis- Elhassan MA Saeed, Mohamed AI Hamid

Literature search- Elhassan MA Saeed, Mohamed AI Hamid

Writing article- Mohamed AI Hamid, Galal Eldin E Mohammed

Critical review- Amel O Bakhiet, Elhassan MA Saeed

Article editing- Mohamed AI Hamid, Elhassan MA Saeed

Final approval- Elhassan MA Saeed, Galal Eldin E Mohammed

REFERENCES

[1] Stanitznig A, Kol JL, Lambacher B, Franz S, Kralik P, et al. Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* and hepatitis E in New world camelids in Austria. Vet. Rec., 2017; http//dx.doi.org/10.1136/vr.104246.

[2] Martinez- Covarrubias AD, Santillan-Flores MA, Cordova- Lope, Guzman-Ruiz CC. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces, using quantitative real time polymerase chain reaction (Q-PCR). J. Vet. Med. animal health., 2017; 9(10): 273–79.

[3] Ayele WY, Machaokova M, Pavlík I. The transmission and impact of paratuberculosis infection in domestic and wild ruminants. Vet. Med. Czech., 2001; 46(7, 8): 205–24.

[4] OIE. Office internaltiond de Epidemiolgie. Paratuberculosis (Johne's diseases) in Terrestrial manual, 2014; Chapter 1.11. pp. 16.

[5] Khol L, Pinedo PJ, Buergelt CD, Neumann LM, Rae DO. Lymphatic fluid for the detection of *Mycobacterium avium* subsp. *Paratuberculosis* in cow by PCR, compared to fecal sampling and

detection of antibodies in blood and milk. Vet. Microbial., 2014; 172: 501–08.

[6] Lilenbaum W, Marassi CD, Olemann WMR. Paratuberculosis: An update. Braz. J. Microbiol., 2007; 38: 580–90.

[7] Perez V, Tellechea J, Badiola JJ, et al. Relationship between serological response and pathological findings in sheep with naturally acquired paratuberculosis. Am. J. Vet. Res., 1997; 58: 799-803.

[8] Clarke CJ, Little D. The pathology of ovine paratuberculosis: Gross and histopathological changes in the intestine and other tissues. J. Comp. Pathol., 1996; 114: 410–37.

[9] Green EP, Tizard ML, Moss HJ, Thompson J, et al. Sequence and characterization of IS900, an insertion element identified in a human Crohn's disease isolate of *mycobacterium paratuberculosis*. Nuc. Acid. Res., 1989; 17: 9063–73.

[10] Kawaji S, Taylor DL, Mori Y, Whittington RJ. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. Vet. Microbiol., 2007; 125: 36-48.

[11] Hoffman EO, Flores TR, Coover J, Garrett HB. Polychrome stains for high resolution light Microsc. Lab. Med., 1983; 14: 779.

[12] Beumer A, King D, Donohue M, Mistry J, Covert T, Pfaller S. Detection of *mycobacterium avium* subsp. *paratuberculosis* in drinking water and biofilms by quantitative PCR. Appl. Environ. Microbiol., 2010; 76(21): 7367–70.

[13] Rajeev S, Zhang Y, Sreevatsan S, Motiwala AS, Byrum B. Evaluation of multiple genomic targets for identification and confirmation of *Mycobacterium avium subsp. paratuberculosis* isolates using real time PCR. Vet. Microbiol., 2005; 105(3, 4), 215–21. doi: 10.1016/j.vetmic.2004.10.018.

[14] Mobius P, Luyven G, Hotzel H, Kohler H. High genetic diversity among *Mycobacterium avium* subsp. paratuberculosis strains from German cattle herds shown by combination of IS900 restriction fragment length polymorphism analysis and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing. J. Clin. Microbiol., 2008; 46: 972–81.

[15] Motiwala AS. Molecular epidemiology of *Mycobacterium avium* subsp. paratuberculosis isolates recovered from wild animal species. J. Clin. Microbiol., 2004; 42: 1703–12. doi: 10.1128/JCM.42.4.1703-1712.2004.

[16] Manning EJ, Collins MT. *Mycobacterium avium* subsp. paratuberculosis: Pathogen, pathogenesis and diagnosis. Rev. Sci. Tech., 2001; 20: 133–50. doi: 10.20506/rst.20.1.1275.

[17] Collins MT. Update on paratuberculosis: Epidemiology of Johne's disease and the biology of Mycobacterium paratubertulosis, Irish Vet. J., 2003, 56: 565–74.

[18] Atif H, Ibrahim HA, Abd El-Rahim, Amr M, Mohamed OB. Clinical and molecular investigations of john's disease among small ruminants in Makkah, Saudi Arabia. Int. J. Bioassays, 2014; 3 (11): 3445-51.