eISSN: 2455-1716
Hamid *et al.*, 2018
DOI:10.21276/ijlssr.2018.4.5.9

Research Article

open access

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

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

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.

How to cite this article

Hamid IMA, Mohammed GEE, Bakhiet AO, Ali Saeed EM. Phenotypic and Molecular Detection of *Mycobacterium avium* subsp. *Paratuberculosis* in Small Ruminants Clinically Suspected with Johne's Disease. Int. J. Life Sci. Scienti. Res., 2018; 4(5): 2019-2024.

Access this article online
www.ijlssr.com

Copyright © 2015–2018 | IJLSSR by Society for Scientific Research under a CC BY-NC 4.0 International License

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 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 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.

Copyright © 2015–2018 | IJLSSR by Society for Scientific Research under a CC BY-NC 4.0 International License

eISSN: 2455-1716
Hamid *et al*., 2018
DOI:10.21276/ijlssr.2018.4.5.9

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

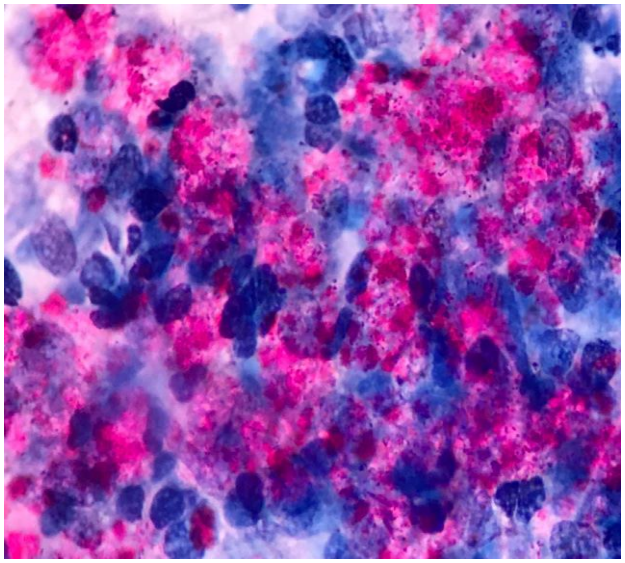

Fig. 1: Direct rectal scrapings smear from sheep shows acid fast clumps, Ziehl-Neelsen Stain X100

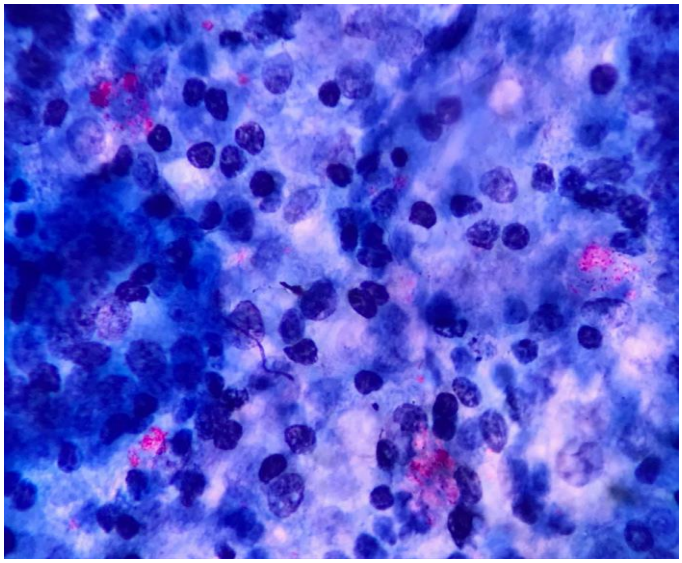

Fig. 2: Direct rectal scrapings smear from goat shows acid fast clumps, Ziehl-Neelsen Stain X100

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

Copyright © 2015–2018 | IJLSSR by Society for Scientific Research under a CC BY-NC 4.0 International License

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**

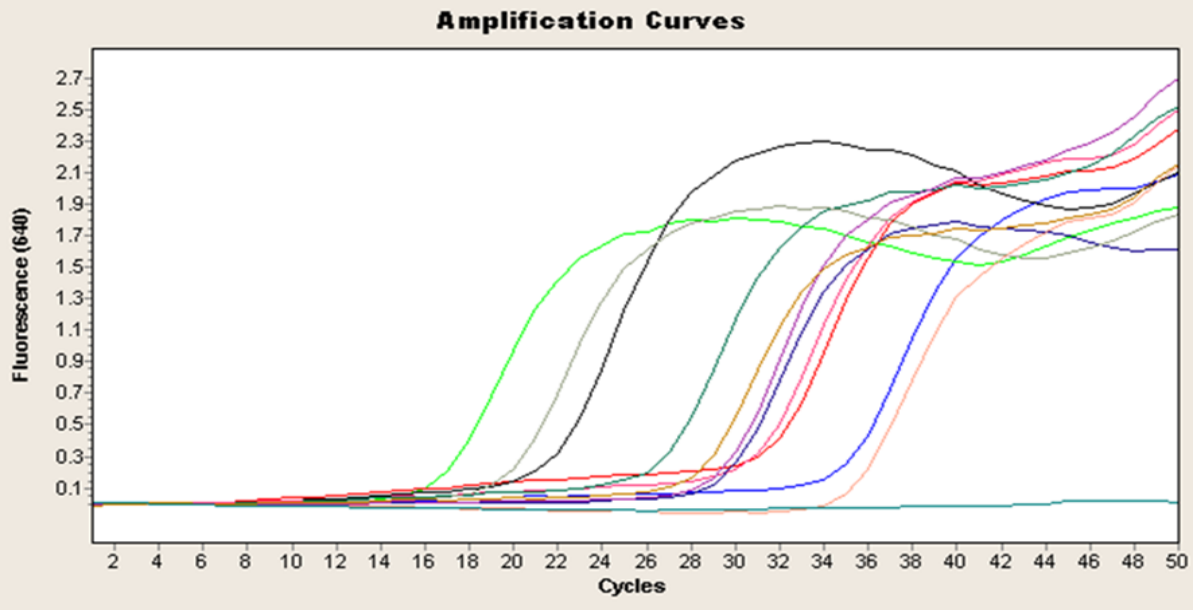

Fig. 3: Displays the amplification curves and crossing points as analyzed by absolute analysis mode in light cycler 2.0

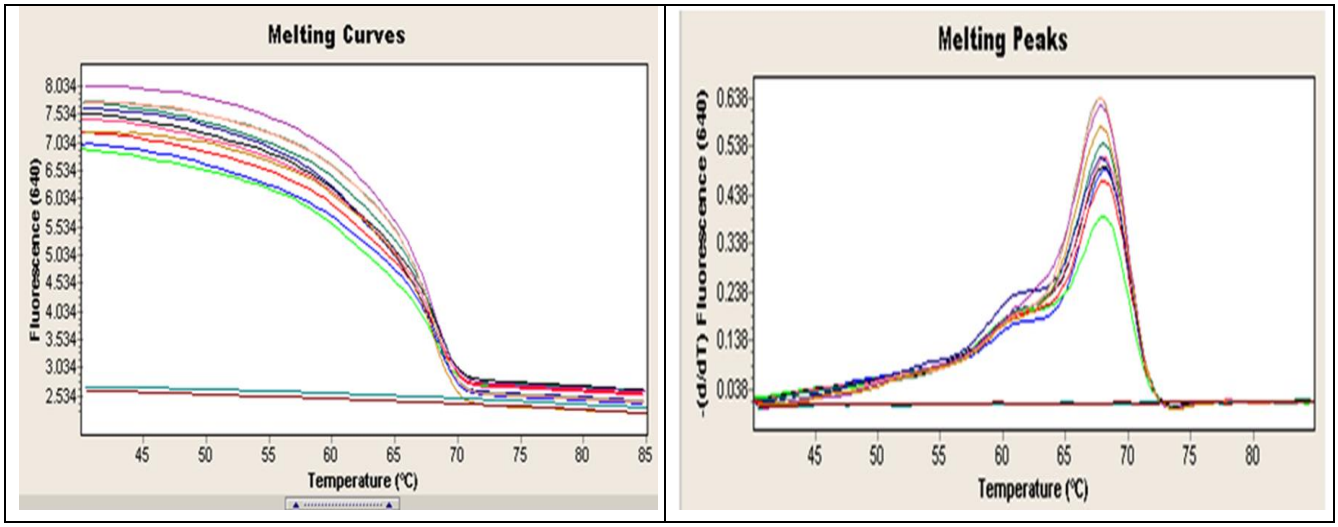

Fig. 4: Displays the melting curves & melting temp. points as analyzed by melting analysis mode in light cycler 2.0

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 Kawaji *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.

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 A. I. 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

Copyright © 2015–2018 | IJLSSR by Society for Scientific Research under a CC BY-NC 4.0 International License

Writing article- Mohamed A. I. Hamid, Galal Eldin E. Mohammed

Critical review- Amel O. Bakhiet, Elhassan M. A. Saeed

Article editing- Mohamed AI Hamid, Elhassan MA Saeed

Final approval- Elhassan MA Saeed, Galal Eldin E. Mohammed

REFERENCES

[1] Stabel JR, Hurd S, Calvente L, Rosenbusch RF. Destruction of *Mycobacterium paratuberculosis, Salmonella* sp., and *Mycoplasma* sp. in raw milk by a commercial on-farm high-temperature, short-time pasteurizer. J. Dairy Sci., 2004; 87 (7): 2177-83.

[2] Ray CG, Ryan KJ. Sherris Medical Microbiology, 4th Edition. McGraw-Hill Medical: 2003; pp. 992.

[3] Jarlier V, Nikaido H. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol. Lett., 1994; 123: 11-8.

[4] Manning EJ, Collins MT. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis, Rev. Sci. Tech., 2001; 20: 133-50.

[5] Behr MH, Collins MD. *Paratuberculosis* Organism, Disease, Control. CAB International, Wallingford, Oxfordshire, UK, 2010.

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

[7] Kumthekar S, Manning EJ, Ghosh P, Tiwari K, Sharma RN, et al. *Mycobacterium avium* subspecies *paratuberculosis* confirmed following serological surveillance of small ruminants in Grenada, West Indies. J. Vet. Diagn Invest., 2013; 25(4): 527-30.

[8] Liapi M, Leontides L, Kostoulas P. Bayesian estimation of the true prevalence of Mycobacterium avium subsp. *paratuberculosis* infection in Cypriot dairy sheep and goat flocks, Small Ruminant Res., 2011; 95(2, 3): 174–78.

[9] Stich RW, Byrum B, Love B, et al. Evaluation of an automated system for nonradiometric detection of *Mycobacterium avium paratuberculosis* in bovine feces. J. Microbiol. Meth., 2004; 56: 267–75.

[10] Rodriguez-Lazaro D, D'Agostino M, et al. Real-time PCR-based methods for detection of *Mycobacterium avium subsp. paratuberculosis* in water and milk. Int. J. Food Microbiol., 2005; 101(1): 93-104.

[11] Stephan R, Schumacher S, Tasara T, Grant IR. Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss raw milk cheeses collected at the retail level. J. Dairy. Sci., 2007; 90(8): 3590-95.

[12] Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC. Mycobacterium species In: Veterinary microbiology and microbial diseases. 1st ed. Iowa State University Press Blackwell Sci.; 1994.

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

[14] Rajeev S, Zhang Y, Sreevatsan S, et al. 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.

[15] Springer B, Stockman L, Teschner K, et al. Two laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol., 1996; 34: 296-303.

[16] Kawaji S, Begg DJ, Plain KM, et al. A longitudinal study to evaluate the diagnostic potential of a direct faecal quantitative PCR test for Johne's disease in sheep. Vet. Microbiol., 2011; 148(1): 35-44.

[17] Sonawane GG, Tripathi BN. Comparison of a quantitative real-time polymerase chain reaction (qPCR) with conventional PCR, bacterial culture and ELISA for detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep showing pathology of Johne's disease. SpringerPlus, 2013; 2(45): 01-09.

Open Access Policy:
Authors/Contributors are responsible for originality, contents, correct references, and ethical issues. IJLSSR publishes all articles under Creative Commons Attribution- Non-Commercial 4.0 International License (CC BY-NC). https://creativecommons.org/licenses/by-nc/4.0/legalcode