INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 18, No. 5, p. 7-13, 2021

RESEARCH PAPER OPEN ACCESS

Comparison of Zielh-Neelsen staining and polymerase chain reaction for the diagnosis of Johne's disease in small ruminants in the district Lahore, Pakistan

Saba Badar[1], Ashiq Ali[2*], Zafar Iqbal Choudary[1], Abdul Jabbar[3], Sidra Altaf[4], Qaisar Tanveer[4], Iqra Asif[5], Asima Tariq[3], Muhammad Saif Ur Rehman Babar[4], Muhammad Umar Amin[4], Muhammad Kashif[2], Akasha Tanveer[5], Kiran Choudhary[2], Sidra Aslam[5], Tehreem Umar[2]

[1]*Department of Pathology, University of Veterinary & Animal Sciences, Lahore Pakistan*

[2]*Department of Pathology, University of Agriculture Faisalabad-38000, Pakistan*

[3]*Institute of Microbiology, University of Agriculture Faisalabad Faisalabad-38000, Pakistan*

[4]*Institute of Pharmacy, Physiology and Pharmacology, University of agriculture Faisalabad, Pakistan*

[5]*Department of Parasitology, University of Agriculture Faisalabad-38000, Pakistan*

Key words: Paratuberculosis, Small intestine, Zeihl-Nelson's staining, PCR.

http://dx.doi.org/10.12692/ijb/18.5.7-13 Article published on May 16, 2021

Abstract

Paratuberculosis is a bacterial disease that affects small and large ruminants and has an impact on the economy of the country in terms of production losses and cost of treatment and control. The objectives of this study were the diagnosis of Paratuberculosis or Johne's disease (JD) in goats of Lahore, Pakistan and a comparison of the two diagnostic techniques for Paratuberculosis. Part of the small intestines and associated mesenteric lymph nodes were collected randomly from goats of all ages and both sexes. Those samples were subjected to the Herrold's Egg Yolk Medium (HEYM) culture and DNA extraction followed by PCR and Ziehl-Neelsen's staining. Acid-fast bacilli were found in the tissues that showed the gross lesions of severe enteritis and lymphadenitis in the collected samples. Results showed that there was a clinical and subclinical infection. This study showed PCR more sensitive in the detection of Insertion Sequence (IS) 900 genomic fragment as compared to the HEYM culture.

* **Corresponding Author:** Ashiq Ali ✉ drashiq3485@gmail.com

Introduction

Paratuberculosis is a chronic, contagious, fatal intestinal enteropathy of domesticated and wild ruminants caused by *Mycobaterium avium* subsp. *paratuberculosis* (MAP). It is an acid-fast organism known to infect primarily all ruminants and primates in the form of Crohn's disease. It causes this fatal disease subclinically and later on clinically**,** which has a long incubation period of about one year or more. (Acharya *et al.,* 2017) reported that the infection differs in different host species in a few aspects and results of the diagnostic tests also vary, although the agent is the same.

The affected animal may shed the organism in feces in the late stages. Shooting or intermittent diarrhea is the main clinical feature in cattle and buffalo, but it is rare in goats (Begg and Whittington, 2020) as they are slaughtered at a younger age. MAP affects all the body tissues including mammary glands, milk, amniotic fluid, uterus and testicles. The organism can sustain in the environment without a host for a year and also can withstand high temperature. In most incidents of paratuberculosis, ruminants lead to loss of weight, low body condition score at the time of slaughter, culling at an early age and unavoidable low milk production (Busatto *et al.*, 2019). Also unbearable economic losses in farm management like poor reproductive efficiency, inefficient feed conservation, low immunity, irregular reproductive health status (prolonged calving intervals, increased infertility rates) and animals not being cost-efficient, made it compulsory to research in the MAP field, additionally the fact that *Mycobacterium avium paratuberculosis* is the probable cause of Crohn's disease and thought to be zoonotic (Eslami *et al.*, 2019) concluded that the uninterrupted spread of infection causes inevitable losses.

The present study details the diagnosis of MAP in the small ruminant population of Lahore, Pakistan through the use of polymerase chain reaction (PCR). Also, the PCR is compared with the centuries-old tested and relied upon the gold standard of HEYM (Herrold's Egg Yolk Medium) culture using the terminal small intestine samples and samples of the associated mesenteric lymph nodes (MLN) instead of milk, feces and blood samples (Chaudhry *et al.* 2012). The use of PCR for the diagnosis of *M. paratuberculosis* has improved the reliability of the diagnosis based on the discovery of specific genetic fragment *IS900* as compared to the time-consuming culture technique (Grant *et al.*, 2001).

This meets the objectives of the study for early and reliable diagnosis of MAP through a comparatively advanced technique of PCR than culture and sees the prevalence of Johne's disease in small ruminant's population where its data is least reported (Karuppusamy *et al.*, 2019).

Materials and methods

Collection of samples

Total of five hundred (n=500) tissue samples i.e. 250 terminal ileum samples and 250 associated mesenteric lymph nodes were collected, from goats of all ages and both sexes, from a slaughterhouse in Lahore. Tissue samples were collected in labeled specimen bags and transported in ice to the laboratory within an hour. There, a small piece of tissue was preserved in 10% neutral buffered formalin in labeled jars. These samples were processed in the Molecular Pathology Laboratory of the Department of Pathology, UVAS, in very fresh form, while the rest of the samples were stored in – 80 degree centigrade for future reference. Sample collection and processing were carried out for a year.

Diagnosis of Mycobacterium paratuberculosis in small ruminants

It was done by conventional methods i.e. Ziehl-Neelsen's staining and culture and advanced method polymerase chain reaction (PCR).

Ziehl-Neelsen's (acid-fast) staining

Smears were made on the slides using deep lesion material from intestinal lesions and the medullary region of lymph nodes. Processed for Ziehl-Neelsen's staining immediately after reaching the lab (Green *et al.*, 1989).

Culture

Lesion material from all samples was inoculated on the slants Herrold's Egg Yolk Medium with Mycobactin J as this separates MAP from the rest of the *Mycobacteria* species. The media was prepared as shown in the OIE Manual of Diagnostic Tests and Vaccines for the Terrestrial Animals, 5th Edition, 2004. The cultured tubes were incubated at 37 °C for eight to twelve weeks. Colony characteristics and growth were recorded.

The smears were made from cultures and stained by the ZN-Staining technique (Quinn *et al.* 1994) to confirm the acid-fast bacilli (Li *et al.*, 2017).

DNA extraction

DNA was isolated from tissue with the help of GENTRA PUREGENE kit, manufactured in the USA which is a DNA purification kit. The method used was that of Meijer, (2005).

Polymerase Chain Reaction (PCR)

Using the following primer sequence for *Mycobacterium paratuberculosis* Insertion Sequence 900 (IS), amplification was carried out by the method of Morales-Pablos *et al.* (2020) with some modifications.

Forward: 5'GTTATTAACGACGCCCAGC3'

Reverse: 5'ACGATGCTGTGTTGGGCGTTAG3'

Purified genomic DNA was subjected to PCR, targeting the Insertion Sequence (IS) 900 of *M. paratuberculosis*. To that end, a 50 µl reaction mixture was formulated, containing 5 µl 10X PCR buffer, 5 µl $MgCl_2$, 5µl 2.5mM dNTPs, 0.5 µl AmpliTaq DNA polymerase, 2µl forward primer, 2µl reverse primer, 5µl DNA sample and 25.5µl distilled water, for each reaction. The cyclic parameters were 94 °C for 4 min followed by 35 cycles of denaturation at 93 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec and a final extension of 72 °C for 7 min.

Gel electrophoresis

The amplified DNA fragments were analyzed by 1.5% Agarose gel electrophoresis. Observed the bands of DNA under ultra-violet trans-illuminator (Okuni *et al.*, 2020) compared with DNA marker ladder.

Results

Culture and polymerase chain reaction

Out of 500 tissue samples, 250 were terminal small intestines (SI) and 250 were associated with mesenteric lymph nodes (MLN). Each sample was processed through both techniques of PCR and culture characteristics of M. paratuberculosis as shown in Table 1.

Table 1. Colony characteristics of *Mycobacterium paratuberculosis*.

Characteristics of *M. paratuberculosis*	Characteristics shown
Growth rate	Very slow (up to 16 weeks)
Optimal incubation temperature	37° C
Atmospheric requirements	Aerobic
Colonial Features	Small, hemispherical; some pigmented
Essential growth supplement	Mycobactin

The selected primers were generating a 626 bp product for IS 900 of *M. paratuberculosis* as shown in Table 2. ZN staining confirms the culture results under the microscope to see the positive and negative samples for the acid-fast bacillus as shown n Fig 2 and Fig 3.

Small intestine

The culture of the small intestine showed fourteen (05.40%) positive samples out of 250. PCR of small intestine showed nineteen (07.6%) positive out of the same 250. Total Thirty Three (06.6%) samples appeared to be positive after all 500 tests (250 PCR and 250 culture examination) as shown in Table 3.

Mesenteric lymph node

The culture of 250 MLN samples demonstrated only twelve (4.80%) positive samples. PCR of seventeen

(6.80%) samples out of 250 MLN found to be positive for *Mycobacterium avium* subsp. *Paratuberculosis*. Twenty nine (05.80%) samples were found positive out of 500 tests (250 PCR and 250 culture examination). As shown from the Table 4, PCR of 250 small intestines detected 17 such samples positive which HEYM culture could not detect and similarly out of 250 MLN samples again HEYM ran short for 12 samples as PCR detected 17 such samples positive that former could not.

Table 2. The sequence of primers used.

Direction	Primer Sequence
Forward:	5-GTTATTAACGACGCCCAGC-3
Reverse:	5-ACGATGCTGTGTTGGGCGTTAG-3

The samples of small intestine detected positive by the tests were both clinical chronic and subclinical carriers. Comparing the laboratory results with the gross findings of carcasses and ante-mortem examinations, 7 samples were chronic clinical cases, 4 samples were subclinical and 4 found to be carriers (on gross examination they had no history of diarrhea ever, but they had been exposed to diarrhoeic animals). The MLNs samples were mostly chronic clinical and subclinical. 17 positive samples of MLN can be categorized as carriers based on the laboratory findings, no history of Johne's disease but exposure and grossly not with a good body condition score at ante-mortem. But HEYM could not detect one of them, PCR detected both of them as shown in Fig 4. Two were clinical with gross lesions present at ante-mortem and postmortem, they are detected by both PCR and HEYM culture.

Table 3. Result of small intestine samples in both techniques.

Techniques used	Positive	Negative	Total
PCR	19 (7.60%)	231	250
Culture	14 (5.40%)	236	250
Total	33 (6.6%)	467	500

Overall results are shown in Table 5, Out of these results, 250 MLN samples and 250 small intestine samples were from the same animals; the rests were different. 62 out of 500 samples were positive in all. 438 samples were negative, although at the gross ante-mortem examination, almost all of them were emaciated, lethargic, diarrhoeic, or with a history of diarrhea but there could be other reasons than that of MAP and ZN staining. The acid-fast bacilli were found as bright or rose-red rods in the blue background.

Table 4. Result of Mesenteric Lymph Node samples in both techniques.

Techniques used	Positive	Negative	Total
PCR	17 (6.80%)	233	250
Culture	12 (4.80%)	238	250
Total	29 (5.8%)	471	500

Discussion

Out of 500 tissue samples, 250 were terminal small intestines (SI) and 250 were associated with mesenteric lymph nodes (MLN). The culture of the small intestine showed fourteen (05.40%) positive samples out of 250. PCR of small intestine showed nineteen (07.6%) positive out of the same 250. Total Thirty-Three (06.6%) samples appeared to be positive after all 500 tests (250 PCR and 250 culture examination). (Acharya *et al.* (2017) reported the similar findings in the sheep and goat. Our results are also in line with the findings of (Li *et al.*, 2017).

Table 5. Showing overall positive results of the study.

Total	PCR	Culture
250 MLN	17 (6.80%)	12 (4.80%)
250 SI	19 (7.60%)	14 (5.40%)
Total	7.20% (36 Out of 500)	5.20% (26 Out of 500)

(Busatto *et al.* (2019) also reported that the PCR is more sensitive as compared to ZN staining for the diagnosis of Paratuberculosis. (Eslami *et al.,* 2019) reported that prevalence of paratuberculosis was 19% by using PCR.

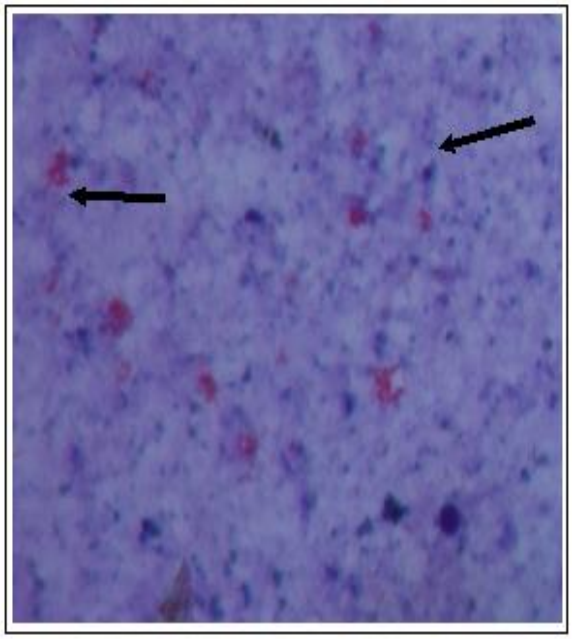

Fig. 1. *Mycobacterium avium* subsp. *paratuberculosis* in ZN stained lymph tissue slide.

The polymerase chain reaction was substantially different from the quick acid staining and single intradermal Johnin test (Busatto *et al.*, 2019). (Begg and Whittington, 2020) noticed that PCR is more sensitive than the bacterial suspension and the testing of the smear. (Karuppusamy *et al.*, 2019) revealed that only a slight proportion of DNA was required for PCR and that the purity of the sample was not always critical. The specific PCR-based detection of MAP was extremely specific and sensitive.

The polymerase chain reaction was capable of detecting specimens that were both culture-negative and detected DNA phentograms (Okuni *et al.*, 2020). (Acharya *et al.,* 2017) examined PCR blood as a rapid, extremely responsive, and accurate test for MAP infection at any point and age of the goat.

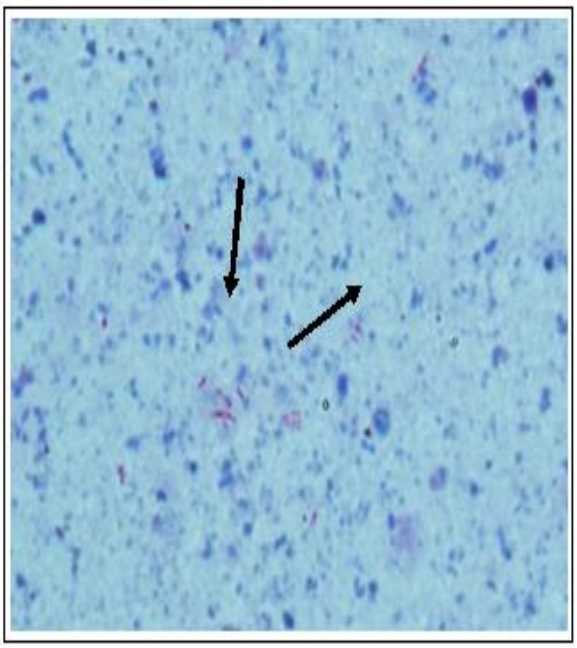

Fig. 2. *Mycobacterium avium* subsp. p*aratuberculosis* in ZN stained culture slide.

The observations of these prior workers offer a valid interpretation of the effects of this research. Outcomes of the study have shown that the culture of 250 MLN samples demonstrated only twelve (4.80%) positive samples. PCR of seventeen (6.80%) samples out of 250 MLN found to be positive for *Mycobacterium avium* subsp. *Paratuberculosis*. Twenty-nine (05.80%) samples were found positive out of 500 tests (250 PCR and 250 culture examination).

The single intradermal Johnin test is ranked as the second-best test after PCR for the diagnosis of MAP. Accordingly, it is inferred from the observations made in this study that PCR is the best diagnostic method tested for early diagnosis of paratuberculosis in goats, for successful slaughter, and hence for the management of paratuberculosis in goats.

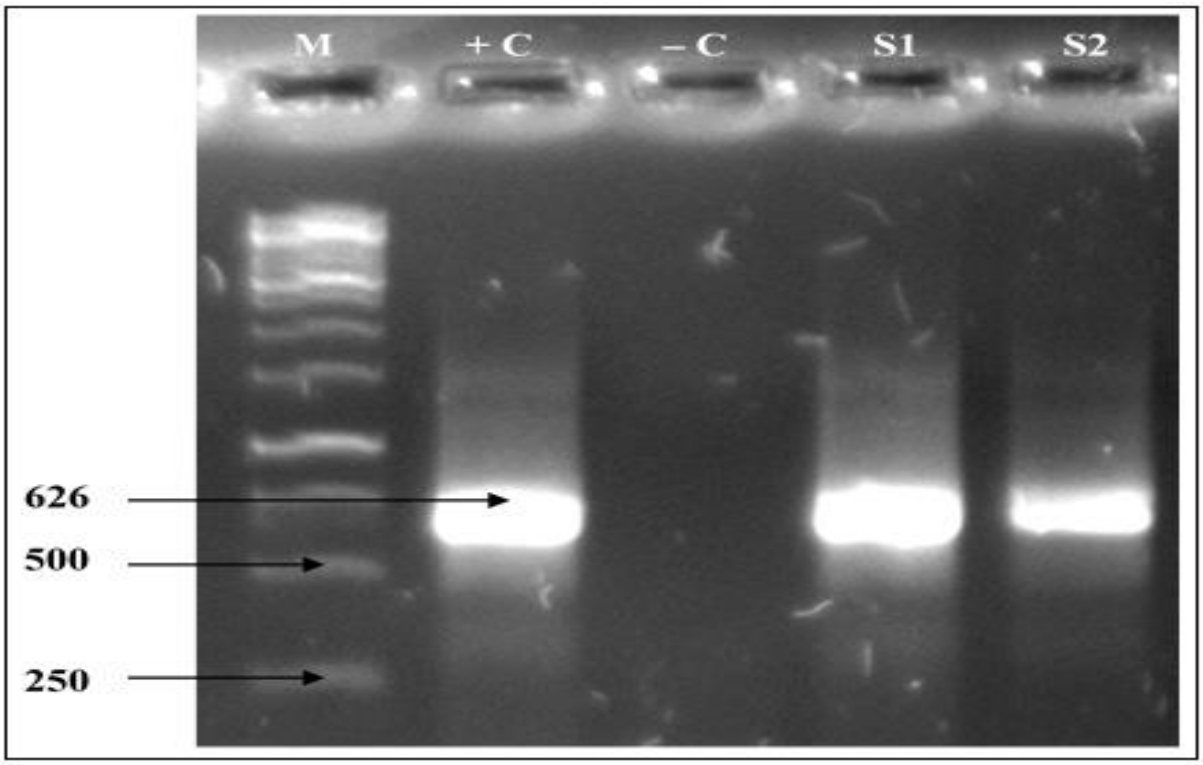

Fig. 3. Results of PCR showing 626 base pair samples of *Mycobacterium avium* subsp. *pratuberculosis*.

M Marker (250 bp)
+C Positive Control (From field ileal tissue sample)
- C Negative Control
S1 & S2 Positive sample 1 and 2.

Acknowledgements

Authors acknowledge the financial support of HEC Pakistan, for providing the funding for the research, laboratory staff, the mentors and University of Veterinary and Animal Sciences, Lahore Pakistan, staff of the slaughter house of Lahore, and companies making all the good chemicals for research and labs.

Conflict of interest

The author has no relevant affiliations or financial involvement with any organization.

References

Acharya KR, Dhand NK, Whittington RJ, Plain KM. 2017. PCR Inhibition of a quantitative PCR for detection of *Mycobacterium avium* subspecies *paratuberculosis* DNA in feces: Diagnostic implications and potential solutions. Frontiers in microbiology **8,** 115. https://doi.org/10.3389/fmicb.2017.00115

Begg D, Whittington R. 2020. 12 Paratuberculosis in Sheep. Paratuberculosis: Organism, Disease, Control. 160.

Busatto C, Vianna JS, da Silva Junior LV, Ramis IB, da Silva PEA. 2019. *Mycobacterium avium*: an overview. Tuberculosis **114,** 127-134. https://doi.org/10.1016/j.tube.2018.12.004

Eslami M, Shafiei M, Ghasemian A, Valizadeh S, Al-Marzoqi AH, Shokouhi Mostafavi SK, Nojoomi F, Mirforughi SA. 2019. *Mycobacterium avium paratuberculosis* and *Mycobacterium avium* complex and related subspecies as causative agents of zoonotic and occupational diseases. Journal of Cellular Physiology **234(8),** 12415-12421. https://doi.org/10.1002/jcp.28076

Grant IR, Rowe MT, Dundee L, Hitchings E. 2001. *Mycobacterium avium* ssp. *paratuberculosis*:

its incidence, heat resistance and detection in milk and dairy products. International Journal of Dairy Technology **54(1),** 2-13
https://doi.org/10.1046/j.1471-0307.2001.00009.x

Green EP, Tizard MLV, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J. 1989. Sequence and characteristics of IS900, an insertion element identified in human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucl. Acids Res. 17 p 9063-9073.
https://doi.org/10.1093/nar/17.22.9063

Karuppusamy S, Kirby GM, Mutharia L, Tripathi BN. 2019. An update on *Mycobacterium avium* subspecies *paratuberculosis* antigens and their role in the diagnosis of Johne's disease. World Journal of Microbiology and Biotechnology **35(8),** 120.
https://doi.org/10.1007/s11274-019-2691-0

Li L, Bannantine JP, Campo JJ, Randall A, Grohn YT, Katani R, Schilling M, Radzio-Basu J, Kapur V. 2017. Identification of sero-reactive antigens for the early diagnosis of Johne's disease in cattle. PloS one. **12(9),** e0184373.
https://doi.org/10.1371/journal.pone.0184373

Meijer ACE. 2019. Prevalence of hepatic epithelioid macrophage microgranulomas due to *Mycobacterium avium* subspecies *paratuberculosis* in Lambs on the North Island of New Zealand (Master's thesis).

Morales-Pablos MI, Mejía-Sánchez P, Díaz-Aparicio E, Palomares-Resendiz EG, Gutiérrez-Hernández JL, Reyna-Granados JR, Luna-Nevárez P, Munguía-Xóchihua JA, Segura-Correa JC, Leyva-Corona JC. 2020. Risk factors associated with the seroprevalence of paratuberculosis in sheep flocks in the hot-arid region of Sonora, México. Tropical Animal Health and Production **52(3),** 1357-1363.
https://doi.org/10.1007/s11250-019-02139-y

Okuni JB, Hansen S, Eltom KH, Eltayeb E, Amanzada A, Omega JA, Czerny CP, Abd El Wahed A, Ojok L. 2020. Paratuberculosis: A Potential Zoonosis and a Neglected Disease in Africa. Microorganisms **8(7),** 1007.
https://doi.org/10.3390/microorganisms8071007

Sachse K, Frey J. 2003. PCR Detection of Microbial Pathogens. Humana Press, Totowa, New Jersey. 289-297.

Shah MM, Mistry M, Marsh SJ, Brown DA. Delmas P. 2002. Molecular correlates of the M-current in cultured rat hippocampal neurons. The Journal of physiology **544(1),** 29-37.

Sivakumar P, Tripathi BN, Singh N. 2005. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in intestinal and lymph node tissues of water buffaloes (Bubalus bubalis) by PCR and bacterial culture. Veterinary Microbiology **108(3-4),** 263-270.
https://doi.org/10.1016/j.vetmic.2005.04.002

Soumya MP, Pillai RM, Antony PX, Mukhopadhyay HK, Rao VN. 2009. Comparison of faecal culture and IS900 PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faecal samples. Veterinary research communications **33(7),** 781.
https://doi.org/10.1007/s11259-009-9226-3

Truyers I, Jennings A. 2016. Management and control of Johne's disease in beef suckler herds. In Practice **38(7),** 347-354.
http://dx.doi.org/10.1136/inp.i3394

Zarei M, Ghorbanpour M, Tajbakhsh S, Mosavari N. 2017. Comparison of ELISA method, PCR and Ziehl-Neelsen staining of the rectal mucosa for detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle **2(13),** 29-37.