Nested PCR and its Comparison with other Diagnostic Test in the Diagnosis of Paratuberculosis (Johne's Disease) in Goats

Karthik K.[1]*, Das P.[2], and Tesfaheywet Zeryehun[1]

[1]College of Veterinary Medicine, Haramaya University, P. O. Box 138, Dire Dawa, Ethiopia
[2]Department of Biological Product, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India- 243122

Abstract: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) causes Johne's disease (JD) in ruminants. There are many methods for diagnosis of Johne's disease in goats. Among these, bacterial isolation was still considered as reference standard for diagnosis of JD in spite of its long turn over time. Molecular targets like *IS900* were routinely used for diagnosis, since it is present only in MAP. But lately presence of *IS900* like sequence in closely related members of *Mycobacterium avium* complex (MAC) make *IS900* based PCR a less sensitive method. Therefore, *IS900* PCR positive sample should be confirmed by PCR assay targeting another gene within the genome of the organism. This led to the discovery of *f57* gene, which is unique to this organism and not present in other members of MAC. The present study was carried out to evaluate the nested PCR method (targeting *IS900* & *f57* gene) to diagnose Johne's disease in goats. The efficacy of this nested PCR was compared with other serological tests like agar gel immunodiffusion (AGID) and absorbed enzyme-linked immunosorbent assay (ELISA). Out of the 265 goat faecal and sera samples, positive results were; AGID 36 (13.59%); absorbed ELISA 51 (19.25%) and nested PCR 58 (21.88%). This nested PCR was also compared with intra-dermal Johnin test in 65 animals, of which, positive results recorded were; Johnin test 21 (32%) and nested PCR 28 (43%). The nested PCR showed higher sensitivity compared to other diagnostic tests. Hence, this method can be used for diagnosis of clinical and sub clinical JD in goats.

Keywords: *Agar Gel Immunodiffusion, ELISA, Nested PCR, Paratuberculosis*

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes paratuberculosis or Johne's disease (JD) in cattle, sheep and goats. Johne's disease is prevalent worldwide and cause substantial economic losses to farming industry. The rate of prevalence may be higher than that of reported due to difficulty in the diagnosis of this disease, particularly during the preclinical stages. Generally, diagnosis of paratuberculosis is based on the detection of the organism or the immune response. Cultivation of bacteria from the faecal samples is considered as reference standard in diagnosis of paratuberculosis, but this method is more time consuming in sheep and goats as compared to cattle (Carrigan and Seaman, 1990; Collins *et al.,* 1993). Furthermore, the sensitivity of faecal culture is also too low (Chiodini *et al.,* 1984).

The host immune response to infection is initially cell mediated (CMI). As the infection progresses from subclinical to clinical disease, CMI responses are replaced by strong humoral responses characterized by the presence of antibodies. So the early identification of MAP infected animals can be detected by delayed type hypersensitivity (DTH) skin test or release of interferon (IFN)-γ in the blood sample (Gwozdz *et al.,* 2000) that can be measured by ELISA (Billman-Jacobe *et al.,* 1992; Stable, 1996). The specificity of these tests is low resulting in many false-positive results (Jungerson *et al.,* 2002; Huda *et al.,* 2003; Manning *et al.,* 2003; Reddacliff and Whittington, 2003). On the other hand, the humoral immune response occurs relatively late in infection and can be detected by various serological test like agar-gel immunodiffusion (AGID), enzyme linked immunosorbent assay (ELISA) and complement fixation test (CFT). The sensitivity and specificity of these assays is relatively high in clinically affected animals, but low in sub clinically infected animals, as antibody generally develop late in this infection (Milner *et al.,* 1987, 1990). In general the absorbed ELISA is considered to have the highest specificity and sensitivity among various serological tests (Hilbink *et al.,* 1994; Rajukumar *et al.,* 2001). In spite of its lower sensitivity and specificity, intradermal Johnin and AGID tests are the diagnostic test available at the field level.

The application of molecular method for the diagnosis of JD is under constant development and modification. A number of genes and sequence unique to MAP have been identified over the years. The insertion element *IS900* has been routinely used to detect the presence of MAP in clinical samples. However, sequence related to *IS900* like *IS902* (Wood pigeon mycobacterium), *IS901* (*Mycobacterium avium* subsp. *avium*), *IS1626* (*Mycobacterium avium* subsp. *avium* and *M. intracellulare*) were reported (Cousins *et al.,* 1999; Puyang *et al.,* 1999; Englund *et al.,* 2002) and hence reduces its specificity. Therefore, a positive *IS900* PCR should be confirmed by subsequent nested PCR or by

*Corresponding Author. E-mail: kartraa@gmail.com

ISSN 2616-8804 (Print)

a PCR assay targeting another gene. Another sequence named *f57* has been identified and this sequence does not have any homology with any known sequences (Poupart *et al.,* 1993; Coetsier *et al.,* 2000). In the present experiment, the samples from the goats were screened for Johne's disease by nested PCR method of both *IS900* and *f57* gene. Furthermore, the efficacy of this nested PCR is compared with other diagnostic tests like AGID, absorbed ELISA and Johnin skin test.

Materials and Methods

Serum and faecal samples from 200 adult goats (>1 year of age) of both sex (male and female) were collected randomly from slaughter house of different regions in India. Additional faecal and serum samples from 65 adult goats of both sexes (male and female) were collected from Indian Veterinary Research institution farm and these 65 animals were also used for Johnin skin testing.

Bacterial Strain and Preparation of Antigens

Standard culture of *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698 and *Mycobacterium phlei* was procured from Biological Product Division, Indian Veterinary Research Institute, India. It was maintained in Lowenstein Jenson (LJ) medium containing mycobactin J. For large scale production of antigen, the culture was grown as a surface pellicle on Watson and Reid synthetic broth containing mycobactin J. and incubated for eight weeks at 37°C. Bacterial growth was killed by heating at 72°C for 2 h and separated by filtration. The cells were washed thrice with PBS and centrifuged to get cell mass. It was re-suspended in PBS containing 0.2mM phenyl methyl sulfonyl fluoride and sonicated at 16μ amplitude for 45 min in ice with intermittent intervals. Sonicated preparation was centrifuged at 12000x g for 1 h at 4°C. The supernatant was filtered through 0.22 μm membrane filter. The protein content of the solution was estimated by the bicinchonimic acid method (Protein estimation kit, Genei, India). Antigen was diluted with PBS up to protein content to 1mg/ml and kept at -20°C in different aliquots.

Raising Hyper-Immune Sera

Two healthy goats free from MAP infection by PCR and ELISA techniques were chosen and used for hyper-immune sera production. Hyper-immune serum was raised as per methods described previously with suitable modifications (Castelnuovo *et al.,* 1969; Johle, 2008). The antigenic mixture consisting of 200 mg of whole cells, 10 mg of sonicated antigen and 4 ml of sonicated sediment of MAP was mixed with equal amounts of Freund's incomplete adjuvant (Difco, USA). Each goat was inoculated with 1 ml of the antigen mixture subcutaneously at weekly intervals for 6 weeks. Antibody titre was monitored by agar gel immunodiffusion test after fifth injection. Serum were collected from goats one week after the last injection and stored at 4°C. The serum collected before immunization was used as negative controls.

Extraction of Genomic DNA from Faecal Samples

Approximately, 200 mg faecal samples were used to isolate genomic DNA by using QIAmp stool DNA kit (Qiagen, Germany) according to manufactures' instruction. Extracted DNA was stored at -20°C until further use.

Nested Polymerase Chain Reaction

The primers which were used to amplify the target gene were taken from published literature (Vansnick *et al.,* 2004). The details of the primers, its target and product length are given in Table 1. Amplification of both sequences was based on the nested PCR approach; PCR product of the first PCR run used as the template for the 2nd amplification (nested). Briefly, for the first PCR run, primers IS900 F and IS900 R were used for amplification of *IS900 gene*, whereas primers F57 F and F57 R for amplification of *f57* gene.

Table 1. Details of the primers, its target and the PCR product size

Primers	Nucleotide sequence	Target gene	Product length
IS900 F	5'-GGGTTGATCTGGACAATGACGGTTA-3'	*IS900*	572
IS900 R	5'-AGCGCGGCACGGCTCTTGTT-3'	*IS900*	
IS900FN	5'-GGAGGTGGTGTGGCACAACCTGT-3'	*IS900*	452
IS900RN	5'-CGATCAGCCACCAGATCGGAA-3'	*IS900*	
F57 F	5'-CCTGTCTAATTCGATCACGGACTAGA-3'	*f57*	432
F57 R	5'-TCAGCTATTGGTGTACCGAATGT-3'	*f57*	
F57 RN	5'-TGGTGTACCGAATGTTGTTGTCAC-3'	*f57*	424

In 2nd amplification (nested), IS900 FN and IS900 RN were used to anneal the amplified product targeting the region within *IS900* from the first PCR run. Similarly, F57 F and F57 RN were used to anneal the amplified product of targeting the region within *f57* from first PCR run.

PCR reaction mixtures for 1st and 2nd amplification of *IS900* and *f57* gene were made in total volume of 25 μl. Final concentrations of different constituents in reaction mixture were made, respectively as tris-Cl (pH 9); 10mM, KCl; 50 mM. MgCl2; 1.6 mM, dNTP mix; 800 μM total, primers; 0.8 μM each, Red Taq polymerase; 0.5 U, triton-X100; 0.01% and DNA

templates 10-20 ng. For positive control, DNA isolated from standard MAP strain ATCC 19698 was used. Whereas nuclease free water was used as non-template control.

Amplification of both sequence were performed in thermocycler (Biometra, India) with amplification condition respectively in sequence as, initial denaturation (94°C) for 4 min; followed by 40 cycles (25 cycles for 2nd PCR run) of denaturation (94°C), annealing (68°C) (the annealing is same for both) and extension (72°C), each for 45 sec and at last, final extension (72°C) for 10 min was given.

PCR products were analyzed by electrophoresis in 1X tris-acetate EDTA (TAE) buffer for 2 h at 50 V. PCR products along with DNA marker were loaded in 2% (w/v) agarose gel, made in 1X TAE containing 0.5 ug/ml (w/v) ethidium bromide. Separation of DNA was visualized by UV at 260nm and documented.

Absorbed Indirect ELISA

The absorbed ELISA was developed by the method previously described (Cox *et al*., 1991). Briefly all the wells in the microtitre plate (NUNC, Maxisorp) were coated with 100 µl of 0.02 µg/µl (2 µg/well) capture antigens (sonicated antigens) in coating buffer (sodium bicarbonate buffer) and incubated at 37°C for 1 h. Unbound antigen in the plate was washed thrice with washing buffer PBS with 0.5% Tween-20 (PBST). Blocking of unbound site was done by adding 100 µl blocking buffer (2% BSA in PBST) and incubated at 37°C for 1 h. Plate was washed thrice with wash buffer. The 100 µl of sera sample were incubated at 37°C with 0.40 µg/µl absorbed antigen (sonicated *M. phlei* antigen) for 30 min. 100 µl of each serum was added in duplicate wells and incubated for 1 h at 37°C. Plate was washed thrice with wash buffer. 100 µl of rabbit anti-goat HRPO conjugate (Genei, Bangalore) was added per well (1:5000) and incubated for 1 h at 37°C. Plate was washed thrice with wash buffer. 100 µl 1x TMB solution (Genei, Bangalore) was added per well and observed for colour development. After 10-20 min the reaction was stopped by adding 100 µl of 1 M sulphuric acid per well and reading was taken at 450 nm in an ELISA reader. All ELISA results were determined by the procedure described previously (Rajukumar *et al.,* 2001).

Agar-Gel Immunodiffusion Assay

One percent agarose gel was prepared in PBS (pH 7.4) containing sodium azide (0.02% w/v). Gel was cast into sterile plastic petriplates and allowed to solidify at 4°C for 1 h in the humid chamber. Wells of 3 mm diameter were punched out in a hexagonal pattern with six peripheral wells for sera and one centre well for the antigen at equidistance of 5 mm between them. The central well was filled with sonicated antigen with optimum concentration (1 mg/ml). The test sera was charged in duplicate in six peripheral wells and incubated at 4°C overnight in the humid chamber along with positive control and negative control. Gels were examined after 24 and 48 h and in suspected cases after 72 h. White precipitation line between antigen and sera wells were taken as positive whereas absence of precipitation line was recorded as negative. Hyperimmune sera were used as positive control whereas distilled water was used as negative control.

Johnin Intradermal Skin Test

This test was carried out by the single intradermal inoculation of 0.1 ml of Johnin purified protein derivatives (1 mg/ml). An increase in skin fold thickness more than 4 mm or more and/or with clinical signs of inflammation and clinical edema was considered as positive reactors.

Data Processing and Analysis

Statistical analysis of the data was accomplished using the Kappa statistic which gives a chance-related measure of agreement (Fleiss, 1981). This statistic can vary from -1 (no agreement) to +1 (perfect agreement). The diagnostic test sensitivity determination for the four tests was also compared by using Mc Nemar's test (Remington and Schork, 1970). The test results were evaluated by binomial distribution (Remington and Schork, 1970). All statistical calculation and interpretation were done using Graphpad QuickCals (San Diego, CA, USA).

Results

Nested PCR of Faecal DNA Sample

Primary PCR with primers targeting IS900 and f57 of MAP resulted in specific amplification of products of 572 bp and 432 bp, respectively (Figure 1). Secondary PCR (nested PCR) with primers targeting the region within *IS900* and *f57* also resulted in specific amplification of products 452 and 424 bp, respectively (Figure 2). Among the DNA samples isolated from 265 fecal samples from different flocks and from institutional animal house, 56 animals were positive for both nested targeted genes (*IS900* and *f57)* (Table 1).

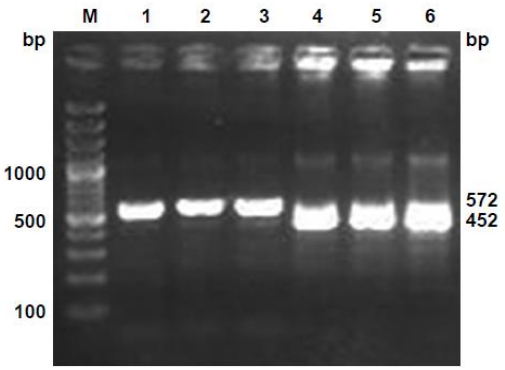

Figure 1. Nested PCR of *IS900* gene for detection of MAP in faecal sample. *Lane M: 100 bp DNA ladder; Lane 1-3: Primary PCR product (572 bp); Lane 4-6: Nested PCR product (452 bp).*

Absorbed Indirect ELISA

The absorbed ELISA (Figure 3) detected a total of 51 animals out of total of 265 serum samples collected from different flock and institutional animal house (Table 2).

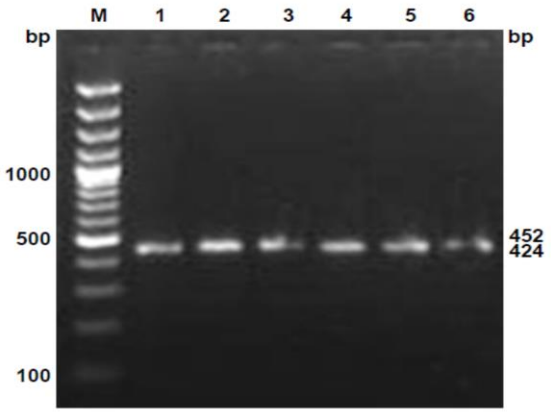

Figure 2. Nested PCR of *f57* gene for detection of MAP in faecal sample. *Lane M: 100 bp DNA ladder; Lane 1-3: Primary PCR product (452 bp); Lane 4-6: Nested PCR product (424 bp).*

Agar Gel Immunodiffusion Assay

A precipitation line (Figure 4) indicating a positive reaction in AGID was observed in 36 animals out of 265 serum samples collected (Table 3).

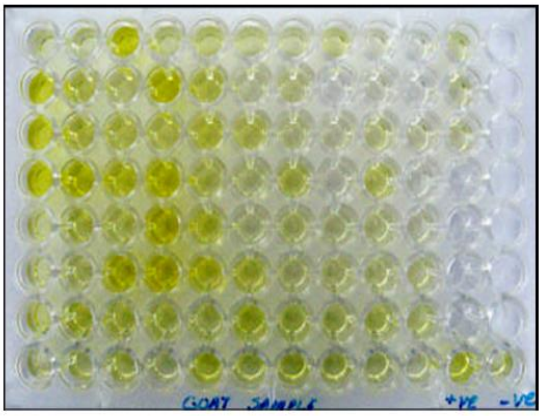

Figure 3. ELISA plate showing the color intensity of various serum samples for detection of antibody response to MAP in infected animals

Table 2. Results of serological tests in 265 sera sample collected from different flock

Flock	Status of flock	No. of samples	No. of animals positive			
			AGID	**ELISA**	**PCR**	**Johnin**
1	Unknown	25	1	3	4	-
2	Negative	23	0	0	0	-
3	Unknown	37	1	3	3	-
4	Unknown	33	1	2	2	-
5	Positive	15	3	5	6	-
6	Positive	33	6	9	10	-
7	Unknown	17	0	3	3	-
8	Negative	17	0	0	0	-
9	Positive	65	24	26	28	21
Total		265	36	51	56	21

Table 3. Result of 65 animals from which both serum and faecal samples were collected and Johnin test was performed

	P+ E+ A+	P+ E+ A-	P+ E- A-	P- E+ A+	P- E- A+	P- E- A-
Johnin +	15	3	-	-	2	3
Johnin -	5	2	2	1	-	45

P= PCR; E= ELISA; A= AGID; += Positive; -= Negative.

Johnin Intradermal Skin Test

An increase in skin fold thickness more than 4 mm or more and/or with clinical signs of inflammation and edema indicating positive reaction was observed in 21 animals out of a total of 65 animals from institutional farm house (Table 4).

Table 4. Statistical analysis of the results of different diagnostics tests for JD using Kappa statistics

Tests	**Kappa value ***	**Interpretation**
Faecal PCR Vs. ELISA	0.961	Perfect agreement
ELISA Vs. AGID	0.872	Perfect agreement
AGID Vs. Faecal PCR	0.834	Perfect agreement
AGID Vs. Johnin	0.774	Substantial agreement
ELISA/PCR Vs. Johnin	0.667	Substantial agreement

*Fleiss (1981).

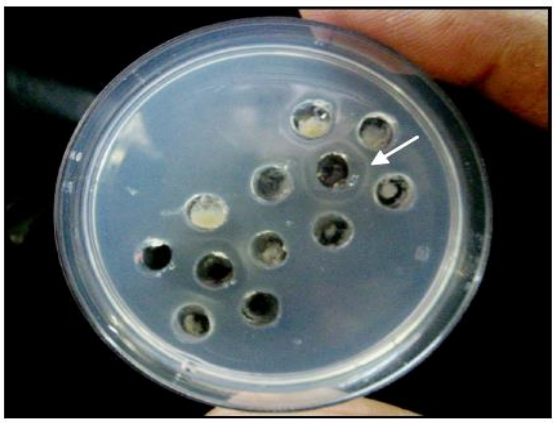

Figure 4. Agar gel immunodiffusion test showing precipitation line

Discussion

The evaluation of the nested PCR in diagnosis of JD was determined by comparing it with other diagnostic tests that commonly used for JD diagnosis. The evaluation of nested PCR with a golden standard is always advisable. Due to more time consumption and the low sensitivity of the faecal culture method (Chiodini *et al*., 1984; Carrigan and Seaman, 1990; Collins *et al*., 1993) absorbed ELISA was used for evaluation. Flocks with negative, unknown and positive status of infection were included in order to determine a proper diagnostic accuracy of the test. When the results of the 65 animals for which all tests (Nested PCR, ELISA, AGID and Johnin test) were performed are compared (Table 3), one animal with negative PCR and positive ELISA result may be due to shedding of bacteria below the detection limit at the time of sampling. Two animals with positive PCR and negative ELISA results indicate that these animals may not have yet sero-converted or that it may be in early stage of infection. AGID test showed lower sensitivity and false positive reaction when compared with nested PCR and ELISA.

The higher sensitivity of nested PCR was due to the ability of this test to detect even low fecal shedders and as the humoral response come to play in later part of the infection. ELISA or AGID was not able to detect these low faecal shedders which are seen during earlier part of infection (Hope *et al.,* 2000; Sergeant *et al.,* 2003). On the other hand, the three animals which were negative by PCR and other serological test were found positive by Johnin test. This indicates the less specificity and cross reactivity of Johnin test with another environmental mycobacterial organism (Huda *et al.,* 2003).

When the different test results were compared statistically using Kappa statistic with 95% confidence interval, the kappa value for these tests varied from 0.667 to 0.961 (Table 4). There was perfect agreement between PCR, AGID and ELISA (K= 0.961). Substantial agreement (K= 0.774) of Johnin test with another test like PCR and ELISA was observed. Furthermore, when the test were compared for sensitivity using Mc Nemar's test, significant difference ($p > 0.01$) was not noticed between the sensitivity of ELISA, AGID and PCR. However, the sensitivity of PCR and ELISA was significantly high ($p < 0.001$) when compared with that of Johnin test. These relationships suggested that absorbed ELISA was better option for screening animal for JD. However, ELISA fails to detect low shedder, which was detected by nested PCR test. When the PCR was compared with the reference test like ELISA, there was no statistically significant difference ($p > 0.01$) in their sensitivity, indicating that ELISA and PCR have similar results in diagnosis of Johne's disease.

Conclusion

The nested PCR was used for diagnosis of Johne's disease in goat and its relative sensitivity and specificity was determined by comparing with other diagnostic tests. Though the test possesses several advantages like rapidity and specificity, it may not be used for screening purpose due to high cost involvement. However, this technique detected earlier stage of infection and low shedder animal. Hence, this method can be used for early diagnosis of Johne's for effective control of the disease.

Acknowledgement

We thank the Director of the Indian Veterinary Research Institute for the use of facilities, providing funds and supporting research work.

Conflict of Interests

The authors declare that they have no competing interests.

References

Billman-Jacobe, H., Carrigan, M., Cockram, F., Corner, L. A., Gill, I. J., Hill, J.F., Jessep, T., Milner, A. R. & Wood, P. R. (1992). A comparison of the interferon gamma assay with the absorbed ELISA for the diagnosis of Johne's disease in cattle. *Australian Veterinary Journal,* 69: 25-28.

Carrigan, M. J. & Seaman, J. T. (1990). The pathology of Johne's disease in sheep. *Australian Veterinary Journal,* 67: 47-50.

Castelnuovo, G., Yamanaka, S., Zeppis, M. & Dotti, E. (1969). The protein components of *M. phlei* fractionation procedures. *Tubercle London,* 50: 194-202.

Chiodini, R. J., Van Kruiningen, H. J. & Merkal, R. S. (1984). Ruminant paratuberculosis (Johne's disease): The current status and future prospect. The *Cornell Veterinarian,* 74: 218-262.

Coetsier, C., Vannuffel, P., Blondeel, N., Denef, J. F., Cocito, C. & Gala, J. L. (2000). Duplex PCR for differential identification of *Mycobacterium bovis*, *M.*

avium and *M. avium* subsp. *paratuberculosis* in formalin fixed paraffin embedded tissue from cattle. *Journal of Clinical Microbiology,* 38: 3048-3054.

Collins, D. M., Gabric, D. M. & De Lisle, G. W. (1993). Comparison of polymerase chain reaction tests and faecal culture for detecting *Mycobacterium paratuberculosis* in bovine faeces. *Veterinary Microbiology,* 36: 289-299.

Cousins, D. V., Whittington, R., Marsh, I., Masters, A., Evans, R. J. & Kluver, P. (1999). Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess *IS900* like sequence detectable by *IS900* PCR: implication for diagnosis. *Molecular and Cellular Probe,* 14: 431-442.

Cox, J. C., Drane, D. P, Jones, S. L., Ridge, S. & Milner, A. R. (1991). Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. *Australian Veterinary Journal,* 68: 157-160.

Englund, S., Bolske, G. & Johansson, K. E. (2002). An IS900 like sequence found in *Mycobacterium* sp. other than *Mycobacterium avium* subsp. *paratuberculosis*. *FEMS Microbiology Letter,* 209: 267-271.

Fleiss, J. L. (1981). Statistical methods for rates and proportions. Wiley, New York, pp: 212-236.

Gwozdz, J. M., Thompson, K. G., Murray, A., Reichel, M. P., Manktelow, B. W. & West, D. M. (2000). Comparison of three serological tests and an interferon-γ assay for the diagnosis of paratuberculosis in experimentally infected sheep. *Australian Veterinary Journal,* 78: 779-783.

Hilbink, F., West, D. M., De Lisle, G. M., Kittelberger, R., Hosie, B. D., Hulton, J., Cooke, M. M. & Penrose, M. (1994). Comparison of a complement fixation test, a gel diffusion test and two absorbed and unabsorbed ELISA for the diagnosis of paratuberculosis in sheep. *Veterinary Microbiology,* 41: 107-116.

Hope, A. F., Kluver, P. F., Jones, S. L. & Condron, R. J. (2000). Sensitivity and specificity of two serological tests for the detection of ovine paratuberculosis. *Australian Veterinary Journal*, 78: 850-856.

Huda, A., Jungerson, G., Christoffersen, A. B. & Lind, P. (2003). Diagnosis of bovine paratuberculosis by interferon gamma (IFN-gamma) test. *Acta Veterinaria Scandinavica,* 44: 281-285.

Johle, D. K. (2008). Experimental paratuberculosis in rabbits and mice. PhD Thesis, Indian Veterinary research Institute, India.

Jungerson, G., Huda, A., Hansen, J. J. & Lind, P. (2002). Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. *Clinical and Diagnostic Laboratory Immunology,* 9: 453-460.

Manning, E. J. B., Steinberg, H., Krebs, V. & Collins, M. T. (2003). Diagnostic testing patterns of natural *Mycobacterium paratuberculosis* infection in pygmy goats. *Canadian Journal of Veterinary Research,* 67: 213-218.

Milner, A. R., Lepper, A. W., Symonds, W. N. & Gruner, E. (1987). Analysis by ELISA and western blotting of antibody reactivities in cattle infected with *Mycobacterium paratuberculosis* after absorption of serum with *M. phlei*. *Research in Veterinary Science,* 42: 104-144.

Milner, A. R., Mack, W. N., Coates, K. J., Hill, J., Gill, I. & Sheldrick, P. (1990). The sensitivity and specificity of a modified ELISA for the diagnosis of Johne's disease from a field trail in cattle. *Veterinary Microbiology,* 25: 193-198.

Poupart, P., Coena, M., Van Heuverswijn, H. & Cocito, C. (1993). Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. *Journal of Clinical Microbiology,* 31: 1601-1605.

Puyang, X., Lee, K., Pawlichuk, C. & Kunimoto, D. Y. (1999). IS1626, a new *IS900* related *Mycobacterium avium* insertion sequence. *Microbiology,* 145: 3163-3168.

Rajukumar, K., Tripathi, B. N., Kurade, N. P. & Parihar, N. S. (2001). An enzyme linked immunosorbent assay using immuno-affinity-purification antigen in the diagnosis of caprine paratuberculosis and its comparison with conventional ELISAs. *Veterinary Research Communication,* 25: 539-553.

Reddacliff, L. A. & Whittington, R. J. (2003). Experimental infection of weaner sheep with S strain *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology,* 96: 247-258.

Remington, R. D. & Schork, M. A. (1970). Chi-square tests for frequency data. In N. J. Englewood Cliffs (Ed.), *Statistics with applications to the biological and health sciences* (pp. 229-252), Prentice-Hall, Inc.

Sergeant, E. S., Marshall, D. J., Eamens, G. J., Kearns, C. & Whittington, R. J. (2003). Evaluation of an absorbed ELISA and an agar-gel immunodiffusion test for ovine paratuberculosis in sheep in Australia. *Preventive Veterinary Medicine,* 61: 235-248.

Stable, J. R. (1996). Production of gamma-interferon by peripheral blood mononuclear cells: an important diagnostic tool for detection of subclinical paratuberculosis. *Journal of Veterinary Diagnostic Investigation,* 8: 345-350.

Vansnick, E., De Rijk, P., Vercammen, F., Geysen, D., Rigouts, L & Portaels, F. (2004). Newly developed primers for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology*, 100 (3&4): 197-204.