Tropical Biomedicine 33(2): 327–334 (2016)

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faecal samples by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) TaqMan Assay

Rasool, S.[1*], Narang, D.[1], Chandra, M.[1], Sharma, N.S.[1] and Tejinder Singh, S.[2]
[1]Department of Veterinary Microbiology,
[2]Department of Animal Genetics and Breeding, COVS, GADVASU, Ludhiana
[*]Corresponding author e-mail: deeptivet@rediffmail.com
Received 9 August 2015; received in revised form 30 October 2015; accepted 3 November 2015

Abstract. *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease is a degenerative chronic granulomatous disease of bovines. In the present study, quantitative real time polymerase chain reaction (qRT-PCR) using TaqMan chemistry targeting the IS*900* sequence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was employed for the molecular diagnosis of the disease in bovine faecal samples. Out of 200 bovine faecal samples processed, 7 samples were tested as positive by IS*900* qRT-PCR. The sensitivity limit of detection of MAP DNA in faecal samples by qRT-PCR TaqMan assay was found to be 0.05pg. No amplification was observed in other Mycobacterial spp. *viz*. *M. phlei*, *M. smegmatis*, *M. intracellulare* and *M. kansasii*.

INTRODUCTION

Paratuberculosis is a degenerative chronic granulomatous infection of the intestinal tract causing diarrhoea, weight loss, reduced reproductive performance. This is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a slow-growing, gram positive, facultative intracellular, mycobactin J dependant, acid-fast bacteria (Harris & Barletta, 2001). The disease is primarily recognized in domestic, wild and zoo animals (Motiwala *et al.*, 2006). Early detection of infection is difficult because of long incubation period, since the fecal culture, the gold standard, can take as long as 4–6 months to confirm the presence of pathogen. Therefore a rapid diagnosis of this pathogen is a high priority task.

PCR has been shown to be a powerful tool in microbiological diagnostics. Molecular detection method based on sequence detection of IS*900*, a 1451 bp repetitive DNA element has been developed and commonly used for the detection of *Mycobacterium avium paratuberculsosis* (Bull *et al.*, 2000). Many of the molecular detection methods are based on the detection of amplification by endpoint PCR (O'Mahony and Hill, 2002; Pillai & Jayarao, 2002). These methods are based on the most widely used primers designed to amplify a 229 bp (Vary *et al.*, 1990) or a 413 bp (Millar *et al.*, 1996) fragment of IS*900*. Among the quantitative PCR methods are the fluorescence detection of SYBR Green bound double stranded DNA (O'Mahony & Hill, 2002); detection of bound fluorescent probes (Fang *et al.*, 2002 and Christopher-Hennings *et al.*, 2003); or detection of the 5V nuclease cleavage of a bound fluorogenic (TaqMan) probe by Taq DNA polymerase (Kim *et al.*, 2002). Molecular beacons and TaqMan probes are highly specific for the detection of target sequences. The presence of 12–20 copies of IS*900* in MAP genome makes this unique genetic element a better target for sensitive detection as compared to the detection of

single copy genes (Chui *et al.*, 2004) like hspX (Stabel *et al.*, 2004) and F57 (Coetsier *et al.*, 2000). Sensitive methods that minimize loss of template DNA during processing are required to detect the low levels of MAP anticipated in environmental samples. Keeping the above points in mind the present study was planned with the objective to detect *Mycobacterium avium subsp. paratuberculosis* in bovine fecal samples using TaqMan probe in qRT-PCR targeting IS*900*.

MATERIALS AND METHODS

Field investigation

The present study was carried out to diagnose paratuberculosis using qRT-PCR Taqman assay on the DNA extracted from faecal samples of cattle and buffaloes suspected for infection with *Mycobacterium avium* subsp. *Paratuberculosis* on the basis of history of chronic intermittent diarrhoea, emaciation and cachectic condition. Standard cultures of *M. phlei*, *M. smegmatis*, *M. intracellulare* and *M. kansasii* were obtained from NuLife, India. Faecal (n=200) samples from cattle and buffaloes with a history of chronic intermittent diarrhea were collected from dairy farms in Ludhiana, Punjab.

Laboratory techniques

DNA Extraction

The extraction of genomic DNA from faecal samples was done using commercially available bacterial genomic DNA Isolation Kit (HiPurA DNA Purification kit) as per the manufacturer's instructions. The primer pair and the TaqMan probe specific for *Mycobacterium avium* subsp. *paratuberculosis* used were those reported by Kim *et al.*, 2002. The forward primer was F2: 5'- AATGA CGGTT ACGGA GGTGG T- 3' ACAGG. The probe was labeled with the fluorescent reporter dye 5-carboxyfluoroscein (FAM) on the 5' end and the quencher dye N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. The primer probe was synthesized by ABI, Life Technologies.

Real time PCR

The real time PCR reaction mixture was made in a final volume of 20µl containing Universal Taqman PCR master mix (ABI), 6 pmol Taqman probe, primer (forward and reverse) 10 pmol each, DNA and nuclease free water. The cycling conditions were 60°C for 30 seconds, followed by initial Denaturation at 95°C for 10 minutes and 40 cycles consist of 95°C for 15 seconds and annealing and extension at 55°C for 1 min and final post PCR read at 60°C for 30 seconds. Real time PCR was carried out in Step-One-Plus RT-PCR machine of ABI, Life Technologies.

Sensitivity of TaqMan Real Time PCR

Serial 10-fold dilutions of standard genomic DNA of concentration 5ng/µl of MAP (provided by Dr. Stephensen, Edinburgh, UK) was carried out up to 5th dilution. (1:1 00 000) and Ct values were obtained as per the software of the instrument.

RESULTS

IS*900* TaqMan assay measures the amount of target IS*900* DNA produced during each cycle of an amplification reaction in a real-time format. Thus, the system is able to quantify the amount of target DNA in contrast to the conventional PCR, which measures only the end-point values with qualitative results. Out of the total 200 fecal samples processed, 7 samples were positive by qRT-PCR Taqman assay. The C_T values of the 7 faecal samples ranged from 20-33 and for rest of the 193 faecal samples, C_T values were greater than 40. The faecal samples whose C_T was between 20 and 33 were considered positive (Table 1, Fig. 1). All the samples were run in duplicate. Quantitation of the amount of target in the unknown samples was accomplished by measuring the threshold cycle (CT) and using a standard curve prepared with a series of known quantities of the target sequence. The C_T is defined as the cycle number at which the copy of the amplified target sequence passes the threshold or baseline. No amplification was observed in other

Table 1. C_T values of DNA extracted from faecal samples in IS*900* qRT-PCR Taqman assay

Sample No	C_T value obtained in IS*900* qRT-PCR
1	20.2
2	20.6
3	22.5
4	28.8
5	31.5
6	32
7	33.6

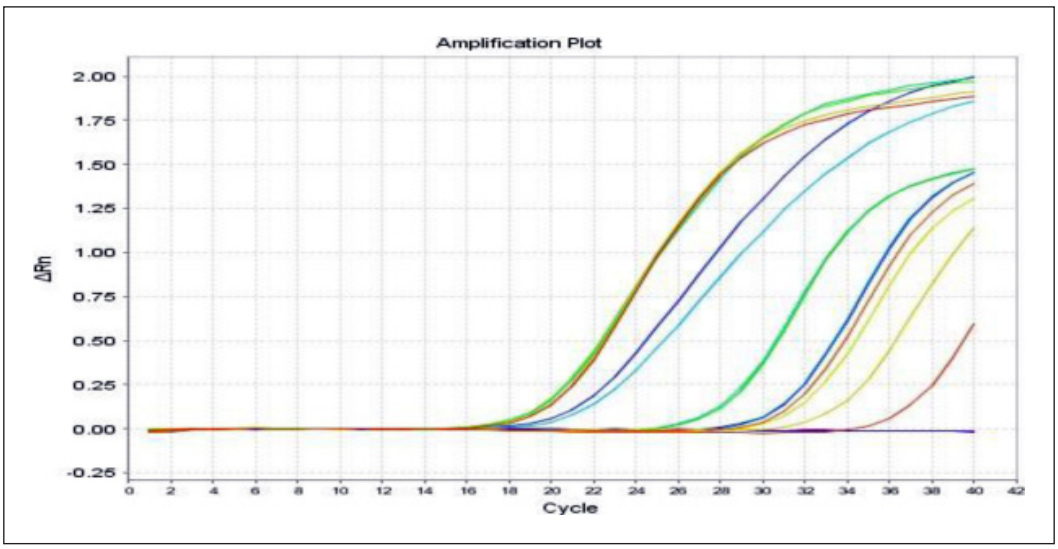

Figure 1. Gene expression profile of IS*900* in DNA extracted from faecal samples by qRT-PCR TaqMan assay (positive samples).

Mycobacterial spp. *viz*. *M. phlei*, *M. smegmatis*, *M. intracellulare* and *M. kansasii*.

Sensitivity of TaqMan real time PCR

Serial 10 fold dilutions of standard genomic DNA of MAP (provided by Dr. Stephensen, Edinburgh, UK) was carried out up to 5th dilution. (1:1 00 000) and Ct values were obtained as per the concentration of the DNA present. Minimum detectable limit of 0.05 picogram of DNA was observed (Table 2, Fig. 2).

DISCUSSION

Paratuberculosis is a chronic infectious disease affecting cattle and buffaloes. The causative organism, *Mycobacterium avium subsp. paratuberculosis* has a long incubation period. Isolation of this organism requires a minimum of 16 weeks, which is very time consuming. With the advent of nucleic acid based diagnostic assays, it becomes easier to diagnose the disease in a shortest possible time. Johne's disease is endemic in cattle in developed countries and

Table 2. C_T values in qRT- PCR Taqman assay in Serial dilutions of known standard DNA (5ng/µl) *Mycobacterium avium paratuberculosis*

Standard DNA Dilutions	Concentration of DNA	Ct Value
1:10	0.5 ng	18.2
1:100	0.05 ng	21.9
1:1000	0.005 ng ~5 pg	25.5
1:10000	0.0005 ng~0.5 pg~5 fg	29
1:100000	0.00005 ng~0.05 pg~50 fg	32.7

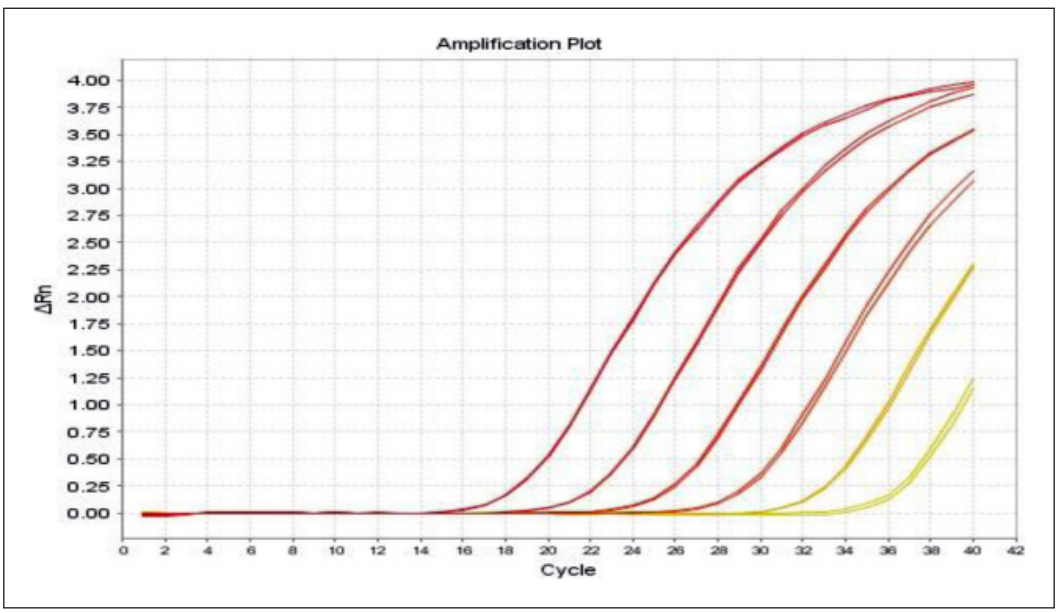

Figure 2. Sensitivity of Real Time PCR using TaqMan probe.

variable prevalence of MAP has been reported to be 5-55 per cent from clinically (Giese & Ahrens 2000) and 2-12 per cent from sub-clinically infected cows (Streeter *et al.*, 1995). According to Singh *et al.*, 2008, prevalence of Johne's disease was 29.0% (28.6% in buffalo and 29.8% in cattle) in Northern India. Animal level prevalence of MAP infection among the European countries were summarized by Nielsen and Toft (2009) and was approximately 20% .

Real-time PCR assays have reached high sensitivity values ranging from 1 to about 50 copies, depending on the number of gene copies targeted by the PCR (Imirzalioglu *et al.*, 2011). In this study, real-time PCR using TaqMan chemistry was employed for the detection of *M. avium* subsp. *paratuberculosis* in bovine fecal samples, though literature is replete with the use of TaqMan assay for the detection of *M. avium* subsp. *paratuberculosis* cultures. The TaqMan assay detects as low as 0.05 pg of MAP specific DNA per assay. All ready-to-use TaqMan reagents enabled the reliable detection of the investigated *M. avium* subsp. *paratuberculosis* strains. No signal for cleavage of probe was detected from controls containing only TaqMan reagents, primers, and probe (*i.e* NTC-No template control). A similar detection level of template DNA was observed in a TaqMan assay using primers

that amplify an 84 bp fragment of IS*900* (Khare *et al.*, 2004). Kim *et al.*, 2002 identified one organism in pure culture by IS*900* TaqMan, and identication of *M. avium subsp. paratuberculosis* with molecular beacons had a sensitivity of 93 to 96 percent. For detection of MAP with high sensitivity, the multi copy genes IS*900* and Mav2 were chosen as targets for quantitative PCR according to Selim & Gaede (2012). Both PCRs showed highly significant positive associations between growth intensity and C_T values, as described by Aly *et al.*, 2010. All the samples were run in duplicate as it increases the sensitivity as also observed by Herthnek & Bolske (2006). In another study, Lazaro *et al.* (2005) developed a real-time PCR assay for quantitative detection of *Mycobacterium avium paratuberculosis*. It targets and amplifies sequences from the IS*900* insertion element which is specific for this bacterium and includes an internal amplification control. The assay was tested against 18 isolates of *M. avium paratuberculosis*, 17 other mycobacterial strains and 25 non-mycobacterial strains, and was fully selective. No false-positive reactions with this or any other sample were obtained with the real-time PCR. Investigation of bovine feacal samples by culture is the gold standard for the estimation of the prevalence of *M. avium* subsp. *paratuberculosis* on the herd level (Roussel *et al.*, 2007). For PCR applications, the extraction of DNA from small amounts of different bacteria from fecal samples is hampered due to different PCR inhibitors (Wilson, 1997). In addition, *M. avium* subsp. *paratuberculosis* is known to form clumps and to be highly resistant to chemical and enzymatic lysis (Bull *et al.*, 2003). The real-time PCR assay was combined with a modified DNA extraction procedure to achieve maximum sensitivity for the detection of *M. avium* subsp. *paratuberculosis* from bovine feacal samples (Schonenbrucher *et al.*, 2008). Alinovi *et al.* (2009) reported that real-time PCR provides results rapidly. Faecal and serum samples collected from dairy cows in northern India were used to estimate the sensitivity and specificity of real-time PCR test for direct feacal detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Results of the real-time PCR were evaluated in parallel with solid and liquid media culture systems and a serum ELISA for detection of MAP antibodies to determine the accuracy of the real-time PCR and the tests' potential usefulness in the field. The accuracy of real-time PCR was comparable to both solid and liquid culture. Because real-time PCR accuracy is comparable to standard culture methods, it is a useful new test. In addition, test results are obtained as rapidly as an ELISA, but are more accurate than the ELISA Kralik *et al.* (2011) compared IS*900* real-time PCR to MAP culture using a cohort of 1906 faecal samples examined from 12 dairy cattle farms. From those samples, 875 were positive by IS*900* real-timePCR and 169 by culture. None of the culture positive samples was negative by IS*900* qPCR although 706 faecal samples which were negative by culture were detected positive by real-time PCR. Hence it was observed that the efficiency of real-time PCR is better than culture technique in detecting MAP in faecal samples. Similar results were also reported by Kawaji *et al.* (2007) in a study in which MAP DNA was detected from some culture negative faecal samples suggesting that the real-time PCR has very high analytical sensitivity for MAP in faecal samples in comparison to other conventional techniques. As culturing of faeces is still the gold standard in the diagnosis of paratuberculosis, Stuber *et al.* (2005) related real-time PCR results to culture data which indicated that in total more samples were scored positive by real-time PCR than by culture (31 *vs* 20 respectively) indicating that real-time PCR to be more sensitive than culture method.

CONCLUSION

The extracted DNA was subjected to qRT-PCR TaqMan assay targeting the IS*900* for the detection of MAP in faecal samples and also for the quantification of the amount of DNA present in the samples. A total of seven

faecal samples (out of 200) were positive by real-time PCR. Sensitivity of the real-time PCR was done by serial dilutions of the standard DNA. Real-time PCR TaqMan assay could detect MAP DNA as low as 0.05 pg. Concentration of the MAP-DNA in faecal samples were determined by comparing the C_T values of the dilutions of standard DNA.

Acknowledgements. The authors are thankful to the Director of Research, GADVASU, Ludhiana for the funding under RKVY Scheme on "Evaluation of mainly molecular diagnostic tests for the development of suitable strategy in diagnosis of Mycobacterial infections (Bovine Tuberculosis and Johne's Diseases) in cattle and buffaloes agro climatic zones of Punjab.

REFERENCES

Alinovi, C.A., Ward, M.P., Lin, T.L., Moore, G.E. & Wu, C.C. (2009). Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. *Veterinary Microbiology* **136**: 177-179.

Aly, S.S., Anderson, R.J., Adaska, J.M., Jiang, J. & Gardner. (2010). Association between *Mycobacterium paratuberculosis* and development of multiplex PCR typing. *Microbiology* **146**: 2185-2197.

Bull, T.J., McMinn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R. & Hermon-Taylor, J. (2003). Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *Journal of Clinical Microbiology* **41**: 2915-2923.

Christopher-Hennings, J., Dammen, M.A., Weeks, S.R., Epperson, W.B., Singh, S.N., Steinlicht, G.L., Fang, Y., Skaare, J.L., Larsen, J.L., Payeur, J.B. & Nelson, E.A. (2003). Comparison of two DNA extractions and nested PCR, real-time PCR, a new commercial PCR assay, and bacterial culture for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. *Journal of Veterinary Diagnostics and Investigations* **15(2)**: 87-93.

Chui, L.W., King, R., Lu, P., Manninen, K. & Sim, J. (2004). Evaluation of four DNA extraction methods for the detection of *Mycobacterium avium* subsp. *Paratuberculosis* by polymerase chain reaction. *Diagnostic Microbiology and Infectious Diseases* **48(1)**: 39-45.

Coetsier, C., Vannuffe, 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 tissues from cattle. *Journal of Clinical Microbiology* **38**: 3048-3054.

Fang, Y., Wu, W.H., Pepper, J.L., Larsen, J.L., Marras, S.A., Nelson, E.A., Epperson, W.B. & Christopher-Hennings, J. (2002). Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine faecal samples. *Journal of Clinical Microbiology* **40(1)**: 287- 291.

Giese, S. & Ahrens, P. (2000). Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. *Veterinary Microbiology* **77**: 291-97.

Harris, N.B. & Barletta, R.G. (2001). *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. *Clinical Microbiology Reviews* **14**: 489-512.

Herthnek, D. & Bolske, G. (2006). New PCR systems to confirm real-time PCR detection of *Mycobacterium avium* subsp. *paratuberculosis*. *BMC Microbiology* **6**: 87.

Imirzalioglu, C., Dahmen, H., Hain, T., Billion, A., Kuenne, C., Chakraborty, T. & Domann, E. (2011). Highly specific and quick detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces and gut tissue of cattle and humans by multiple real-time PCR assays. *Journal of Clinical Microbiology* **49**: 1843-52.

Kawaji, S., Taylor, D.L., Mori, Y. & Whittington, R.J. (2007). Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine faeces by direct quantitative PCR has similar or greater sensitivity compared to radiometric culture. *Veterinary Microbiology* **125**: 36-38.

Khare, S., Ficht, T.A., Santos, R.L., Romano, J., Ficht, A.R., Zhang, S., Grant, I.R., Libal, M., Hunter, D. & Adams, L.G. (2004). Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and faeces by a combination of immunomagnetic bead separation conventional PCR and real-time PCR. *Journal of Clinical Microbiology* **42**: 1075-81.

Kim, S.G., Shin, S.J., Jacobson, R.H., Miller, L.J., Harpending, P.R., Stehman, S.M., Rossiter, C.A. & Lein, D.A. (2002). Development and application of quantitative polymerase chain reaction assay based on the ABI 7700 system (TaqMan) for detection and quantification of *Mycobacterium avium subsp. paratuberculosis*. *Journal of Veterinary Diagnostics and Investigations* **14**: 126-131.

Kralik, P., Slana, I., Kralova, A., Babak, V., Whitlock, R.H. & Pavlik, I. (2011). Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. *Veterinary Microbiology* **149**: 133-138.

Lazaro, D.R., D'Agostino, M., Herrewegh, A., Pla, M., Cook, N. & Ikonomopoulos, J. (2005). Real-time PCR-based methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. *International Journal of Food Microbiology* **101**: 93-104.

Millar, D., Ford, J., Sanderson, J., Withey, S., Tizard, M., Doran, T. & Hermon-Taylor, J. (1996). IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurised cows' milk in England and Wales. *Applied Environmental Microbiology* **62**: 3446-3452.

Motiwala, A.S., Li, L.L., Kapur, V. & Sreevatsan, S. (2006). Current understanding of the genetic diversity of *Mycobacterium avium* subsp *paratuberculosis*. *Microbes and Infection* **8**: 1406-1418.

Nielsen, S.S. & Toft, N. (2009). A review of prevalences of paratuberculosis in farmed animals in Europe. *Preventive Veterinary Medicine* **88**: 1-14.

O' Mahony, J. & Hill, C. (2004). Rapid real-time PCR assay for detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* in artificially contaminated milk. *Applied Environmental Microbiology* **70**: 4561-4568.

Pillai, S.R. & Jayarao, B.M. (2002). Application of IS*900* PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* directly from raw milk. *Journal of Dairy Science* **85**: 1052-1057.

Roussel, A.J., Fosgate, G.T., Manning, E.J. & Collins, M.T. **(**2007). Association of faecal shedding of mycobacteria with high ELISA-determined seroprevalence for paratuberculosis in beef herds. *Journal of American Veterinary Medical Association* **230**: 890-895.

Schonenbrucher, H., Abdulmawjood, A., Failing, K. & Bulte, M. (2008). New Triplex Real-Time PCR Assay for Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Bovine Faeces. *Applied And Environmental Microbiology* **74(9)**: 2751-2758.

Selim, A. & Gaede, W. (2012). Optimization of DNA extraction for identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. *Journal of Veterinary Research* **16**: 163-71.

Singh, S.V., Singh, A.V., Singh, R., Sharma, S., Shukla, N., Mishra, S., Singh, P.K., Sohal, J.S., Kumar, H., Patil, P.K., Misra, P. & Sandhu, K.S. (2008). Sero-prevalence of bovine Johne's disease in buffaloes and cattle population of north India using indigenous ELISA kit based on native *Mycobacterium avium* subsp. *paratuberculosis* 'Bison type' genotype of goat origin. *Comparative Immunology, Microbiology and Infectious Diseases* **31**: 419-33.

Stabel, J.R., Bosworth, T.L., Kirkbride, T.A., Forde, R.L. & Whitlock, R.H. (2004). A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from faecal samples for polymerase chain reaction. *Journal of Veterinary Diagnostics and Investigations* **16 (1)**: 22-30.

Streeter, R.N., Hoffsis, G.F., Bech-Nielsen, S., Shulaw, W.P. & Rings, D.M. (1995). Isolation of *Mycobacterium paratuberculosis* from colostrums and milk of subclinically infected cows. *American Journal of Veterinary Research* **56**: 1322-24.

Stuber, K.B., Kohler, C., Seitert, G., Glanemann, B., Antognoli, M.C., Salman, M.D., Wittenbrink, M.M., Wittwer, M., Wassenaar, T., Jemmi, T. & Choisat, B.B. (2005). Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss dairy cattle by real-time PCR and culture: a comparison of the two assays. *Journal of Applied Microbiology* **99**: 587-597.

Vary, P.H., Anderson, P.R., Green, E., Taylor, J.H. & McFadden, J.J. (1990). Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *Journal of Clinical Microbiology* **28(5)**: 933-937.

Wilson, I.G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied Environmental Microbiology* **63**: 3741-3751.