Recombinant 20.8-kDa protein of *Mycobacterium avium* subsp. *paratuberculosis*-based sero-diagnosis of paratuberculosis

P. P. Goswami¹ · Gokul Chand¹ · N. S. Prasad¹ · R. Deb¹ · S. H. Basagoudanavar¹

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Abstract Johne's disease or paratuberculosis is a chronic infectious enteric disease of ruminants caused by the intracellular pathogen. The control of the Johne's disease is hampered by lack of specific diagnostic tests. In this study, we have cloned and expressed the N-terminal region of the locus tag Map 1637c encoding 20.8-kDa (r20.8) protein of *Mycobacterium avium* subsp. *paratuberculosis*. The recombinant protein r20.8 was expressed in high levels in *Escherichia coli*. The protein r20.8 was purified by single-step chromatography using Ni-NTA agarose. The protein r20.8 was reacted with anti-r20.8 antibodies as well as cattle sera infected with Map on Western blot. ELISA using well-characterized sera (both positive and negative; $n = 60$ each) Map-infected and non-infected cattle, respectively, yielded a sensitivity of 73.3% and a specificity of 98.3%. The 20.8 kDa protein expressed in the present study will prove useful as reagent in diagnostic test.

Keywords 20.8 kDa · *Mycobacterium avium* subsp. *paratuberculosis*

Introduction

Mycobacterium avium subspecies *paratuberculosis* (Map) is an intracellular pathogen causing paratuberculosis (Johne's disease), a chronic enteric disease of domestic and wild ruminants including primates (Chiodini et al. 1984). Johne's disease is widespread throughout the world and

causes substantial economic losses to the farmers and the dairy industry (Hasonova and Pavlik 2006). Prevalence of bovine paratuberculosis in European countries was reported as 3.5–71.0% (Boelaert et al. 2000). Singh et al. (2005) reported sero-prevalence of 29.05 and 23.63% paratuberculosis in bovines and goats, respectively, in northern India.

To date, effective therapeutics or vaccine candidates are not available that can completely eliminate viable Map from the host. Early detection along with good management practices is the only way to control paratuberculosis (Harris and Barletta 2001).

Control of Map at the herd level requires identification of infected animals, at early subclinical level, and their removal from the herd (Van Roermund et al. 2007). A number of methodologies have been employed, including detection of Map by PCR in faeces and milk, bacterial culture of faecal samples, measurement of delayed-type hypersensitivity response, gamma interferon (IFN- γ) assay and serodiagnosis by ELISA. However, variables such as antigen specificity and sensitivity, reproducibility, PCR inhibitors in faeces, and cross-reactivity among the antigens limit the efficacy of these methods (Wadhwa et al. 2012).

Previously, serological tests based on commercial ELISA kits for paratuberculosis infection and various studies have been compared for the accuracy (Fry et al. 2008; van Schaik et al. 2007). ELISA studies with different antigens have shown discrepancies in the ability of these tests to identify all infected animals (Costanzo et al. 2012).

It is universally accepted that major improvements are needed in currently available serodiagnostic tests for efficient control programs (Collins et al. 2005; Sweeney et al. 2006). Various laboratories have, therefore, chosen to improve the efficiency of serological assays by replacing

✉ P. P. Goswami
ppgnbc@yahoo.co.in

¹ Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar 243122, India

the “crude antigens” by “Map-specific antigens” (Dupont et al. 2005; Willemsen et al. 2006). Bannantine et al. (2008) generated a partial protein array representing the Map coding sequences and showed that some Map-specific proteins were strongly detected in clinical sera early post infection in experimental model. To date, a number of Map-specific immune targets have been identified and the antigenicity of some of these proteins has been evaluated (Mon et al. 2012; Facciuolo et al. 2013).

The protein tag Map 1637c encodes for immunodominant antigen of 53.2 kDa and contain epitopes specific for Map. The 40.8-kDa antigen from the tag Map 1637c was used for the detection of Map antibodies in sera from 18 Map-infected cattle with the sensitivity and specificity of 82 and 98%, respectively (Leroy et al. 2009). In the present study, we describe a cloning strategy for the N-terminus region of Map 1637c encoding 20.8-kDa protein, its purification and a preliminary evaluation using ELISA for its use as a diagnostic reagent in the detection of bovine paratuberculosis.

Materials and methods

Bacterial strains and plasmid

Mycobacterium avium subsp. *paratuberculosis* (Map) strain 316F was obtained from Central Diengenees Kundig Tieh Institute, Lelystad, The Netherlands, and maintained at Gene Expression Laboratory, Veterinary Biotechnology Division, IVRI, Izatnagar, India. The organism was grown and maintained at 37 °C on Middlebrook 7H10 agar (Difco laboratories, Detroit, USA) enriched with 0.1% glycerol (v/v) and 10% oleic acid–dextrose–catalase (Difco laboratories and supplemented with of 20 mg/L mycobactin J (Allied Monitor, Fayette, USA). The *Escherichia coli* strain (M15, pREP4) purchased from Qiagen (Valencia, USA) was maintained on Luria–Bertani agar containing kanamycin (25 µg/mL), as the strain carries kanamycin-resistant plasmid. The plasmid pQE30 containing an ampicillin resistance marker was used as expression vector supplied by Qiagen.

DNA isolation, polymerase chain reaction and gene cloning

The genomic DNA from *M. a. paratuberculosis* was isolated from the grown culture by the method of Del Portillo et al. (1991). Plasmid DNA extraction from *E. coli* was carried out by the alkaline lysis method (Sambrook and Russel 2001). To amplify a gene fragment encoding 20.8-kDa protein of Map, a set of oligonucleotide primers 5'-TACGGATCC ATGCAACGGCAACGCGGC-3' and 5'-

GTACTGCAGTCACCGATCATTTTGGGC-3' containing *Bam*HI/*Pst*I restriction endonuclease sites, respectively, were designed on the basis of sequence information of Map strain k10, complete genome GenBank Accession No. AE016958 tag Map 1637c. The primers were used to amplify about 546-bp PCR product encoding nearly 182 amino acids of the N+ terminus of the whole gene encoding 53.2 kDa protein. For PCR amplification, 1 µL (100 ng) DNA of Map was added to 5 µL of Taq DNA polymerase buffer 10 mM/L Tris. HCl (pH 9.0), 50 mM/L KCl, 1.5 mM/L MgCl₂ and 0.01% (w/v) gelatin; 200 µM of each dNTP; 0.5 µM each primers; 2 units of Taq DNA polymerase (New England Biolabs Inc.). The final volume was made up to 50 µL with sterile distilled water. After the initial denaturation step (5 min at (94 °C), 30 cycles of amplification were performed as follows: denaturation at 94 °C for 1 min, annealing for 1 min at 58 °C, and extension at 72 °C for 1 min followed by final extension at 72 °C for 3 min. The reaction was carried out in a PTC-100 reactor (MJ Research Inc., Waltham, USA). The PCR product was resolved in 1% agarose gel and stained with ethidium bromide. The purified PCR product was cut with *Bam*HI/*Pst*I and inserted in frame into the plasmid pQE30 digested with the same enzymes. The resulting plasmid pQ20.8 was transformed and recombinant clones were selected on LB agar containing ampicillin (75 µg/mL) and kanamycin (25 µg/mL) and subjected to direct colony PCR screening by insert-specific primers mentioned above.

Expression and purification of the recombinant protein and raising of antisera

Escherichia coli M 15 cells harbouring the plasmid pQ20.8 were grown in LB medium containing ampicillin (75 µg/mL) and kanamycin (25 µg/mL), and induced with 1.0 mM IPTG for 4–6 h. The purification of recombinant 20.8-kDa His fusion protein under denaturing conditions was carried out by one-step Ni–NTA (nickel–nitrilotriacetate) agarose (Qiagen) affinity chromatography and renatured as described previously (Basagoudanavar et al. 2004). The protein was analysed by SDS-PAGE. The protein solution was sterilized by filtration, and aliquots were stored at –70 °C, until used. Concentration of the protein was determined spectrophotometrically (Lowry et al. 1951). The hyperimmune sera against the recombinant 20.8-kDa protein were raised in rabbits as described previously (Basagoudanavar et al. 2004). All the sera used in this study were preabsorbed with *E. coli* antigens.

Characterization of recombinant protein

The purified recombinant 20.8-kDa protein was separated on 12% (w/v) gel along with appropriate coloured protein

marker range from 7 to 175 kDa (New England Biolab, USA). The resolved proteins from SDS-PAGE were transferred to nitrocellulose membranes (0.45 μM) using semi-dry electroblotting (Atto, Tokyo, Japan) at 0.8 mA/cm². The blots were blocked with 5% skimmed milk powder in PBS-T buffer (PBS containing 0.1% Tween-20) for 2 h at room temperature. After washing thrice with PBS-T buffer, the membranes were incubated at 37 °C for 2 h with antisera against 20.8-kDa protein (1:10,000 in PBS) raised in rabbit. Following further washing, the blots were incubated with a 1:10,000 dilution of HRP-labelled goat anti-rabbit IgG (Sigma) for 1 h. The blot was washed and dipped in substrate solution (0.02% diaminobenzidine suspended in PBS containing 0.03% hydrogen peroxide in PBS pH 7.4), till brown colour appeared.

Serum samples

A total of 120 samples including 60 bovine paratuberculosis-positive serum and 60 negative samples were measured with the use of ELISA based on the r20.8 protein to determine the sensitivity of the assay. Healthy bovine serum samples ($n = 60$) were used to determine the specificity of the assay. Serum samples were collected from a total of 140 (cows/calves) from Gausadan, Ghumanhera village, New Delhi. The animals were tested by PPD as well as ELISA using commercial kit.

ELISA for the detection of anti-Map antibodies

Checkerboard titration was used to determine the optimum concentration of protein (0.1, 0.2, 0.3, 0.4 or 0.5 $\mu\text{g}/\text{well}$) and serum dilution for use in an indirect ELISA. The purified recombinant 20.8-kDa protein was diluted to 4 $\mu\text{g}/\text{ml}$ in 0.1 M Na₂CO₃/NaHCO₃ buffer, pH 9.6, and used for coating 96-well ELISA plates (Greiner, Germany) 50 $\mu\text{l}/\text{well}$ and incubated at 4 °C overnight. The coated wells were washed thrice in PBS-T (0.05% Tween 20 in PBS, pH 7.2) and blocked with 5% skim milk powder in PBS for 2 h at 37 °C to remove excess unabsorbed antigen. The wells were washed again with PBS-T and once with PBS and incubated for 1 h at 37 °C with 50 μl of bovine sera samples at 1:100 dilution in PBS-T. Plates were washed with PBS-T thrice followed by incubation with 100 μl HRP-conjugated goat anti-bovine immunoglobulins (1:10,000 dilution in 5% skimmed milk) for 1 h at 37 °C. After a final wash with PBS-T, colour was developed using chromogenic substrate 100 μl solution/well containing 1 mg OPD (*o*-phenylenediamine) and 10 μl of 30% H₂O₂ in sodium phosphate/citric acid buffer, pH 5.0. The plate was incubated in dark at room temperature for 10–15 min in dark. The reaction was stopped using 2.5 M sulfuric acid and the plate was read at A492 using an ELISA reader

(Tecan, Austria). The cut-off value for the assay was calculated as the mean specific absorbance plus 3 SD for 60 sera from healthy cattle assayed at a dilution of 1:100 and it was arbitrarily fixed at an A492 of 0.35.

Statistical analysis

The Kappa statistic was calculated using the standard formula (Cohen 1960). The kappa value was interpreted as described previously (Viera and Garrett 2005). Sensitivity (true positive rate) was calculated by the formula, $\text{sensitivity} = \text{true positive} / (\text{true positive} + \text{false negative})$. The specificity (true negative rate) was calculated by the formula, $\text{specificity} = \text{true negative} / (\text{false positive} + \text{true negative})$. Confidence intervals for sensitivity and specificity were calculated using free online tool at the website: https://www.medcalc.org/calc/diagnostic_test.php.

Results and discussion

Mycobacterium avium subsp. *paratuberculosis* (Map) is the etiologic agent of bovine paratuberculosis, also called Johne's disease. However, despite several years of development, currently available diagnostic tests still lack performance to be used in control programs. The early and specific diagnosis of Map is still a challenge. Experiments have revealed that antibodies appear much earlier, and therefore, the ELISA could be used as a diagnostic tool (Waters et al. 2003; Begg et al. 2011). Hence, it is necessary to characterize Map antigens to increase the sensitivity and specificity of the ELISA test for paratuberculosis diagnosis. To resolve this issue, we developed an antibody-based method using r20.8 antigen for diagnosis of paratuberculosis in cattle.

In the present study, we have used an efficient expression system, based on pQE30 vector to produce 20.8-kDa protein of *M. a. paratuberculosis* in *E. coli* so as to facilitate further characterization of the protein. Based on the sequence information of the locus tag 1637c N-terminus region of 546 bp encoding the 20.8-kDa protein of *M. a. paratuberculosis* and also the information on the multiple cloning site of the plasmid vector pQE30, restriction sites for *Bam*HI and *Pst*II were incorporated into the specific primers, to facilitate the directional in-frame cloning into the pQE30 vector under the control of T5 promoter. The recombinant pQ20.8 clones were confirmed by colony PCR using specific primers. Earlier, a similar strategy of cloning in pQE expression vector system was used to produce 35-kDa protein as well as PPE protein of Map (Basagoudanavar et al. 2004; Deb and Goswami 2010). Production of r20.8 protein was induced by IPTG which binds to the *lac* repressor protein, inactivating it leading to transcription

Fig. 1 Analysis of the recombinant 20.8-kDa Map protein by SDS-PAGE (a) and Western blot (b). Lane M pre-stained protein weight molecular marker; lane 1 whole-cell extract of *E. coli* M15; lane 2 whole-cell extract of *E. coli* M15 harbouring construct pQ20.8 (IPTG

uninduced); lane 3 whole-cell extract of *E. coli* M15 harbouring construct pQ20.8 (IPTG induced); lane 4 purified His-tagged r20.8 kDa protein

of sequences downstream of the promoter. High-level expression of the recombinant protein was observed in plasmid pQ20.8, 6 h post induction with 1 mM IPTG as resolved by predominant bands 20.8 kDa corresponding to that predicted for r20.8 protein on SDS-PAGE. No such protein band was observed with *E. coli* M15 cells or in uninduced *E. coli* M15 cells harbouring recombinant plasmid pQ20.8. Using pQE vector, Basagoudanavar et al. (2004) achieved maximum expression in 6 h post induction of IPTG. Therefore, it was decided to purify the proteins 6 h post induction from *E. coli* culture. The presence of 6xHis residues at N-terminal of the recombinant protein enabled us to purify the protein by single-step affinity purification using Ni-NTA resin. The 6xHis tag with 840 Da size is poorly immunogenic and may not interfere with structure and function of recombinant protein (Sisk et al. 1994). The yield of the pure recombinant protein was about 15–20 mg/l of culture at shake-flask level (Fig. 1a). High-level expression of the r20.8 protein indicates that the expression system is highly efficient.

Purified recombinant 20.8-kDa protein as well as lysate from IPTG-induced *E. coli* harbouring pQ20.8 plasmid, were probed with the rabbit polyclonal serum raised against the purified r20.8 kDa protein. It reacted strongly with the expressed protein on Western blot (Fig. 1b). No such band was found in *E. coli* cell M15 cells as well as in uninduced *E. coli* cell lysate harbouring plasmid pQ20.8 indicating the immunogenic nature of the r20.8 protein, which was not affected by expression in heterologous system. The immunoblot assays clearly identified the expression of the recombinant protein.

We have examined the ability of recombinant 20.8-kDa protein to detect Map infections in cattle using an ELISA. A total of 120 bovine sera samples (60 positive for Map

and 60 negative for Map) were present in the laboratory earlier been tested by fecal examination, PCR, PPD and ELISA using commercial kit. The result demonstrated that the sensitivity of ELISA was 73.33% (95% CI 60.34–83.93%) and specificity was 98.33% (95% CI 91.06–99.96%), relative to infection status as indicated by the commercial kit test (Table 1). The ELISA did not have a reaction with sera from non-infected control.

Screening of serum 140 serum samples (cows/calves) from Gausadan with PPD and r20.8 protein revealed 11 and 18 positive reactors, respectively (Table 2). The sensitivity was 100% (95% CI 71.51–100%), while the specificity was 94.57% (95% CI 89.14–97.79%). ELISA-based serological tests are easy to perform, cheap to produce and do not require dedicated facilities. In these current commercial ELISAs, antigens used for plate coating are different, and crude bacterial extracts require *M. phlei* pre-absorption of sera to remove cross-reactive antibodies. This pre-absorption step is not only time-consuming but also affects test sensitivity (McKenna et al. 2005). This is the main reason why we have chosen to focus our effort on the development of a sero-diagnostic test based on one or a handful of purified antigens rather than on a Map extract.

Table 1 Specificity and sensitivity of recombinant Map antigen ELISA

	Commercial ELISA test		Total
	Positive	Negative	
Map r20.8 antigen ELISA			
Positive	44	1	45
Negative	16	59	75
Total	60	60	120

Kappa agreement = 0.72

Table 2 Comparison of recombinant Map antigen ELISA using individual serum samples and PPD skin test

	PPD test		Total
	Positive	Negative	
Map r20.8 antigen ELISA			
Positive	11	7	18
Negative	0	122	122
Total	11	129	140

Kappa agreement = 0.78

In the present study, we have selected an N-terminus region of 546 bp from the locus tag MAP 1637 c because of having the most probable B-cell epitopes. A similar approach of using protein from locus tag MAP 1637 c has been used by Leroy et al. (2009) using 40.8-kDa protein and less number of serum samples. Obviously, the use of a 'golden' antigen should be able to discriminate all infected animals regardless of their clinical stage and with a very small false-positive rate. But it is now clear that such an antigen probably does not exist and that the most efficient diagnostic test would be based on a combination of purified antigens. Moreover, Bannantine et al. (2008) have demonstrated, using an experimental infection model, that diagnostic efficiency of an antigen could vary and decrease over the course of the infection. Thus, antigens effective in detection of clinical cases could be inefficient for early diagnosis. The ELISA test based on Map r20.8 protein shows substantial kappa value agreement with commercial ELISA kit ($k = 0.72$) as well as when compared with PPD assay ($k = 0.78$) (Viera and Garrett 2005). Therefore, the r20.8 protein is a good candidate to formulate an antigen combination useful to develop a highly- efficient ELISA-based serological test.

Conclusion

The use of gene expression techniques in the production of recombinant protein also completely eliminated the preparation and use of protein mixtures. The design of the r20.8 and its ease of expression and purification have the potential to make this a highly effective approach in the development of reagents for the diagnosis of bovine paratuberculosis. Comparing with the PPD skin test as the standard method, this ELISA method showed high specificity and sensitivity and can be used for routine diagnosis of paratuberculosis in cattle.

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Compliance with ethical standards

Conflict of interest Authors declared that there was no conflict of interests for the present study.

References

- Bannantine JP, Paustian ML, Waters WR, Stabel JR, Palmer MV, Li L, Kapur V (2008) Prolong bovine antibody responses to *Mycobacterium avium* subsp. *paratuberculosis* infection by using protein arrays. *Infect Immun* 76:739–749
- Basagoudanavar SH, Goswami PP, Tiwari V, Pandey AK, Singh N (2004) Heterologous expression of a gene encoding a 35 kDa protein of *Mycobacterium avium paratuberculosis* in *Escherichia coli*. *Vet Res Commun* 28:209–224
- Begg DJ, De Silva K, Carter N, Plain KM, Purdie A, Whittington RJ (2011) Does a Th1 over Th2 dominancy really exist in the early stages of *Mycobacterium avium* subspecies *paratuberculosis* infections? *Immunobiology* 216(7):840–846
- Boelaert F, Walravens K, Biront P, Vermeersch JP, Berkvens D, Godfroid J (2000) Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population. *Vet Microbiol* 77(3):269–281
- Chiodini RJ, Van Kruiningen HJ, Merkal RS, Thayer WR, Coutu JA (1984) Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. *J Clin Microbiol* 20(5):966–971
- Cohen J (1960) A coefficient of agreement for nominal scales. *Educ Psychol Meas* 20:37–46
- Collins MT, Wells SJ, Petrini KR, Collins JE, Schultz RD, Whitlock RH (2005) Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. *Clin Diagn Lab Immunol* 12(6):685–692
- Costanzo G, Pinedo FA, Mon ML, Viale M, Gil A, Illia MC, Gioffré A, Arese A, Travería G, Romano MI (2012) Accuracy assessment and screening of a dairy herd with paratuberculosis by three different ELISAs. *Vet Microbiol* 156(1):183–188
- Deb R, Goswami PP (2010) Expression of a gene encoding 34.9 kDa PPE antigen of *Mycobacterium avium* subsp. *paratuberculosis* in *E. coli*. *Mol Biol Int* 2010:628153
- Del Portillo P, Murillo LA, Patarroyo ME (1991) Amplification of a species-specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. *J Clin Microbiol* 29(10):2163–2168
- Dupont C, Thompson K, Heuer C, Gicquel B, Murray A (2005) Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies *paratuberculosis*. *J Med Microbiol* 54(11):1083–1092
- Facciolo A, Kelton DF, Mutharia LM (2013) Novel secreted antigens of *Mycobacterium paratuberculosis* as serodiagnostic biomarkers for Johne's disease in cattle. *Clin Vaccine Immunol* 20(12):1783–1791
- Fry MP, Kruze J, Collins MT (2008) Evaluation of four commercial enzyme-linked immunosorbent assays for the diagnosis of bovine paratuberculosis in Chilean dairy herds. *J Vet Diagn Invest* 20(3):329–332
- Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clin Microbiol Rev* 14(3):489–512
- Hasonova L, Pavlik I (2006) Economic impact of paratuberculosis in dairy cattle herds: a review. *Vet Med* 51(5):193–211
- Leroy B, Viart S, Trincherio N, Roupie V, Govaerts M (2009) Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding

- sequences for serodiagnosis of bovine paratuberculosis. *Vet Microbiol* 135:313–319
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1):265–275
- McKenna SLB, Keefe GP, Barkema HW, Sockett DC (2005) Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Vet Microbiol* 110(1):105–111
- Mon ML, Viale M, Baschetti G, Alvarado Pinedo F, Gioffre A, Travería G, Willemsen P, Bakker D, Romano MI (2012) Search for *Mycobacterium avium* subspecies *paratuberculosis* antigens for the diagnosis of paratuberculosis. *Vet Med Int* 2012:860362
- Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York
- Singh SV, Singh AV, Singh R, Sandhu KS, Gupta VK (2005) Survey of ruminant population of Northern India for the incidence of *Mycobacterium avium* subsp. *paratuberculosis* infection. In: Eighth international colloquium on paratuberculosis, Denmark, DFVF
- Sisk WP, Bradley JD, Leipold RJ (1994) High-level expression and purification of secreted forms of herpes simplex virus type 1 glycoprotein gD synthesized by baculovirus-infected insect cells. *J Virol* 68:766–775
- Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. *Res Vet Sci* 80(3):253–259
- Van Roermund HJW, Bakker D, Willemsen PTJ, De Jong MCM (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. *Vet Microbiol* 122(3):270–279
- van Schaik G, Pradenas M, Mella A, Kruze J (2007) Diagnostic validity and costs of pooled fecal samples and individual blood or fecal samples to determine the cow-and herd-status for *Mycobacterium avium* subsp. *paratuberculosis*. *Prev Vet Med* 82(1):159–165
- Viera AJ, Garrett JM (2005) Understanding interobserver agreement: the kappa statistic. *Fam Med* 37(5):360–363
- Wadhwa A, Hickling GJ, Eda S (2012) Opportunities for improved serodiagnosis of human tuberculosis, bovine tuberculosis, and paratuberculosis. *Vet Med Int* 2012:674238
- Waters WR, Miller JM, Palmer MV, Stabel JR, Jones DE, Koistinen KA, Steadham EM, Hamilton MJ, Davis WC, Bannantine JP (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. *Infect Immun* 71(9):5130–5138
- Willemsen PT, Westerveen J, Dinkla A, Bakker D, Van Zijderveld FG, Thole JE (2006) Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. *Vet Microbiol* 114(3):337–344