



Evaluation of single intra-dermal test and commercial ELISA for diagnosis of paratuberculosis in Indian cattle population

B J TRANGADIA¹, B M DEC BRONSVOORT², I G HANDEL³, K NAGMANI⁴, S K RANA⁵,
DEV CHANDRAN⁶ and V A SRINIVASAN⁷

National Dairy Development Board, Indian Immunologicals Limited,
Hyderabad, Andhra Pradesh 444 103 India

Received: 13 August 2013; Accepted: 3 February 2014

ABSTRACT

Between May 2008 and March 2009, serum samples (1,170) were collected from indigenous purebred and crossbred cattle housed in 15 organized farms located across seven states of India. These sera were tested for the presence of antibodies against *Mycobacterium avium paratuberculosis* (*Map*). After blood collection, all these animals were screened for paratuberculosis by single intra-dermal injection of Johnin protein purified derivatives. The Hui-Walter, no gold standard approach under the assumption of conditional independence was used to estimate sensitivity and specificity of these 2 tests and the prevalence of paratuberculosis in these populations. The estimated sensitivities were 18.2 and 26.6% for SIT and ELISA, respectively, whereas, specificities were 98.0 and 92.4% for SIT and ELISA, respectively. The results indicated that both tests were very insensitive but, the SIT was reasonably specific. The sero-prevalence estimates of *Map* varied from 7.0% in Uttar Pradesh to 77.9% in Tamil Nadu. As it is difficult to estimate the full impact of either of these tests for use in control or surveillance of this disease, there is a need to develop improved tests for field use in India.

Key words: Cattle, ELISA, Intra-dermal test, Paratuberculosis

Paratuberculosis or Johne's disease (JD) caused by *Mycobacterium avium paratuberculosis* (*Map*) is a fatal chronic granulomatous enteritis of ruminants characterized by progressive wasting, emaciation and death. Limited reports available on the prevalence of the disease in various states of India, indicated 15.6 to 22.5% sero-positivity of *Map* in cattle and buffaloes, respectively (Garg *et al.* 2007, Tripathi *et al.* 2007, Trangadia *et al.* 2012). In India, diagnostic testing for *Map* is mainly limited to use of the intra-dermal test using purified protein derivatives (PPD), which evaluates the delayed-type hypersensitivity reaction. However, this test has potential poor specificity due to antigenic cross-reactivity with environmental mycobacteria (Collins 1996). Therefore, the present study was carried out to screen an Indian cattle population using both the single intra-dermal test (SIT) and ELISA tests; and the Hui-Walter, no gold standard approach, was used to estimate the sensitivity (Se) and specificity (Sp) of these 2 tests and the prevalence of *Map* in these cattle populations.

Present address: ¹ Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari, Gujarat (drbjt@rediffmail.com). ⁴⁻⁷R&D Centre (ranask_1960@yahoo.co.in, dev@indimmune.com, srini@indimmune.com). ^{2,3}The Roslin Institute at The Royal (Dick) School of Veterinary Studies (mark.bronsvort@ed.ac.uk, ian.handel@ed.ac.uk), University of Edinburgh, Roslin, Midlothian, EH25 9RG, United Kingdom.

MATERIALS AND METHODS

Study population: The study was carried out between May 2008 and March 2009. Indigenous purebred (*Bos indicus*) and crossbred cattle (1,170) were randomly selected from 15 different organized herds located in the states of Andhra Pradesh (4 herds), Maharashtra (3 herds), Gujarat (3 herds), Odisha (2 herds) and 1 herd each in the state of Uttar Pradesh, Tamil Nadu and Haryana. All animals housed in these farms were screened for paratuberculosis by the single intra-dermal test and ELISA. Prior to injection of Johnin PPD, a single sample of blood (approximately 5 ml) was collected from these animals for separation of serum and were stored at –20°C until used for ELISA.

Single intra-dermal test (SIT): Single intra-dermal test with Johnin (procured from Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India) was carried out as per manufacturers' instructions and Bapat and Bangi (1985). The animal was considered as a positive reactor to Johnin PPD if palpable swellings with the presence of diffuse oedema with or without local heat.

ELISA testing: A commercial *Map* ELISA test kit (CattleType MAP Ab) was used to screen these animals for the presence of antibodies to *Map* in serum according to manufacturer's instructions and Jark *et al.* (1997). Briefly, 5 µl of test sera was diluted to 345 µl of buffer containing inactivated *Mycobacterium phlei* extract and incubated for

60 min at room temperature; 100 µl from this mixture and 100 µl each of ready to use positive and negative controls were added to the plates coated with non-infectious *Map* antigen and incubated for another 30 min at room temperature. Plates were washed 3 times with supplied washing buffer. One hundred microlitres of ready-to-use anti-IgG-HRP conjugate was added to plates and incubated for 30 min at RT and the washing steps were repeated. After washing, 100 µl of TMB substrate solution was added to plates and incubated for 10 min at RT in dark. Finally the reaction was stopped by adding 100 µl of stop solution and plates were read at 450 nm filter against air as blank to calculate optical density (OD) of the samples. The S/P ratio was calculated using the following formula.

$$\text{S/P ratio} = \frac{\text{OD}_{\text{sample}} - \text{meanOD}_{\text{negative control}}}{\text{meanOD}_{\text{positive control}} - \text{meanOD}_{\text{negative control}}}$$

Samples with an S/P ratio less than 0.3 were classified as negative and an S/P ratio greater than 0.4 were classified as positive, otherwise classified as doubtful and such samples were repeated for testing by ELISA and classified either as negative or positive.

Statistical analysis: The apparent prevalence is estimated using the observed number of test positives divided by the total sample. The Se is the conditional probability that given an animal is truly positive the test correctly identifies it as positive; and the Sp is the probability that a truly negative animal tests negative on the test. The true prevalence is the apparent prevalence adjusted for the sensitivity and specificity. The Se, Sp and true prevalence were estimated using the Hui-Walter no gold standard latent class model (Hui and Walter 1980). The Hui-Walter paradigm requires 2 (or more) tests evaluated in 2 (or more) populations. This model assumes that: (i) the prevalence of the disease is different within each population; (ii) the tests have the same properties across populations; and (iii) the tests are conditionally independent given the disease status. This Bayesian implementation of the Hui-Walter model assumes that for the i th sub-population the counts (O_i) of the different combinations of test results, +/+, +/-, -/+ and -/- for the 2 tests, follow a multinomial distribution:

$$O_i | \text{Se}_j, \text{Sp}_j, p_i \sim \text{Multinomial}(\text{Pr}_i, n_i) \text{ for } i = 1, 2, \dots, S \text{ and } j = 1, 2, \dots, T$$

where S is the number of subpopulations, T is the number of tests and Pr_i is a vector of probabilities of observing the individual combinations of test results. Conditioning on the (latent) disease status, these probabilities can be specified using the Se_j and Sp_j of the tests and the true prevalence (p_i) in the sub-populations. The probabilities of observing each test result combination in the i th subpopulation are given by:

$$\begin{aligned} \text{Pr}(T_1+, T_2+) &= \text{Se}_1 \text{Se}_2 p_i + (1-\text{Sp}_1)(1-\text{Sp}_2)(1-p_i) \\ \text{Pr}(T_1-, T_2+) &= (1-\text{Se}_1) \text{Se}_2 p_i + (1-\text{Sp}_2) \text{Sp}_1(1-p_i) \\ \text{Pr}(T_1+, T_2-) &= \text{Se}_1 (1-\text{Se}_2) p_i + \text{Sp}_2(1-\text{Sp}_1)(1-p_i) \\ \text{Pr}(T_1-, T_2-) &= (1-\text{Se}_1)(1-\text{Se}_2) p_i + \text{Sp}_1 \text{Sp}_2(1-p_i) \end{aligned}$$

In a Bayesian analysis all parameters are expressed as random variables. The prevalence in each of the 7 Indian States were given flat (Uniform (0,1) priors) as there were no published data to inform these estimates. Prior distributions of Beta (2+1, 35+1) and Beta (62+1, 3+1) for the sensitivity and specificity of the SIT were used respectively based on estimates from De Lisle *et al.* (1980). Prior distributions of Beta (10+1, 26+1) and Beta (751+1, 83+1) for the sensitivity and specificity of the ELISA were used respectively based on estimates from McKenna *et al.* (2005).

The model was estimated using the JAGS (Just Another Gibbs Sampler) software (Plummer 2003) using the Runjags package (Denwood 2009) of the R statistical system. Convergence of the chains after the initial burn-in was assessed by visual inspection of the time-series plots for the parameter samples as well as Gelman-Rubin diagnostic plots using 3 sample chains with dispersed starting values (Brooks and Gelman 1998). The R package CODA (Plummer *et al.* 2009) was used for analysis and graphing of the MCMC output. In a classical analysis the single point estimates of the diagnostic test's sensitivity and specificity are used in these estimators.

RESULTS AND DISCUSSION

A major component of effective control of paratuberculosis depends on the availability of rapid and accurate diagnostic tests. In the present study, SIT and ELISA tests were used to screen cattle herds for paratuberculosis in India. Prevalence, Se and Sp of SIT and ELISA tests for paratuberculosis in dairy cattle were estimated using Hui-Walter no gold standard approach. Assumption of conditional independence was considered reasonable given the very different types of test where SIT is based on the cellular immune response and the ELISA on the humoral response.

The apparent sero-prevalence (ap) in cattle (1,170) across

Table 1. The 2x2 table of test results and state-wide apparent seroprevalences (ap) for the SIT and ELISA

States			SIT +	SIT -	Total
Andhra Pradesh	ap _{ELISA} =0.070	ELISA +	3	5	8
	ap _{SIT} =0.035	ELISA -	1	106	107
Gujarat	ap _{ELISA} =0.078	ELISA +	6	51	57
	ap _{SIT} =0.029	ELISA -	15	657	672
Haryana	ap _{ELISA} =0.059	ELISA +	0	1	1
	ap _{SIT} =0.000	ELISA -	0	16	16
Maharashtra	ap _{ELISA} =0.014	ELISA +	1	0	1
	ap _{SIT} =0.029	ELISA -	1	68	69
Odisha	ap _{ELISA} =0.057	ELISA +	0	3	3
	ap _{SIT} =0.075	ELISA -	4	46	50
Tamil Nadu	ap _{ELISA} =0.375	ELISA +	6	0	6
	ap _{SIT} =0.375	ELISA -	0	10	10
Uttar Pradesh	ap _{ELISA} =0.065	ELISA +	1	10	11
	ap _{SIT} =0.024	ELISA -	3	156	159
Total			41	1129	1170

Ap, apparent prevalence; ELISA, enzyme linked immunosorbent assay; SIT, single intra-dermal test.

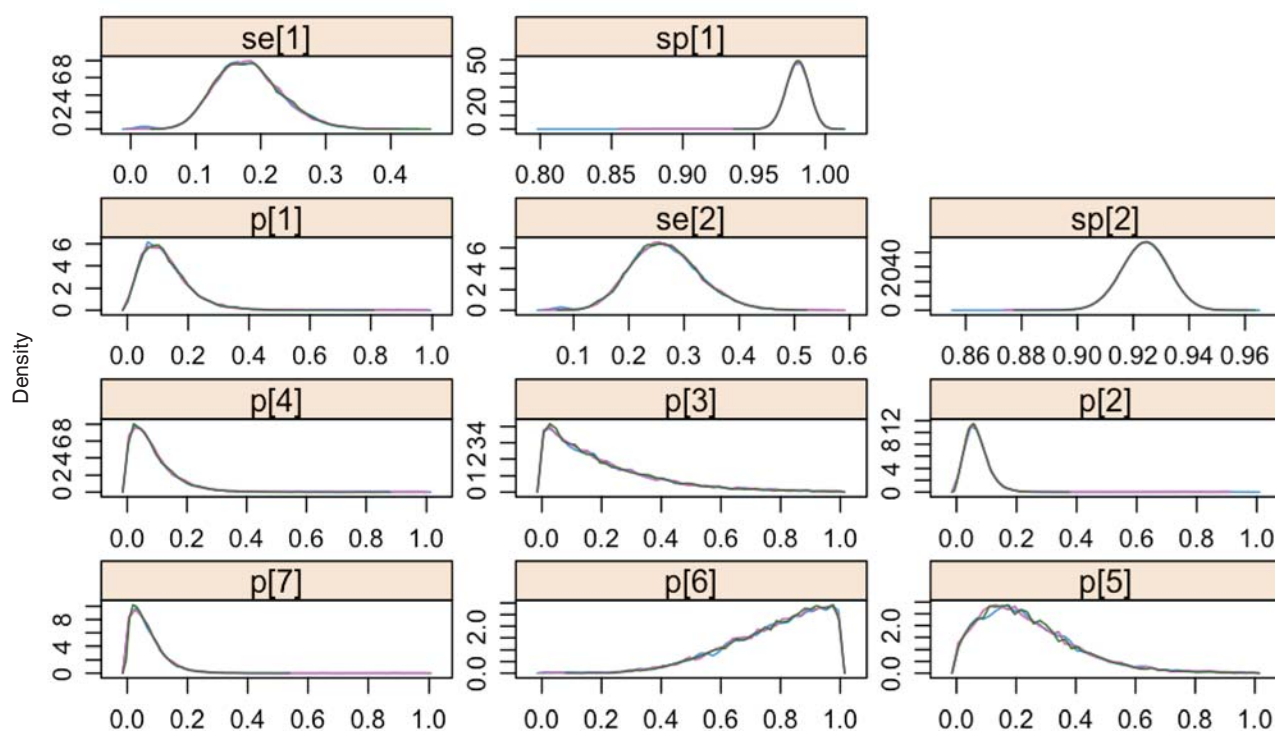


Fig. 1. Posterior distributions of test parameters and seroprevalence of paratuberculosis in 7 Indian states.

7 states in India is given in Table 1; ap of SIT varied from 0% in Haryana to 37.5% in Tamil Nadu, whereas ap of ELISA was 1.4% in Maharashtra to 37.5% in Tamil Nadu. Results indicated that ELISA is classifying more animals as sero-positive than the SIT. Considerable variation in ap between states may be due to the very small sample size and small number of investigated herds in some states (van Schaik *et al.* 2003) and collection of these samples from selected breeding farms also.

The unbiased estimates of the mean and 95% highest density intervals (HDI) of the posterior probabilities (i.e. the interval that captures 95% of the most likely estimates) for the test parameters and the true sero-prevalence (p) from the Hui-Walter no gold standard model are given in Table 2 and their full posterior distributions are visualized in Fig. 1. The seroprevalence estimates of *Map* varied from 7.0% (95% HDI, 0.3–21.5%) in Uttar Pradesh to 77.9% (95% HDI, 39.7–99.1%) in Tamil Nadu. Earlier workers reported an apparent prevalence of *Map* 15.6% by serum ELISA in Punjab (Garg *et al.* 2007), 22.5% from different parts of the India using a commercial ELISA (Tripathi *et al.* 2007). Pozzato *et al.* (2011) recorded a prevalence of 70% in Lombardy and 71% in Veneto, Italy. However, Gasteiner *et al.* (1999) reported a low prevalence of antibody to *Map* between 1.99 and 3.51% in Austria. Estimates of the within-herd true prevalence distributions differed between the regions possibly due to the different age distribution within the herds from the different regions (Pozzato *et al.* 2011). However the sampling was not random and only small number were sampled from some states reflected in the wide credibility intervals.

In the present study, the ELISA was estimated to be more

sensitive and less specific than the SIT. The estimated sensitivity of the SIT was 18.2% (95% HDI, 9.2–29.2%) compared to the ELISA which was 26.6% (95% HDI, 15.3–39.9%). The specificities of the SIT and ELISA were estimated to be 98.0% (95% HDI, 96.7–99.2%) and 92.4% (95% HDI, 91.0–93.7%) respectively. As mentioned in earlier estimates for the Se of the ELISA varied from 37–74% and Sp from 98.9–100% in dairy herds in Argentina (Costanzo *et al.* 2012). Se of ELISA was 26% and Sp was

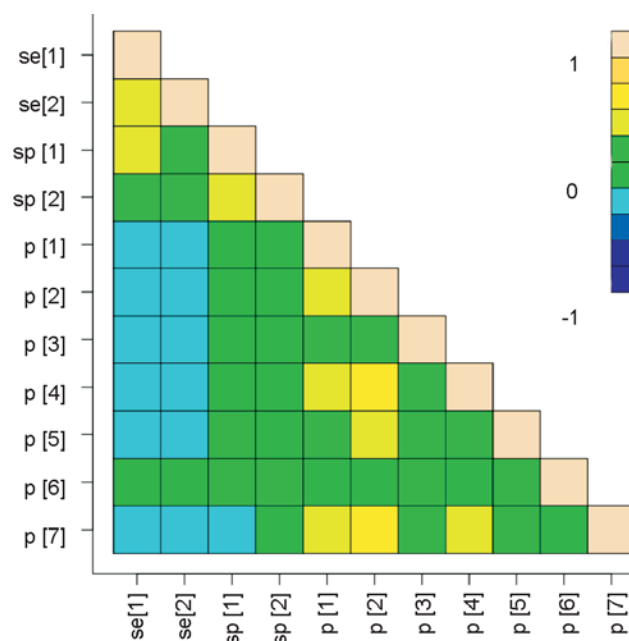


Fig. 2. Cross correlation plot between the two test parameters and the seroprevalences of 7 Indian states.

Table 2. Posterior point estimates of the mean and 95% highest density intervals for the two paratuberculosis tests in Indian cattle and the estimated seroprevalence in each of the 7 states

Parameter	Mean	2.5% HDI	97.5% HDI
Se _{SIT}	0.182	0.092	0.292
Se _{ELISA}	0.266	0.153	0.399
Sp _{SIT}	0.980	0.967	0.992
Sp _{ELISA}	0.924	0.910	0.937
P _{Andhra Pradesh}	0.128	0.016	0.334
P _{Gujarat}	0.071	0.001	0.163
P _{Haryana}	0.211	0.006	0.730
P _{Maharashtra}	0.089	0.003	0.286
P _{Orissa}	0.243	0.018	0.656
P _{Tamil Nadu}	0.779	0.397	0.991
P _{Uttar Pradesh}	0.070	0.003	0.215

p, prevalence; Se, sensitivity; Sp, specificity; HDI, high density interval.

98.5% was estimated by Bayesian model in Chile (van Schaik *et al.* 2007). The sensitivity of the tests for *Map* depend on the stage of infection and age of animals (Kawaji *et al.* 2012) and therefore the estimates of sensitivity will be affected by the composition of the infection stages in the sample and how much representative this is of the underlying population from which it was drawn. Differences in the specificities are likely between different areas with different environmental mycobacteriological flora (Nielsen *et al.* 2002). However, Hermel (1998) reported the Se and Sp of the SIT as 54 and 79%, respectively, and also found poor correlation with the infection status of the animal. The prior estimates of Se and Sp for the 2 tests were 7.8% and 94.1% for the SIT and 27.1% and 90.1% for the ELISA based on non-Indian populations but this does not appear to have overwhelmed the posteriors.

The results indicated that both tests are very insensitive, particularly the SIT (~18%). These were, however, reasonably specific particularly the SIT (~98%). The small numbers of animal classified positive by either test are reflected in the low apparent sero-prevalence. The wide highest density interval reflects the uncertainty in the sero-prevalence estimates but also results in wide intervals for the estimates of sensitivity of the tests compared to the specificities. The model was considered to have satisfactorily converged with a Gelman-Rubin statistic of 1.01 overall and good mixing of the 3 chains. The cross correlation plot (Fig. 2) does not indicate any serious correlation problems that would indicate a problem with the specification of the model or identification of the parameter estimates.

In the present study, the Se and Sp of the SIT and ELISA were determined using the Hui-Walter no gold standard latent class model under the assumption of conditional independence. Both the tests were insensitive but the SIT was reasonably specific while the ELISA was only moderately specific. The ELISA in particular has been suggested as a herd-level test, however although the

sensitivity limitations can be overcome by testing more animals this will have an impact on the herd-level specificity as this will decline to the power of the number tested per herd. The SIT is therefore probably the more useful one in many situations as herd-level test but in low prevalence situations generally found with paratuberculosis false positives will be problematic to resolve. Hence, there is a need to develop improved tests for field use in India as current tools may not be capable of achieving control in a useful time frame.

REFERENCES

- Bapat J A and Bangi M I. 1985. Tuberculosis and Johne's disease among cattle and buffaloes in Maharashtra state. *Indian Journal of Animal Sciences* **55**:1022–23.
- Brooks S P and Gelman A. 1998. Alternative methods for monitoring convergence of iterative simulations. *Journal of Computational and Graphical Statistics* **7**: 434–55.
- Collins M T. 1996. Diagnosis of paratuberculosis. *Veterinary Clinics of North America. Food Animal Practice* **12**: 357–71.
- Costanzo G, Pinedo F A, Mon M L, Viale M, Gil A, Illia M C, Gioffré A, Arese A, Traveria G and Romano M I. 2012. Accuracy assessment and screening of a dairy herd with paratuberculosis by three different ELISAs. *Veterinary Microbiology* **156**:183–88.
- De Lisle, G W, Seguin P, Samagh B S, Corner A H and Duncan J R. 1980. Bovine paratuberculosis: 1. A herd study using complement fixation and intradermal tests. *Canadian Journal of Comparative Medicine* **44**: 177–82.
- Denwood K. 2009. Runjags: Run Bayesian MCMC Models in the BUGS syntax from within R. Available from <http://cran.r-project.org/web/packages/runjags/>
- Garg R, Patil P, Singh R, Sharma S, Mahajan V, Verma S and Sandhu K S. 2007. Epidemiology of Johne's disease in organized dairy farms of Punjab and analysis of associated risk factors. *Proceedings of International Association for Paratuberculosis*. Tsukuba, Japan. pp. 101. (Ed.) Nielsen S S.
- Gasteiner J, Wenzl H, Fuchs K, Jark U and Baumgartner W. 1999. Serological cross-sectional study of Paratuberculosis in cattle in Austria. *Zentralbl Veterinarmed B*. **46**(7): 457–66.
- Hermel S R. 1998. Testing for Johne's Disease. *Angus Journal* **3**: 194.
- Hui S L and Walter S D. 1980. Estimating the error rates of diagnostic tests. *Biometrics* **36**: 167–71.
- Jark U, Ringena I, Franz B, Gerlach G F, Beyerbach M and Franz B. 1997. Development of an ELISA technique for serodiagnosis of bovine paratuberculosis. *Veterinary Microbiology* **5**: 189–98.
- Kawaji S, Nagata R, Whittington R J and Mori Y. 2012. Detection of antibody responses against *Mycobacterium avium* subsp. *paratuberculosis* stress-associated proteins within 30 weeks after infection in cattle. *Veterinary Immunology and Immunopathology* **150**: 101–11.
- McKenna S L B, Keefe G P, Barkema H W and Sockett D C. 2005. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Veterinary Microbiology* **110**: 105–11.
- Nielsen S S, Gronbaek C, Agger J F and Houe H. 2002. Maximum-likelihood estimation of sensitivity specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. *Preventive Veterinary Medicine* **53**: 191–204.

- Plummer M. 2003. JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. Proceedings of the 3rd International Workshop on Distributed Statistical Computing.
- Plummer M, Best N, Cowles K and Vines K. 2009. Output analysis and diagnostics for MCMC. R package version 1, 3–4.
- Pozzato N, Capello K, Comin A, Toft N, Nielsen SS, Vicenzoni G and Arrigoni N. 2011. Prevalence of paratuberculosis infection in dairy cattle in Northern Italy. *Preventive Veterinary Medicine* **102**: 83–86.
- Tripathi B N, Sonawane G G, Munjal S K, Bind R B, Gradinaru D, Dubey S C, Mondal D, Paliwal O P and Singh, N. 2007. Seroprevalence of paratuberculosis in selected population of ruminants in India. *Proceedings of International Association for Paratuberculosis*. Tsukuba, Japan. pp. 104. (Ed.) Nielsen S S.
- Trangadia B J, Rana S K, Naymami K and Srinirasan V A. 2012. Serological investigation of bovine brucellosis, Johne's disease and infections bovine rhinotracheitis in two states of India. *Journal of Advanced Veterinary Research* **2**: 38–41.
- van Schaik G, Haro F, Mella A and Kruze J. 2007. Bayesian analysis to validate a commercial ELISA to detect paratuberculosis in dairy herds of southern Chile. *Preventive Veterinary Medicine* **79**: 59–69.
- van Schaik G, Schukken Y H, Crainiceanu C, Muskens J and VanLeeuwen J A. 2003. Prevalence estimates for paratuberculosis adjusted for test variability using Bayesian analysis. *Preventive Veterinary Medicine* **60**: 281–95.