



Evaluation of ELISA kits for brucellosis in naturally infected Indian cattle population by latent class analysis

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ABSTRACT

Brucellosis is endemic in India and the disease is confirmed by various laboratory tests. Among the tests available, ELISA offers several advantages such as ease, cost effectiveness and possibilities of automation for screening large number of serum samples at any given period of time. This study was performed to evaluate 5 commercially available indirect ELISA kits and 1 competitive ELISA kit in naturally infected cattle population in India. Six organized cattle herds located in Andhra Pradesh, Gujarat and Odisha were selected. Sensitivity (Se) and specificity (Sp) of the tests were estimated in absence of gold standard using latent class analysis. Nearly perfect Sp was achieved by the CEDI test 99.8% (95%; credibility posterior interval (CPI) = 98.9-100%), Chekit test 99.4% (95%; CPI = 98.3-99.9%) and Svanova test 99.2% (95%; CPI = 99.2-99.9%). Se of the 5 indirect ELISA was ranged between 75.0 and 77.3%. However, a Se of 56.1% (95%; CPI= 36.1-75.1%) and Sp of 91.7% (95%; CPI = 88.7-94.2%) were recorded for cELISA.

Key words: Brucellosis, Competitive ELISA, Indirect ELISA

Bovine brucellosis, caused by *Brucella abortus*, is a highly infectious and zoonotic disease responsible for economic losses in livestock worldwide. While it is largely eradicated in many developed countries, it is endemic in most parts of the world, including India. The serological evidence of infection has been reported from various states of the country (Trangadia *et al.* 2010, Trangadia *et al.* 2012, Jagapur *et al.* 2013, Singh *et al.* 2014). Isolation and identification of *Brucella* spp. is performed as the gold standard for diagnosis of brucellosis (OIE 2008). In the absence of the bacterial isolation, other methods such as serological test or combination of tests with known sensitivity and specificity estimates can be used to define the status of animals. There are several serological techniques available, the most common being Rose Bengal test (RBT), complement fixation test (CFT), fluorescent polarization assay (FPA) and indirect and competitive enzyme-linked immunosorbent assay (ELISA). Among these tests, ELISA offers several advantages such as ease,

cost effectiveness and possibilities of automation for screening large number of serum samples at any given period of time. Usually the performance of a diagnostic test is evaluated by the sensitivity (the proportion of true positives among infected) and the specificity (the proportion of true negatives among non-infected).

Thus, to evaluate the diagnostic performance of several ELISAs for brucellosis in naturally infected cattle it is preferable to avoid the assumption of a perfect reference test. Muma *et al.* (2007) evaluated the performance of serological tests for brucellosis using latent class analysis (LCA) (Hui and Walter 1980) which under certain assumptions permit evaluation of performance of the diagnostic test in the absence of a gold standard. Therefore, the objective of this study is to evaluate the diagnostic performance of commercially available 5 indirect and 1 competitive ELISA for diagnosis of brucellosis in naturally infected cattle in absence of 'gold standard'.

MATERIALS AND METHODS

Animals and serum samples: Blood samples (404) were randomly collected from indigenous and crossbred cattle housed in 6 organized farms located in Gujarat (3 farms), Andhra Pradesh (2 farms) and Odisha (1 farm) during 2007-08. These animals were not vaccinated against brucellosis. Approximately 5 ml blood was collected from the jugular vein and allowed to clot. At the laboratory, the samples

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were centrifuged at 1,500 rpm for 15 min for separation of serum. Serum samples were stored at -20°C until used for analysis.

Diagnostic tests: The serum samples were tested for detection of antibodies against *Brucella abortus* using 6 commercially available ELISA kits (5 indirect and 1 competitive ELISA). The samples were screened as per manufacturers' instructions of the respective ELISA kits.

Principle of indirect ELISA: Microtitre ELISA plates coated with inactivated *Brucella abortus* antigen were used for detection of antibodies against *B. abortus* present in the test serum. The bound antibodies were detected using anti ruminant IgG conjugated to the enzyme horseradish-peroxidase. Subsequently, the bound conjugate was visualized by adding a chromogen/substrate solution. Colour development occurs only when specific antibodies against *Brucella abortus* are present in the test sample. Using the above protocol, the following indirect ELISA systems were used

ELISA kit 1: A commercial indirect-ELISA form was used for detection of antibodies against *Brucella* in serum samples. The test serum samples were added in microtitre ELISA plates pre-coated with *Brucella* LPS antigen, after a final dilution of 1:200 in diluting buffer. After the performing conjugate and chromogen (ABTS) /substrate steps, the optical density (OD) was recorded at 405 nm in an ELISA reader. The positive and negative control samples were also supplied by the manufacturer. A test sample giving an OD equal to or above 10% of the mean OD of the control positive sample was considered as positive.

ELISA kit 2: Indirect immunoenzymatic assay kit 2 was used for specific detection of antibodies to *Brucella abortus* in cattle sera. The ELISA plates were supplied pre-coated with purified extract of LPS of *Brucella*. Test serum samples were diluted 1:25 and added to each well. After incubation and washing, a monoclonal antibody specific to bovine immunoglobulin labeled with peroxidase conjugate was added. Finally presence of brucella specific antibodies in the test sera was detected by use of chromogen (TMB)/ substrate reaction and OD was measured at 450 nm. Cut-off for positive result was calculated as 40% of the OD of positive control sample. Cut-off for negative was determined as 35% of the OD of positive control sample. Hence, the sample was deemed as positive if OD is higher than positive cut-off and negative if OD is lower than negative cut-off. Samples with OD values between these 2 cut-offs were considered as doubtful.

ELISA kit 3: In ELISA kit 3 commercially procured brucellosis serum from Nederland was used for detection of antibodies against *Brucella abortus* in serum samples. Test serum samples were added to ELISA micro-titre plate pre-coated with inactivated brucella antigen, after 1:10 dilution in diluents buffer. Positive and negative controls were added in duplicates. Value (%) were calculated as per manufacturers' instructions and samples had <80 % of value were considered as negative and 80 % and above were considered as positive.

$$\text{Value (\%)} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{negative}} / \text{OD}_{\text{positive}} - \text{OD}_{\text{negative}}) \times 100$$

ELISA kit 4: In indirect ELISA kit 4 serum samples were added to coated plate after 1:10 dilution in dummy plate and test was performed as per manufacturers' instructions. OD was measured at 450 nm. Corrected OD of the reference sera and test samples were measured by subtracting the mean OD blank from their original OD values. Percent positivity (PP) values were calculated as per manufacturers' recommendations. The samples which had PP values less than 45 % were considered as negative and PP values 45% and above were considered as positive. Test performance is checked by the use of 3 reference serum samples on each plate. The 3 references (reference serum 1, 2 and 3) represent a positive, a negative and a weak positive serum sample, respectively.

$$\text{PP} = (\text{corrected OD}_{450} \text{ test sample} / \text{corrected OD}_{450} \text{ reference serum1}) \times 100$$

ELISA kit 5: Brucella-Ab I-ELISA kit 5, an indirect ELISA kit, was used to determine *Brucella* specific antibody in serum. Pre-coated ELISA plates were received and serum samples were added at 1:25 dilution in single well. Positive and negative controls were also added in duplicate wells and finally OD was measured at 450 nm. Results were interpreted by calculation of percent positivity (PP) values as recommended by the manufacturer. The samples with PP values lower than 25 were considered as negative and 25 or above were considered as positive.

$$\text{Percent positivity (PP)} = \frac{\text{Test sample/ negative control (OD)}}{\text{Positive control OD}} \times 100$$

Principle of competitive ELISA: In this test system, serum samples were exposed to *Brucella abortus* smooth lipopolysaccharide (S-LPS) coated wells on microtitre plates together with a mouse monoclonal antibody (MAb) specific for an epitope on the o-polysaccharide portion of the S-LPS antigen. After an incubation period the microplate was washed and goat anti-mouse IgG antibody conjugate with horseradish-peroxidase was added which binds to any MABs bound to the S-LPS on the plate. Unbound materials were removed by rinsing before the addition of substrate solution.

In the absence of anti-*Brucella* antibody in the test serum, the MAB may bind to the o-polysaccharide epitope of the S-LPS antigen and which indicated by colour development. In case of brucella positive serum, the antibodies would compete with the MAB for the epitope sites and inhibit the MAB binding to the o-polysaccharide portion of the S-LPS antigen. Hence, less colour development indicated more antibodies in the test serum samples and vice versa.

cELISA kit 6: Brucella-Ab c-ELISA kit 6, a competitive ELISA kit, was used to determine anti-*Brucella* spp. antibody titres and conducted according to the manufacturers' instructions. Sera and controls were run in duplicates. The OD was measured at 450nm in a microplate

reader. The threshold for determining sero-positivity was based upon the manufacturers' recommendations (<30%), with antibody titres recorded as percentage inhibition (PI) defined by the ELISA kit supplier as:

$$PI = 100 - \frac{(\text{Mean OD}_{\text{samples/control}} \times 100)}{\text{Mean OD}_{\text{conjugate control Cc}}}$$

Reading of all 96 well microtitre plates were taken at wavelength recommended by kit manufacturers.

Statistical analyses: To estimate the Se and Sp of the 6 ELISAs in the absence of a gold standard, we used a modified version of the LCA proposed by Hui and Walter (1980). Furthermore, we adopted a Bayesian formulation to accommodate the relatively small sample size of this study (Toft *et al.* 2005). It did not seem appropriate to assume that all ELISAs were conditionally independent given disease status, thus a model allowing for correlation between the 5 indirect ELISA and conditional independence between the competitive ELISA and the indirect ELISAs was specified. A correlation structure between 5 tests can be modeled in many ways; however, our focus was mostly in accounting for possible correlation and not on exploring these structures. Hence we adopted and extended the random effects model used by Baadsgaard and Jørgensen (2003) to assess the accuracy of clinical raters. Thus for the i th test for i th cow in the j th population the following holds

Model:

$$ap_{ij} = Se_i * p_j + (1 - Sp_i) * (1 - p_j)$$

$$pos_{ij} \sim \text{Bern}(ap_{ij})$$

$$\text{logit}(Se_i) = \mu_{Se} + v_{Se} \text{ for } i=1, \dots, 5$$

$$\text{logit}(Sp_i) = \mu_{Sp} + v_{Sp} \text{ for } i=1, \dots, 5$$

Priors:

$$Se_i \sim \text{Beta}(1,1) \text{ for } i=1, \dots, 6$$

$$Sp_i \sim \text{Beta}(1,1) \text{ for } i=1, \dots, 6$$

$$p_j \sim \text{Beta}(1,1)$$

$$v_{Se} \sim N(0, \sigma_{Se})$$

$$v_{Sp} \sim N(0, \sigma_{Sp})$$

$$\mu_{Se} \sim N(0,10)$$

$$\mu_{Sp} \sim N(0,10)$$

$$\sigma_{Se} \sim \text{Gamma}(0.01, 0.01)$$

$$\sigma_{Sp} \sim \text{Gamma}(0.01, 0.01)$$

where ap_{ij} is the probability of a test positive result for the i th test at the i th cow in the j th population, $pos_{ij} = 1$ for test positive and 0 for test negative, μ and v are the parameters for the random effects model for the 5 indirect ELISA (designated test 1 to 5, test 6 is the competitive ELISA).

The model was implemented in OpenBUGS (Lunn *et al.* 2009), which uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution. The first 10,000 MC samples were discarded as a burn-in to allow convergence and the following 90,000 iterations were used for posterior inference. Convergence of the MCMC chain after the initial burn-in was assessed by visual inspection of the time-series plots of selected variables as well as Gelman-Rubin

diagnostic plots using 3 sample chains with different initial values (Toft *et al.* 2007).

In a Bayesian analysis, all parameters are modelled using distributions, where prior distributions are provided to reflect what is known about the tests. However, as we used a model capable of estimating all parameters from data alone, we chose to use uninformative or vague priors for all parameters. Posterior inference was based on medians and 95% posterior credibility intervals (PCI, the Bayesian analogue of a confidence interval) of the prevalence in the 3 populations and the Se and Sp of the 6 tests as well as the parameters describing the random effects. To assess the model assumptions, the specified model was compared to a model without the random effects using the sampled deviance. Furthermore, the analysis was rerun on subsets of tests where each of the tests was excluded one at a time.

RESULTS AND DISCUSSION

The present study determined the Se and Sp of 5 iELISAs and 1 cELISA from various commercial manufacturers for detection of antibodies against bovine brucellosis in a population of naturally infected cattle in absence of a perfect reference test (gold standard) to classify the animals as truly infected and non-infected using LCA. Thus, the findings of this study are free from the bias often inherent from the procedure used to establish the true disease status and the results therefore better reflects the true properties of the 6 tests when applied to the Indian cattle population.

The test results for each of the 6 tests, stratified by the 3 sampling populations are given in Table 1. Using data summarized in Table 1, the posterior true prevalence (p), the Se and Sp of each test were estimated. The median and 95% CPI are given in Table 2. The association between the Se and Sp of the 6 ELISAs are illustrated in an ROC type plot in Fig 1. All indirect ELISAs have a more than 96% Sp, with comparable Se. However, cELISA has the lowest Se and Sp as compared to iELISAs. The deviance highly

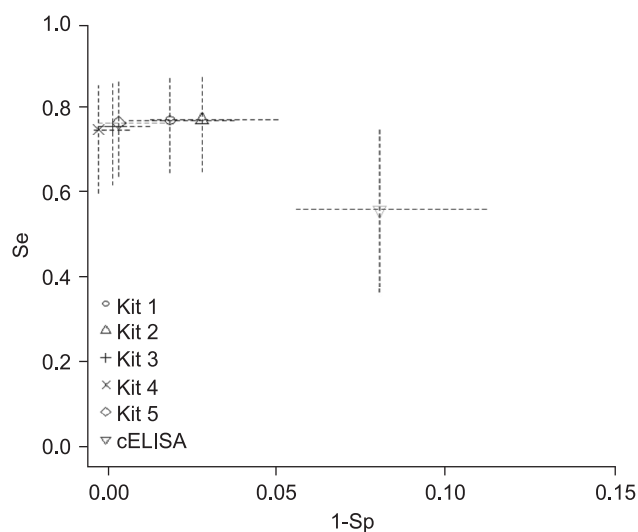


Fig. 1. ROC-plot of posterior median Se against 1-Sp for each of the 6 ELISA (indirect ELISA in black), lines give the 95% credible posterior interval.

Table 1. The tabulated results of each test (% positive), stratified by the sampling populations (each comprised herds sampled within an Indian state). Nuer of test positive (%)

State	Indirect ELISAs					cELISA	Total number tested
	1	2	3	4	5		
AP	10 (5.9)	8 (4.8)	7 (4.2)	7 (4.2)	9 (5.4)	16 (9.5)	168
Gujarat	14 (8.6)	14 (8.6)	11 (6.8)	8 (4.9)	11 (6.8)	26 (16.0)	162
Odisha	4 (5.4)	10 (13.5)	1 (1.4)	1 (1.4)	1 (1.4)	2 (2.7)	74

AP, Andhra Pradesh.

Table 2. The estimated posterior median and 95% credible posterior interval (CPI) for the Se and Sp of the 7 ELISAs as well as the true prevalence (p) in each population.

ELISA	Se	95% CPI	Sp	95% CPI
Kit 1*	77.3	[64.7; 87.8]	97.8	[95.9; 98.9]
Kit 2*	77.3	[65.0; 87.8]	96.8	[94.7; 98.3]
Kit 3*	75.7	[62.0; 86.0]	99.4	[98.3; 99.9]
Kit 4*	75.0	[60.0; 85.4]	99.8	[98.9; 100]
Kit 5*	76.7	[64.1; 86.6]	99.2	[99.2; 99.9]
cELISA	56.1	[36.1; 75.1]	91.7	[88.7; 94.2]
Population	p	95% CPI		
Andhra Pradesh	6.0	[3.0; 11.2]		
Gujarat	8.2	[4.5; 13.2]		
Odisha	2.3	[0.3; 7.3]		

*Indirect ELISA, posterior median estimates of random effects components ($\mu_{Se, S_{Se}}$) = (1.2; 0.22) and ($\mu_{Sp, S_{Sp}}$) = (4.7; 1.4)

avored the random effects model, however formal testing was not done, as the number of degrees of freedom is somewhat debatable for the model. Sensitivity analysis using subsets of tests did not suggest any important deviations from the underlying modeling assumptions.

The estimated true prevalence of brucellosis in Andhra Pradesh, Gujarat and Odisha was 6.0, 8.2 and 2.3% respectively. Renukaradhya *et al.* (2002) recorded prevalence in cattle 6.6% in Gujarat, 4.3% in Andhra Pradesh and 1.0% in Odisha with 5.0% as national average of brucellosis.

There were notable differences between the tests with the indirect ELISAs having the best Sp and moderate Se; however, the cELISA had sub-optimal Se as well as Sp. The median posterior Sp of 5 indirect ELISA ranged from 96.8 to 99.8%, whereas median posterior Se varied from 75.0 to 77.3%. McGiven *et al.* (2003) reported mean Se = 97.8% and mean Sp = 97.2% for iELISA from VLA (UK). Similarly, Boqvist *et al.* (1998) reported Se and Sp as 92.3 and 88.9%, respectively, in relation to RBPT for SVANOVIR Brucella-Ab iELISA. Munir *et al.* (2008) recorded Se as 100, 100, 97.53 and 86.42% and Sp as 84.03, 84.87, 85.71 and 87.39% respectively for in-house

developed iELISA at various cut-off values. Thus, compared to the findings of this study, higher Se and comparatively lower Sp of the various iELISAs were reported by various authors. However, comparing ELISAs across studies is notoriously difficult as several factors contribute to the accuracy of an ELISA, the fact that higher Se is associated with lower Sp compared to our findings is somewhat reassuring and suggested some of the difference might be due to different cut-offs used in the reported studies.

Comparatively lower medium posterior Se of 56.1% and Sp of 91.7% were reported for cELISA. However, perusal of literature reported the Se of cELISA ranging from 83.9 to 100% where as Sp were from 54 to 100% (Nielsen *et al.* 1995, Gall *et al.* 1998, Stack *et al.* 1999, Fosgate *et al.* 2003, Muma *et al.* 2007). It is generally recommended to optimize the test performance within the population where test is to be applied and also consider the purpose of the test in particular area. Further, different environmental conditions may affect the interpretation of the results (Greiner and Gardner 2000). However, the low Se is accompanied by the best Sp for all iELISAs, suggesting the test is optimized for confirmation of negative status of infection.

To conclude, among the kits evaluated in this study, all indirect ELISAs were reported to be highly specific and moderately sensitive. To identify negative animals from infected herds or from brucellosis endemic areas, any of indirect ELISA tests can be used.

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