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Evaluation of fluorescence polarization assay for the diagnosis of brucellosis in cattle and buffaloes in India

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ABSTRACT

Fluorescence polarization assay (FPA) was evaluated for serological diagnosis of brucellosis in cattle and buffaloes. Sera samples from 1397 cattle, comprising 120 indirect ELISA (iELISA) positive and 1277 iELISA negative; and 937 from buffaloes (88 iELISA positive and 849 iELISA negative) were tested by Rose Bengal test (RBT) and FPA in parallel. Receiver operating characteristic (ROC) curve was used for evaluation of FPA, using iELISA as gold standard to determine the cut-off offering the highest performance index (PI) and to compare its performance with RBT. In cattle, sensitivity (Sn) and specificity (Sp) of FPA were 46.7% and 96.6% respectively at a cut-off of 75 millipolarization (mP) in relation to iELISA, while Sn and Sp of RBT were 35.8% and 99.6% respectively. In buffaloes, Sn of FPA was 47.7% and Sp was 96.7% at a cut-off of 77.1 mP, whereas Sn and Sp of RBT were 44.3% and 99.2% respectively in relation to iELISA. McNemar's chi-square test for independent data (with Yates' correction) revealed that there was no significant difference in the proportion of positive samples between the FPA and RBT in cattle and buffaloes. Results indicated that FPA is as efficient as RBT for diagnosis of brucellosis. FPA may be preferred over RBT because of its characteristic of cut-off adjustments useful in different epidemiological situations and its potential application in field. However, further study with large number of known positive samples would be required for endorsement of FPA as a routine diagnostic test under the field condition.

Key words: Brucellosis, ELISA, FPA RBT

Bovine brucellosis is one of the most important bacterial zoonoses worldwide and particularly in developing countries. In India a high sero-prevalence (17.00 to 22.18%) of the disease is reported and the organism has been identified from aborted material as well as from milk (Isloor *et al.* 1998, Chahota *et al.* 2003, Trangadia *et al.* 2010).

Control and prevention of brucellosis mainly depends on the use of rapid and sensitive diagnostic tests. Efficiency and limitations of various diagnostic techniques for brucellosis, viz. ELISA (enzyme-linked immunesorbent assay), STAT (standard tube agglutination test), RBT (Rose bengal test), MRT (milk ring test) and CFT (complement fixation test) have been described (Alton *et al.* 1988, Nielsen *et al.* 1996a, Muma *et al.* 2007). Laboratory based ELISA technique is sensitive, but it is time consuming. A suitable test, which is affordable, rapid, diagnostically sensitive and specific and convenient to use at the pen-side would be the test of choice. RBT and FPA seem to have such features. RBT is reported to have high sensitivity but a low specificity, and has been used to diagnose brucellosis despite of its limitation (OIE 2008). The accuracy of FPA is comparable to cELISA and superior to RBT (Nielsen and Gall 2001, Montagnaro *et al.* 2008). FPA is suitable for detection of antibodies to *Brucella* spp. in multiple species in the field condition.

The present study aims in evaluating FPA for the serodiagnosis of brucellosis infection in cattle and buffaloes, using i-ELISA as gold standard and to compare its performance with that of RBT.

MATERIALS AND METHODS

Serum samples: Serum samples from 1397 cattle and 937 buffaloes were collected from various parts of the country and tested for brucellosis by FPA, iELISA and RBT. The iELISA was considered as gold standard and hence samples positive by this test were considered as truly positive.

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Serological tests

RBT: RBT antigen was procured from the Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh (UP), India. RBT was performed according to procedure described by World Organization for Animal Health (OIE 2008). The result was recorded after the mixture was rocked gently for 4 min at room temperature. Any sign of agglutination was considered as positive.

ELISA: Diagnosis of brucellosis from serum on the basis of detection antibodies against *Brucella abortus,* was carried out by using iELISA kit.

Fluorescent polarization assay (FPA): The principle of this test relies on a fluorescent dye attached to a small antigen (or antibody fragment) that is excited by plane-polarized light at the appropriate wavelength. The rate of rotation of the antigen molecule is reduced when its molecular size is increased through binding to an antigen (or antibody). This change in rate can be measured. The assay which can be completed in a few min, first require a one-step serum dilution, then the assessment of background fluorescence, followed by the addition of a labeled antigen and finally the measurement of antigen-antibody interaction (Lucero *et al.* 2003).

The FPA was performed in a single tube testing mode by using *Brucella abortus* antibody test kit. Sentry 100, a FPA instrument was set up and calibrated as per manufacturers specifications by using Sentry standard prior to use. Briefly, 10 µl of serum sample and controls were added to 1 ml of diluted reaction buffer into a glass tube. Buffer and samples were vortexed and incubated for at least for 5 min at room temperature. After initial incubation, the tube was put in reader to obtain a blank intensity reading. Subsequently, 10 µl of conjugate (O-polysaccaharide conjugated fluorescein) was added to the tube, mixed and further incubated for two min as above. The tube was read again as before to obtain the result in mP (millipolarization) units. The mP values were calculated by using the following formula:

$$mP = 1000 \times (V\text{-}GH)/(V+ GH)$$

where V, vertical reading; H, horizontal reading; G, correction factor for optics balancing.

The samples were declared positive where mP values were > 20 mP above the mean negative control and declared negative where, mP values recorded as < 10 mP above the mean negative control; otherwise suspected.

Data analysis: The sensitivity (Sn) and specificity (Sp) of the FPA and RBT as compared to iELISA (at 95% confidence interval) and performance index (PI) were calculated by the receiver operator characteristic (ROC) analysis curve with the aid of a software (Shoonjans 2005). Results were expressed in a ROC curve analysis graph that plotted the true positive rate related to the false positive rate at different cut-off points (Schoonjans 2005). ROC analysis provides information about the Sn (the proportion of true positives that are detected by the test), Sp (the proportion of true negatives that are detected by the test) (Thrusfield 2003), the area under the ROC curve (AUC), which is indicative of the accuracy of the test (Nielsen *et al.* 1996b, Ramirez-Pfeiffer *et al.* 2006), and the cut-off value of the test. In addition, software provides a dot graph, a diagram in which the paired observations are plotted as dots against two axes, displaying the results of individual tests according to the value of the classiûcation variable and a horizontal line that corresponds to the cut-off value, which represent the minimum number of false classification (positive and negative) by the test. Finally, Sn and Sp of FPA and RBT were statistically compared by McNemar's chi-square test with the aid of software (Shoonjans 2005).

RESULTS AND DISCUSSION

Bovine brucellosis is highly prevalent in India and causes significant economic losses to the livestock industry. Rapid and accurate screening tests are essential for the successful implementation of control programme. Taking into account several challenges encountered in testing animals under prevailing Indian rural condition, both the RBT and FPA could be effectively used in diagnosis of brucellosis because both the tests allow single animal contact, cow-side field testing of animals and on-spot reporting of the testing result. Hence, these tests have been chosen in many other countries for the diagnosis and eradication of brucellosis infection in livestock (Montagnaro *et al.* 2008).

Although RBT is recommended under field condition, false negative reaction can occur due to prozone phenomenon, where sera with high levels of antibody results in non-visible reactions with the RBT antigen (Alton *et al.* 1988). Thus a strong *Brucella* positive serum may be classified as negative in compare to results of other serological assays. In contrast, primary binding assays such as FPA do not exhibit prozone effect and make it more preferable over RBT. Additionally, diagnostic sensitivity and specificity of FPA reported to be superior to that of RBT and CFT (Nielsen and Gall 2001, Minas *et al.* 2005). FPA showed high Sp for bovines vaccinated by *Brucella abortus* S-19 vaccine (Nielsen *et al.*1996a). Further, FPA is host-species independent and can also be conducted on whole blood (Nielsen *et al.* 2001a) and milk (Nielsen *et al.* 2001b).

In the present study, ROC analysis was used to evaluate the use of FPA for the diagnosis of brucellosis in cattle and buffaloes, to determine the cut-off offering the highest performance index and to compare its performance with that of RBT.

Comparison of FPA and RBT with iELISA results

Cattle: Cattle sera samples (1397), comprising 120 positive and 1277 negative samples by iELISA, were evaluated by RBT and FPA. Sn and Sp of FPA at different cut-off values calculated by ROC analysis, positive likelihood ratios (+LR), negative likelihood ratios (-LR) and PI are

presented in Table 1. The cut-off of 75 mP (Table 1) provided the highest combined Sn and Sp values. Sn and Sp of FPA as compared to iELISA were 46.7% (CI 95%, 37.5–56.0%) and 96.6% (CI 95%, 95.5–97.6%) respectively at a cut-off of 75 mP, with a performance Index (PI) of 143.3 (Fig. 1a). The dot diagram for FPA compared with the iELISA is shown in Fig. 1b, where every square represents a single FPA result and the horizontal line located at 75 mP represents the cut-off line for Sn and Sp.

RBT was also evaluated by MedCalc and the data revealed that Sn and Sp of RBT in cattle (relative to iELISA) were 35.8% (C.I. 27.3–45.1) and 99.6% (C.I. 99.1– 99.9), respectively, with a PI of 135.4. Finally, McNemar's chi-square test for independent data (Yates' correction) revealed that there was no significant difference in the proportion of positive samples between the FPA and RBT (P = 0.0500 with 95% C.I.).

Buffaloes: Sera sample, comprising 88 positive and 849 negative samples by iELISA, were evaluated by RBT and FPA. Sn and Sp of FPA at different cut-off values calculated by ROC analysis as well as positive likelihood ratios (+LR), negative likelihood ratios (LR), and PI are presented in Table 2, where the cut-off of 77.1 mP provided the highest combined Sn and Sp values. As depicted in Fig. 2a, Sn of FPA was 47.7% (CI 95%, 37.0–58.6%) and Sp was 96.7% (CI 95%, 95.3–97.8%) at a cut-off of 77.1 mP, with a performance index (PI) of 144.4. The dot diagram for FPA compared with the iELISA is shown in Fig. 2b.

The results of RBT were also analyzed by MedCalc and the data showed that Sn and Sp of RBT in buffalo (relative to iELISA) were 44.3% (CI 33.7–55.3) and 99.2% (CI 98.3–99.7), respectively, with a PI of 143.5. However, McNemar's chi-square test for independent data (with Yates' correction) revealed that there was no significant difference in the proportion of positive samples between the FPA and RBT (P = 0.1859 with 95% CI).

The PI of FPA in cattle and buffaloes were higher than that of RBT. Although no statistically significant difference was recorded in the detection of positivity of samples by RBT and FPA, but the sensitivity of FPA was recorded higher than that of RBT in cattle and buffaloes. However, RBT was found to be more specific than FPA in both species. Contrary, available report indicated that FPA is more sensitive than RBT but does not significantly differ in specificity (Montagnaro *et al.* 2008).

Several workers have determined the Sn and Sp of FPA at various cut-off in relation to different tests, viz. CFT, RBT, cELISA, iELISA for various species, and differences in Sn and Sp of FPA were observed by Nielsen *et al.* (2005), Nielsen and Gall (2001) and Minas *et al.* (2005). Dajer *et al.* (1999) reported a cut-off of 90mP with the Sn and Sp of 99.0% and 96.9%, respectively, for FPA in relation to CFT. A higher cut-off value of 117mP was reported by Montagnaro *et al.* 2008 with Sn and Sp of 92.6% and 91.2% respectively in

Table 1. Sensitivity (Sn) and specificity (Sp) of fluorescence polarization assay (FPA) at different cut-off values calculated by receiver operating characteristic (ROC) analysis in cattle sera

mP Units	% Sn	95% CI^	% Sp	95% CI^	+LR	-LR	PI
74.6	46.7	37.5–56.0	96.1	94.9–97.1	11.92	0.56	142.8
74.7	46.7	37.5–56.0	96.2	95.0–97.2	12.42	0.55	142.9
74.8	46.7	37.5–56.0	96.4	95.2–97.4	12.96	0.55	143.1
75*	46.7	37.5–56.0	96.6	95.5–97.6	13.86	0.55	143.3
75.2	45.8	36.7–55.2	96.9	95.8–97.8	14.63	0.56	142.7
75.3	45.8	36.7–55.2	96.9	95.8–97.8	15.01	0.56	142.7
75.5	45.8	36.7–55.2	97.1	96.0–98.0	15.82	0.56	142.9

95% CI^,95% confidence interval, *,cut-off,% Sn, per cent sensitivity. % Sp, per cent specificity.

Table 2. Sensitivity (Sn) and specificity (Sp) of fluorescence polarization assay (FPA) at different cut-off values calculated by receiver operating characteristic (ROC) analysis in buffaloes sera

mP Units	% Sn	95% CI^	% Sp	95% CI^	+LR	-LR	PI
73.5	48.9	38.1–59.8	90.3	88.2–92.2	5.06	0.57	139.2
75	48.9	38.1–59.8	94.6	92.8–96.0	9.02	0.54	143.5
75.1	47.7	37.0–58.6	94.8	93.1–96.2	9.21	0.55	142.5
77.1*	47.7	37.0–58.6	96.7	95.3–97.8	14.47	0.54	144.4
77.6	46.6	35.9–57.5	96.8	95.4–97.9	14.65	0.55	143.4
78.1	46.6	35.9–57.5	97.1	95.7–98.1	15.82	0.55	143.7
78.3	45.5	34.8–56.4	97.1	95.7–98.1	15.44	0.56	142.6

95% CI^, 95% confidence interval; *,cut-off;% Sn, per cent sensitivity;% Sp, per cent specificity.

comparison to CFT. In comparison to cELISA, Sn and Sp of FPA were reported as 95.3% and 97.3% respectively (Nielsen *et al.* 2001a). The lower Sn of FPA determined in this study may be attributed to the number of positive serum samples used for testing, where number of positive samples was far less than the negative samples. However, the Sp of FPA determined in the present study was comparable to the above reports.

To compare the performance of diagnostic tests, ROC curve can be used (Griner *et al.* 1981). In this study, AUC in cattle for FPA and RBT were 0.701 and 0.677 respectively, where as AUC in buffaloes was 0.652 for FPA and 0.717 for RBT. The results indicated that both the tests have reasonably good disseminating ability for infected and uninfected animals.

Our results indicated that FPA is as efficient as RBT for diagnosis of brucellosis in cattle and buffaloes. The result of RBT is known to be more influenced by the presence of cross reacting antibodies (Nielsen 2002). Whereas cut-off of FPA can be adjusted, so that desirable Sn and Sp could be achieved in different epidemiological situations. However, for using FPA as a routine diagnostic test under the field condition, further studies would be required to screen a large number of serum samples from known infected and negative animals.

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