Note

Lateral flow assay for brucellosis testing in multiple livestock species

Rajeswari Shome[a],[*], Triveni Kalleshamurthy[a], Bibek Ranjan Shome[a], Swati Sahay[a], Krithiga Natesan[a], Rajendra Gulabrao Bambal[b], Lipi Sairiwal[b], Nagalingam Mohandoss[a], Sukhadeo B. Barbuddhe[c]

[a] Indian Council Agricultural Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Yelahanka, Bengaluru 560064, India
[b] Department of Animal Husbandry, Dairying & Fisheries, Krishi Bhavan, New Delhi 110001, India
[c] ICAR-National Research Centre on Meat, Chengicherla, Hyderabad 500092, India

ABSTRACT

Lateral flow assay (LFA) for brucellosis was standardized and evaluated. The test showed high diagnostic sensitivity, specificity and accuracy for diagnosis of brucellosis in bovines, small ruminants and swine. The study emphasized the importance of LFA as a useful, rapid, and easy-to-perform tool for the testing of brucellosis.

Brucellosis, a well-documented zoonotic pathogen which has been isolated from almost all mammals of either land living or marine living. *Brucella* is a member of Gram-negative facultative intracellular pathogen having six classical species *Brucella abortus* (bovine brucellosis), *B. melitensis* (small ruminant brucellosis); *B. canis* (canine brucellosis), *B. ovis* (ram epididymitis), *B. suis* (swine) and *B. neotomae* (in desert rats). Brucellosis has been recognized as one of the seven neglected, under-detected and under-reported zoonoses (OIE, 2014). The global picture of the disease is continuously changing due to intensification of farming, sanitary, socioeconomic and political reasons and increased international travel (WHO, 2010). The disease is a major trade barrier to socioeconomic progress, food security and threat to public health in most of the endemic countries including India (ILRI, 2012). Brucellosis is diagnosed by isolation, serological tests and molecular techniques. Isolation is a gold standard test but it is less sensitive (15–70%), time consuming, requires biocontainment facility and poses risk to laboratory personnel (Habtamu et al., 2013). Serological tests such as rose bengal plate test (RBPT), serum agglutination test (SAT), complement fixation test (CFT), enzyme linked immune sorbent assay (ELISA), fluorescent polarization assay (FPA) are recommended for diagnosis (Morata et al., 2003; Muma et al., 2009). All these tests require vigorous evaluations at country level, sophisticated laboratory facilities, refrigeration of reagents, technical skill and at times not cost effective for routine use in the developing countries.

RBPT is routinely used for multiple livestock species in endemic countries including India. It is simple, cost effective but requires refrigeration of diagnostic antigen and has limitations of false positive results due to cross reacting antibodies against many Gram negative bacteria (Poester et al., 2013). Recently, point-of-care tests [immuno chromatographic flow assays/lateral flow assays (LFA)] have shown better sensitivity (Sn) and specificity (Sp) than RBPT are being recognized as important diagnostics for brucellosis worldwide (Elshemey and Abd-Elrahman, 2014; Genc and Buyuktanr, 2011).

India has huge and diverse livestock populations reared in intensive, semi-intensive, extensive and mixed farming at different agro-climatic conditions. The transmission of disease between herds and flocks, market places, unrestricted movement of animals across states and international borders and lack of pre-tests before purchase are resulting in increased prevalence of the disease in bovines, small ruminants, swine, camels, equines, mithun and yak populations (NAVS, 2013). The major challenge in diagnosis is early detection of infection at market places, farm level, hospitals, during outbreaks and slaughter houses. The present study envisages standardization and evaluation of LFA for brucellosis testing in bovines, small ruminants and swine which can be used at every level of the animal production system in India.

Smooth lipopolysaccharide (sLPs) antigen extracted from *B. abortus* S99 was used as an antigen in the LFA as described earlier (Shome et al., 2015). LFA test devices were developed in collaboration with Ubio Biotechnology Systems Pvt. Ltd. (Kerala, India). The test device contains two distinct lines on a nitrocellulose strip, test line with *Brucella*-sLPS antigen and the control line containing goat anti-mouse IgG. The recombinant protein-G conjugated with 40 nm colloidal gold nanoparticles (Innova Biosciences, UK) was used as a detection reagent (Smits et al., 2003). The test was standardized by titrating antigen
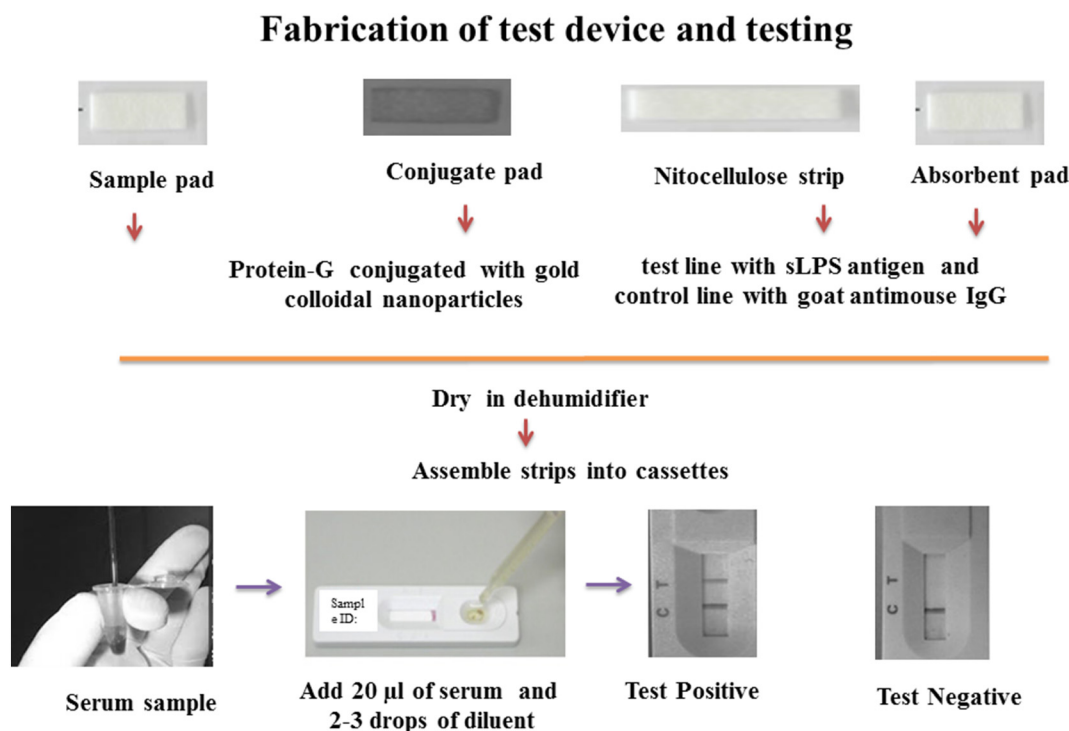
Fabrication of test device and testing

Fig. 1. A schematic work flow diagram depicting the procedure for assembling and testing of the samples.

Table 1
Concordance of the LFA test using known status of sera panel.

Species	Total samples	Positive concordant	Negative concordant	Discordant	X^2 value	p value	Unweighted kappa	Results of agreement[a]
Bovine	100	17	80	3	81.92	< 0.001**	0.90	Very good
Small ruminants	100	15	78	7	59.60	< 0.001**	0.76	Good
Swine	100	17	77	6	66.01	< 0.001**	0.81	Very good

** p-Value < 0.05 is considered as significant.

[a] K value of < 0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.0, indicate the strength of agreement as poor, fair, moderate, good and very good, respectively.

Table 2
Evaluation of the LFA test employing field sera samples.

Species	No. of farms	No. of samples	RBPT	Protein-G iELISA	LFA
Bovine	5	309	91 (29.45)	92 (29.78)	85 (27.50)
Small ruminants	6	298	26 (8.72)	29 (9.73)	25 (8.38)
Swine	4	136	40 (29.41)	36 (26.47)	37 (27.20)

Values in parenthesis represented in percentage.

against conjugate dilution with different volumes of neat positive and negative serum samples. Test was performed by the addition of 20 μl serum to the sample well followed by 2–3 drops of sterile phosphate-buffered saline (pH 7.6) containing 1.67% bovine serum albumin and 3% Tween 20. Test results were read within 3–5 min by visual inspection for staining of the test and control lines. Tests were scored negative when no stained band was observed at the test line and scored positive when the test line stained band was observed along with the control line (Fig. 1). The test was rejected or retested if the control line in the test was absent. The developed LFA was evaluated using a panel of 300 serum samples constituting 20 positive and 80 negative each from bovine, small ruminants and swine. Positive samples were confirmed by the presence of anti-*Brucella* antibodies by RBPT and iELISA. These samples were sourced from brucellosis infected farms with no history of brucellosis vaccination. Similarly, negative serum samples were confirmed negative by RBPT and iELISA at intervals of 3 months duration. To rule out the cross-reactivity of the sLPS antigen used in the assay, *E.*

coli (O157 H7), 17 *Salmonella* (VI; polyvalent O; polyvalent O1; O1, 3, 19; O2; O3, 10; O4; O6, 14; O7; O8; O9; O9, 46; O11; O13; O16; O18; O35; O21) and 5 *Yersinia enterocolitica* (O1 & 2; O3; O5; O8; O9) serotype specific reference sera (Denka Seiken Co, Tokyo, Japan) were tested. Tests were evaluated with standard bovine origin strong positive (OIEELISA$_{SP}$SS), weak positive (OIEELISA$_{WP}$SS) and negative (OIEELISA$_N$SS) and National (Indian Veterinary Research Institute) standard positive and negative serum samples.

After preliminary evaluation, field sera samples were collected from dairy farms with a history of abortions and reproductive failures, orchitis and frequent abortions in swine herds and small ruminant flocks. A total of 309, 298 and 136 field serum samples were collected from five bovine, six small ruminants and four swine farms, respectively. Samples were tested by RBPT, iELISA and LFA for detection of anti-*Brucella* antibodies. The diagnostic agreement between the tests was quantified by kappa (K) or weighted kappa (Kw) statistics for the measurement of agreement with RBPT and iELISA. The K value of < 0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.0, indicated the strength of agreement as poor, fair, moderate, good and very good, respectively. The significance difference determined by chi square test and value p < 0.05 was considered statistically significant. Diagnostic statistics viz., sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were computed using statistical software, SPSS 22.0 (IBM, India) and MedCalc 9.0.1.

Brucellosis in livestock is resulting in total median losses of US $3425.3 million in Indian livestock. The individual economic losses

Table 3
Comparative results of LFA with RBPT and protein-G based iELISA.

Species		Bovine				Small ruminants				Swine			
Diagnostic test		RBPT		Protein-G iELISA		RBPT		Protein-G iELISA		RBPT		Protein-G iELISA	
		+	-	+	-	+	-	+	-	+	-	+	-
LFA	+	84	01	82	03	21	04	21	4	35	02	34	03
	-	07	217	10	214	05	268	08	265	05	94	02	97
Kappa		0.93		0.89		0.80		0.75		0.87		0.90	
Sensitivity (Se)		92.31%		89.13%		80.77%		72.41%		87.50%		94.44%	
		(84.79 – 96.85)		(80.92 – 94.66)		(60.65 – 93.45)		(52.71 – 87.27)		(73.20 – 95.81)		(81.34–99.32)	
Specificity (Sp)		99.54%		98.62%		98.53%		98.51%		97.20%		97.00%	
		(97.47 – 99.9)		(96.01 – 99.71)		(96.28 – 99.60)		(96.24 – 99.59)		(92.68 – 99.75)		(91.48 – 99.38)	
PPV		98.82%		96.47%		84.00%		84.00%		94.59%		91.89%	
		(93.62 – 99.67)		(90.03 – 99.27)		(63.92 – 95.46)		(63.92 – 95.46)		(81.81 – 99.34)		(78.09 – 98.30)	
NPV		96.88%		95.54%		98.17%		97.07%		94.95%		97.98%	
		(93.67 – 98.73)		(91.94 – 97.84)		(95.78 – 99.40)		(94.31 – 98.73)		(88.61 – 98.34)		(92.89–99.75)	
Diagnostic accuracy (%)		97.41%		95.79%		96.97%		95.97%		94.85%		91.89%	

PPV-positive predictive value; NPV-negative predictive value.
Values in parenthesis indicates 95% confidential interval.

estimated are US $ 918.3; 1357.1; 71.6; 48.9 and 7.1 million for cattle, buffaloes, goats, sheep and pigs, respectively (Singh et al., 2015). As per the estimation, the prevention of brucellosis will add 2.63 million female calves and 5% increased milk production which were valued at US $387.4 million and US $1162.3 million per annum, respectively (NAVS, 2013). These economic estimates clearly emphasize the huge economic impact of the disease on animal husbandry growth in addition to the economic and social consequences of the disease on humans (Thrusfield, 2007). Brucellosis in humans depends mainly on disease status in livestock and several publications have indicated increasing human brucellosis prevalence ranging from 2.26 to 34% from different parts of the country (OIE, 2014; Mantur et al., 2006; Mantur and Amarnath, 2008). For surveillance, RBPT, serum based iELISA and milk ring test (MRT) were suggested. Conducting these tests in remote areas which are inaccessible to laboratory facilities in a vast country like India was a constraint. Penside test promises to overcome these problems and facilitate diagnosis at every place to uncover chains of infection in animals (Abdoel et al., 2008; Sturenburg and Junker, 2009).

In diagnostic tests, different antigens have been evaluated for the diagnosis of brucellosis (Corbel and Beeching, 2004). To avoid test ambiguities associated with different types of antigens, sLPS antigen which is being used in many commercially available ELISA kits and also in our laboratory has been adopted to the test as per OIE (Shome et al., 2015). One of the constraints associated with LFA or iELISA is that each species requires its own anti-immunoglobulin detection reagent. Hence, common conjugate (protein-G) having a property of binding with immunoglobulins of many livestock species was used in the test (Shome et al., 2011; Corbel et al., 2005). The tests showed positive test results with OIE and National strong positive standard sera. The cross reacting sera against many biovars of *Salmonella*, *Yersinia*, and *E. coli* did not show any stained band in test line indicating no cross reactivity in the standardized test. The LFA test with panel sera samples scored 0.90, 0.76 and 0.81 K agreements for bovine, small ruminants and swine samples, respectively (Table 1). With field samples, very good for bovine (0.93 and 0.89) and swine (0.87 and 0.90) and comparatively good (0.80 and 0.75) K agreements were recorded in comparison to RBPT and iELISA tests (Tables 2, 3). The Sp of LFA for all species was > 97% for both RBPT and iELISA and was highly correlated to the earlier reports (Irmak et al., 2004; Zeytinoglu et al., 2006; Sturenburg and Junker, 2009; Smits and Kadri, 2005).

The promising test results observed with panel, field bovine, swine and small ruminant samples suggested the usefulness of LFA for brucellosis screening. Veterinary officers rarely seek laboratory confirmation for suspected brucellosis cases as the diagnostic facilities are far away from the veterinary hospitals. The LFA test does not require laboratory facilities, technical skill or storage conditions and hence makes the test useful for on-spot testing. The tests should be made available to the veterinary departments, colleges and institutions through government or private agencies. The easy access to the tests will definitely aid quick diagnosis and segregation of infected animals to prevent disease transmission to other animals and strengthen the disease reporting from livestock species in the country.

Acknowledgements

References

Abdoel, T., Dias, I.T., Cardoso, R., Smits, H.L., 2008. Simple and rapid field tests for brucellosis in livestock. Vet. Microbiol. 130, 312–319.

Corbel, M.J., Beeching, N.J., 2004. In: Harrison's Textbook of Internal Medicine, 16th ed. McGraw-Hill, New York, pp. 914–917 (Brucellosis, Ch 141).

Corbel, M.J., Banai, M., 2005. Genus I. *Brucella* Meyer and Shaw 1920, 173AL. In: Brenner, D.J., Krieg, N.R., Staley, J.T. (Eds.), Bergey's manual of systematic bacteriology, vol. 2. Springer, New York, pp. 370–386.

Elshemey, T.M., Abd-Elrahman, A.H., 2014. Evaluation of a rapid Immunochromatographic test for detection of *Brucella abortus* antibodies in Egyptian cattle sera and milk. Alex. J. Pharm. Sci. 40, 24–28.

Genc, O., Buyuktanr, N.Y., 2011. Development of an individual rapid test based on enzymatic immunofiltration assay for detection of anti–*Brucella abortus* antibody in bovine sera. J. Vet. Diagn. Investig. 23, 49–56.

Habtamu, T.T., Rathore, R., Dhama, K., Karthik, K., 2013. Isolation and molecular detection of *Brucella melitensis* from disease outbreak in sheep and *B. abortus* from cattle farm by IS711 and omp2a gene based PCR. Int. J. Curr. Res. 5, 1920–1925.

International Livestock Research Institute (ILRI), 2012. Institute of Zoology & Hanoi School of Public Health Mapping of Poverty and Likely Zoonoses Hotspots. Zoonoses Project 4. Report to the Department for International Development, UK. ILRI, Nairobi Available at: www.dfid.gov.uk/r4d/Output/190314/Default.aspx.

Irmak, H., Buzgan, T., Evirgen, O., Akdeniz, H., Demiroz, A.P., Abdoel, T.H., Smits, H.L., 2004. Use of the *Brucella*IgM and IgG flow assays in the serodiagnosis of human brucellosis in an area endemic for brucellosis. Am. J. Trop. Med. Hyg. 70, 688–694.

Mantur, B.G., Amarnath, S., 2008. Brucellosis in India - a review. J. Biosci. 33, 539–547.

Mantur, B.G., Biradar, M.S., Bidri, R.C., Mulimani, M.S., Veerappa, K.P., 2006. Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 years' experience in an endemic area. J. Med. Microbiol. 55, 897–903.

Morata, P., Queipo-Ortuno, M.I., Reguera, J.M., García-Ordoñez, M.A., Cárdenas, A.A., Colmenero, J.D., 2003. Development and evaluation of a PCR–Enzyme-Linked Immunosorbent assay for diagnosis of human brucellosis. J. Clin. Microbiol. 41,

144–148.

Muma, J.B., Lund, A., Nielsen, K., Matope, G., Munyeme, M., Mwacalimba, K., Skjerve, E., 2009. Effectiveness of rose bengal test and fluorescence polarization assay in the diagnosis of *Brucella* spp. infections in free range cattle reared in endemic areas in Zambia. Trop. Anim. Health Prod. 41, 723–729.

NAVS, 2013. Brucellosis in Animals: Strategies for Prevention and Control. Policy Paper No. 1. National Academy of Veterinary Sciences (India), New Delhi (16 pp.).

OIE, 2014. World Organization for Animal Health-Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. (Chapter 1.1.5. and Chapter 2.4.3).

Poester, F.P., Samartino, L.M., Santos, R.L., 2013. Pathogenesis and pathobiology of brucellosis in livestock. Rev. Sci. Tech. 32, 105–115.

Shome, R., Gangadar, N.L., Rao, N.K., Shome, B.R., Prabhudas, K., 2011. Diagnosis of brucellosis in the equines by serological tests and PCR: a clinical report. Indian J. Anim. Sci. 81, 137–139.

Shome, R., Padmashree, B.S., Triveni, K., Sahay, S., Krithiga, N., Shome, B.R., Rahman, H., 2015. Spatial distribution of brucellosis in small ruminants of India using indigenously developed ELISA kit. J. Pure Appl. Microbiol. 9, 2285–2291.

Singh, B.B., Dhand, N.K., Gill, J.P.S., 2015. Economic losses occurring due to brucellosis in Indian livestock populations. Prev. Vet. Med. 119, 211–215.

Smits, H.L., Kadri, S.M., 2005. Brucellosis in India: a deceptive infectious disease. Indian J. Med. Res. 122, 375–384.

Smits, H.L., Abdoel, T.H., Solera, J., Clavijo, E., Diaz, R., 2003. Immunochromatographic *Brucella*-specific immunoglobulin M and G lateral flow assays for rapid sero diagnosis of human brucellosis. Clin. Diagn. Lab. Immunol. 10, 1141–1146.

Sturenburg, E., Junker, R., 2009. Point-of-Care testing in microbiology-the advantages and disadvantages of immunochromatographic test strips. Dtsch Arztebl Int 106, 48–54.

Thrusfield, M., 2007. Diagnostic testing. In: Thrusfield, M. (Ed.), Veterinary Epidemiology, 3rd ed. Blackwell Publishing, Oxford, U K (ISBN0632048514).

World Health Organization (WHO), 2010. The control of neglected zoonotic diseases. In: Report of the 3rd WHO Conference on the Control of Neglected Zoonotic Diseases, Community-based Interventions for Prevention and Control of Neglected Zoonotic Diseases, Geneva. WHO, Geneva Available at: www.who.int/neglected_diseases/zoonoses/en/ (23–24 November).

Zeytinoglu, A., Turhan, A., Altuglu, I., Bilgic, A., Abdoel, T.H., Smits, H.L., 2006. Comparison of *Brucella* immunoglobulin M and G flow assays with serum agglutination and 2-mercaptoethanol tests in the diagnosis of brucellosis. Clin. Chem. Lab. Med. 44, 180–184.