Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.105

RESEARCH ARTICLE

Comparative Diagnostic Efficacy of Commonly used Serological Assays for Brucellosis

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ARTICLE HISTORY (23-388)

Received: August 30, 2023
Revised: November 21, 2023
Accepted: November 24, 2023
Published online: December 09, 2023

Key words:
Brucellosis
Egypt
Cattle
Zoonoses
ELISA

A B S T R A C T

Brucellosis is a popular, zoonotic disease that has detrimental implications on human, animal, and economic health. This study compared the efficiency and effectiveness of various serological tests for the seroprevalence of brucellosis in cattle in four Egyptian governorates, including the buffered acidified plate antigen (BAPA) assay, Rose Bengal Plate Test (RBPT), indirect enzyme-linked immunosorbent assay (i-ELISA), and competitive enzyme-linked immunosorbent assay (c-ELISA). A total of 240 sera samples were collected from asymptomatic cattle from Cairo (n = 58), Ismailia (n = 62), Beni Suif (n = 60), and Fayom (n = 60) in Egypt. According to PABA, RBT, i-ELISA, and c-ELISA assay results, the overall prevalence of brucellosis was 141 (58.75%), 141 (58.75%), 169 (70.4%), and 160 (66.66%), respectively. i-ELISA as the gold standard: the sensitivity, specificity, and accuracy of RBT were evaluated as 99, 70 and 59%, respectively with a 95% confidence interval (CI) of 95 to 100%, 60 to 79% and 52 to 65%. While considering c-ELISA as the gold standard: the sensitivity, specificity, and accuracy of RBT were evaluated as 100, 81 and 59%, respectively with a 95% CI of 97 to 100%, 72 to 88% and 52 to 65%, respectively. In conclusion, both ELISA tests were proven to be superior serological tests than PABA and RBPT, and they may be suggested for use in Egypt in screening cattle for brucellosis. A national campaign to control and prevent brucellosis should also get underway to lower its incidence. To do this, veterinary practitioners and cattle owners should exert more effort to inform the public about the economic effects and zoonotic potential of the disease.

To Cite This Article: Ahmed EM, Naguib D, Mazeed AM, Ahmed AE and El-tarabili RM, 2023. Comparative diagnostic efficacy of commonly used serological assays for brucellosis. Pak Vet J, 43(4): 665-670. http://dx.doi.org/10.29261/pakvetj/2023.105

INTRODUCTION

Brucellosis is a widespread neglected zoonotic disease that impacts veterinary, economic, and public health issues (Hassan *et al.,* 2020). It is a contagious disease of ruminant livestock, swine, rodents, canine, equine, and humans caused by *Brucella* spp. Brucellosis is a severe public health problem in Sub-Saharan Africa, resulting in enormous economic losses estimated at approximately 427 million USD annually (Mangen *et al.,* 2002). Brucellosis continuously represents a public health issue in the Egyptian population (Samaha *et al.,* 2009). The annual occurrence of Egyptian human brucellosis has recorded an increase ranging from 0.5 to 70 cases for each 100.000 population from 1994 to 2003 (Refai, 2002; Jennings *et al.,* 2007). Humans are infected either through direct contact with infected animals or by consuming unhygienic foods, particularly non-pasteurized dairy products (Pal *et al*., 2017; Hull and Schumaker, 2018). *Brucella* is a Gram-negative, facultative intracellular bacterium. Brucellaceae family within the Alpha proteobacteria class includes twelve species within the genus *Brucella* are recognized, four of which are zoonotic as *Brucella abortus* causes infection in cattle and

buffaloes, *Brucella melitensis* in sheep and goats, and *Brucella suis* in pigs. and *Brucella canis* in dogs (Khan *et al*., 2020a).

The major manifestations of brucellosis include reproductive failures such as abortion or congenital disability and infertility (Radostits *et al.,* 2010). The subregion includes occasional brucellosis cases up to 41% in some regions (Bayemi *et al.,* 2009; Scolamacchia *et al*., 2010; Mazeri *et al.,* 2013). Several *Brucella* infections remain undetected in many Sub-Saharan countries due to a lack of surveillance (Ladbury *et al.,* 2017). Consequently, the disease is neglected, posing a significant public health threat (Akakpo *et al.,* 2009). Abortion is the predominant clinical characteristic of this illness, thus clinical diagnosis undiscriminating to all age groups, especially non-pregnant heifers and male cattle, and some symptoms are absent, making it difficult to distinguish brucellosis from other febrile diseases. Therefore, an ultimate diagnosis must be reinforced by other laboratory tests (Manishimwe *et al*., 2015; Zakaria *et al.,* 2018). The isolation of the organism confirms infection, but bacteriological testing is hazardous and costly.

Serological animal testing is essential to Brucellosis surveillance and eradication (Nielsen, 2002; Dadar *et al*., 2021). Serological testing supports epidemiology and diagnosis. However, it is essential to note that a conclusive serological test has not yet been developed. Serological tests now lack the ability to yield positive results for sera obtained from different stages of infection, and there is presently no single serological test capable of accurately identifying every stage of the disease. Therefore, the utilization of both screening and confirmatory testing methods is typically more accurate in determining the infection status (Nielsen, 2002; McGiven *et al.,* 2003; Gall and Nielsen, 2004; Poester *et al*., 2010). However, it is possible to evaluate the diagnostic efficiency and discriminatory abilities of a test through an analytical difference appraisal of the sensitivity and specificity of different tests (Nielsen and Gall, 1994).

Brucellosis seroepidemiology is currently carried out using some serological tests as Rose Bengal Card Test (RBT) (Pfukenyi *et al*., 2020), Serum Agglutination Test (SAT), *Brucella* Antibody Test, complement fixation test, indirect Hemolysis Test and ELISA (Gall and Nielsen, 2004). The World Organization for Animal Health (OIE) has endorsed an indirect ELISA (i-ELISA) and competitive ELISA (c-ELISA) for the evaluation of serum and milk (Gall and Nielsen, 2004). RBPT is one of the essential tests mainly used in domestic animals as a screening test for brucellosis detection in sub-Saharan Africa, including countries such as Egypt; because of its low cost and simplicity, The RBPT is commonly regarded as having lower sensitivity compared to alternative tests (Mangen *et al.,* 2002). i-ELISA has proved to be an extremely sensitive and effective method for large-scale bovine brucellosis screening (García-Bocanegra *et al.,* 2014; O'Grady *et al.,* 2014). Although i-ELISA is a highly sensitive test of dairy herds, Commercial kits for routine screening in developing countries are expensive for routine screening in countries, which is considered a big issue. The c-ELISA has been demonstrated to eradicate some but not all FPSR produced by cross-reacting microorganisms (Praud *et al.,* 2012). In some circumstances, FPSR can be detected in c-ELISA but not in other S-LPS-based assays in ruminants or pigs. Due to variations in sensitivity and specificity among different procedures of c-ELISA, the comparability of results from many assays may be impaired. The c-ELISA employed for the detection of anti-*Brucella* antibodies in small ruminants and pigs is fundamentally identical to the c-ELISA utilized for the diagnosis of anti-*Brucella* antibodies in cattle. ELISA has been reported to be an effective screening test, whether employed independently or in combination with the RBPT (Gall and Nielsen, 2004; Legesse *et al.,* 2023). In recent years, innovative molecular methodologies, including polymerase chain reaction (PCR) and gene sequencing, have emerged as valuable tools to identify *Brucella* DNA in body fluids containing a limited quantity or non-viable *Brucella* microorganisms (Neha *et al*., 2014; Ciftci *et al.,* 2017). Indirect-ELISA demonstrates a significant advantage when compared to alternative serological techniques employed for the diagnosis of brucellosis within an endemic geographic area (Ciftci *et al.,* 2017; Saadat *et al.,* 2017). Hence, the current investigation was intended to evaluate and compare the efficacy and efficacy of serological techniques such as Rose Bengal Plate Test (RBPT), indirect enzyme-linked immunosorbent assay (i-ELISA) and competitive enzyme-linked immunosorbent assay (c-ELISA) tests used for the detection of brucellosis in Egypt. This study is one type of Egypt's evaluation of diagnostic tests for brucellosis that has significant implications for disease monitoring and long-term control strategies.

MATERIALS AND METHODS

Ethical approval: Our research approach was carried out in accordance with the OIE Diagnostic Tests and Vaccine of Terrestrial Animals standards for animal care and usage. The owners of the farms gave their consent before any blood samples were collected. Samples were securely transported to the laboratories, and the work was conducted following OIE guidance in *Brucella* diagnosis, biosafety measures, and testing quality standards.

Study area and samples: A total of 240 sera samples were collected from cattle in four Egyptian provinces (Cairo (n = 58), Ismailia (n = 62), Beni Suif (n = 60), and Fayom (n = 60) obtained during (2020–2021) to investigate brucellosis prevalence in asymptomatic animals with comparative analysis of diagnostic efficiency of serological tests mostly used for brucellosis detection either in the field or veterinary laboratories in Egypt. Aseptically collecting 10 ml of blood from cows' jugular veins with a jack and labeled evacuated test tube, the samples were left at room temperature for 2 hours before being transported to the lab for serum separation by centrifugation at 3000 rpm for 10 min and storage at -20°C.

Antigens and controls: Buffered acidified plate antigen (a crystal violet, brilliant green stained, killed *Brucella abortus* strain 99 antigen, at a concentration of 11% in

lactate buffer, pH 3.7±0.03), Rose Bengal antigen (Rose Bengal stained, killed *Brucella abortus* strain 99 antigen in lactate buffer, pH 3.65±0.05) from Veterinary Sera and Vaccine Research Institute, VSVRI, Abasia, Cairo, Egypt, positive and negative controls for the i-ELISA commercial available kit (ID Screen® Brucellosis Serum Indirect Multi-species kit) and c-ELISA commercial available ELISA kits (SVANOVIR Brucella- Ab C-ELISA) were supplied by with the kits by the manufacture.

Serological tests: All sera were screened for antibodies against *Brucella* by BPAT as previously mentioned (Pfukenyi *et al*., 2020) and RBT as previously recorded (Dadar *et al*., 2021), i-ELISA, and c-ELISA according to the manufacturer instructions manual for testing, results calculation, and interpretation.

Statistical investigation tests: All statistical investigation tests were implemented by using R-software (version 4.0.2; https//www.r-project.org/) and Graph pad prism software (version 8; San Diego, CA, USA).

RESULTS

Prevalence of *Brucella*: A total of 240 serum samples were collected (either officially brucellosis-free or not brucellosis-free) obtained from four governates (Cairo, Ismailia, Beni Suif, and Fyoum), in Egypt. All serum samples were blinded and evaluated for *Brucella* infection using four different assays: PABA, RBT, i-ELISA, and c-ELISA. The prevalence of brucellosis varied as exhibited in (Table 1). The whole prevalence of brucellosis was 141 (58.75%), 141 (58.75%), 169 (70.4%), and 160 (66.66%) as determined by PABA, RBT, i-ELISA, c-ELISA assays, respectively as exhibited in Table (1). Statistically, By ANOVA, there is a substantial difference between brucellosis serological assays (i-ELISA and both RBT and PABA), between (c-ELISA and both RBT and PABA), while no significant difference between (RBT and PABA) and (i-ELISA and c-ELISA) (Fig. 1).

Comparison of sensitivity and specificity of serological tests: This study evaluated 240 serum samples from several serological tests, including positive values and percentages for each test and animal (Table 2). By ANOVA, there is a substantial difference of brucellosis serological assays between Beni Suif and other governorates (Fig. 2).

Comparison of RBT and PABA test to i-ELISA: From (n=169) positive i-ELISA up to (n=141) were positive and (n=19) samples were negative to both RBT and PABA respectively as exhibited in (Table 3). i-ELISA is considered statistically as the gold standard: the sensitivity, specificity, and accuracy of RBT were evaluated as 99, 70, and 59%, respectively with a 95% confidence interval (CI) of (95 to 100%), (60 to 79%) and (52 to 65%). Moreover, the positive likelihood ratio (PLR) for the RBT test was 3.25, while the negative likelihood ratio (NLR) was evaluated as 0.02. For the RPT and PABA, a positive predictive value (PPV) of 82% was evaluated, while a negative predictive value (NPV) was evaluated as 97% as exhibited in Table 4.

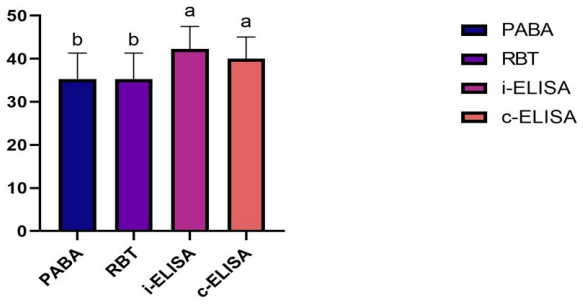

Fig. 1: Comparison of diagnostic test of brucellosis in asymptomatic animals.

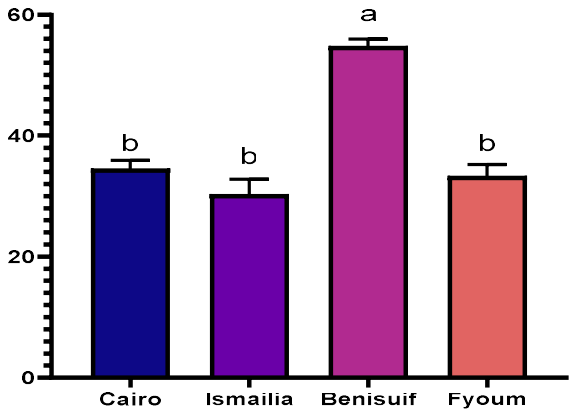

Fig 2: Comparison of brucellosis in asymptomatic animals per test used for detection among different governates.

Table 1: Prevalence of brucellosis in asymptomatic animals by number and percentage of positive results per test used for detection.

Sample (N)	RBT	PABA	i-ELISA	c-ELISA
240	141(58.75%)	141(58.75%)	169 (70.4%)	160 (66.66%)

Table 2: Prevalence of brucellosis in asymptomatic animals by number and percentage of positive results per test used for detection among different governorates.

Serological test	Cairo	Ismailia	Beni Suif	Fyoum
PABA	32 (55.2%)	26 (41.9%)	53 (88.3%)	30 (50%)
RBT	32 (55.2%)	26 (41.9%)	53 (88.3%)	30 (50%)
i-ELISA	37 (63.8%)	36 (58.1%)	58 (96.6)	38 (63.3)
c-ELISA	37 (63.8%)	33 (53.2%)	55 (91.7%)	35 (58.3%)

Table 3: Raw data obtained from RBT, PABA, and i-ELISA where true positives = (a), true negatives= (d), false positives= (b), and false negatives= (c)

i-ELISA	RBT and PABA		
	Positive	Negative	Total
Positive	(a) 139	(b) 30	(a+b) 169
Negative	(c) 2	(d) 69	(c+d) 71
Total	(a+c) 141	(b+d) 99	240

Comparison of RBT and PABA test to c-ELISA: From (n=160) positive c-ELISA up to (n=141) were positive and (n=19) samples were negative to both RBT and PABA respectively as exhibited in (Table 5). c-ELISA is considered statistically as the gold standard: the sensitivity, specificity, and accuracy of RBT were evaluated as 100, 81 and 59%, respectively with a 95% (CI) of 97 to 100%, 72 to 88% and 52 to 65%. Moreover, the PLR for the RBT test was 5.21, while the NLR was evaluated as 0.00. For RBT and PABA, a positive predictive value (PPV) of 88% was evaluated, while NPV was evaluated as 100% as exhibited in Table 6.

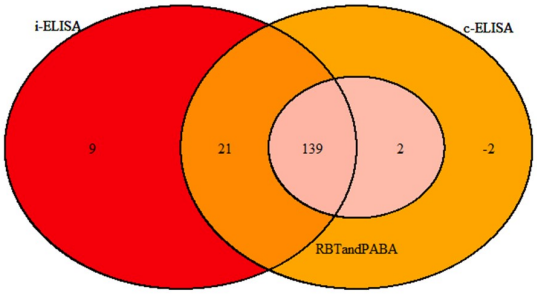

Fig. 3: Venn diagram showing the agreement of positive results of all serological tests.

Table 4: Statistical data of RBT and PABT for diagnosis of bovine brucellosis in comparison to i-ELISA in a 95% confidence interval (CI).

Statistic parameter	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	99%	95% to 100%
Specificity	$\frac{d}{c+d}$	70%	60% to 79%
Positive Likelihood Ratio	$\frac{Sensitivity}{1-Specificity}$	3.25	2.41 to 4.39
Negative Likelihood Ratio	$\frac{1-Sensitivity}{Specificity}$	0.02	0.01 to 0.08
Brucellosis prevalence	$\frac{a+b}{a+b+c+d}$	70%	64% to76 %
Positive Predictive Value	$\frac{a}{a+c}$	82%	76% to 88%
Negative Predictive Value	$\frac{d}{b+d}$	97%	90% to 100%
Accuracy	$\frac{a+d}{a+b+c+d}$	59%	52% to %65

Table 5: Raw data obtained from RBT, PABA, and c-ELISA where true positives = (a), true negatives= (d), false positives= (b), and false negatives= (c).

c-ELISA	RBT and PABA		
	Positive	Negative	Total
Positive	(a) 141	(b) 19	(a+b) 160
Negative	(c) 0	(d) 80	(c+d) 80
Total	(a+c) 141	(b+d) 99	240

Table 6: Statistical data of RBT and PABT for diagnosis of bovine brucellosis in comparison to c-ELISA in a 95% confidence interval (CI).

Statistic parameter	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	100%	97% to 100%
Specificity	$\frac{d}{c+d}$	81%	72% to 88%
Positive Likelihood Ratio	$\frac{Sensitivity}{1-Specificity}$	5.21	3.48 to 7.81
Negative Likelihood Ratio	$\frac{1-Sensitivity}{Specificity}$	0.00	0
Brucellosis prevalence	$\frac{a+b}{a+b+c+d}$	67%	60% to73 %
Positive Predictive Value	$\frac{a}{a+c}$	88%	82% to 93%
Negative Predictive Value	$\frac{d}{b+d}$	100%	95% to 100%
Accuracy	$\frac{a+d}{a+b+c+d}$	59%	52% to %65

Statistical Analysis: Comparing serological test findings for positive results, the Venn diagram illustrates that 2, -2, and 9 were detected as positives by RBT and PABA, i-ELISA, and c-ELISA only (Fig. 3). All serological assays confirmed 141 positive animals. Indirect ELISA and c-ELISA had 21 positive results (Fig. 3).

DISCUSSION

The relationship between livestock and humans in low and middle-income countries, consisting of Egypt is inextricably linked, which directed the attention of the United Nations to the important part of livestock in the livelihood of persons from these settings (Upton, 2004). This relationship represents high risks for those populations because of nigh closeness, low hygiene resource access, habits of food consumption related to culture, and high incidence of zoonotic diseases (Penakalapati *et al.,* 2017). The public health issues of brucellosis in these countries are attributed to it is epidemic in animals, as emphasized by the World Health Organization (Godfroid *et al.,* 2005). So, any prospective strategies for improving cost-efficient public health management should include knowledge of disease prevalence and its epidemiology. The seroprevalence of brucellosis in healthy cattle in four provinces in Egypt was assessed.

In this study, the seroprevalence of 240 cattle sera was 141 (58.8%), 141 (58.8%), 169 (70.4%), and 160 (66.7%) as determined by PABA, RBT, i-ELISA, c-ELISA assays, respectively. PABA and RBT gave a lower incidence of seroprevalence compared to the two ELISA. This might be ascribed to the ability of the acidic pH of antigen to inhibit the non-specific agglutinins (Oomen and Waghela, 1974). Similar to our results, the seroprevalence of brucellosis in cattle in the Nile Delta, Egypt was 67.9% by ELISA and 53.7% by RBT (El-Ashker *et al.,* 2015). Our findings were in accordance with another study that recorded that ELISA could be greater specific and of considerable sensitivity than RBT and could be a propitiate test for extensive surveillance of bovine brucellosis (Gall and Nielsen, 2004). Moreover, our results agree with that of Konstantinidis *et al.* (2007) who reported an equal extent of sensitivity between ELISA and RBT. Contrary to our results, the seroprevalence of brucellosis in cattle in the Menufiya governorate of Egypt was 11%. In another study, the seroprevalence of cattle in Gujarat, India, was 21.67% and 14.55% by RBPT and i-ELISA, respectively (Shrimali *et al.,* 2019). It was known that the ELISA for detecting brucellosis was more effective as compared to traditional tests like RBT and complement fixation test (CFT) (El-Razik *et al.,* 2007). The high incidence of seropositive in cattle in this study might be due to either the association of *Brucella* LPS with that of other microorganisms, such as *Yersinia enterocolitica* O: 9 and *E. coli* serotype O: 157, or inclusion of high-hazard cattle groups in this study. This may be attributed to the spread of the disease from infected animals to healthy ones (Khan and Zahoor, 2018; Ghobrial *et al.,* 2023).

In our study, the difference in the seroprevalence of brucellosis determined by using the four tests among four governments was non-significant. Small sample sizes in each governorate might be responsible for these insignificant differences. The nearly similar distribution of seroprevalence of *Brucella* in all governorates might indicate the locative allocation of the disease and the possibility of sustaining the disease within and between governorates that might result from contact between animals and increased densities. A previous study

mentioned that the combining of livestock at grazing and watering locations posed a disease transmission threat *(*Shirima and Kunda, 2016).

Multiple indicators can be employed to assess the diagnostic efficacy of serological tests, encompassing sensitivity and specificity, positive and negative predictive values, as well as diagnostic accuracy (Simundic, 2009). From our study, we found that the sensitivity, specificity, and accuracy of RBT were 99, 70, and 59%, respectively, as compared with the gold standard test i-ELISA for seroprevalence of brucellosis in cattle. In contrast to our results, RBT was found to have more performance in sensitivity, specificity, and accuracy than ELISA (Zakaria *et al.,* 2018). Interestingly, different reports indicate that there is variation in the sensitivity of RBT which ranges from 68.6-100% (Ahmed *et al.,* 2016). A test with a convenient PPV is substantial for guaranteeing the existence of the disease, besides a test with NPV value to eliminate the infection (Chachra *et al.,* 2009). For the RPT and PABA in our work, the PPV was 82%, while the NPV was 97%.

In our study, 21 samples tested positive for i-ELISA and c-ELISA but negative for RBT. This might be attributed to either more sensitivity of i-ELISA due to the application of cytosolic S-LPS fragments, thus diminishing an association with other Gram-negative bacteria, or prozoning phenomena that are frequently observed in acidified antigens in RBT (Meena *et al.,* 2023). For many years, ELISA has been esteemed for its superior sensitivity to discovering anti-*Brucella* antibodies in all species. Various reports indicated that either i-ELISA or c-ELISA is more sensitive than agglutination tests (Nielsen *et al.,* 2004). Since it can detect antibodies directly and yields fewer false positives than the i-ELISA and traditional tests, the c-ELISA is a highly sensitive and specific diagnostic tool (Nielsen *et al.,* 2004). These findings contrast with our results where i-ELISA was greater sensitive than c-ELISA. In agreement with our findings, the sensitivity of i-ELISA was higher than c-ELISA for sero-prevalence of camel brucellosis in Egypt (Khan *et al.,* 2020b).

Conclusions: Both ELISA tests were found to be better serological tests when compared with RBPT and PABA, and they could be recommended for screening cattle brucellosis in Egypt. Further studies will be needed for evaluating serum samples from positive bacteriology animals. Besides this, programs of control and prevention should be begun everywhere in-country for diminishing the incidence of brucellosis. To do that, an increased education effort about the risk factors of disease, economic, and zoonotic significance of the disease should be announced especially in areas of high risk, among veterinary practitioners and livestock owners of livestock.

Authors contribution: EMA, DN, RME designed the experiment. EMA, DN, AMM, AEA, and RME conducted research and collected the data. EMA, DN, AMM, AEA, and RME analyzed the data and finalized the write-up of this manuscript. All authors approved and finalized the manuscript.

Acknowledgments: The authors are grateful to the deanship of scientific research at King Khalid University for supporting this work under the grant number (R.G.P2/326/44).

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