Research Article

Sensitivity and Specificity of the commonly used Diagnostic Procedures of Bovine Brucellosis

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Abstract | The present study was carried out for investigation of the sensitivity and specificity of some diagnostic procedures used for diagnosis of bovine brucellosis on serological, bacteriological and molecular basis. A total of 141 cows from brucella infected farms under quarantine of the veterinary authorities were employed. Serological examination using BPAT, RBT and CFT in 141 cows revealed 109 (77. 3), 105 (74.47) and 104 (73.76) seropositive respectively. Relative sensitivity, relative specificity, positive predictive value and negative predictive value of BPAT, RBT and CFT were estimated as, (98.04%, 76.92%, 91.74% and 93.75%); (94.33%, 85.71%, 95.24% and 83.33%) and (93.46%, 88.23%, 96.15% and 88. 24%) respectively. Different tissues specimens of 104 confirmed seropositive cows under investigation including, retropharyngeal, prescapular, prefemoral, internal iliac, supramammary lymph nodes, udder and spleen as well as milk of 46 lactating cows were subjected for bacteriological studies for isolation and identification of Brucella organisms. *Brucella melitensis biovar 3* could be recovered from 64 (61.5%) tissue specimens and 28 (60.9%) milk samples. Brucella cultures were further identified on molecular basis using universal and Bruce ladder PCR.

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Introduction

Brucellosis is a highly contagious zoonotic disease of animals and humans, caused by bacteria of the genus Brucella that presently involving 11 Brucella species, (Whatmore et al., 2015). Brucella is an intracellular facultative gram- negative bacterium survives and multiplies in phagocytic cells, (Godfroid et al., 2005; Nicolletti and Tanya, 1993). As soon as brucellosis is transmitted to livestock, it causes severe economic losses (Holt et al., 2011) due to abortion, stillbirth, mastitis, metritis and placental retention in females and orchitis in males. Also the disease represents a major public health concern as a zoonotic disease (Jung et al., 2010; OIE, 2016a).

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (OIE), brucellosis remains one of the most widespread zoonoses, (Lopes et al., 2010; Schelling et al., 2003).Annually, there are about 500,000 new human cases of brucellosis reported wide, (Seleem et al., 2010). Moreover, the WHO (2006) considers brucellosis as a neglected zoonosis because it is not ranked by national and international health systems as well as the lack of public awareness (WHO, 2009).

Diagnosis of brucellosis depends upon detection of

Brucella spp. and demonstration of specific antibody or cell-mediated immune responses. Serological tests are decisive for laboratory diagnosis of brucellosis since control and eradication programs counts on these tests. Usually, the minimum requirement for diagnosis of brucellosis is a combination of a quick screening test and a confirmatory test.

The value of a diagnostic test depends on a combination of sensitivity and selectivity tasks. Sensitivity and Specificity are evaluated against a reference standard test referred to as a 'Gold Standard'.

The most widely used methods of diagnosis are based on serology. Serological tests are however liable to false-positive results due to other cross-reacting bacteria, and are not beneficial in the detection of rough Brucellae (Kaltungo et al., 2014).

Bovine Brucellosis is usually caused by *B. abortus* biovars and occasionally by *B. melitensis***.** In Egypt, *Brucella melitensis* biovar 3 is considered the predominant Brucella type recovered from animals and humans (Refai, 2002; Menshawy et al., 2014). The isolation of Brucella is absolute evidence that the animal is infected; however, not all infected animals give a positive culture. Detection of antibody is the most practical and economic tool of diagnosis (WHO, 2006).

The overall objective of this study was to evaluate the commonly used diagnostic procedures and estimation of their sensitivities, specificities, and positive and negative predictive values in order to provide information for appropriate control strategies.

Materials and Methods

Animals and clinical samples

Blood sera were collected from 141 cows from brucella infected farms during the test and slaughter national program of brucellosis applied by the Egyptian veterinary authorities. Blood serawere employed for estimation of relative sensitivity, relative specificity, positive predictive value and negative predictive value of BPAT, RBT and CFT. Different tissues specimens of 104 confirmed seropositive cows including retropharyngeal, prescapular, prefemoral, internal iliac and supramammary lymph nodes, udder and spleen as well as milk samples of 46 sero-positive cows were used for isolation and identification of brucella organisms.

Serological examination

Buffered plate antigen test (BPAT) using Buffered acidified plate test antigen (killed Brucella abortus strain 99 antigen, at concentration of 11% in lactate buffer, pH 3.7±0.03), Rose Bengal test (RBT) using Rose Bengal test antigen (Rose Bengal stained, 8% cells killed Brucella abortus strain 99 antigen in lactate buffer, pH 3.65±0.05) and Complement fixation test, Warm micro technique (CFT), using complement fixation test antigen (Brucella abortus biovar 1 strain 1119-3 cells in phenol saline, at a concentration of 4.5%, pH 6.8) were conducted according to Alton et al. (1988) and OIE (2016a).

Estimation of relative sensitivity and specificity: sensitivity, specificity, Positive predictive value and Negative predictive value of the test under evaluation were calculated according to Parikh et al., (2008) from the following equations.

Sensitivity= True positive / True positive + false negative x 100

Specificity= True negative / True negative + false positive x 100

Positive predictive value= True positive / True positive + false positive x 100

Negative predictive value= True negative/True negative + false negative x 100

Where;

True positive or negative reactions are those confirmed as being positive or negative by other two or more tests. False positive or negative reactions are those confirmed as being positive or negative by other one or non-tests.

Bacteriological examination: Tissue homogenates and milk cream-sediment mixture were cultured on tryptose agar medium with antibiotics selective antibiotic supplement (Ewalt et al., 1983), (Oxoid) according to Alton et al. (1988). Plates were incubated at 37 ˚C in an atmosphere of 10% CO2 and examined daily for 10 days for growth. Isolates were identified as *Brucella* according to the methods described by Alton et al. (1988), Ewalt et al. (2001), OIE (2016a).

DNA extraction from brucella cultures: Few colonies were harvested and suspended in 200 µl of sterile, DNase, RNase-free deionized water. Bacterial cells were inactivated by heating the tubes at 100˚C for 10 minutes. Killed bacterial cells were centrifuged at 15,

700 × g for 10 minutes. The supernatant containing crude DNA template was pipetted into new sterile Eppendorf tubes and the sediment was discarded.

Table 1: *Primer sets for universal PCR.*

Primer	Sequence (5'–3')	Amplicon size (bp)
BMEI0535f BMEI0535r	GCG-CAT-TCT-TCG-GTT-ATG-AA CGC-AGG-CGA-AAA-CAG-CTA-TAA	450

PCR: Universal PCR was performed for molecular identification of *Brucella* in DNA extracts from *Brucella* cultures, at the genus level according to the procedures of Bricker (2002), using PCR master mix (Jena bioscience Gmbh, Germany) in a total volume 25µl/reaction. The PCR primers were developed for Amplification of target gene (Immunodominant antigen, gene *bp26),* (Table 1). Bruce ladder multiplex PCR) was carried out for molecular identification of *Brucella* in DNA extracts from *Brucella* cultures at the species level according to Garcia-Yoldi et al. (2006) using INgene Bruce ladder (INgene Bruce ladder VR: Batch No 180515, Ingenasa, Madrid, Spain). The PCR amplicons were analyzed by running 10 µl of the PCR products in 1% agarose gel stained with ethidium bromide (0.5µg/ml). Thereafter, gels were photographed under UV illumination using gel documentation and analysis system.

Table 2: *Results of serological tests.*

No. of examined animals	Serological examination		
	BPAT	RBT	CFT
141	109 (77. 3%)	105 (74.47%)	104 (73.76%)

Table 3: *Relative sensitivity and specificity and positive and negative predictive values of BPAT, RBT and CFT in cattle.*

No. of animals 141	BPAT	RBT	CFT
100	+	+	+
3	+	+	-
2	+	-	+
4	+	-	-
2	-	+	+
30	-	-	-
Relative sensitivity	98.04 %	94.33 %	93.46%
Relative specificity	76.92%	85.71 %	88.23 %
Positive predictive value	91.74%	95.24%	96.15 %
Negative predictive value	93.75 %	83.33%	88. 24 %

Results and Discussions

1. Serological examination using BPAT, RBT and CFT in 141 cowsrevealed 109 (77. 3), 105 (74.47) and 104 (73.76) seropositive respectively (Table2).
2. Relative sensitivity, relative specificity, positive predictive value and negative predictive value of BPAT were estimated as, 98.04%, 76.92%, 91.74% and 93.75% respectively, while for RBT, relative sensitivity, relative specificity, positive predictive value and negative predictive value were estimated as 94.33%, 85.71%, 95.24% and 83.33% respectively. Concerning CFT, relative sensitivity, relative specificity, positive predictive value and negative predictive value were estimated as 93.46%, 88.23%, 96.15% and 88. 24% respectively (Table 3).
3. Estimated true positives were 100 (21. 28%) and estimated true negatives were 30 (70.92%) for the three employed tests. Estimated false positives of BAPT, RBT and CFT were 9 cases (6.38%), 5 cases (3.54%) and 4 cases (2.83%) respectively. While false negatives were 2 cases (1.42%), 6 cases (4.26%) and 7 cases (4.96%) respectively.
4. Bacteriological examination of tissue specimens of 104 cows and 46 milk samples of sero-positive cows revealed isolation of 64 (61.5%) and 28 (60.9) brucella isolates respectively that were identified as *Brucella melitensis* biovar 3, on bacteriological basis, Tables 5 and 6 and molecular basis (Figure 1).
5. Agreement between Brucella isolation from clinical samples (tissue specimens and milk) and serological status, Table 4 showed that64 (61.5%) and 28 (60.9%) respectively gave positive bacteriological and serological results. Sensitivity of culture technique in tissue specimens and milk was estimated as (61.5%) and (60.9%) respectively. On the other hand bacteriological examinations failed to classify 40 (38.5%) and 18 (39.1) serologically positive cows respectively and were culture negative.
6. Universal PCR in this study confirmed the presence of genetic material of genus Brucella in culture DNA extracts. The assay has amplified the target gene (Immunodominant antigen, gene bp*26)* with amplification of the fragment of 450 bp (Figure 1) characteristic for the Genus *Brucella*. Bruce ladder multiplex PCR detected the presence of genetic material of *Brucella melitensis*

on species level in culture DNA extracts as the test has recognized the three characteristics fragments; 587 bp, 1071 bp and 1682 bp, (Figure 1).

Evaluation of BPAT, RBT and CFT in 141 slaughtered cows in the test and slaughter national program, revealed 109 (77. 3%), 105 (74.47%) and 104 (73.76%) seropositive respectively (Table 2). Considering the CFT as the recommended confirmatory test that should be used following screening tests as recommended by the OIE (2016a), the overall of sero-prevalence of brucellosis is (73.76%). The obtained results indicate that among the employed tests, no test has functioned in all infected animals. This poor performance is a reason that eradication programs is demanding to achieve, Therefore, several procedures are suggested to be used to overcome the problem of evasion of some infected animals from diagnosis.

Table 4: *Agreement between culture technique and serological status.*

Samples	No of cases	Isolation	Serological status
104 tissue specimens	64 (61.5%)	+	+
	40 (38.5%)	-	+
46 Milk samples	28 (60.9%)	+	+
	18 (39,1)	-	+

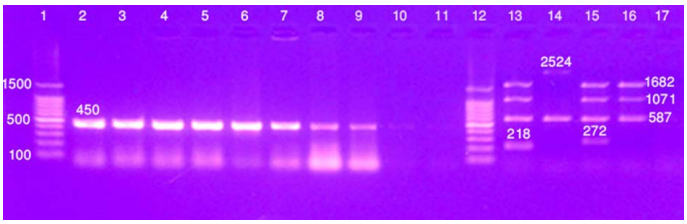

Figure 1: *universal PCR and Bruce ladder assay.*
Lane 1: *100bp DNA ladder;* **Lane 2:** *Brucella melitensis;* **Lane 3:** *RB51;* **Lane 4:** *Rev1;* **Lane 5:** *S19;* **Lane 6 to 11:** *Tissue samples;* **Lane12:***100 bp ladder;* **Lane 13:** *Rev1 (Bruce-ladder kit control);* **Lane 14:** *RB51 (Bruce-ladder kit control);* **Lane15:** *Brucella suis (Bruce-ladder kit control);* **Lane16:** *Brucella isolate;* **Lane17:** *Control negative.*

The performance of a diagnostic test is defined by its sensitivity and the specificity, each relating the ability of the test to reveal the "true" disease status, (Speybroeck et al., 2013). Relative sensitivity, relative specificity, positive predictive value and negative predictive value of BPAT were estimated as, 98.04%, 76.92%, 91.74% and 93.75% respectively, while for RBT, the results revealed 94.33%, 85.71%, 95.24% and 83.33% respectively and concerning CFT, the results revealed 93.46%, 88.2 %, 96.15% and 88.24% respectively (Table 3).

The high number of false positives detected by BPAT, 9 cases (6.38%) and RBT, 5 cases (3.54%) may be attributed to non-specific antibodies. In conclusion, BPAT and RBT revealed the highest rates of sensitivity and lowest rates of specificity 76.92% and 85.71%, respectively, the matter that suggests the use of these tests for screening of animal brucellosis. Such results clarify that the buffered *Brucella* antigen tests, BPAT and RBT have a higher sensitivity but also have a fewer reliable specificity resulting in a reduced number of false negatives and a significant number of false positive. Therefore a confirmatory test with higher specificity is desirable (Nielson, 2002; OIE, 2016a).

The RBT and BPAT use acidified antigens to reduce the binding of IgM antibodies and to encourage the IgG1 binding. Differnet bacteria, in particular Yersinia enterocolitica O:9, may cause false positive serological reactions in brucellosis tests, hindering accurate diagnosis (OIE, 2016a). The RBT is one of a group of tests known as the buffered Brucellaantigen tests which depend on the fact that the capacity of IgM antibodies to bind to antigen is distinctly decreased at a low pH. Therefore the IgG is measured instead and at the buffered pH of 3.65 the test measures only IgG1.The test is an excellent screening test but may be oversensitive for diagnosis in individual animal particularly vaccinated ones (WHO, 2006).

In comparison to other serological tests employed in this study, the CFT proved to have the highest rate of specificity 88.23%, Tables 3 and also showed the least false positives, 4 cases (2.83%) that bearing in mind that the BPAT and RBT positive samples should be confirmed by this test. Al Dahouk et al. (2003) considered that CFT should be used only as a confirmatory test WHO (2006) considered the CFT superior to agglutination methods but its sensitivity and specificity are limited and it should be regarded as a complementary rather than confirmatory test. Finally for the control of brucellosis at the national or local level, the World Organization for Animal Health (OIE) prescribes the use of a buffered *Brucella* antigen tests namely the buffered plate antigen test and the Rose Bengal Test (RBT) as approved screening tests, and complement fixation test as the confirmatory test (OIE, 2016a; OIE, 2016b).

Bacteriological examination of tissue specimens of 10

Table 5: *Phenotypic characteristics of Brucella isolates (Brucella melitensis biovar 3).*

Brucella isolates	CO_2	H_2S	Urease	Growth on dyes				Lysis by Tb phage		Monospecific sera			Conclusion
				Thionin		Fuchsin		RTD	RTD 10^4	A	M	R	
				a	b	a	b						
64 isolates from tissues 28 isolates from milk	-	-	+ in 20 hrs	+	+	+	+	-	-	+	+	-	B. melitensis 3
Reference strains													
B.melitensis Ether	-	-	+ in 18-24 hrs	+	+	+	+	-	-	+	+	-	B. melitensis 3
B.abortus544	-	+	+ in 2 hrs	-	-	+	+	+	+	+	-	-	B. abortus 1
B.Suis1330	-	+++	++ in < 15min.	+	+	-	-	-	+	+	-	-	B. suis 1

RTD: *routine test dilution;* **Tp :** *Tbilisi (Tb);* **a:** *1:50000;* **b:** *1:100000;* **A:** *anti Brucella abortus;* **M:** *anti Brucella melitensis;* **R:** *rough brucella antiserum.*

-4 cows and 46 milk samples of sero-positive cows during the test and slaughter national program applied by the Egyptian veterinary authorities revealed isolation of 64 (61.5%) and 28 (60.9) brucella isolates respectively that were identified as *Brucella melitensis* biovar 3 (Table 5).

Amplification of target gene (Immunodominant antigen, gene *bp26)* usinguniversal PCR confirmed on genus level brucella cultures with amplification of the fragment of 450 bp. (Figure 1) characteristic for the Genus *Brucella*. Bruce ladder multiplex PCR established the genetic material of *Brucella melitensis* on species level in culture DNA extracts as the test has recognized the three characteristics fragments; of 587 bp, 1071 bp and 1682 bp (Figure 1).

Brucella melitensis biovar 3 was considered as the prevalent biovar in Egypt as recorded by Affi et al. (2015), Hosein et al. (2016), Menshawy et al. (2014), Salem and Hosein (1990). Characterization of *Brucella melitensis* from tissues and milk of cattle on bacteriological and molecular basis is of epidemiological importance and clarify the danger for which humans are exposed. *Brucella melitensis*is the type most frequently reported as a cause of human disease and the most virulent type and associated with severe acute disease as reported by the WHO (2006). As a zoonotic disease, brucellosis is almost consistently transmitted by both direct or indirect contact with infected animals and their products. Excretion of brucella in genital discharges and milk of brucella infected animals is common (Jung et al., 2010) and is a major source of human infection that causes a serious ailment in humans especially those contact with infected animals and those consume infected animal products (Marei et al., 2011; Shimol et al., 2012). The results obtained in this study indicated that transmission of Brucella through infected milk is an increasing hazard for humans particularly in enzootic countries as reported by Wareth et al. (2014). Many of the *Brucella* species are highly pathogenic in humans and some species of brucellae are extremely infectious with as few as 10 organisms capable of causing disease in humans (Godfroid et al., 2011) with *B. melitensis* referred to as the agent commonly blamable for human cases (Pappas et al., 2005).

Agreement between Brucella isolation from clinical samples (tissue specimens and milk) and serological status, Table 4 showed that64 (61.5%) and 28 (60.9%) respectively gave positive bacteriological and serological results. On the other hand bacteriological examinations failed to classify 40 (38.5%) and 18 (39.1) serologically positive cows respectively and were culture negative. Such lower sensitivity of culture technique from tissues (61.5%) and milk (60.9%) may be attributed to the fastidious nature of brucella organisms as reported by Alton et al., (1988). False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (Poster et al., 2010). The specificity of a serological test cannot usually be evaluated by bacteriological findings because some animals with negative cultures maybe infected. Reasons for this may be selecting samples from uninfected tissues. Also, failure may occur if the number of viable Brucella organisms in a test sample is low and when the sample is contaminated with other bacteria especially milk samples (Seleem et al., 2010). Intermittent shedding of brucella in milk is another limiting factor, (Wernery et al., 2007). Difficulty of isolation from milk may be the outcome in animals that might not be in active state of shedding of Brucella in their milk (Terzi et al., 2010).

Conclusions and Recommendations

Serology remains the most practicable method for diagnosis of brucellosis and no currently available single test is reliable for the detection of brucellosis at the level of individual animal. BPAT and RBT are strongly recommended for screening purposes and CFT is recommended for confirmation of infection in individual animals. *Brucella melitensis* biovar3 remains the prevalent brucella type among cattle in Egypt and responsible for significant economic losses for animal industry and constitutes hazards to public health.

Conflicts of Interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. All authors read and approved the final manuscript.

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Authors' Contributions

All authors contributed extensively to the work presented in this paper. All authors discussed the results and approved the final manuscript.

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