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## Research Article

### COMPREHENSIVE SEROLOGICAL, BACTERIOLOGICAL, AND MOLECULAR PERSPECTIVE OF BRUCellosIS IN CATTLE AND BUFFALOES IN SOME GOVERNORATES

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#### ABSTRACT

For the aim of validation, 347 known positive and negative serum sample of large ruminants with a history of *Brucella melitensis* infection were selected. The highest relative sensitivity (rSe) was achieved by the buffer acidified plate agglutination test (BAPA). The assessed kappa( $\kappa$ ) agreement in both species indicated a substantial agreement ( $p < 0.05$ ) in case of the BAPA, RBPT, iELISA and rivanol (Riv. T) tests. According to the data obtained from the receiver operating characteristics curves, the area under the ROCs and diagnostic odd ratio, the diagnostic performance of serological tests in cattle was arranged in descending order as follows; BAPAT, Riv.T, RBPT, iELISA, EDTA-modified micro-agglutination test (EDTA-mMAT) and MAT. The equivalent picture in buffaloes was, Riv.T, RBPT, BAPAT, iELISA, EDTA-mMAT and MAT. Eleven *Brucella* field isolates were recovered, whereas four isolates were recognized as *Brucella abortus* biovar 1 from cattle and seven as *Brucella melitensis* biovar 3 from cattle and buffaloes using phenotypic bacteriological typing and molecular speciation (Bruce-ladder PCR). As a result of better diagnostic performance offered by EDTA-mMAT over MAT under investigation, the authors recommended switching from MAT version locally adopted to EDTA-mMAT, and to a limited extent, Riv.T could be used to confirm reactors identified by screening tests. As a result of the frequent isolation of *Brucella melitensis* from the liver of slaughtered seropositive ruminants, it is necessary to amend the ministerial decree No. 1329 of 1999 to contain an explicit clause of liver condemnation as it poses hazards on public health.

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#### INTRODUCTION

Brucellosis is the common name used for the animal and human infections triggered by several species of the genus *Brucella* (OIE, 2016). *Brucellae* show a wide range of host preference. Currently, twelve *Brucella* species exist (Whatmore *et al.*, 2014; Scholz *et al.*, 2016) including three that have been reported in Egypt (Menshawy *et al.*, 2014; Sayour and Sayour, 2015), viz. *B. abortus*, *B. melitensis*, and *B. suis*. *B. melitensis* infection of small ruminants is relatively similar in both pathological and epidemiological viewpoints to *B. abortus* infection of cattle. The main symptoms of brucellosis in ruminants are reproductive disorders in form of abortion or birth of weakoff-springs that do not survive, low milk yield (20-25% reduction), orchitis, epididymitis and less commonly arthritis. *B. melitensis* causes no abortion storms in pregnant cattle. Moreover, brucellosis is famous for its latent infection which hinder any control programmes.

The diagnostic method that offers a conclusive evidence of brucellosis is the isolation and typing of *Brucella* microorganisms from the suspected animal. Yet, this method has an inadequate sensitivity and has in addition a difficulty of being unpractical to apply at a wide scale in control strategies (Gall and Nielsen, 2004).

The detection of specific immunoglobulins to *Brucella* in serum or milk samples still the most practical means of the diagnosis. The most proficient and cost-effective method is usually screening all samples using an inexpensive and rapid test which is sensitive enough to detect a high proportion of infected animals. Reactors to screening tests are then confirmed using standard, accurate and specific tests for the final diagnosis to be made (Corbel, 2006).

Serological results must be interpreted against the circumstantial of disease incidence, the degree of false positive serum reactions due to cross reactions with related Gram-

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negative bacteria or due to vaccination (Gall and Nielsen, 2004; Corbel, 2006).

*Brucella* sp. Infection in buffaloes has almost a similar course to that in cattle and the same serological procedures adopted for cattle may be used for these animals, but each test should be validated for its fitness (OIE, 2016).

In this view, the current research was designed to detect the predominate species and biovars of *Brucella* isolates by conventional bacteriology and by Bruce-Ladder PCR which recovered from different tissue samples of large ruminant as well as, evaluating the diagnostic performance of some serological tests used for the diagnosis of large ruminant brucellosis.

## MATERIALS AND METHODS

### Samples

A total of 347 selected positive and negative serum samples (95% confidence interval; 2% error) were selected from the previously examined samples for the validation of some serological tests used in this research according to the regulations of (OIE, 2013). These samples were selected from examined samples of 1089 cattle and 1135 buffaloes belong to individual animals in small and large herds located at some Nile Delta governorates. These ruminants had a previous history of *B. melitensis* (Abdel-Hamid et al., 2012) infections and late term of abortion.

**Table 1** Animal species, breed, age, population and numbers of Nile Delta ruminants used in this investigation

Species	Breed	Age*	Population	Governorate	Number
Cattle	Hybrid/Friesian	1-4	Small/ large herds	Beheira	500
Cattle	Native	1-3	Individuals/ small herds	Gharbia	589
Buffaloes	Native	1-5	Individuals/ small herds	Sharkia	611
Buffaloes	Native	1-4	Individuals/ small herds	Kafr-Elsheikh	524
Total animals					2224

\* Age in years

Milk and tissue samples (supramammary and retropharyngeal lymph nodes, liver, fetal stomach contents and fetal livers) were collected from live and slaughtered serologically positive animals in some Governorates for the aim of isolation and typing of *Brucella* microorganisms.

### Serological tests

Serum samples were serologically examined against brucellosis using 1. Screening tests; RBT, BAPA and iELISA, 2. Supplementary tests; MAT and EDTA-modified microagglutination tests and 3. Confirmatory tests; CFT and Riv. T.

RBT, BAPA and Rivanol antigens were purchased from (NVSL/DBL, USDA, USA). RB, BAPA tests were performed according to (OIE, 2016). Riv.T was done according to the (Alton et al., 1988). 2.2.3. The Egyptian antigen for MAT and EDTA-mMAT was obtained from the Veterinary Serum and Vaccine Research Institute, Abbasseya, Cairo. Both tests were performed according to Alton et al., (1988).

Antigen for the American CFT was purchased from NVSL/DBL, USDA, USA. Hemolysin and complement were prepared, preserved according to (Alton et al., 1988) and titrated according to (Hennager, 2004). CFT was done according to (Hennager, 2004). Results of CFT were considered as positive at a cutoff point of  $\geq 20$  ICFTU/ml (OIE, 2016).

Bacteriological isolation, identification and typing of *Brucella*: Bacteriological typing at genus (colonial morphology, microscopic appearance, catalase, oxidase and urease), biovar level (CO<sub>2</sub> requirement, H<sub>2</sub>S production, growth on dyes (thionin and basic fuchsin) and agglutination with monospecific sera) was done according to (Alton et al., 1988).

PCR amplification of target genes (Garcia et al, 2006): A standardized PCR assay named Bruce-ladder was performed. Primer pairs (Bioneer, Germany) (Table, 2), designed on the strain-specific genetic differences, and were used in this PCR reaction for isolation of omp31 and eryC genes. The PCR amplification was carried out using GeneAmp<sup>®</sup> PCR system 9700 thermal cycler (Applied Biosystems, USA). PCR assay was done to identify *Brucella* isolates at species level since phage lysis was unfortunately unavailable.

**Table 2** Different specific primers of *Brucella*

Primer	Sequence (5'-3')	Amplicon size (bp)	DNA targets	Source of genetic difference
BMEII0843F	TTT-ACA-CAG-GCA-ATC-CAG-CA	1071	Outer membrane protein, gene <i>omp31</i>	deletion of 25,061 bp in BMEII826- BMEII0850 in <i>B. abortus</i>
BMEII0844R	GCG-TCC-AGT-TGT-TGT-TGA-TG			
BMEII0428F	GCC-GCT-ATT-ATG-TGG-ACT-GG	587	Erythritol catabolism, gene <i>eryC</i> (D-erythrulose-1 phosphate dehydrogenase)	deletion of 702 bp in BMEII0427- BMEII0428 in <i>B. abortus</i> S19
BMEII0428R	AAT-GAC-TTC-ACG-GTC-GTT-CG			
BR0953F	GGA-ACA-CTA-CGC-CAC-CTT-GT	272	ABC transporter binding protein	deletion of 2653 bp in BR0951 BR0955 in <i>B. melitensis</i> and <i>B. abortus</i>
BR0953R	GAT-GGA-GCA-AAC-GCT-GAA-G			
BMEI0752F	CAG-GCA-AAC-CCT-CAG-AAG-C	218	Ribosomal protein S12, gene <i>rpsL</i>	point mutation in BMEI0752 in <i>B. melitensis</i> Rev.1
BMEI0752R	GAT-GTG-GTA-ACG-CAC-ACC-AA			

**Statistical analyses:** All the following analyses were performed using SPSS<sup>®</sup> Statistics, Version 21 under the environment of Windows<sup>®</sup> 10, Microsoft Corporation:

Kappa ( $\kappa$ ) agreement and relative sensitivity/ specificity: The kappa ( $\kappa$ ) agreement of agglutination tests with CFT was used to measure the matching of results at  $p < 0.05$

Receiver operating characteristics (ROC) curves: Considering the CFT as the serological gold standard, ROC curves were plotted for all agglutination tests. Data were obtained from ROC curves including the area under the curve (AUC) representing accuracy and ROCS and AUCS were done according to Hanley and McNeil (1982).

**Diagnostic odds ratio (DOR):** The diagnostic odds ratio was estimated for the serological tests used in the diagnosis large ruminant brucellosis according to (Kraemer, 1992 cited in Glas et al., 2013)

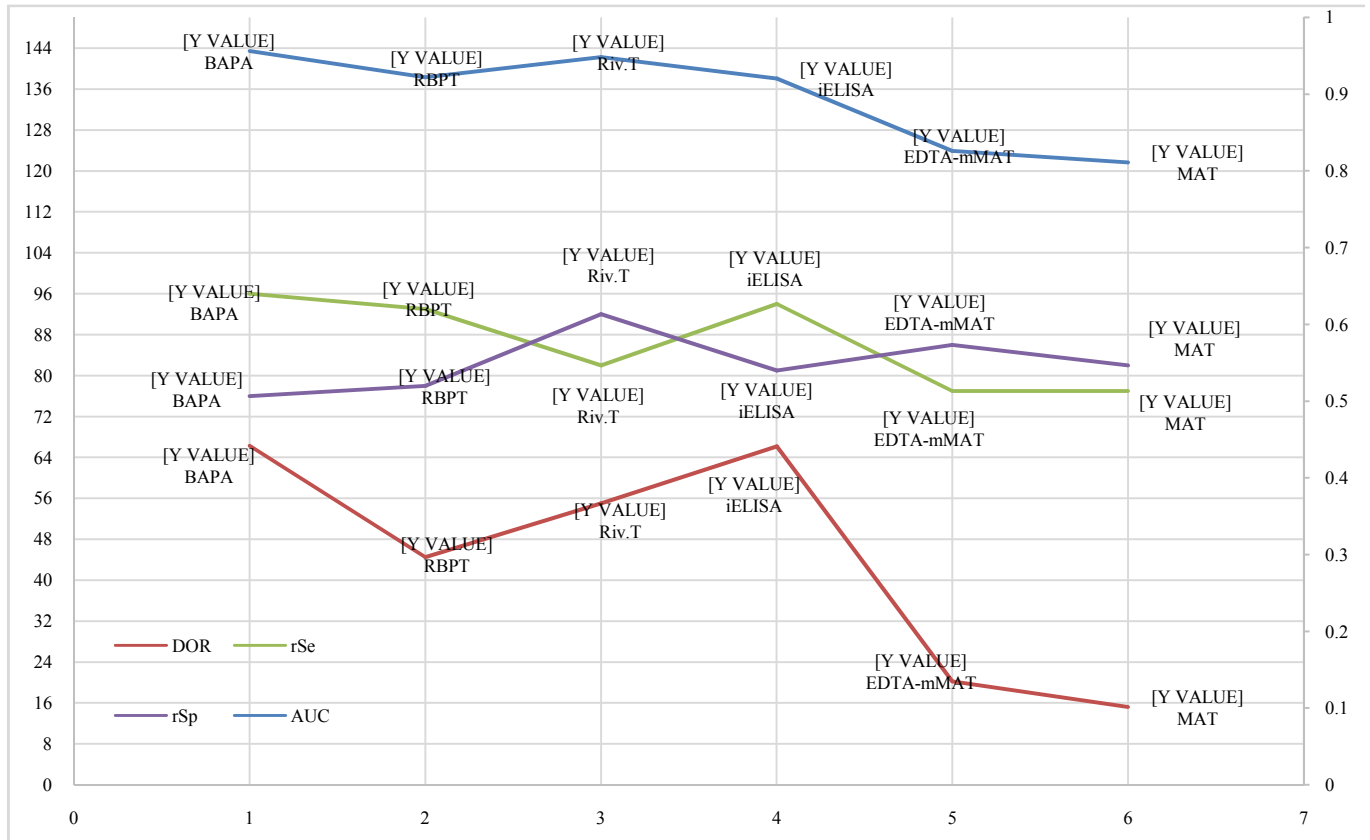
organism in the cultured tissues or insufficient numbers of the micro-organism present to grow on specific media. (Gall and Nielsen, 2004). Beside improper storage of tissues, not selecting appropriate variety of tissues, or insufficient amount of tissues, and selecting samples from uninfected tissues.

**RESULTS AND DISCUSSION**

**Table 3** Agreement with CFT of serological tests and their relative sensitivity/ specificity in cattle

Diagnostic performance of serological tests					Relative sensitivity (%) (SPSS)	Relative specificity (%) (SPSS)	Area under the ROC curve (AUC) (SPSS)	Diagnostic odd ratio (DOR) (TP/FN)/(FP/TN)	Kappa agreement (* κ value) (SPSS)
BAPA	TP	TN	FP	FN	96%	76%	0.956	66.25	0.737 ± 0.049**
	128	59	19	6					
RBPT (8%)	TP	TN	FP	FN	93%	78%	0.922	44.51	0.721 ± 0.050**
	124	61	17	10					
Riv. T	TP	TN	FP	FN	82%	92%	0.948	55	0.682 ± 0.050**
	110	72	6	24					
iELISA	TP	TN	FP	FN	94%	81%	0.92	66.15	0.762 ± 0.047**
	126	63	15	8					
EDTA-mMAT	TP	TN	FP	FN	77%	86%	0.826	20.25	0.596 ± 0.055**
	103	67	11	31					
MAT	TP	TN	FP	FN	77%	82.1%	0.811	15.2	0.564 ± 0.057**
	103	64	14	31					

-: number of negative cases, +: number of positive cases. \*: agreement with CFT at p < 0.05 with confidence interval of 95%, \*\*: κ value ± standard error. The abbreviations TP, FP, FN, and TN symbolize the number of respectively, true positives, false positives, false negatives, and true negatives in view of CFT as a gold standard. **DOR** = diagnostic odds ratio, **AUC** = area under the ROC curve estimated at confidence interval of 95%.



**Figure 1** Diagnostic performance of different serological tests based on rSe, rSp, AUCs and DORs considering CFT as the gold standard in cattle

Validation is a process that determines the fitness of an assay, which has been properly developed, optimized and standardized, for a planned purpose (OIE, 2013). All diagnostic immunoassays either laboratory or field should be verified for the species in which they will be used and should include estimates of diagnostic performance of each a test (OIE, 2013). The sensitivity of a test cannot usually be determined by bacteriological isolation as false negative culture results can occur for many reasons, including the absence of the micro-

Furthermore, it takes days to weeks to produce a result, making the bacteriological isolation impractical for field testing or testing where livestock health authorities shall make rapid decisions. In the absence of bacterial isolation as the gold standard in this study, another serological test with known sensitivity and specificity estimates can be used to define the status of animals.

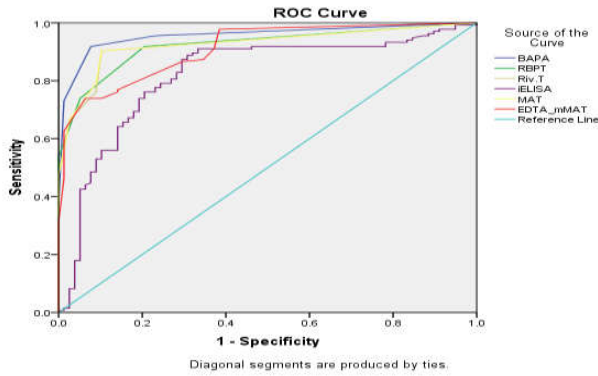


Figure 2 ROC curves reflecting diagnostic accuracy of serological test categories in Cows

For this reason, CFT was selected to be a gold standard in the current study, providing the necessary reference to determine the sensitivity and specificity of the tests being evaluated (Jacobson, 1985; Martin, 1988; Elbauomy et al., 2014a; Elbauomy et al., 2014b).

**Diagnostic performance of serological tests used for the diagnosis of brucellosis in cattle and buffaloes:**

No single serological test is appropriate under all the epidemiological circumstances; all have limitations especially when it comes to screening individual animals (Godfroid et al., 2002; Moriyon et al., 2004; Corbel, 2006). Therefore, samples that are positive in screening tests should be confirmed using an established standard confirmatory test.

Table 4 Agreement with CFT of serological tests and their relative sensitivity/ specificity in buffalo

Diagnostic performance of serological tests					Relative sensitivity (%) (SPSS)	Relative specificity (%) (SPSS)	Area under the ROC curve (AUC) (SPSS)	Diagnostic odd ratio (DOR) (TP/FN)/(FP/TN)	Kappa agreement (* κ value) (SPSS)
BAPA	TP	TN	FP	FN	95%	70%	0.908	42.4	0.648 ± 0.094**
	109	14	6	6					
RBPT (8%)	TP	TN	FP	FN	92%	75%	0.945	53	0.620 ± 0.092**
	106	15	5	9					
Riv. T	TP	TN	FP	FN	88%	90%	0.987	65	0.624 ± 0.084**
	101	18	2	14					
iELISA	TP	TN	FP	FN	91%	80%	0.886	42	0.634 ± 0.089**
	105	16	4	10					
EDTA- mMAT	TP	TN	FP	FN	81%	85%	0.821	24	0.473 ± 0.085**
	93	17	3	22					
MAT	TP	TN	FP	FN	78%	80%	0.789	14.4	0.406 ± 0.085**
	90	16	4	25					

-: number of negative cases, +: number of positive cases. \*: agreement with CFT at p < 0.05 with confidence interval of 95%, \*\*: κ value ± standard error. The abbreviations TP, FP, FN, and TN symbolize the number of respectively, true positives, false positives, false negatives, and true negatives in view of CFT as a gold standard. DOR = diagnostic odds ratio, AUC = area under the ROC curve estimated at confidence interval of 95%.

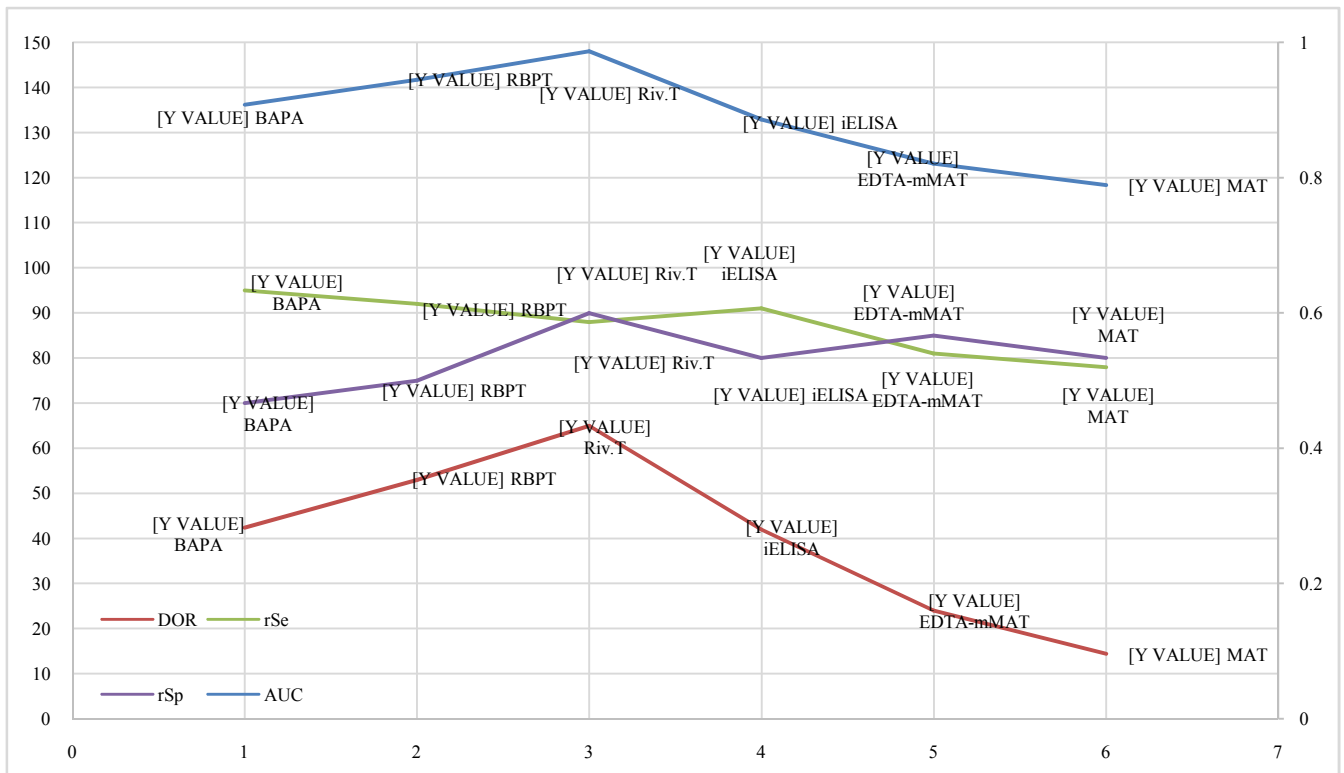


Figure 3 Diagnostic performance of different serological tests based on rSe, rSp, AUCs and DORs considering CFT as the gold standard in buffaloes.

Table (3), Table (4), Figure (1), Figure (2), Figure (3) and Figure (4) show different diagnostic performance in terms of relative sensitivities, specificities, Kappa agreement, diagnostic odds ratios and areas under the receiver operating characteristic curves (AUCs) of different serological tests used in the diagnosis of large ruminant brucellosis namely, BAPA, RBP, Riv. T, iELISA, EDTA-mMAT and MAT tests considering CFT as the gold standard.

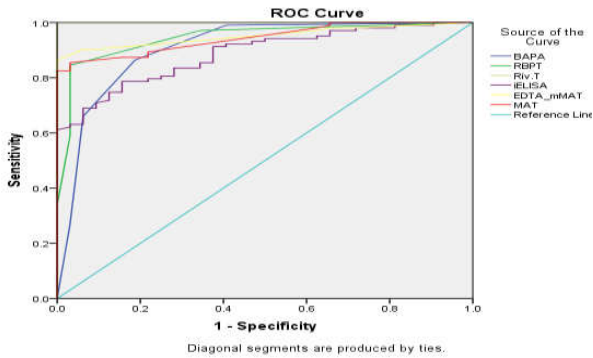


Figure 4 ROC curves reflecting diagnostic accuracy of serological test categories in Buffalo

The recognition of specific antibody in serum samples remains the most applied and practical means of the diagnosis of brucellosis. The most efficient and cost-effective method is usually screening all samples using a cheap and rapid test which is sensitive enough to detect a high proportion of infected animals (Corbel, 2006).

There has always been a challenge to the serological tests for brucellosis in terms of sensitivity and specificity. Reduced sensitivity is associated with under detection of infected animals, a matter that can lead to serious breakdowns in the disease control. On the other hand, impaired specificity results in over condemnation of animals that are actually non-infected. The pH of the serological reaction and the antigen-antibody ratio are two main determinants of sensitivity in presumptive/screening tests. The highest relative sensitivities of the presumptive BAPAT in cattle (96%) and buffaloes (95%) and the RBPT in cattle (93%) and buffaloes (92%) as revealed by (Table 3 and 4) can be attributed to the acidic pH of lactate buffer at which the antigens were preserved. The acidic pH alters the isoelectric point of IgM, thus reducing its agglutinability usually responsible for nonspecific serological reactions (Corbel, 1972).

Table 5 Detailed identification of 7 field isolates as *Brucella melitensis* biovar 3

Identification of <i>Brucella</i> at the genus level									
Colonial morphology Smooth colonies			Microscopic appearance Gram negative coccobacilli, weak acid fast				Catalase +		
Multiplex PCR assay (Bruce-ladder) DNA product at both 587 bp and 1071 bp			B- Identification of <i>Brucella</i> at the species level Urease activity + (slow)				Oxidase +		
C- Identification of <i>Brucella</i> species at the biovar level									
Growth on dyes		Agglutination with monospecific sera							
CO <sub>2</sub> requirement	H <sub>2</sub> S production	1:25000	Thionin 1:50000	1:100000	Basic Fuchsin 1:25000	1:50000	A	M	R
-	-	-	+	+	+	+	+	+	-
Conclusion <i>Brucella melitensis</i> biovar 3									

Table 6 Detailed identification of 4 field isolates as *Brucella abortus* biovar 1

Identification of <i>Brucella</i> at the genus level									
Colonial morphology Smooth colonies			Microscopic appearance Gram negative coccobacilli, weak acid fast				Catalase +		
Multiplex PCR assay (Bruce-ladder) DNA product at 587 bp.			B- Identification of <i>Brucella</i> at the species level Urease activity + (intermediate rate)				Oxidase +		
C- Identification of <i>Brucella</i> species at the biovar level									
Growth on dyes		Agglutination with monospecific sera							
CO <sub>2</sub> requirement	H <sub>2</sub> S production	1:25000	Thionin 1:50000	1:100000	Basic Fuchsin 1:25000	1:50000	A	M	R
+	+	-	-	-	+	+	+	-	-
Conclusion <i>Brucella abortus</i> biovar 1									

Table 7 predominating *Brucella* species and biovars isolated from large ruminant in some governorates

Species	Samples	Governorates	Isolates	Identification
Cows	Supra-mammary L.N.		1	<i>B. abortus</i> bv. 1
Cows	Retro pharyngeal L.N.	Sharkia	1	<i>B. melitensis</i> bv. 3
Cows	Liver		1	<i>B. melitensis</i> bv. 3
Cows	Liver	Gharbia	1	<i>B. melitensis</i> bv. 3
Cows	Spleen		2	<i>B. abortus</i> bv. 1
Buffaloes	Milk	KafrElsheikh	1	<i>B. abortus</i> bv. 1
Buffaloes	Fetal stomach contents		1	<i>B. melitensis</i> bv. 3
Buffaloes	Milk	Beheira	1	<i>B. melitensis</i> bv. 3
Buffaloes	Liver		1	<i>B. melitensis</i> bv. 3

The acidic pH of the lactate buffer enhances the agglutinability of IgG<sub>1</sub> which is non-agglutinogenic at neutral pH. Likewise, the final pH after addition of serum in BAPAT is (4.02) and (3.8) in RBPT and the final packed cell volume in case of BAPA is (3%) while that of RBPT is (4%) enhancing sensitivity (Alton et al., 1988). The low final packed cell volume of BAPA compared with RBPT in addition to slightly low final acidic pH of BAPA relative to RBPT are the key reasons why the BAPAT is to somewhat sensitive than the RBPT in both species. Also, the sensitivity of screening tests is affected in apart by the potential technical human errors. Where analyst with healthy eye sight of 6/6 might misidentify several repetitions of a single weak positive sample to include some or few false negative ones. Moreover, individual humans might often have different perspectives regarding the cutoff between positive and negative samples based on visual matching of the test result with the positive and negative controls (Sayour et al., 2017).



**Figure 5** Differentiation of *Brucella* species by Bruce-ladder multiplex PCR. Lane 1 control negative; lane 2, *B. abortus* reference strain 544; lane 3, *B. melitensis* reference strain 16M; lane (4-7) *B. abortus* field isolates; lane (8-14) *B. melitensis* field isolates

Table (3), (4), Figure (1) and (3) reveal low relative specificity percentages of BAPAT (76% and 78%) and RBPT (70% and 75%) in cattle and buffaloes respectively. High sensitivities of such tests were at the expense of specificities and this fits with their use as screening tests specially for detecting infected flocks or for ensuring the absence of the disease in brucellosis-free flocks (OIE, 2009).

The highest sensitivity of iELISA in large ruminants as shown in Table (3), (4), Figure (1) and (3) of 94% and 91% in cattle and buffaloes respectively are mainly endorsed to their primary binding nature that detect the attendance of all antibodies regardless to their class or biological activity (Tizard, 2004; Crowther, 2009). Moreover, for indirect ELISA versions, the enzyme-substrate reaction consequences in intensification of the signal indicating the presence of the analyte, where one molecule of an enzyme can act on numerous molecules of the substrate (Crowther, 2009).

Because iELISAs are largely unable to distinguish *B. abortus*S19 vaccinal antibody and cross-reacting antibody, the specificity can be slightly lower as in the present study by 81% and 80% of cattle and buffaloes respectively as shown in Table (3) and (4). iELISA kits, as well as in-house versions, are excellent screening assays for the diagnosis of brucellosis (Nielsen, 2010).

Table (3), (4), Figure (1) and (3) show low relative sensitivities (77%, 77%, 81% and 78%) and moderate relative specificities (86%, 82%, 85% and 80%) of EDTA-mMAT and micro-agglutination test in cattle and buffaloes respectively. Micro-agglutination test (MAT) is performed at a near neutral pH and therefore detects IgM efficiently and is therefore very sensitive to recent infection. But under the environment of this study, the sensitivity of the test was low as a result of the endemic situation of the disease, where the main immunoglobulin class is IgG<sub>1</sub> inefficiently detected by the test. In addition, high titer of IgG<sub>1</sub> in serum samples is accountable for prozone phenomenon (absent of agglutination in the first lowest dilution/s of the test) affecting the sensitivity of the test (high false negative results) (Nielsen, 1984; Alton, et al., 1988). Therefore, the MAT is generally not used as a single test but rather in combination with other tests. The detection of significant levels of agglutinating antibody especially IgM by MAT in response to cross-reacting antigens causes specificity problems in the MAT (OIE, 2009; Nielsen, 2010).

The better specificity of EDTA-mMAT over the MAT in large ruminants as shown in Table (3) and (4) may be attributed to the chelating agent, EDTA. EDTA reduces non-specific IgM binding thus reducing false positive reactions. The mechanism by which EDTA reduces non-specificity is not yet known; however, it appears to eliminate attachment of immunoglobulins to the *Brucella* cell wall via the Fc piece. The action of EDTA is assumed to be a result of its competition with a receptor site on the *Brucella* antigen cells for binding of the non-specific IgM (Nielsen, 2010; Poester et al., 2010; Kaltungo et al., 2013).

The rivanol test detects principally IgG<sub>1</sub>, and to a lesser extent IgG<sub>2</sub>, due to the initial treatment of sera with rivanol solution (2-ethoxy-6,9-diaminoacridine lactate), This cationic acridine dye forms a complex with high molecular weight serum glycoproteins (IgM) that precipitate, reduces the reactivity of IgG<sub>2</sub>, and promotes the reactivity of IgG<sub>1</sub>. This gives the rivanol test low sensitivity but high specificity (Alton et al., 1988; Mikolon et al., 1998) and for these reasons the relative specificity of Riv.T was high in both species (92% in cattle and 90% in buffaloes) and the relative sensitivities were slightly low in cattle and buffaloes of 82% and 88% respectively (Table 3, 4, Figure 1 and 2).

The kappa ( $\kappa$ ) agreement of CFT with the serological tests in large ruminants (cattle and buffaloes) as shown by Table (3) and (4) was used to evaluate matching of results. Landis and Koch (1977) categorized values of (< 0), (0- 0.20), (0.21- 0.40), (0.41- 0.60), (0.61- 0.80), and (0.81- 1) as demonstrating no agreement, slight agreement, fair agreement, moderate agreement, substantial agreement and almost perfect agreement respectively. All the serological tests under the validation of this study in both species agreed significantly with CFT at  $p < 0.05$ . The estimated  $\kappa$  agreement values with the CFT in cattle and buffaloes as shown in Table (3) and Table (4) indicated substantial agreement in the case of the BAPA, RBPT, iELISA and Riv. T where the values ranged from 0.682 to 0.762 in cattle and from 0.620 to 0.648 in buffaloes. Then again, the estimated  $\kappa$  agreement values indicated moderate agreement in the case of the EDTA-mMAT and MAT, where the  $\kappa$  values

ranged from 0.564 to 0.596 in cattle and from 0.406 to 473 in buffaloes.

Good agreement achieved under the current investigation between CFT and Riv.T in large ruminants are attributed to the fact that both tests detect almost the same immunoglobulin class IgG<sub>1</sub> (MacMillan, 1990; Mikolon *et al.*, 1998; Nielsen, 2010).

The main reason stands behind the good agreement estimated between screening tests (RBPT, BAPA and iELISA) and CFT is the ability of these tests to apparently detect IgG<sub>1</sub> and IgG<sub>2</sub> (Corbel, 1972; Angus and Barton, 1984, Crowther, 2009). However, the moderate agreement of CFT with both EDTA-mMAT and MAT is attributed to different immunoglobulin isotypes detected by these tests.

The ROCs were shaped by drawing the sensitivity against the false positive rate (FPR) at different possible cutoff values of the tests under evaluation as shown in Figure (2) and (4). The false-positive rate is also known as (1 -specificity). The closer the ROC curve to the vertical axis, the better the overall test performance (Fawcett, 2006).

The area under the curve obtained (AUC) can subsequently be calculated as an alternative single indicator of test accuracy and a measure of how well a test can distinguish between the infected and healthy group of animals (Hanley and McNeil, 1982). The AUC takes values between 0 and 1, with higher values indicating better accuracy of the test.

Based on the ROCs and AUCs, the performance of serological tests in cattle can be arranged in descending order as follows, BAPAT, Riv.T, RBPT, iELISA, EDTA-mMAT and MAT of 0.956, 0.948, 0.922, 0.92, 0.826 and 0.811 (Figure 1 and 2). The equivalent picture in buffaloes was as follows, Riv.T, RBPT, BAPAT, iELISA, EDTA-mMAT and MAT of 0.987, 0.945, 0.908, 0.886, 0.821, 0.789 (Figure 3 and 4).

The overall performance of serological tests in large ruminants based on both ROCs and AUCs is very good being  $\geq 0.9$  and is a reflection of how good the tests are distinguishing between *Brucella* infected and healthy animals. MAT and EDTA-mMAT recorded the lowest AUCs figures with acceptable performance and therefore both tests are generally not used as a single test in the diagnosis but rather in combination with other tests (Nielsen, 2010). The main reasons behind the better performance of screening tests as well as a confirmatory test (Riv.T) in cattle and buffaloes are attributed in part to the better sensitivities and/or specificities estimated under the umbrella of the current investigation.

Another diagnostic performance parameter is the diagnostic odd ratio (DOR) which is the ratio of the odds of positivity in the diseased animals relative to the odds of positivity in non-diseased one (Kraemer, 1992). It reviews the diagnostic accuracy of the test as a single number that describes how many times higher the chances are of obtaining a test positive result in *Brucella* infected animals rather than a non-diseased animal). The value of a DOR ranges from 0 to infinity, with higher values indicating discriminatory test performance. A value of 1 means that a test does not distinguish between infected and healthy group of animals. Values lower than 1 refer to inadequate test interpretation (more negative test among the diseased). The DOR increases sharply when relative

sensitivity or specificity becomes near perfect (Glas *et al.*, 2003).

The DOR result of serological tests in cattle as shown in (Table 3) was arranged in descending order as follows BAPAT, iELISA, Riv.T, RBPT, EDTA-mMAT and MAT of 66.25, 66.15, 55, 44.5, 20.25 and 15.2 respectively. The corresponding picture in buffaloes (Table 4) was Riv.T, RBPT, BAPAT, iELISA, EDTA-mMAT and MAT of 65, 53, 42.4, 42, 24 and 14 respectively.

The results of DOR reflected the capability of serological tests in large ruminants to discriminate between diseased and non-diseased animals being over one and to a lesser extent in EDTA-mMAT and MAT. The reason why low but acceptable DOR results of both EDTA-mMAT and MAT in cattle and buffaloes is the lowest relative sensitivities and/or specificities recorded by both tests in comparison with other serological tests.

#### **Isolation and identification of *Brucella* isolates among different animal species at different Egyptian governorates:**

Bacteriological trials for the isolation of *Brucella* from large ruminants in the five governorates under investigation namely; Sharkia, Dakahlia, KafrElsheikh, Beni-Suef and Gharbia (Table 7) resulted in the recovery of 11 field isolates including 7 from cows and 4 from buffaloes.

conventional bacteriological identification at the genus (colonial morphology, microscopic appearance, biochemical tests), species (molecular speciation by multiplex PCR) and biovar levels (CO<sub>2</sub> requirement, H<sub>2</sub>S production, growth on the dyes (thionin and fuchsin), agglutination with monospecific sera) as shown by (Table 5 and Table 6) resulted in the recognition of 4 isolates as *Brucella abortus* biovar 1 from cattle and the rest (7 isolates) as *Brucella melitensis* biovar 3 from cattle (3 isolates) and buffaloes (4 isolates). *B. melitensis* biovar 3 is the almost sole biovar which has been reported over the last 14 years in Egypt (Sayour, 2004; Abdel-Hamid, 2007; Afifi *et al.*, 2011; Abdel-Hamid *et al.*, 2012) until re-emerging of *B. abortus* biovar 1 again (Menshawy *et al.*, 2014). The preference hosts for *Brucella melitensis* are the small ruminants (OIE, 2016).

The four *Brucella* isolates that identified as *B. abortus*bv. 1 were recovered from supra mammary lymph node, spleen and liver. While the rest seven isolates identified as *B. melitensis*bv. 3 were recovered from retro pharyngeal lymph node, milk, liver and fetal stomach contents.

Frequent isolation of *Brucella melitensis* biovar 3 from the liver of slaughtered seropositive ruminants, beats the alarm about potential hazards on human health and the consumers as a results of uncondemned edible offal (liver) release (Zahran, 2004; Samaha *et al.*, 2008; Fatma and Mahdey, 2010; Abdel-Hamid *et al.*, 2012; Sayour and Sayour, 2015; Abdel-Hamid *et al.*, 2016). Therefore, the ministerial decree No. 1329 of 1999 must be re-amended to contain an explicit clause of liver condemnation that belongs to infected bovine carcasses with brucellosis as it poses hazards on public health.

The recovery of *Brucella melitensis* from large ruminants is undoubtedly a proof of vital role of small ruminants (preference host) in cross-species infection and an evidence

that small ruminants are concerned more than ever given that sheep and goats graze after cattle and that they are kept in the household with cattle and buffaloes (Hegazy et al., 2009; Elbauomy et al., 2014a).

No matter how many *Brucella* isolates were recovered from different animal species, since the isolation of micro-organism from a single animal is a definitive proof to establish the infection status of a herd or flock (Gall and Nielsen, 2004; Elbauomy et al., 2014a) and supporting the serological results. Multiplex PCR (Bruce-ladder) has been implemented in this study for molecular typing of *Brucella* at species level as shown in Figure (5). However, one of the challenges of using DNA-based techniques for differentiating the various *Brucella* species and strains is their high degree of genetic homology (Grimont et al., 1992).

Bruce-ladder PCR assay cannot differentiate among biovars from the same species. Bruce-ladder was species specific and all the strains and biovars from the same *Brucella* species gave the same profile (Lopez-Goni et al., 2008; Nagalingam et al., 2012) and for that reason, it was applied to differentiate the *Brucella* isolates at the species level and to differentiate the vaccinal strain from *Brucella* field strains. The practical interest of Bruce-ladder for typing purposes is obvious since some of the cumbersome, hazardous and long-lasting microbiological procedures currently used could be avoided.

## CONCLUSIONS AND RECOMMENDATIONS

Under the field of this investigation authors concluded and recommended the following:

It is recommended that the screening BAPA and RBPT, low cost and better performance than iELISA, shall be used in any sero-prevalence programmes implemented for the control and eradication of the disease.

To a limited extent Riv.T could be used in large ruminants to confirm reactors identified by screening tests if CFT is not available. As a result of better diagnostic performance offered by EDTA-mMAT in large ruminants under investigation, it is an appropriate time to shift from MAT formats locally adopted to EDTA-mMAT to avoid bias in results unfitting the native epizootological condition.

Regarding meat inspection regulations for slaughtered animals infected with *Brucella*, it is absolutely urgent to re-amendment the ministerial decree No. 1329 issued in 1999 in keeping with the policy of safeguarding humans from the public health hazards. This old decree necessitated the condemnation of blood, lymph nodes and all internal organs with the exception of the liver from being condemned.

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