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Bacteriological Examination and Diagnostic Performance of Some Serological Tests Used In Diagnosis of Small Ruminant Brucellosis

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Abstract

A total of 249 serum samples was collected from 120 ewes and 129 goatsfrom the Nile delta Governorateswith no history of vaccination against brucellosis and with sporadic cases of late-term abortions.Collected serum samples were serologically examined for brucellosis using the screening tests;buffered acidified plate antigen (BAPA), modified Rose-Bengal (mRBT) and indirect enzyme linked immunosorbent assay (iELISA). Alsothe supplementary micro-agglutination (MAT), EDTA modified micro-agglutination tests, as well asthe confirmatory complement fixation (CFT) and Rivanol-precipitation plate agglutination tests (Riv. T) were used. The aim was to evaluate the diagnostic performance of these testsas regards to relative sensitivity/specificity, area under the receiver operating characteristic curve (AUC), diagnostic odds ratio (DOR) and kappa agreement (κ) with complement fixation test (gold standard).Good diagnostic significancewas proved with BAPA, mRBT and iELISA in the form of high relative sensitivities on the expense of relative specificities, substantial agreement with CFT, high DORs and better performance based on ROCs and AUCs.Questionable diagnostic results were recordedby MAT render itunreliable for diagnosis of small ruminant brucellosis.The diagnostic performance of Riv.T didn't fulfil the requirements for the confirmatory test particularly in sheep where the test recorded the lowest relative sensitivity and specificity of 73% and fair agreement (0.224) with the CFT, lowest DOR (7.35)and AUC (0.8). These results subsequently reclassified the testas a supplementary rather than a confirmatory test.Bacteriological examination of samplesfromsmall ruminants resulted in the recovery of 9 isolates ofBrucella melitensis biovar 3.

Keywords: Bacteria- Brucellosis – serological – smallruminants

Introduction

Brucellosis is a contagious disease of different animal species caused by bacteria of the genus Brucella. It causes reproductive losses in animals in the form of late stage abortion, retained placenta, weak or dead birth and infertility in females and orchitis and epididymitis in males with hygroma and arthritis. Additionally, brucellosis is notorious for its latent infection (**OIE, 2009a**).

Similar toB. abortus infection in cattle, B. melitensis can be transmitted congenitally in sheep and goats. Most latent infections in cattle take place through in utero transmission. However, only a small proportion of lambs and kids are infected in utero, and the majority of B. melitensis latent infections are probably acquired through colostrum or milk (**Grillo et al., 1997**). Such latent infection increases the difficulty of eradicating this disease, as the bacteria persist in the animal without inducing detectable immune responses(**Corbel, 2006**).

Currently, twelveBrucellaspecies are recognised: B. abortus (affecting mainly cattle), B. melitensis (sheep and goats), B. suis (swine), B. neotomae (desert rats), B. ovis (sheep), B. canis (dog), B. ceti (cetaceans), B. pinnipedialis (pinnipeds), B. microti (common voles), B. inopinata (human breast implant) and B. papionis (baboons) (**Whatmore et al., 2014**) and lately B. vulpis (red foxes) (**Scholz et al., 2016**)..With the exception of B. ovis and B. neotomae, all these species are pathogenic for humans (**Blasco, 2010**).

In small ruminants, the brucella buffered antigen tests and the CFT are the most widely used serological methods for diagnosis, control and eradication of the disease (**European Commission, 2001; Godfroid et al., 2002 and OIE, 2009a**). All these tests are prescribed for international trade. The buffered acidified plate antigen test (BAPAT) and Rose-Bengal test (RBT) are not completely specific, but are adequate as screening tests for detecting infected flocks or guaranteeing the absence of infection. However, due to the relative lack of

sensitivity of both tests, discrepancies between results obtained using the card or Rose-Bengal test (RBT) and the CFT are not rare in infected sheep and goats (**OIE, 2009b**). The results of these two tests should, therefore, be considered simultaneously to increase the likelihood of detecting infected individuals and to improve control of the disease in areas where it has not been completely eradicated (**Blasco, 1992**). There is a potential limitation in diagnostic performance of the secondary binding assays of the buffered antigen tests and the CFT for their indirect dependence on the phenomena of acidified agglutination and fixation of complement resulting from antigen antibody reaction, and not direct dependence on the presence of antibodies(**Nielsen, 2010**).

The aim of the current research was to detect the predominating species and biovars of Brucella microorganisms from small ruminants in some governorates and to evaluate the diagnostic performance characteristics of some serological tests used for the diagnosis of brucellosis in small ruminants in term ofrelative sensitivity/specificity, area under the receiver operating characteristic curve (AUC), diagnostic odds ratio (DOR) and kappa agreement (κ) with complement fixation test (gold standard).

1. Materials and methods:

1.1. Samples

1.1.1. A total of 249 blood samples was collected from 120 ewes and 129 goats as individual animals and small flocks with age range of 1-4 years and from animals sent to abattoir for slaughter.All animals were from the Nile delta Governorates; including Kafr El-Sheikh, Gharbia, Beheira, Sharkia and Giza. There was no history of vaccination against brucellosis. Some animals were reported to have late-term abortions. Blood Samples were kept overnight at 4° C to allow for separation of serum then centrifuged at 1000 xg. for 10 minutes to separate serum. Sera were divided into aliquots and kept at -20° C till examined.

1.1.2. 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 Governoratesfor the isolation and typing of Brucella microorganisms.

1.2. Serological tests

1.2.1. Serum samples were serologically examined against brucellosis using 1. Screening tests, namely BAPA, mRBT and iELISA, 2. Supplementary tests, namely MAT and EDTA modified micro-agglutination tests and 3. Confirmatory tests, specifically CFT and Riv. T.

1.2.2. mRB, BAPA and Rivanol antigens were purchased from (NVSL/DBL, USDA, USA). Riv. and BAPA tests were performed according to **Alton et al. (1988)**. mRBT was performed according to the **Blasco et al. (1994)** as recommended by the **OIE (2009b)**. Although qualitative, the BAPA and RBT results were recorded as scores from 0 to 4+ according to the degree of agglutination.

1.2.3. Antigen for the CFT was imported from NVSL/DBL, USDA, USA. Complement, hemolysin and antigen were prepared and preserved according to **Alton et al. (1988)**. Sheep RBCswere collected on Alsever's solution from an adult healthy ram serologically negative to brucellosis. The sheep RBCs standardized to 3% suspension in veronal buffer diluent. Warm fixation of complement was adopted as cold fixation was unacceptably slow and the test was performed according to **Alton et al. (1988)**. Results of CFT were converted to ICFTU/ml and interpreted as positive at a cutoff point of ≥ 20 ICFTU/ml.

1.2.4. Brucella-AB-I-ELISA O/C kit for the detection of Brucella IgG in ovine and caprine serum and plasma. It was manufactured by (Boehringer Ingelheim Svanova, Box 1545, SE-751 45 Uppsala, Sweden). This kit uses noninfectious Brucella antigen coated microtitre plates, lyophilized HRP conjugate (horseradish peroxidase conjugated. anti-ruminant IgG monoclonal antibodies), and substrate solution – (tetramethyl-benzidine in substrate buffer containing H2O2).The kits was validated according to the kit instructions, the validation guidelines of the **ISO/IEC 17025 (2005), Crowther (2009)** and **OIE (2013a)**. The percent positive (PP) was calculated for iELISA kit from the formula: PP = (Mean OD samples×100)/ (Mean OD Positive control) PP Status ≥ 15% was considered positive. If <15%, the test was negative.

1.3. Bacteriologicalisolation,identification and typing of Brucella

Bacteriological identification at genus (colonial morphology, microscopic appearance, catalase, oxidase and urease), species level (phage lysis) and biovar level (CO_2 requirement, H_2S production, growth in the presence of thionin and basic fuchsin and agglutination with monospecific antisera) was done according to **(Alton et al., 1988).**

1.4. Statistical analyses

All the following analyses were performed using IBM® SPSS® Statistics, Version 21, IBM Corporation (2012) under the environment of Windows® 8.1, Microsoft Corporation:

1.4.1. Kappa (κ) agreement and relative sensitivity/ specificity:

The kappa (κ) agreement of agglutination tests with CFT was used to assess the matching of results at $p < 0.05$. Relative sensitivity/ specificity pairs were also calculated.

1.4.2. Receiver operatingcharacteristics (ROC) curves:

Considering the CFT as the serological gold standard, ROC curves expressing the sensitivity (true positive rate) versus the false positive rate 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).**

1.4.3. Diagnostic odds ratio (DOR):

The diagnostic odds ratio of a test is the ratio of the odds of positivity in disease relatively to the odds of positivity in the non-diseased and DORs were estimated for the serological tests used in the diagnosis of small and large ruminant brucellosis according to **(Kraemer, 1992** cited in **Glas et al., 2013)**

Results

1.5. Table (1), Figure (1) and Figure (2) reveal the estimated relative sensitivities of different serological tests used in the diagnosis of brucellosis in ewes and they were as follows: BAPAT, mRBT, Riv.T, iELISA, EDTA-mMAT and MAT of 96%, 96%, 73%, 95%, 80% and 74%, respectively. The corresponding relative specificities of the same tests were recorded as follows: BAPAT (73%), mRBT (82%), Riv.T (73%), iELISA (82%), EDTA-mMAT (82%) and MAT (73%).

All the serological tests used to diagnose brucellosis in ewes agreed significantly with CFT at $p < 0.05$. The estimated κ agreement values in ewes were 0.663, 0.722, 0.224, 0.688, 0.339 and 0.308 for BAPAT, mRBT, Riv.T, iELISA, EDTA-mMAT and MAT.

Areas under the receiver operating characteristic curves (AUCs) in ewes were recorded as follows: BAPAT (0.967), mRBT (0.975), Riv.T (0.8), iELISA (0.973), EDTA-mMAT (0.853) and MAT (0.703).The DOR results of serological tests in ewes were recorded as follows: BAPAT (70), mRBT (118), Riv.T (7.35), iELISA (93.61), EDTA-mMAT (17.8) and MAT (7.7).

1.6. Table (2), Figure (3) and Figure (4) revealed the estimated relative sensitivities of different serological tests used in the diagnosis of brucellosis in goats and they were as follows: BAPAT, mRBT, Riv.T, iELISA, EDTA-mMAT and MAT of 94%, 93%, 83%, 94%, 80% and 74% respectively. The corresponding relative specificities of the same tests were recorded as follows: BAPAT (70%), mRBT (75%), Riv.T (80%), iELISA (80%), EDTA-mMAT (75%) and MAT (70%).

All the serological tests used to diagnose brucellosis in goats agreed significantly with CFT at $p < 0.05$. The estimated κ agreement values in goats were 0.623, 0.638, 0.479, 0.693, 0.407 and 0.339 for BAPAT, mRBT, Riv.T, iELISA, EDTA-mMAT and MAT.

Areas under the receiver operating characteristic curves (AUCs) in goats were recorded as follows: BAPAT (0.930), mRBT (0.946), Riv.T (0.793), iELISA (0.961), EDTA-mMAT (0.760) and MAT (0.723).The DOR results of serological tests in goats were recorded as follows: BAPAT (34), mRBT (37.9), Riv.T (18.9), iELISA (58.3), EDTA-mMAT (9.2) and MAT (6.7).

1.7. Tables (3) and (4) show the results of bacteriological isolation of Brucellaspeciesfrom small ruminants in four Governorates under investigation namely;Al-Dakahlia, Kafr Elsheikh, Beni-Suef and El-Gharbia. Nine field isolates of Brucella spp. were recovered from ewes (7) and goats (2).

Phenotypic bacteriological identification at the genus and species and biovar levels resulted in the identification of all the 9 isolates as Brucella melitensis biovar 3.The nine Brucella isolates that identified as B. melitensis were recovered from milk and livers, fetal stomach contents and fetallivers

Table (1) Results of relative sensitivity, specificity, DORs and AUCs of different serological tests used for diagnosis of brucellosis in ewe as compared with standard CFT.

Results			CFT δ -	CFT δ +	Relative sensitivity (%)	Relative specificity (%)	Diagnostic odd ratio (DOR) (TP/FN)/(FP/TN)	Areaunder the ROCcurve(AUC)	Kappa agreement (* κ value)
Ewe	BAPA	-	8	4	96%	73%	70	0.967	0.663 ± 0.118**
		+	3	105					
	mRBT	-	9	4	96%	82%	118	0.975	0.722 ± 0.107**
		+	2	105					
	Riv. T	-	8	29	73%	73%	7.35	0.8	0.224 ± 0.084**
		+	3	80					
	iELISA	-	9	5	95%	82%	93.61	0.973	0.688 ± 0.110**
		+	2	104					
	EDTA-mMAT	-	9	22	80%	82%	17.8	0.853	0.339 ± 0.096**
		+	2	87					
	MAT	-	8	28	74%	73%	7.7	0.703	0.308 ± 0.099**
		+	3	81					

-: number of negative cases, +: number of positive cases, δ: gold standard. *: agreement with CFT at $p < 0.05$ with confidence interval of 95%, **: κ value ± standard error. The abbreviations TP, FP, FN, and TN denote the number of respectively, true positives, false positives, false negatives, and true negatives. **DOR** = diagnostic odds ratio (summarizes the diagnostic accuracy of the test as a single number that describes how many times higher the odds are of obtaining a test positive result in a diseased rather than a non-diseased animal), **AUC** = area under the ROC curve representing accuracy at confidence interval of 95%.

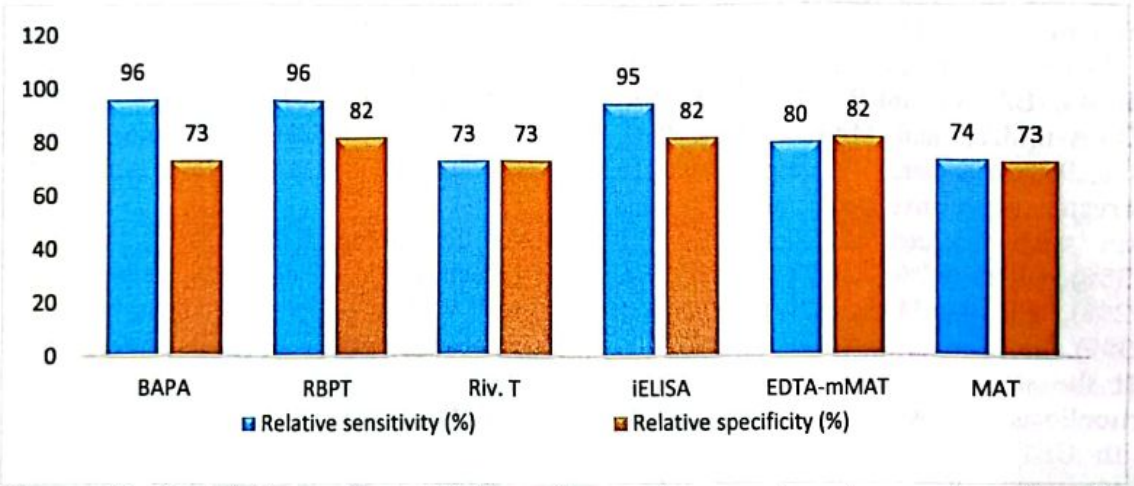


Figure (1) Sensitivities and specificities of screening, supplementary and confirmatory serological tests used in the diagnosis of brucellosis in ewes as compared with CFT (gold standardtest).

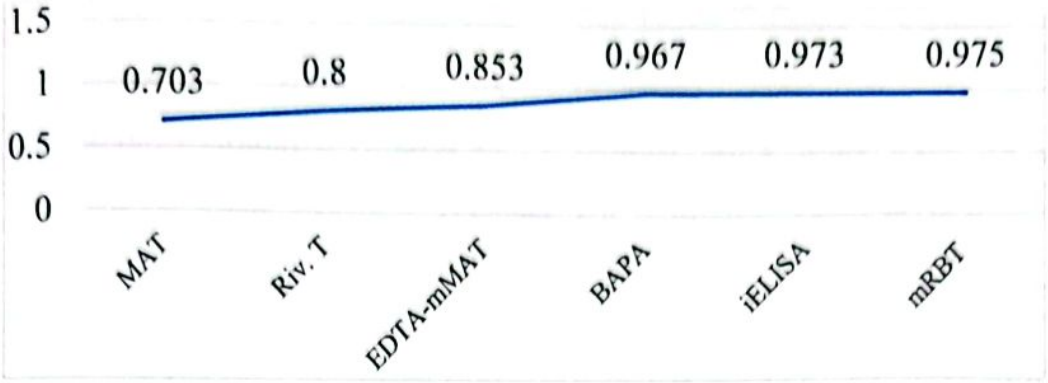
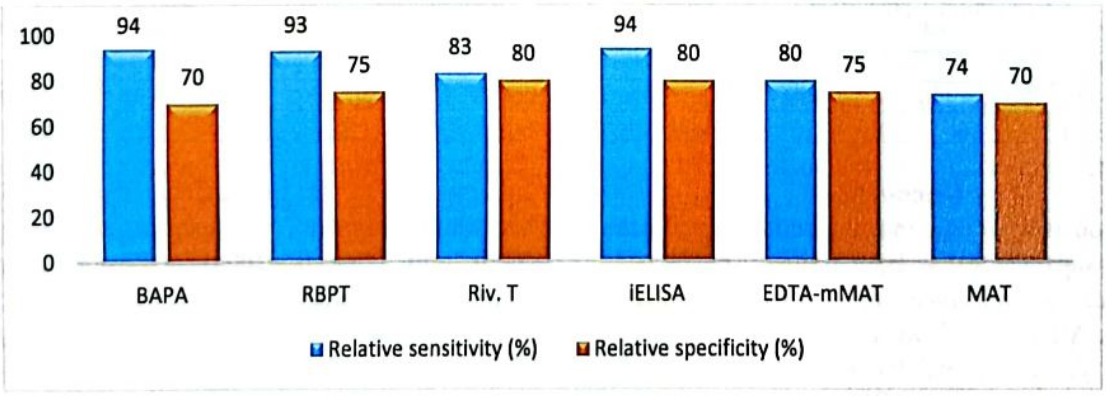


Figure (2) Accuracy of different serological tests arranged in ascending order based on AUCs considering CFT as the gold standard in ewes.

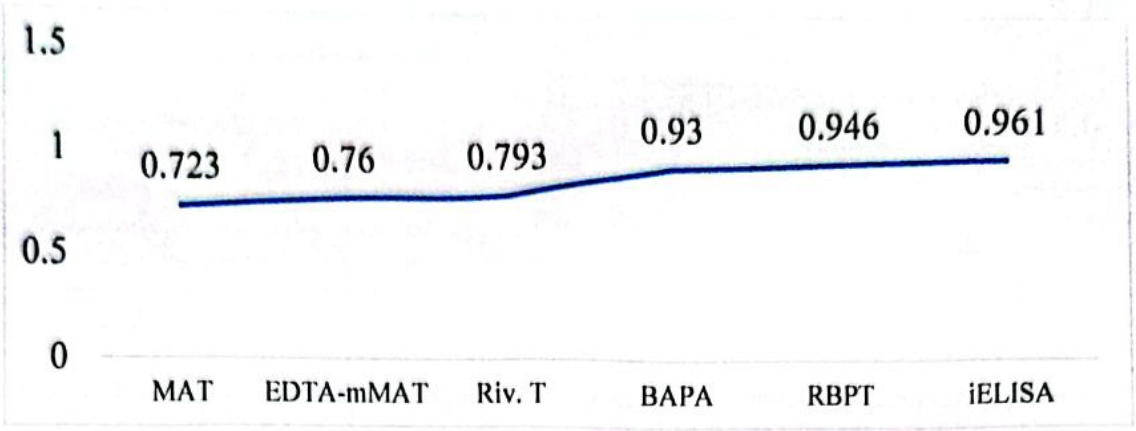
Table (2) Results of relative sensitivity, specificity, DORs and AUCs of different serological tests used for diagnosis of brucellosis in goats as compared with standard CFT.

Results			CFT δ -	CFT δ +	Relative sensitivity (%)	Relative specificity (%)	Diagnostic odd ratio (DOR) (TP/FN)/(FP/TN)	Area under the ROC curve (AUC)	Kappa agreement (* κ value)
Goat	BAPA	-	14	7	94%	70%	34	0.930	0.623 ± 0.095**
		+	6	102					
	mRBT	-	15	8	93%	75%	37.9	0.946	0.638 ± 0.092**
		+	5	101					
	Riv. T	-	16	19	83%	80%	18.9	0.793	0.479 ± 0.089**
		+	4	90					
	iELISA	-	16	7	94%	80%	58.3	0.961	0.693 ± 0.086**
		+	4	102					
	EDTA- mMAT	-	15	22	80%	75%	9.2	0.760	0.407 ± 0.090**
		+	5	87					
	MAT	-	14	28	74%	70%	6.7	0.723	0.339 ± 0.089**
		+	6	81					

-: number of negative cases, +: number of positive cases, δ: gold standard. *: agreement with CFT at $p < 0.05$ with confidence interval of 95%, **: κ value ± standard error. The abbreviations TP, FP, FN, and TN denote the number of respectively, true positives, false positives, false negatives, and true negatives. **DOR** = diagnostic odds ratio (summarizes the diagnostic accuracy of the test as a single number that describes how many times higher the odds are of obtaining a test positive result in a diseased rather than a non-diseased animal), **AUC** = area under the ROC curve representing accuracy at confidence interval of 95%.



Figure(3) Sensitivities and specificities of screening, supplementary and confirmatory serological tests used in the diagnosis of brucellosis in goats as compared with CFT (gold standard test).



Figure(4) Accuracy of different serological tests arranged in ascending order based on AUCs considering CFT as the gold standard in goats.

Table (3) Brucella species and biovars recovered from small ruminants in some Governorates.

Species	Samples	Governorates	Isolates	Identification
Ewes	Milk	Dakahlia	1	B. melitensis bv. 3
			2	B. melitensis bv. 3
Ewes	Liver	Kafr Elsheikh	1	B. melitensis bv. 3
Ewes	Liver		1	B. melitensis bv. 3
Ewes	Fetal stomach contents	Beni-Suef	1	B. melitensis bv. 3
Ewes	Milk		1	B. melitensis bv. 3
Goats	Fetal liver	Gharbia	1	B. melitensis bv. 3
Goats	Milk		1	B. melitensis bv. 3

Table (4): In depth identification of 9 field isolates as Brucella melitensis biovar 3

A-Identification of Brucella at the genus level							
Colonial morphology	Microscopic appearance			Catalase	Oxidase	Urease	
Smooth, convex, honey coloured	Gram negative coccobacilli, weak acid fast			+	+	+	
B- Identification of Brucella at the species level							
Lysis by phage							
Tbilisi	Izatnagar			Rough/Canis			
-	+			-			
C- Identification of Brucella species at the biovar level							
CO_2 requirement	H_2S production	Growth on dyes			Reaction to monospecific sera		
		Thionin	Basic Fuchsin				
		1:50000	1:25000	1:50000	A	M	R
-	-	+	+	+	+	+	-
Conclusion							
Brucella melitensis biovar 3							

Discussion

Validation is a process that determines the fitness of an assay, which has been properly developed, optimized and standardized, for an intended purpose. All diagnostic assays (laboratory and field assays) should be validated for the species in which they will be used **(OIE, 2013b)**. Validation includes estimates of diagnostic performance characteristics of a test.

The sensitivity of a test cannot usually be determined by bacteriological isolation as false negative culture results can occur for many reasons, including absence of the bacterium in the cultured tissues or insufficient numbers of the bacterium present to reproduce on growth media. **(Gall and Nielsen, 2004).** Beside improper storage of tissues, not selecting an appropriate variety of tissues, not selecting a sufficient amount of tissues, or selecting samples from uninfected tissues. Furthermore, it takes days to weeks to produce a result, making the bacteriological isolation impractical for field testing or testing where livestock health authorities must make immediate decisions.

In the absence of bacterial isolation as the gold standard in this study, another serological test or

combination of tests with known sensitivity and specificity estimates can be used to define the status of animals(**OIE, 2009a**). CFT was considered as gold standard in the current study, providing the necessary reference to determine the sensitivity and specificity of the test being evaluated (**Jacobson, 1985; Martin, 1988; Elbauomy et al., 2014a; Elbauomy et al., 2014b**). In species other than cattle, the same serological procedures may be used for these animals (**Nicoletti, 1992**), but each test should be validated in the animal species under study (**Gall et al., 2001**), where all immunoassays are primarily standardized to cattle.

In small ruminants, the RBPT and the CFT are the most widely used methods (**Garin-Bastuji et al. (2006)**. All these tests are prescribed for international trade. The RBPT is not completely specific, but is satisfactory as a screening test for detecting infected flocks or for ensuring the absence of infection in brucellosis-free flocks. However, due to the relative deficiency of sensitivity of both tests, discrepancies between results obtained using the Rose Bengal test (RBT) and the CFT are not rare in infected sheep and goats (**Blasco et al., 1994**).

For the previous reasons, it is recommended to improve the sensitivity of the RBT by using three volumes of serum and one volume of antigen in place of an equal volume of each. The recorded superior sensitivity of mRBT as screening test in both ewes and goats (96% and 93% respectively) in this investigation was attributed to this modification. This simple modification increased RBT sensitivity (by decreasing false negative cases) without affecting its specificity and minimized the discrepancies between RBT and CFT results (**Blasco et al., 1994**).

The receiver operating characteristic curves were created by plotting thetrue positive rate(TPR) against the false positive rate (FPR) at different possible cutoff values of the tests under evaluation. The true-positive rate is also known as sensitivity. 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 performance and a measure of how well a parameter can distinguish between infected and healthy group of animals (**Hanley and McNeil, 1982**). The AUC takes values between 0 and 1, with higher values indicating better test performance.

Based on the ROCs and AUCS, the performance of serological tests in ewes can be arranged in descending order as follows: mRBT, iELISA,BAPAT, EDTA-mMAT, Riv. T and MAT of 0.975, 0.973, 0.967, 0.853, 0.8 and 0.703 (**Figure 2**). The equivalent picture in goats was as follows: iELISA, mRBT, BAPAT, Riv.T,EDTA-mMAT and MAT of 0.961, 0.946, 0.930, 0.793, 0.760 and 0.723 (**Figure 4**).

Another diagnostic performance characteristics 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**). 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 discriminate between diseased and non-diseased. 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**).

Good diagnostic results have been obtained in sheep and goats with BAPA, mRBT and iELISA in the form of high relative sensitivities on the expense of relative specificities being acceptable for these screening tests, substantial agreement with CFT (gold standard), high DORs and finally better performance of used tests based on ROCs and AUCs.

The highest relative sensitivities of the presumptive BAPAT in ewes (96%) and goats (94%)as shown by **Table (1), Table (2), Figure (1) and Figure (3)** can be attributed tothe 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**). The acidic pH also enhances the agglutinability of IgG_1 which is non-agglutinogenic at neutral pH. Likewise, the final pH after addition of serum in BAPAT is (4.02) and 3.65 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 highest relative sensitivities of iELISA in large ruminants as shown in**Table(1),Table (2), Figure (1) and Figure (3)** of 95% and 94% in ewes and goats respectively are mainly attributed to their primary binding nature that detect the presence of all antibodies irrespective to their isotype or biological activity (**Tizard, 2004; Crowther, 2009**). Moreover, for indirect ELISA versions, the enzyme-substrate reaction results in

intensification of the signal indicating the presence of the analyte, where one molecule of enzyme can act on several molecules of the substrate (**Crowther, 2009**).iELISA kits as well as individually developed assays are excellent screening assays for the diagnosis of brucellosis, especially in individual animals (**Nielsen, 2010**).

Questionable diagnostic results have been obtained by MAT in both sheep and goats as shown by **Table (1),Table (2), Figure (1) and Figure (3)** based on the overall low relative sensitivities of 74% each and specificities of 73% and 70% respectively. Low relative sensitivities of MAT in both speciesmay beas a result of the endemic situation of the disease, where the main immunoglobulin class is IgG_1 inefficiently detected by the test (detects maily IgM). In addition, high titer of IgG_1 in serum samples is responsible for prozone phenomenon (lacking 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, 2009a; Nielsen, 2010**). In addition to fair agreement with CFT (0.308 and 0.339 respectively), very low DORs (7.7 and 6.7) and low AUCS (0.703 and 0.723 respectively) if compared with other tests. This bad performance rendered the MAT as unsatisfactory for the purposes of international trade and not reliable to be used in the diagnosis of small ruminant brucellosis (**OIE, 2009b**).

The better specificity of EDTA-mMAT over the MAT in small ruminants as shown in**Table (1)** and **(2)** 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 understood; however, it appears to eliminate attachment of immunoglobulins to the Brucella cell wall via the Fc piece. The action of EDTA is hypothesized 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; Poiester et al., 2010; Kaltungo et al., 2013**).

The main reason behind the good agreement recorded between screening tests (RBPT, BAPA and iELISA) and CFT in small ruminants as shown by **Table (1)** and **Table (2)** is the ability of those tests to apparently detect IgG_1 and IgG_2(**Corbel, 1972; Angus and Barton, 1984, Crowther, 2009**).However, the fair agreement of CFT with both EDTA-mMAT and MAT is attributed to different immunoglobulin isotypes detected by these tests.

The diagnostic performance characteristics of Riv.T didn't fulfil the requirements for the confirmatory test under the umbrella of the current study in small ruminants particularly in sheep where the test recorded the lowest relative sensitivity and specificity of 73% and fair agreement (0.224) with the CFT test, in addition to lowest DOR (7.35), AUC (0.8) and ranked last after EDTA-mMAT makes its use a confirmatory test in sheep questionable and classified it as a supplementary (**Alton et al., 1988; Corbel, 2006**) rather than a confirmatory test.

On the other side, the picture in goat seems better than that of sheep, where the test recorded better sensitivity of 83% and better specificity of 80% which still unacceptable for the requirement of confirmatory tests, in addition to moderate agreement (0.479) with CFT, DOR of 18.9 and AUC of 0.793.

The labour intensiveness of the CFT, inability to test hemolyzed samples beside routine daily titration of complement and the necessity of doing a number of controls for all the reagents and reactions render, the test time-consuming and technically challenging (**Gall and Nielsen, 2004**). This leadssome labs to choose time-saving, unstandardized easily performed tests like Riv.T in the diagnosis of brucellosis even if it is not recommended by the OIE for the diagnosis of ruminant brucellosis and it is not advised to replace standard confirmatory tests (**Corbel, 2006**).

Phenotypic bacteriological identification at the genus (colonial morphology, microscopic appearance, biochemical tests), species (phage) and biovar levels (CO_2 requirement, H_2S production, growth in the presence of the dyes thionin and fuchsin, agglutination with monospecific antisera) as shown by (**Table 3, Table 4**) resulted in the recognition of 9 isolates as Brucella melitensis biovar 3. The almost sole biovar reported over the last 15 years in Egypt in small ruminants (**Sayour, 2004; Abdel-Hamid, 2007; Affifi et al., 2011; Abdel-Hamid et al., 2012**) which are the primary hosts for Brucella melitensis(**OIE, 2009b**).

The recovery of Brucella melitensis from all animal species (as reported in different article in Egypt) including large ruminants is undoubtedly a proof of vital role of small ruminants in cross-species infection and an evidence that small

ruminants are implicated more than ever given that sheep and goats graze after cattle and that they are kept in the household with cattle and buffaloes, in addition to free movement of small ruminants between the various governorates in Egypt **(Hegazy et al., 2009; Elbauomy et al., 2014a;Hegazy et al., 2016).**

Conclusion and recommendation:

Conclusion

Based on the results obtained from this study in terms of relative sensitivities, specificities, DORs, ROCs, AUCs and Kappa agreement, it is concluded that:

- Low diagnostic performance characteristics of Riv.T in small ruminants specially in ewes if compared to other serological tests, makes it unwise to replace the standard confirmatory CFT test recommended by OIE, European commissions, USDA for international trade by Riv.T even in large ruminants
- Nine field isolates of B. melitensis were recovered from four governorates (Dakahlia, Kafr Elsheikh, Beni-Suef and Gharbia). All the B. melitensis were recovered from native breeds of ewes and goats and identified as B. melitensis bv.3.
- Improper diagnostic performance characteristics of serological tests in small ruminantrender it unreliable to be used in the diagnosis of small ruminantbrucellosis.
- Diagnostic performance characteristics of EDTA-mMAT were better than MAT in both sheep and goats.

It doesn't matter how many Brucella isolates were recovered from small ruminants in different governorate under investigation, since bacteriological isolation 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

Recommendation:

- Taking into consideration the relative sensitivities of screening tests used (BAPAT and mRBT over iELISA) in small ruminants, as well as the cost-benefit effect of these tests, it is recommended that the screening BAPA and mRBT, low cost and better performance, shall be used in any sero-prevalence programmes implemented for the control and eradication of the disease.
- It is not recommended to confirm reactors identified by screening tests by Riv.T in small ruminants.
- Good diagnostic performance of mRBT compared with its peers, and its substantial agreement with CFT, it is recommended to use this modification to replace RBPT in the diagnosis of small ruminant brucellosis.
- As a result of better diagnostic performance characteristics offered by EDTA-mMAT in all small ruminants under investigation, it is high time to switch from MAT formats locally adopted to EDTA-mMAT to avoid bias in results unfitting the native epizootological condition.

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الملخص العربى

الفحص البكتريولوجي والأداء التشخيصي لبعض الاختبارات السيرولوجية المستخدمة في تشخيص مرض الإجهاض المعدي في المجترات الصغيرة

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أجريت هذه الدراسة على عدد 249 عينة مصل جمعت من 120 نعجة و129 ماعز من محافظات دلتا النيل دون تاريخ سابق للتحصين ضد البروسيلا مع وجود بعض الإجهاضات في الفترة الأخيرة من الحمل، وقد تم فحص عينات المصل المجمعة سيرولوجياً لمرض الإجهاض المعدي باستخدام الاختبارات المسحية مثل اختبار الأنتيجين المُحمّض المجمد الشريحي واختبار الروزبنجال المُعدّل واختبار الإليزا،كما تم فحص تلك العينات باستخدام الاختبارات المكملة مثل اختبار التلزن فى أطباق مايكروتايتر في وجود مادة الإديتا وبدونها، فضلاً عن تأكيد النتائج باختبارى تثبيت المُكمّل والريفانول، وكان الهدف من إجراء البحث هو تقييم الأداء التشخيصي لهذه الاختبارات فيما يتعلق بالحساسية والخصوصية النسبيةوالمساحاتتحت منحنيات خصائص أداء المستخدم ونسبة احتمالات التشخيصوالتوافق(كابا)مع اختبار تثبيت المكمل (المعيار الذهبي)، وقد ثبت وجوداداء تشخيصي معنوي جيدلكل من اختبار الأنتيجين المُحمّض المُجمّد الشريحي واختبارالروزبنجال المعدل واختبار الإليزا غير المباشرفي شكل الحساسيات النسبية المرتفعة على حساب الخصوصيات النسبية، وتوافق جوهري مع اختبار تثبيت المكمل، ونسبة احتمالات تشخيصيةعالية وأداء أفضل على أساسمنحنيات خصائص أداء المستخدموالمساحاتتحت منحنيات خصائص أداء المستخدم، هذا وقد سجلت نتائج مثيرة للجدلإختبار التلزن في أطباق مايكروتايترمماجعله لا يمكن الاعتماد عليه في تشخيص مرض الإجهاض المعدي في المجترات الصغيرة،ولم يحققالأداء التشخيصي لاختبار الريفانولالهدف المرجو منهكاختبار تأكيدي خاصة في الأغنام حيث سجل الاختبار أدنى حساسية نسبية وخصوصية نسبية بنسبة 73٪ لكل منهما،وتوافق ضعيف بقيمة (0.224) مع اختبار تثبيت المكمل، وأدنى نسبة احتمالات تشخيصية بقيمة (7.35) ومساحةتحت منحنى خصائص أداء المستخدم بقيمة(0.8)،وبناءً على هذه النتائج يرجح إعادة تصنيف الاختبار كاختبارتكميليبدلاً من كونه اختبار تأكيدي، وقد أدى الفحص البكتريولوجي لعينات من المجترات الصغيرة إلى عزل عدد 9 معزولات من البروسيلا صنفت كبروسيلا ميليتنسيز النوع الحيوي 3.