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Research Article

Efficiency of Different Preparations of Rapid Slide Agglutination Antigens for the Diagnosis of Bovine and Ovine Brucellosis

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Abstract

Background: Brucellosis is an important zoonotic bacterial disease of global health importance affecting different animals and man. Brucellosis control and eradication procedures are highly depending on accurate diagnostic tools and effective and safe vaccination programs. Rapid slide agglutination tests using different antigens as rose bengal antigen and Buffered Acidified Plate Antigen (BAPA) are considered as cheap, quick and effective tests for diagnosis and screening of brucellosis. **Materials and Methods:** With respect to packed cells volume and pH, specificity and sensitivity of 18 different slide agglutination antigens (rose bengal and buffered acidified plate agglutination antigens) prepared in Veterinary Serum and Vaccine Research Institute were evaluated. In the absence of bacteriological isolation, complement fixation test was used as a Gold Standard test. **Results:** No satisfactory differences with the results of rapid slide agglutination antigens with different pH and this may be due to narrow range of pH used in this study. However, when antigens of different packed cells volume were used, a high degree of sensitivity appeared especially when buffered acidified plate agglutination and modified rose bengal tests were carried out using antigens of cell concentration of 4 and 6%. In contrast specificity was decreased with antigens of less packed cells volume. Modified rose bengal and buffered acidified plate agglutination tests using antigens with cell concentration 4 and 6% showed lowest specificity. **Conclusion:** Results revealed that modified rose bengal and Buffered Acidified Plate Agglutination (BAPA) tests using antigens with lower cells concentrations than that of international standard are recommended to be used especially in animals of low anti-brucella titers and endemic areas with brucellosis especially when *Brucella melitensis* is the main causative agent. Modified rose bengal test and BABA test using antigens of cell concentration of 4-6% is recommended for diagnosis of *B. melitensis* infection.

Key words: *Brucella*, brucellosis, SAT, rose bengal, CFT

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Brucellosis is an important zoonotic bacterial disease of global importance affecting different mammals including man, sheep, goats, cattle, swine, rodents and marine mammals worldwide. The disease primarily affects the reproductive system with significant loss of productivity and re-productivity of affected animals. In human, the symptoms characterized by recurrent febrile episodes called undulant fever causing economic and public health importance. The severity of human brucellosis and lack of vaccines for man makes *Brucella* as important agents for bioterrorism[1]. Vaccines to be used for human are not yet available and so eradication of human brucellosis have been largely depend on the eradication of the disease in animals, therefore eradication of animal brucellosis has been a target for many countries. In order to control brucellosis, comprehensive vaccination, surveillance and quarantine programs should be implemented. Both of control and prevention procedures are highly depending on accurate diagnostic tools and effective and safe vaccination programs[2].

Rapid Slide Agglutination (RSA) tests are well known as a pilot, cheap, rapid and effective screening test for *Brucella* diagnosis. It can be performed with minimum facilities. Because of its apparent simplicity, high level of standardization of antigen and accuracy of reading is needed[3].

Many tests were compared to sensitivity and specificity of slide agglutination tests SAT as I-ELISA which was a better serological test as compared to slide agglutination tests in the sense of sensitivity and specificity for diagnosis of ovine brucellosis[4] but modified rose bengal test is a suitable test as a screening test for ovine brucellosis caused by *B. melitensis*[5,6] where ICA was more specific than conventional RBPT for bovine brucellosis diagnosis while the latter was more sensitive[7].

Previous studies have shown that there are many differences in the levels of antigen standardization used around the world. In addition, there is marked variations in standardization which lead to disagreement in results between different countries and even between different laboratories, therefore different results were obtained with antigens tested in the study at CVL[8] and in the study of Blasco *et al*.[6]. When antigens standardized in one laboratory and used in another, quite different results may be obtained, this may be due to many factors, different experiences between people because different persons have different ability to see finer agglutination than others. Many factors affect RSA reactions and their reading as storage temperature and temperature of antigens during performing the test and period of reading reaction, antigens Packed Cell Volume (PCV) and pH, *Brucella* strains used in production of antigen and main causative agent of brucellosis.

The aim of the study is to evaluate the specificity and sensitivity of different RSA antigens used for diagnosis of bovine brucellosis in animals of known history of vaccination and relates this to the sensitivity and specificity of Complement Fixation Test (CFT).

MATERIALS AND METHODS

Serum: Randomized bovine and ovine serum samples: One hundred and ninety eight bovine sera and 66 ovine sera collected from non-vaccinated animals of infected flocks. *Brucella* free and infected animals were determined using conventional rose bengal test as a pilot method for diagnosis[9].

Antigens: Nine rose bengal antigens, nine buffered acidified plate agglutination antigens of different PCV and pH and one complement fixation antigen were used in this study as shown in Table 1. All RSA antigens were prepared in VSVRI using *B. abortus* S99 where complement fixation antigen is American antigen which prepared using *B. abortus* S1119. All antigens were prepared according to international rules[9,10].

Rose Bengal (RB) test was performed, following the procedure described by Alton *et al*.[9] in which 25 µL of tested sera was mixed with 25 µL of the antigen. The plates were shaken for 4 min and any agglutination appeared within this time was recorded as a positive reaction.

Modified Rose Bengal (MRB) test was performed following the procedure described by Blasco *et al*.[6] in which 75 µL of tested sera was mixed with 25 µL of the antigen. The plates were shaken for 4 min and any agglutination appeared within this time was recorded as a positive reaction.

The BAPA test was carried out following the procedure described by Alton *et al*.[9] in which 80 µL of tested sera was mixed with 30 µL of the antigen. The plates were shaken for 8 min and any agglutination appeared within this time was recorded as a positive reaction.

Complement Fixation Test (CFT) was performed on a microplate, following the procedure described by Alton *et al*.[9]. Complement fixation at a dilution of 3log2 (1:8), the level recommended by the ABAH[11], was regarded as a positive reaction. Serum samples were titrated 1:4 to 1:128 in the CFT. Titers determined by CFT were expressed as log2 of the reciprocal of the last dilution at which a positive reaction occurred[11].

Table 1: Antigens used in this study

Antigens	PCV (%)	pH	Antigen's strain
Rose Bengal Antigens	4	3.65	*Brucella abortus* S99
	6		
	8		
	10		
	11		
	8	3.3	
		3.5	
		3.65	
		4	
		4.5	
BAPA antigens	4	3.7	
	6		
	8		
	10		
	11		
	11	3.3	
		3.5	
		3.7	
		4	
		4.5	
CFT			*Brucella abortus* S1119

Table 2: Calculation of sensitivity and specificity with respect of gold standard test

	Gold standard test (CFT)		
Test under evalution	Positive	Negative	Total
Positive	A	B	A+B
Negative	C	D	C+D
Total	A+C	B+D	N (264)

Relative sensitivity: A/A+C, Specificity: D/D+B, True positive (Positive predictive value): A/A+B, False positive (B): B/A+B, True negative (Negative predictive value): D/D+C, False negative (C): C/D+C

With respect of CFT as a gold standard test[12], antigens sensitivity, relative sensitivity and specificity were calculated (http://vassarstats.net/clin1.html) as shown in Table 2.

RESULTS AND DISCUSSION

The rose bengal plate agglutination, Buffered Acidified Plate Agglutination (BAPA), indirect ELISA and complement fixation tests are usually used for testing animals against brucellosis. The complement fixation test is the only test recommended for confirmation and international trade, but other tests as the agar gel precipitation test and competitive ELISA, are used for confirmation purposes. The slide and tube Serum Agglutination Test (SAT) is not considered accurate and reliable for use in ovine brucellosis[10] while modified rose bengal test is considered reliable for use in small ruminant brucellosis where the causative agent is Brucella melitensis and commercial RSA antigens are prepared from *B. abortus* strains[5,6]. Because of the cross-reaction of the lipopolysaccharide (LPS) of *B. abortus*, *B. melitensis* and *B. suis*, only one antigen can be used for serological diagnosis of brucellosis caused by these three species. All over the world, all agglutination antigens were prepared by using *B. abortus* strain 99 or 1119 antigens although in some cases different strains were used as *B. melitensis* local isolate[13] which used for rapid diagnosis of brucellosis in goats in Malaysia, *Brucella melitensis* and *Brucella suis* S2 antigens[3] and *B. suis* was used in production of Chinese antigen[6]. The SAT are considered as reliable screening tests for *Brucella* infection, followed by confirmatory testing, but the antibodies resulting from *B. abortus* S19 and *B. melitensis* Rev-1 vaccination will react in these tests[10,14].

In this study 198 bovine and 66 ovine serum were tested against all prepared rapid slide agglutination antigens and CFT. Complement fixation tests were considered as a gold standard test to determine the sensitivity and specificity of tested antigens in absence of bacteriological isolation. Statistics in this study were considered the 95% confidence intervals (95% CI). Serum with suspected results was excluded from the study. All prepared antigens were tested against international standard anti-*Brucella abortus* serum (ISABS) (Veterinary Laboratories Agency (VLA), Weybridge, Surrey, UK) as shown in Table 3.

Carrying out traditional RBT, There were some differences in sensitivity of tested antigens of different PCV against ISABS where rose Bengal antigen of 4 and 6% cell concentration had high sensitivity which agreed with Blasco *et al.*[6] and Macmillan[8]. In contrast, performing MRBT and BAPA using antigens of different PCV, sensitivity was higher than that of traditional RBT but no satisfactory differences observed using different antigens. Sensitivity of different antigens against ISABS was not affected by pH which not agreed with Nielsen and Yu[15] and Smit[16] which may be due to narrow range of pH used in this study. Differences of sensitivity against ISABS may be do not able to tell what difference this would make in a diagnostic situation, as it would depend on the prevalence of the disease[8] and also this sensitivity may differ from batch to another.

In the present study, 129 (48.86%) and 66 (25%) sera samples tested by RBT, MRBT, BAPA and CFT using different antigens types were found to be negative and positive, respectively by all the tests. Number of serum samples that showed positivity with all or some of antigens showed differences in the period needed to show agglutination with faster reaction in case of using antigens with 4 and 6% PCV. Ovine serum samples gave same results by all tests with 42 and 15 of ovine sera were found to be negative and positive, respectively by all the tests. Nine ovine sera were reacted positively with MRBT and BAPA tests only. Results revealed that there is no satisfactory differences were observed in specificity and sensitivity of antigens with different pH as sensitivity were 25, 40.91 and 38.64% and specificity were 100, 79.7 and 82.8% using RBT, MRBT and BAPA techniques, respectively. Results of antigens with different pH against bovine and ovine sera samples not agreed with Nielsen and Yu[15] and Smit[16] whom explained that acidic PH increases specificity of antigens. This may be attributed to narrow range of pH (3.3-4.5) in this study but anyhow standard pH needed to achieve optimum sensitivity and specificity of RSA tests was not clear even with Blasco *et al.*[6] and Macmillan[8] whom not used antigens of different pH and constant PCV. In contrast, there was agreement with Blasco *et al.*[6] and Macmillan[8] as satisfactory differences were observed in case of different antigens with different PCV (Table 4, 5 and 6).

Table 3: Titration of different antigens preparations against ISABS (1000 IU mL^{-1})

				Dilutions of ISABS								
Tests	Antigens	PCV (%)	pH	1:35	1:40	1:45	1:47.5	1:50	1:55	1:60	1:80	1:100
Rose bengal test	Rose bengal antigens	4	3.65	+	+	+	+	+	+	-	-	-
		6		+	+	+	+	+	+	-	-	-
		8		+	+	+	+	+	-	-	-	-
		10		+	+	+	+	+	-	-	-	-
		11		+	+	+	+	+	-	-	-	-
Modified rose bengal test		4		+	+	+	+	+	+	+	+	-
		6		+	+	+	+	+	+	+	+	-
		8		+	+	+	+	+	+	+	+	-
		10		+	+	+	+	+	+	+	+	-
		11		+	+	+	+	+	+	+	+	-
BAPA test	BAPA antigens	4	3.7	+	+	+	+	+	+	+	+	-
		6		+	+	+	+	+	+	+	+	-
		8		+	+	+	+	+	+	+	+	-
		10		+	+	+	+	+	+	+	+	-
		11		+	+	+	+	+	+	+	+	-
RBT	Rose bengal antigens	8	3.3-4.5	+	+	+	+	+	-	-	-	-
MRBT				+	+	+	+	+	+	+	+	-
BAPA test	BAPA antigens	11		+	+	+	+	+	+	+	+	-

Table 4: Sensitivity of RBT, MRBT and BABA tests for diagnosis of ovine and bovine brucellosis

Tests	Antigens	PCV (%)	No. of positive animals	*Sensitivity (%)	95% CI
Rose Bengal test	Rose bengal antigens	4	72	27.27	27.27 : 27.27
		6	69	26.14	26.04 : 26.4
		8	66	25	24.81 : 25.19
		10	66	25	24.81 : 25.19
		11	66	25	24.81 : 25.19
Modified rose bengal test		4	132	50	48.05 : 51.95
		6	124	46.97	45.28 : 48.66
		8	108	40.9	39.74 : 42.08
		10	102	38.64	37.67 : 39.61
		11	102	38.64	37.67 : 39.61
BAPA test	BAPA antigens	4	126	47.73	45.98 : 49.48
		6	114	43.18	41.82 : 44.54
		8	102	38.64	37.67 : 39.61
		10	102	38.64	37.67 : 39.61
		11	102	38.64	37.67 : 39.61
CFT			72	27.27	

*Sensitivity (%): Positive animals/total number of tested animals×100

Considering CFT as a gold standard test[12], antigens sensitivity, relative sensitivity and specificity were calculated using (Table 2).

The sensitivity of traditional RBT[9] was reported to be 27.27, 26.14, 25 and 25% using antigens of cell concentration of 4, 6, 8, 10 and 11%, respectively but these sensitivities were elevated to be 50, 46.97, 40.91, 38.64 and 38.64%, respectively, when modified RB technique were performed[6]. Sensitivities were 47.73, 43.18, 38.64, 38.64 and 38.64% when BAPA test was carried out using antigens of PCV 4, 6, 8, 10 and 11%, respectively, according to Alton *et al.*[9] as shown in Table 4. Using same antigens PCV, no significant difference in the sensitivity of modified RB techniques and BAPA test where final cell concentration after mixing with serum samples is nearly equal. Results revealed that sensitivity of rose bengal and BAPA antigens were increased with reducing PCV with end results that antigens with 4% showed highest sensitivity.

As shown in Table 5 and 6, no satisfactory differences in relative sensitivity of BAPA and MRB tests where conventional RB test using antigens with PCV of 8, 10 and 11% had lowest relative sensitivity. Specificity was inversely proportional with sensitivity and directly proportional with PCV. Lowest specificity was showed with MRBT and BAPA tests using antigens with cell concentration of 4 and 6% where RBT with antigens of cell concentration of 6, 8, 10 and 11% showed highest specificity (100%). Anyhow, results had shown that even with the most sensitive antigen preparations demonstrated 100% specificity with brucellosis-free animals. Increasing antigens sensitivity at the expense of the share of specificity as in this study may be needed according to situation of disease in Egypt, absences of accurate survey

Table 5: Results of RBT, MRBT and BABA tests against CFT as a Gold Standard test

				CFT	
Tests	Antigens	PCV (%)		+ve	-ve
Rose bengal test	Rose bengal antigens	4	+ve	69	3
			-ve	3	189
		6	+ve	69	0
			-ve	3	192
		8	+ve	66	0
			-ve	6	192
		10	+ve	66	0
			-ve	6	192
		11	+ve	66	0
			-ve	6	192
Modified rose bengal test		4	+ve	69	63
			-ve	3	129
		6	+ve	69	54
			-ve	3	138
		8	+ve	69	39
			-ve	3	153
		10	+ve	69	33
			-ve	3	159
		11	+ve	69	33
			-ve	3	159
BAPA test	BAPA antigens	4	+ve	69	57
			-ve	3	135
		6	+ve	69	45
			-ve	3	147
		8	+ve	69	33
			-ve	3	159
		10	+ve	69	33
			-ve	3	159
		11	+ve	69	33
			-ve	3	159

Table 6: Relative Sensitivity and specificity of RBT, MRBT and BABA tests for diagnosis of ovine and bovine brucellosis

Tests	Antigens	PCV (%)	Sensitivity (%)	95% CI	Specificity (%)	95% CI	True positive (positive predictive value) (%)	False positive (%)	True negative (negative predictive value) (%)	False positive (%)
Rose bengal test	Rose bengal antigens	4	95.8	(87.5 : 98.9)%	98.4	(95.1 : 99.6)%	95.8	4.2	98.4	1.6
		6	95.8	(87.5: 99)%	100	(97.6 : 100) %	100	0	98.5	1.5
		8	91.7	(82.1 : 96.6)%	100	(97.6 : 100)%	100	0	97	3
		10	91.7	(82.1 : 96.6)%	100	(97.6 : 100)%	100	0	97	3
		11	91.7	(82.1 : 96.6)%	100	(97.6 : 100)%	100	0	97	3
Modified rose bengal test		4	95.8	(87.5 : 98.9)%	67.2	(60 : 73.3)%	52.3	47.7	97.7	2.3
		6	95.8	(87.5 : 98.9)%	71.9	(64.9 : 78)%	65.1	43.9	97.9	2.1
		8	95.8	(87.5 : 98.9)%	79.7	(73.2 : 85)%	63.9	36.1	98.1	1.9
		10	95.8	(87.5 : 98.9)%	82.8	(76.6 : 87.7)%	67.6	32.4	98.1	1.9
		11	95.8	(87.5 : 98.9)%	82.8	(76.6 : 87.7)%	67.6	32.4	98.1	1.9
BAPA test	BAPA antigens	4	95.8	(87.4 : 98.9)%	70.3	(63.2 : 76.6)%	54.8	45.2	97.8	2.2
		6	95.8	(87.5 : 98.9)%	76.6	(69.8 : 82.2)%	60.5	39.5	98	2
		8	95.8	(87.5 : 98.9)%	82.8	(76.6 : 87.7)%	67.6	32.4	98.1	1.9
		10	95.8	(87.5 : 98.9)%	82.8	(76.6 : 87.7)%	67.6	32.4	98.1	1.9
		11	95.8	(87.5 : 98.9)%	82.8	(76.6 : 87.7)%	67.6	32.4	98.1	1.9

and epidemiological studies, polices aims (control of brucellosis) and also the most common causative agents of brucellosis in Egypt which is *B. melitensis*[17] biovar 3 and this agree with results of Mammeri[18]. Ferreira *et al*.[5] and Blasco *et al*.[6] who evaluate the antigens sensitivity and specificity against *Brucella melitensis* infection in sheep and goat while antigens were prepared from *B. abortus*. This may explained with Shahaza *et al*.[13] as an in-house rose bengal plate agglutination test (RBPT) was performed for a rapid diagnosis of brucellosis in goats with high sensitivity results and also Sadhu *et al*.[4] who used rose bengal antigen prepared from *B. melitensis* for diagnosis of *B. melitensis* infection. Symmetry in increasing sensitivity of antigens from PCV 4-11% which not agreed with Blasco *et al*.[6] and Macmillan[8] may be contributed to different manufacturer know how (for example strains used and concentration of stain) in the pervious studies but in this study, all antigens were prepared in VSVRI using same stain concentration and *B. abortus* S99 as a strain for antigen productions. The results confirmed the high specificity of traditional rose bengal technique tests and CFT.

CONCLUSION

In conclusions, sensitivity of tested antigens were increased with reducing PCV and BAPA test and modified RB techniques was recommended to be used according to situation of disease in Egypt. More studies are needed to be done on large animal's populations and different animal's species including especially camels, sheep, goats, cattle and baffoles accompanied with bacteriological isolation.

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