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Brucella ovis as a Common Antigen for Rapid Diagnosis of Rough Brucellosis in Cattle and Sheep

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ABSTRACT

Brucellosis still represents a serious public health problem that results in significant morbidity and economic losses and regarded as one of the major zoonotic infections worldwide. Rough *Brucella ovis* strain (REO, 198) was used as antigens in routine and modified Rapid Slide Agglutination Test (RSAT) stained with Rose Bengal stain, Complement Fixation Test (CFT) and Agar Gel Precipitation Test (AGPT) for diagnosis of rough *Brucella* infected and vaccinated cattle and sheep in comparison with conventional RSAT incorporated with smooth S99 strain as antigen. The Rough RSAT using *Brucella* ovis as antigen showed a high sensitivity in identifying all experimentally *B. ovis* infected sheep and RB51 vaccinated cattle. Moreover, this antigen prepared with 0.1 M PBS (not 10% saline) showed negative results in 28 out of 30 naturally smooth *Brucella* infected sheep. On the contrary, cattle and sheep vaccinated with S19 and Rev-1 vaccines reacted positively with the rough as well as the conventional (smooth) Rose Bengal antigens. AGPT with rough antigens yielded the same results of RSAT in vaccinated RB51 cattle and experimentally *B. ovis* infected sheep whereas CFT was less sensitive. The current study advise the use of conventional RSAT either by modified or traditional way in parallel with the rough RSAT (prepared with 0.1 M PBS) as screening tests for routine diagnosis of ovine brucellosis followed by the AGPT as confirmatory test and moreover the rough RSAT can be used for identifying of RB51 vaccination in cattle.

Key words: Brucellosis, cattle, sheep, rough RSAT, AGPT, CFT

INTRODUCTION

Brucellosis is a worldwide zoonotic infectious disease caused by Gram-negative bacteria from the genus *Brucella*. The infection in animals has not been controlled and where the transmission of the disease to humans is still common acquired by consumption of unpasteurized dairy products or by contact with infected animals (Atluri *et al*., 2011).

Ruminant brucellosis has been greatly reduced in many developed countries; however, this is far from being a reality in non-developed countries and the infection was reported in almost all domestic animals (Holt *et al*., 2011). The species of *Brucella* which infect ruminants are *B. abortus* (cattle), *B. melitensis* (sheep and goats) and *B. ovis* (sheep) (Meyer, 1990).

Brucellosis decreases productivity of infected cattle and sheep by causing abortions, reducing fertility and decreasing milk yield, resulting in substantial economic losses (Ghosh and Nanda, 1988). Ovine brucellosis caused by *B. ovis* is a global disease of economic

importance that has been one of the common causes of epididymitis in rams and infrequent cause of abortion in ewes and neonatal mortality in lambs (Lopez *et al.*, 2005).

The diagnosis of brucellosis in cattle and small ruminants requires the use of more than one serological test. The Complement Fixation Test (CFT), the Rose Bengal Test (RBT) and the Serum Agglutination Test (SAT) are among the most useful tests for routine diagnosis (Baum *et al.*, 1995). *B. ovis* infection diagnosis in sheep depends on clinical examination that is not sensitive enough because only about 50% infected rams show epididymitis (Blasco, 1990). Besides, it is nonspecific due to the presence of many other bacteria causing clinical epididymitis (Bulgin and Anderson, 1983; Webb, 1983).

The traditional slide agglutination tests as Rose Bengal test (RBT) used for diagnosis of brucellosis in cattle and small ruminants except ovine brucellosis caused by *B. ovis* as it is a rough strain that lack O-LPS chain which found in all smooth strains and upon which the idea of agglutination tests for diagnosis of brucellosis are depended (OIE, 2008). So currently, the most commonly used serological tests for diagnosis of ovine brucellosis due to *B. ovis* are the Complement Fixation Test (CFT), the double Agar Gel Immuno Diffusion test (AGID) and the indirect Enzyme-Linked Immuno Sorbent Assay (ELISA) (Estein *et al.*, 2002).

AGID was reported to be as sensitive as CFT and easier to run but the only test prescribed by the OIE and the European Union (EU) for international trade is the CFT (Nielsen *et al.*, 2007). Because of the inherent problems with anti-complementary activity of rough antigens, the *B. ovis* cellular antigen has a short half-life, possibly due to shedding of rough lipopolysaccharides (R-LPS) into the liquid phase which cause direct activation of complement (OIE, 2008).

Cattle and small ruminants are the limited hosts can be vaccinated against brucellosis, the most commonly used vaccines are smooth *B. abortus* S19 and *B. melitensis* Rev.1 vaccines while, rough *B. abortus* RB51 vaccine is used in some countries on small scale and the vaccine strains can be distinguished from field strains by their growth characteristics and sensitivity to antibiotics and other additives (Schurig *et al.*, 2002).

As the major problem of ruminant brucellosis and no data about the current situation of *B. ovis* infection in Egypt in local as well as imported sheep, therefore, the objectives of the present study were to develop a rapid screening RSAT using rough *Brucella ovis* Ag for diagnosis of both *Brucella* infected sheep and vaccinated cattle and sheep in comparison with conventional RSAT and also with CFT and AGPT.

MATERIALS AND METHODS

Strains: ACO_2, serum-independent, avirulent *B. ovis* (REO198) and *E. coli* (O: 157) strains were used in this study.

Serum samples

Naturally *Brucella* infected sera: Thirty naturally infected sera were collected from Egyptian cattle, sheep and Somalian sheep which confirmidely positive with traditional slide agglutination tests and B. melitensis biovar3 was isolated from Lymph node samples.

***Brucella* free sera:** Seventy five cattle and sheep sera were reacted negatively with traditional slide agglutination tests and no *Brucella* were isolated from L.N. samples.

Vaccinated sera: Twenty sera each from cattle vaccinated with *B. abortus* RB51, S19 vaccines and sheep vaccinated with *B. melitensis* Rev-1 vaccine. The sera collected over period of ten weeks post-vaccination.

Experimentally infected sera: Twenty and 2 sera collected from experimentally inoculated sheep with killed *B. ovis* (REO198) and killed *E. coli* (O: 157) strains, respectively.

Antigens preparation

***B. ovis* REO 198 rough antigen used in Rose Bengal test:** *B. ovis* REO 198, a CO_2-independent avirulent strain, used as Ag with modification of (Alton *et al.*, 1988), as the *B. ovis* (REO, 198) strain was grown on Triptycase Soy Agar (TSA) for 48 h at 37°C. The cultures were harvested with modified phenol saline solution (10% NaCl and 0.5% phenol) and incubated for 24 h and then incubated in water bath at 68°C for 1 h for complete killing of *Brucella*. The harvest was centrifuged 3000 rpm/15 min and pellet was suspended in phenol saline and Rose Bengal stain was added followed by filtration. Final product was prepared at PH 7.2 and PCV 6%. Another rough rose bengal antigen was prepared by the same way above but the culture and antigen was harvested and suspended in 0.1 M PBS instead of saline.

***B. ovis* REO198 rough antigen used in CFT and AGPT:** Hot saline extracted antigen according to Myers and Varela-Diaz, 1979, a phosphate-buffered saline suspension (pH 7.2) of freshly harvested *B. ovis* cells that had been kept at 80°C in a water bath for 2 hrs was centrifuged. The supernatant was frozen, thawed and re-centrifuged. The clear antigenic preparation was then dialyzed against phosphate-buffered saline at 4°C and stored in small frozen or lyophilized portions until use.

Detection of Abs against *Brucella*: All collected serum samples were tested with RSAT, CFT and AGPT according to OIE (2008) and Modified RSAT through increasing slightly the amount of sera for the test dose from 25-30 μL to 75-90 μL at the same time maintaining the antigen volume (25-30 μL) according to (Blasco *et al.*, 1994).

RESULTS

Rapid Slide Agglutination Test (RSAT): All naturally *Brucella* infected sera were reacted positively with conventional and rough Rose Bengal Antigens (RBA) prepared with 10% saline but positive in two cases with rough RBA prepared with 0.1 M PBS while the *Brucella* free cattle and sheep sera gave negative results with both antigens with the exception of 2 bovine samples in the RBT (0.1M PBS and 10% saline) which had unknown history of vaccination with RB51 (commonly used nowadays in Egypt). Both vaccinated sheep and cattle with Rev-1 and S19 vaccines reacted positively with the entire RSAT using smooth and rough antigens with the exception of 4 sheep samples using rough modified RSAT prepared with 0.1M PBS while vaccinated cattle with RB51 reacted positively with rough RBA and negatively with the conventional smooth one. The all sera of experimentally inoculated sheep with killed *B. ovis* REO198 reacted positively with rough and negatively with conventional smooth RBA, whereas, experimentally inoculated sheep with killed *E. coli* O: 157 reacted negatively with rough antigen (using both buffers) while one out of the two samples reacted positively with conventional RBA (Table 1).

Table 1: Results of RSAT using rough rose bengal antigens (10% saline and 0.1M PBS) and conventional rose bengal antigen

	Rough Rose Bengal antigens		
Sera	Prepared with 10% saline	Prepared with 0.1M PBS	Conventional smooth rose bengal antigen
Naturally *Brucella* infected sheep and cattle+ sera	30/30*	2/30**2/30***	30/30
Brucella free sheep and cattle sera	2+++/75	2+++/75	0/75
Vaccinated RB51 cattle sera (over 10 weeks PV++)	20/20	20/20	0/20
Vaccinated S19 cattle sera (over 10 weeks PV)	20/20	20/20	20/20
Vaccinated Rev-1 sheep Sera (over 10 weeks PV)	20/20	16/20**20/20***	20/20
Experimentally *B. ovis* infected sheep sera	20/20	20/20	0/20
Experimentally E. coli O:157 infected sheep sera	0/2	0/2	1/2

* No. +ve / No. tested samples; ** Rose Bengal test ***; Modified Rose Bengal test; + *Brucella* melitensis biovar3 were isolated from these animals; ++ PV = Post-vaccination; +++ The 2 samples are bovine samples

Table 2: Results of AGPT and CFT using rough *Brucella* antigens

	Using rough antigen	
Sera	CFT	AGPT
Brucella free sheep and cattle sera	0/75 (0%)	0/75 (0%)
Vaccinated RB51 cattle sera (over 10 weeks PV*)	20/20 (100%)	20/20 (100%)
Experimentally *B. ovis* infected sheep sera	16/20 (80 %)	20/20 (100%)

*PV = post-vaccination

Complement Fixation (CFT) and Agar Gel Precipitation Tests (AGPT): CFT using rough Hot Saline Extract antigen (HSE) yielded positive results with sera from all RB51 vaccinated cattle (20), negative results with all *Brucella* free sheep and cattle (75) and positive results in 16 out of 20 *B. ovis* experimentally infected sheep. AGPT using rough LPS (R-LPS) gave the same results of CFT in all the RB51 vaccinated cattle and *Brucella* free sheep and cattle but it was positive in all the 20 *B. ovis* experimentally infected sheep (Table 2).

DISCUSSION

The main serological test used for diagnosis of brucellosis is Rapid Slide Agglutination Test (RSAT) which has very high sensitivity but low specificity and low positive predictive value so it is required to be confirmed by some other more specific test like CFT, AGID and ELISA (Cadmus *et al.*, 2006). In the present study, it is the first time to use *B. ovis* rough Rose Bengal antigen in the diagnosis of *B. ovis* infection in sheep and also it is the first time to investigate the results of this test in S19, Rev.1 and RB51 vaccinated animals.

RSAT (Table 1), showed positive reaction of naturally infected animals with smooth *Brucella* infection using rough Rose Bengal antigen prepared with 10% NaCl and negative with that one prepared with 0.1 M PBS (except 2 sample). This was the first study about the response of the RSAT with animals naturally infected with smooth *Brucella.* While negative results given by

Brucella free animals in all 75 (Except two), suggesting that if sera are negative by RSAT, no further testing is required and the animal is considered not to be infected with rough *Brucella* strains as agreed by Badakhsh *et al.* (1982).

The low level of false results of the RSAT (6.66%) (2 out of 75) in *Brucella* free animals and animals infected with *E. coli* O: 157, came to some extent with previous reports (Mateu-de-Antonio *et al.*, 1994; Lopez *et al.*, 2006; Keid *et al.*, 2009) confirming that as many as 50-60% false positives do occur when using *B. ovis* antigen in RSAT for diagnosis of *B. canis* infection in dogs.

Regarding the positive results of the sera of all 20 experimentally *B. ovis* infected sheep and RB51 vaccinated cattle, this suggests the high sensitivity of RSAT and came in agreement with that of Lopez *et al.* (2005) and Escobar *et al.* (2010), On the contrary, the two rough antigens gave positive results with sera of animals vaccinated with smooth *Brucella* strains (Rev1 and S19 vaccines) regardless of the using of classical or modified RBT, this may be due to the high number of *Brucella* colonies inoculated in the animals during vaccination (1-2×10^9 CFU/dose in case of Rev.1 and 6-10×10^9 CFU/dose in case of S19) which may resulted in high concentration of cell wall proteins on which rough antigen depend on in agglutination test due to the absence of O-LPS as agreed by Hollett (2006).

Although, both CFT and AGPT are widely used for the diagnosis of *B. ovis* infection in sheep using R-LPS antigens, they have some disadvantages as in case of AGPT include low sample capacity, long turn around times, labour intensive work and high cost (Hollett, 2006). Whereas CFT have frequent anti complementary results, labor intensity, prozone phenomena, serum inactivation and false positives and negative (Searson, 1982; Marin *et al.*, 1989).

Both AGPT and CF tests (Table 2), showed similar results to that of RSAT in all the 75 *Brucella* free sheep and cattle, whereas the CF test showed a lower sensitivity (80%) than both the RSAT and AGPT tests (100%) in experimentally *B. ovis* infected sheep. The high sensitivity of AGPT in our results came in agreement with that of Cerri *et al.* (2000). On the contrary, Lopez *et al.* (2006) reported a lower sensitivity of the AGPT than RSAT, whereas Lopez *et al.* (2005) confirmed equal sensitivity of AGPT and CF tests using the strain (M) variant of *B. canis* as antigen. Moreover, Estein *et al.* (2002) recorded a lower sensitivity of AGPT than CF tests using *B. ovis* antigen for diagnosis of ovine brucellosis caused by *B. ovis*.

Because *B. ovis* and *B. canis* are rough strains that cross-reacts serologically with each other. Therefore, *B. canis* and *B. ovis* antigens have been used for detection of ovine and canine infections. Agglutination tests are generally not used for the diagnosis of infection with *B. ovis*, *B. canis* and rough species of *Brucella*, as the whole cell antigens tends to auto-agglutinate. However, micro-agglutination tests (Kimura *et al.*, 2008) as well as Rapid Slide Agglutination Tests (RSAT) or card tests were developed in as an easy and practical screening test. The serum is mixed with a Rose-Bengal stained heat-killed either *B. ovis* or canis suspension as shown in Table 3.

Table 3: Previous investigations of the RSAT using rough *B. ovis* and B. canis antigens for the diagnosis of ovine and canine brucellosis

Used antigen	Targeted infection	Species	Reference
B. ovis B. canis	*B. canis*	Dogs	Mateu-de-Antonio *et al.* (1994)
B. ovis	*B. canis*	Dogs	Hollett (2006), Keid *et al.* (2009)
B. canis	*B. ovis*	Sheep	Lopez *et al.* (2006, 2005)
B. canis	*B. canis*	Dogs	Escobar *et al.* (2010)

CONCLUSIONS

It is suggested the use of conventional RSAT (using rose Bengal antigen prepared from smooth strain S99) either by modified or traditional method in parallel with the rough RSAT (prepared with 0.1 M PBS) as screening tests for routine diagnosis of rough brucellosis followed by the AGPT as confirmatory test. Also it is possible the use of rough RSAT for identifying of RB51 vaccination in cattle. More investigations and standardization are needed by using larger number of animals to measure the sensitivity and specificity of the current antigens for accurate diagnosis and control of the disease in animals and to manage the risk of human exposure.

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