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Development of a Rapid and Specific Latex Agglutination Test for the Serodiagnosis of Camel Brucellosis Using a *Brucella melitensis* Periplasmic Protein Antigen

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Abstract: This study aimed to coat latex beads with a soluble *Brucella melitensis* periplasmic protein (SBPP50), then evaluate and compare the efficacy of latex agglutination test (LAT) using SBPP50 with RBPT, BPAT, SAT and I-ELISA serological tests in the field diagnosis of camel brucellosis. Blood samples (n=600) were collected from seven camel herds and tested using the five serological tests. Milk samples (n=78) were collected from she-camels for bacteriological culture. Test performance was analyzed by calculating sensitivity and specificity. No false positive results were detected by LAT, which was significantly lower than the other four tests in respect to test agreement. Additionally, the percentage of false positive was significantly higher in I-ELISA (18.52) than BPAT, SAT and RBPT (4.35, 8.33 and 13.37 respectively). With respect to bacteriological culture, the diagnostic sensitivity of LAT (81.81%) and I-ELISA (81.81%) is superior numerically to that of RBPT (72.72%), BPAT (72.72%), SAT(63.63%).Moreover, the diagnostic specificity of LAT (88%) and I-ELISA (88%) is superior numerically to RBPT (84%), BPAT (84%) and SAT (76%). In conclusions, LAT using SBPP50 of *B. melitensis* is a successful and rapid serodiagnostic field test for diagnosis of camel brucellosis with high sensitivity and specificity.

Key words: *Brucella melitensis* Periplasmic Protein · Latex Agglutination Test · Camel Brucellosis

INTRODUCTION

Brucellosis, especially caused by *Brucella melitensis* (*B. melitensis*), is considered the most-widespread re-emerging zoonosis in the world with more than 500, 000 human cases reported annually [1, 2]. The disease may have considerable economic, veterinarian and public health impacts in various countries. The eradication of brucellosis in animals is a necessary step to control the human disease [3]. Camel plays vital socioeconomic roles in the semi dry and arid zones of Asia and Africa [4]. Camels are highly susceptible to brucellosis caused by *B. melitensis* and *B. abortus*. The camels are always herded together with sheep and goats and to a lesser

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extent with cattle. Clinical diagnosis of *B.melitensis* infection in camel is based on the observation of clinical findings that is few and may include abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk. The incidence of these reproductive disorders might reached up to 12% [4, 5]. The clinical diagnosis is confirmed by isolation of *Brucella* and/or positive serology. In spite of *Brucella* isolation is the golden standard, this method is expensive, cumbersome and unpractical to apply at a large scale in control campaigns and only few laboratories have the expertise and the facility to culture *Brucella* from clinical specimens. Accordingly, the indirect diagnosis of brucellosis based on serological tests is of choice especially in eradication and control [6, 7].

The prescribed serological tests that widely used for diagnosis of brucellosis are the Rose Bengal test (RBT), the buffered plate agglutination test (BPAT), an indirect enzyme-linked immunosorbent assay (I-ELISA), the complement fixation test (CFT) and the serum agglutination test (SAT) [5, 7, 8]. RBT, BPAT and CFT tests are not specific enough to distinguish serological reactions due to *B. melitensis* from the false-positive reactions due to cross-reacting bacteria such as *Yersinia enterocolitica* O:9. Moreover, the RBT and CFT cannot detect all infected animals in a flock when used for individual testing. While good diagnostic results have been obtained with indirect or competitive enzyme-linked immunosorbent assays (ELISAs) using various antigens [5, 9]. ELISAs also are unable to differentiate *B.melitensis*-infected animals from those recently vaccinated with the Rev.1 vaccine or those infected with cross-reacting bacteria [10]. Additionally, these serological tests depend on the detection of *B.abortus* whole bacterial cells or smooth lipopolysaccharide (LPS) as antigens to detect antibodies in serum that give rise to false-positive reactions because of cross-reactivity with LPS from other bacteria [5]. None of the available serological tests has been shown to be reliable in routine individual diagnosis to be used as a gold standard test. This and other drawbacks of anti-LPS antibodies have developed an increasing interest in the detection of antibodies to alternative antigens, mainly outer membrane proteins and cytoplasmic proteins [11, 12]. A periplasmic protein has been identified as an immunodominant antigen of the cytosoluble protein extract of *B. melitensis* in sheep [13].In humans, latex agglutination assay was used for the serodiagnosis of brucellosis by coating colored latex beads with *brucella* lipopolysaccharides [14]. The present study aimed at (i) developing simple, practical, quick agglutination test for diagnosis of brucellosis in camel by coating latex beads with a hot saline extract soluble *B.melitensis* periplasmic protein (SBPP50) and (ii) evaluating the efficacy of latex agglutination test (LAT) for the diagnosis of brucellosis in camels compared to other serological tests including I-ELISA, RBPT, BPAT and SAT.

MATERIALS AND METHODS

All manipulations were in compliance with the guidelines for the welfare of animals and those of the concerned ethical authorities.

Animals: Six hundred camels of both sexes (110 males and 490 females) at different ages were included in the present study. The study was performed during the period of May 2013 to December 2014. These camels were belonged to seven herds located on the center and western regions of KSA. The herds were subjected to careful clinical and laboratory investigations. The inclusion criteria include (i) the herds that have never been vaccinated and they were considered as free or not free of infection, so any positive reaction in the serological tests was considered to be due to infection with a field strain of *Brucella,* (ii) animals with a history of abortion or stillbirth, (iii) group of clinically healthy animals to be used as a control in bacteriological examination. The exclusion criteria include (i) the herds vaccinated with any type of *brucella* vaccines, (ii) animals with surgical interference.

Samples: Five ml of blood was collected from each animal (n=600), that represented all camels in the respective herds, by jugular venipuncture with disposable needles and venoject tubes. Sera were separated by centrifuging at 5000×g for 7 min and stored in aliquots at -20°C until tested by the five selected serological tests (LAT, RBPT, BPAT, SAT and I-ELISA). Serum samples were heat inactivated at 56°C for inactivation of non-specific antibodies. Positive and negative control sera are the national reference sera standardized according to OIE [5]. Moreover, serum samples were collected from the naturally infected and the non-infected camels, depending upon bacteriological culture, for evaluating the sensitivity and specificity of the tests. A pretested questionnaire designed to collect animal and herd level data during blood sampling was administered.

Approximately 40 ml of milk were collected from each serologically positive (n=53, positive for at least 3 tests) and seronegative (n=25, negative for at least three tests) she-camels in sterile tubes for bacteriological examination. Additionally, abortion materials or vaginal discharges were collected from aborted animals (n=9/78) for bacterial culture.

Serological Tests: The serological tests used were LAT, I-ELISA, BPAT, RBPT and SAT. They applied in accordance with the Office International des Epizooties (OIE) Manual of Standards for Diagnostic Tests [5].

LAT

Preparation of Hot Saline Extract Containing Soluble *B. melitensis* Periplasmic Protein (SBPP50): Soluble *B.melitensis* periplasmic protein was prepared as previously reported [15] with some modifications. Five ml of *B. melitensis* serotype 3 suspension (10^{10}/ml) were added to 800 ml of sterile Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C in a Co_2 incubator for 48 h. *B. melitensis* was harvested and washed once with normal saline. Hot saline extracts were obtained by suspending *B. melitensis* in normal saline and autoclaving at 121°C for 20 min. The autoclaved suspension was centrifuged at 12000×g for 20 min at 4°C. Then the ammonium sulphate was added to obtain 50% saturationof supernatant and the solution was centrifuged at 10000×g for 20 min. The precipitate was dissolved in 0.01 M phosphate buffered saline (PBS) (pH 7.2). Dialysis occurred against PBS (pH 7.2) in dialysis bag overnight at 4°C. This preparation was designated SBPP50. The protein concentration was determined by the Micro BCA protein assay reagent kit using bovine serum albumin (BSA) as standard (Pierce, Rockford, USA). The SBPPs were stored at -80°C until use.

Sensitization of Latex Microspheres: The following procedure for sensitization of latex microspheres is slightly modified from previous work [16]. A latex polystyrene microspheres particle of 0.81µm diameter (LB8; Sigma) was used in the covalent binding reaction. Prior to use, the beads were suspended in deionized water and vigorously vortexed to ensure even distribution to break up any large particles and obtain a 10% suspension. An equal volume of latex particle and SBPP50antigenin PBS 0.01M (0.6 mg protein/ml) was incubated over night at 37°C. The mixture was centrifuged at 6000×g for 10 min. The supernatant was removed and the pellet was washed and brought to an original volume in glycine sodium chloride buffer (final pH 8.2) containing 1:500 bovine serum albumin to keep the latex well suspended (7.505g of glycine, 5.85g of sodium chloride and 0.1% sodium azide in 1 liter of deionized water, adjust the pH to 8.2 with 1N NaOH). The latex particles were colored by methylene blue (0.005g%) and the latex suspension kept at 4°C.

Agglutination Reactions: The purified hot saline extract SBPP50 was used as antigen that coated the latex beads. Agglutination reactions were performed by using the following procedure. A drop of latex solution and sample was placed on a microscope glass slide. The reaction was mixed by a side-to-side movement. The agglutination reaction appears in less than 2 minutes at room temperature in positive cases [14].

I-ELISA: Commercial LPS-based I-ELISA kit was used (Pourquier, France).The results were recorded on an ELISA reader (Reader 270, BioMerieux) at 450 nm. The color development is directly proportional to the bound antigen-antibody complex. Samples with a value twice the optical density (O.D.) of the mean negative control were considered positive.

RBTand BPAT: BT and BPAT was conducted as described in the Manual of Standards for Diagnostic Tests and Vaccines [5] using antigen obtained from Institute Pourquier, France.

SAT: SAT was performed in microtiter plates [17]. Samples showing more than 30 I.U. per milliliter were considered positive.

Bacteriological Isolation: Milk samples (n=78, 53 from serologically positive and 25 from seronegative she-camels) were cultured on *brucella* agar selective media. The species and biovars of *brucella* were identified according to [5] using cultural and serological criteria.

Sensitivity and Specificity: Depending upon bacteriological examination, the sensitivity of all tests were assessed with sera from naturally infected she-camels (culture positive). While, the specificity was assessed with sera from non-infected (culture negative) she-camels.

Test performance was analyzed using classical means of calculating sensitivity and specificity with respect to the test agreement [18] and the infected and brucella-free

groups [17]. In the case of test agreement, the diagnostic sensitivity was estimated as true positive/true positive +false negative and the diagnostic specificity estimated as true negative /true negative +false positive. The sample was considered true negative or true positive when it gave negative or positive results for at least three tests. Culturing and the serological tests were performed at our central research laboratory in order to limit bias linked to the test performance.

Statistical Analysis: Data were entered in Microsoft Excel and the software package Statistical Products and Service Solutions (SPSSv. 17.0, SPSS Inc., Chicago, IL, USA). The program was used for all analyses. Comparisons among serological tests were done using the χ^2 (Chi-square) test. A difference was considered to be significant when $p \leq 0.05$.

RESULTS

Clinically, only few clinical signs were appeared in some camels (n=9) including reduced appetite and abortion or stillbirth.

The evaluation of RBPT, BPAT, SAT, I-ELISA and LAT for diagnosis of brucellosis in camel was showed in Table 1. Figure 1 showed LAT using SBPP50 antigen, a clear agglutination reaction appears in less than 2 minutes at room temperature in case of a positive control serum (a), with no agglutination in case of a negative control serum (b). The percentages of total positive sera were significantly lower than the total negative sera in all five serological tests. There was an insignificant different between the percentages of total positive sera or the percentages of total negative sera in all selected serological tests (8.5%, 7.67%, 8%, 9% and 7.33% in RBPT, BPAT, SAT, I-ELISA and LAT respectively). No false positive results were detected in LAT, which made it significantly lower than the four other tests. Additionally, the percentage of false positive was significantly higher in I-ELISA than RBPT, BPAT and SAT. While, no significant difference was detected in between RBPT, BPAT and SAT or in between RBPT and I-ELISA. Additionally, no false negative results were detected in the five serological tests. There was no significant difference was detected between the serological tests for diagnostic sensitivity or diagnostic specificity with respect to the test agreement.

Bacteriologically, the natural infection was confirmed by isolation and identification of the *B.melitensis* serotype 3 from 11 of 53 (20.7%) she-camels. While, the 25 seronegative she-camels were bacteriologically negative. The sensitivity of the LAT using periplasmic protein antigen of *B. melitensis* assessed with sera from naturally infected she-camels (culture positive) was 81.81% whereas its specificity when assessed with sera from non-infected (culture negative) she-camels was 88%.

Table 1: Evaluation of LAT, RBT, BPAT, SAT and I-ELISA for diagnosis of camel Brucellosis and their sensitivity and specificity (percentage).

Parameters		RBPT	BPAT	SAT	I-ELISA	LAT	Chi square *p* value
Positive	Total	8.5	7.67	8	9	7.33	0.8416
	False	13.73^{ab}	4.35^{bc}	8.33^{ab}	18.52^{a}	0^{c}	0.0151
Negative	Total	91.5	92.33	92	91	92.67	0.8416
	False	0	0	0	0	0	1
Sensitivity	Test agreement	100	100	100	100	100	1
	Bacteriological	72.72	72.72	63.63	81.81	81.81	0.8543
Specificity	Test agreement	98.74	99.64	99.28	98.2	100	0.6458
	Bacteriological	84	84	76	88	88	0.6379

Percentage carry different superscript within the same row was significantly higher at probability ≤ 0.05

Table 2: The correlation among LAT, RBT, BPAT, SAT and I-ELISA for diagnosis of camel Brucellosis.

Number of cases	RBPT	BPAT	SAT	I-ELISA	LAT	Final decision result
543	Negative	Negative	Negative	Negative	Negative	Negative
1	Positive	Positive	Negative	Negative	Negative	Negative
3	Negative	Negative	Positive	Positive	Negative	Negative
5	Positive	Negative	Negative	positive	Negative	Negative
1	Negative	Positive	Negative	Positive	Negative	Negative
1	Negative	Negative	positive	Negative	Negative	Negative
1	Positive	Negative	Negative	Negative	Negative	Negative
1	Negative	Negative	Negative	positive	Negative	Negative
44	Positive	Positive	Positive	Positive	Positive	Positive

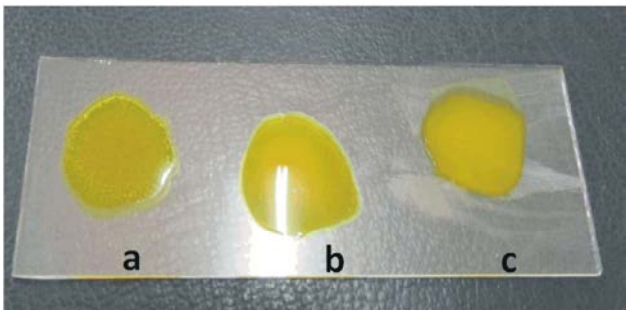

Fig. 1: Latex agglutination test using SBPP50 antigen. A clear agglutination reaction appears in less than 2 minutes at room temperature in case of a positive control serum (a), with no agglutination in case of a negative control serum (b) and latex SBPP50 alone (c)

Whereas, the sensitivity was insignificantly differ between RBPT, BPAT, SAT and LAT or between RBPT, BPAT, I-ELISA and LAT. Additionally, no significant difference was detected between all selected serological tests related to specificity.

The correlation between RBT, BPAT, SAT, I-ELISA and LAT for the diagnosis of camel Brucellosis was presented in Table 2. There was complete agreement among the five tests in 587 cases (543 negative and 44 positive), with some disagreement in 13 cases.

DISCUSSION

The present study developed a rapid and specific field test, LAT, for the serodiagnosis of camel brucellosis using a soluble *B.melitensis* periplasmic protein (SBPP50) as an antigen. It is very easy to perform and to read, requires neither training nor electricity and, apart from a micropipette to dispense the serum sample, the procedure does not require expensive equipment. In addition, the stability of the test reagent and simplicity of the assay procedure make the test suitable for use outside the established laboratory. A similar assay was developed for serodiagnosis of human brucellosis [14], but that assay used the *Brucella* lipopolysaccharide (LPS) as an antigen that gave false-positive reactions because of cross-reactivity with LPS from other bacteria [5].

No false positive result was detected in LAT, which made it significantly lower than the other four serological tests. The other four tests (RBPT, BPAT, SAT and I-ELISA) are mainly based on the detection of antibodies directed against the *B.abortus* whole bacterial cells or LPS portion of the cell membrane [5], which gives false positives because of cross-reactivity with other Gram-negative bacteria like *Escherichia coli* [19], *Yersinia enterocolitica* O:9 [20] and *Salmonella enterica* serotype urbana [21]. Additionally, it is difficult to differentiate between vaccinated and infected animals using LPS-based serological tests [22]. Hence, a major goal in immunological studies of brucellosis has been the identification of non-lipopolysaccharide antigens, which could be useful to circumvent drawbacks of LPS antigen. For this reason, we used SBPP50 of *B. melitensis* as a protein antigen in LAT and confirmed that it did not lead tofalse positive results.

The protein antigens, other than non-LPS antigens, would permit a better standardization of the assay compared to the more complex whole-cell antigen preparations currently in use. In this study, hot saline extracts of *B. melitensis* serotype 3 were separated by precipitation with ammonium sulfate, yielding a SBPP50 (proteins of 36 kDa) that couple to the latex beads (15). Additionally, the latex coagglutination assay can substitute advantageously for the current anti-*Brucella* (R) rabbit monospecific serum. Bowden and coworkers used latex beads and protein A coated with either an anti-Brucella rough-lipopolysaccharide (R-LPS) monoclonal antibody (MAb) or an anti-Brucella 25-kDa outer membrane protein (Omp25) MAb and were able to discriminate *B.ovis* isolates from other S and R *Brucella* isolates [23]. The test was determined to be specific for the genus *Brucella* as no reactivity was observed with other genetically or antigenically related organisms [23]. Preliminary C-ELISAs studies with a periplasmic protein from *B. melitensis* (BP26) as the antigen have been applied in sheep and reported to be promising in differentiating Rev.1 vaccinated from *B. melitensis* infected animals [13, 24]. Furthermore, our results showed complete agreement among the five tests in 587 cases (543 negative and 44 positive), with some disagreement in 13 cases (Table 2). No reference test is currently available and, to date, none of the available serological tests has been reliable enough in routine individual diagnosis to be used as the only test [5].

Clinically, the natural infection of camel was confirmed by isolation and identification of the *B. melitensis* from 11 (20.75%) of 53 camels with history of abortion or stillbirth. A close contact between infected and susceptible camels in a herd promotes the spread of diseases. The appearance of brucellosis depends on the *Brucella* species being prevalent in other animals sharing their habitat (cross transmission between species) and on the husbandry system. The camels are always herded together with sheep and goats and to a lesser extent with cattle and they share the same watering points and pastures and so it is not surprising to find a higher incidence of the *B. melitensis* among camels [25]. It is possible that the tendency of Saudis to raise large flocks of sheep along with the camel herds contributed towards the spread of *B. melitensis* among camels [26]. Malta fever due to *B. melitensis* was diagnosed in 30% of the camel handlers and milkers on large camel farm in Riyadh, Saudia Arabia. The abortion rate in the farm reaching 12% and *B. melitensis* biovars 1, 2 and 3 were isolated from aborted camel fetuses [26]. Moreover, different biovars of *B. melitensis* were isolated from camel milk [26, 27], which indicates that this organism is excreted through milk. Therefore, there is a real need for cooperation between public health officials and veterinary officers to reduce the circulation of human brucellosis in endemic areas [28]. However; the prevalence of brucellosis is increasing in many developing countries due to various sanitary, socioeconomic and political factors [29].

Importantly and with respect to the infected and *brucella*-free groups, both the sensitivity and the specificity of the LAT using SBPP50 from *B. melitensis* are relatively high (Table 1). The sensitivity of LAT is superior numerically to that of RBPT, BPAT and SAT. Moreover, the specificity of LAT is superior to RBPT, BPAT and SAT. We chose culture for calculating sensitivity and specificity because the culture provides definite proof of infection. It is considered the gold standard and is reliable for calculation; in our study there was no numerical difference in the sensitivity of the five tests with respect to test agreement (Table 1). The sensitivity and specificity of LAT using SBPP50 of *B. melitensis* were slightly similar that obtained in another study [13]. Because sensitivities and specificities of screening tests in camels were not well known, none of the commonly used serological test can be perceived as a perfect test for *Brucella* diagnosis in camel and most serological tests used for camels have been directly transposed from cattle without adequate validation. Hence, our results support the use of LAT using SBPP50 of *B. melitensis* for diagnosis of brucellosis in camel.

In conclusion, this is the first study, for the best of our knowledge, to identify serum reactivity to a purified hot saline extract SBPP50 of *B.melitensis* in camel brucellosis. Moreover, we have developed a simple and rapid screen field test for the serodiagnosis of camel brucellosis, LAT using SBPP50 of *B. melitensis* as antigen. This test may well be ideal in field condition as, (i) it is very easy to perform and to read, (ii) it does not require expensive equipment and electricity, (iii) it does not require access to a diagnostic laboratory and can be used in places where health care has no access and (iv) it is sensitive and specific as the four other tests used in this study and other proteins in other studies. This test may be suitable for clinics in endemic areas that do not have the expertise, facility and resources to perform or order any of the routine tests used in well-equipped clinics. The primary objective in the control and eradication of brucellosis is to have a sensitive and specific serological test to detect infected animals. The results of our study support the use of LAT using SBPP50 of *B. melitensis* not only for diagnosis of brucellosis in camel but also in its control and eradication. The identification of protein components of *Brucella* bacteria that elicit an antibody response in the majority of infected animals would improve the diagnosis of the disease and contribute to the development of new vaccine strategies. Hence, further studies will needed to evaluate the SBPP50 of *B. melitensis* as vaccine candidate using the molecular methods for developing a safe recombinant vaccine [30, 31].

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Conflict of Interest: No conflict of interest to declare.

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