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BACTERIOLOGY AND VIROLOGY RESEARCH

Research Article

Development, Optimization and Standardization of a Diagnostic Immunoassay for Camel Brucellosis

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Received: December 17, 2015; **Published:** January 07, 2016

Abstract

This research was carried out to develop a Tube Agglutination Test with greater sensitivity and specificity for serological diagnosis of camel brucellosis. Classical Tube Agglutination Test (TAT) antigen and Buffered Tube Agglutination Test (BTAT) antigen were prepared. In the Tube Agglutination Test (TAT), the classical neutral pH antigen had to be replaced by a buffered antigen (pH 3.65).

The Buffered Tube Agglutination Test (BTAT) demonstrates a better combination of diagnostic sensitivity and diagnostic specificity than the classical Tube Agglutination Test (TAT) in current use. The sensitivity and specificity of the Buffered Tube Agglutination Test (BTAT) were high (73.74% and 91.73%, respectively). The high sensitivity and specificity values make the Buffered Tube Agglutination Test (BTAT) an excellent immunoassay for the routine diagnosis of camel brucellosis.

The Buffered Tube Agglutination Test (BTAT) is a suitable immunoassay to solve the problem of formation of prozone reaction. On the other hand, the classical Tube Agglutination Test (TAT) was found to be associated with formation of more prozone reactions.

Keywords: *Buffered Agglutination; Tube Agglutination; Sensitivity; Specificity; Camel Brucellosis; Prozone Reaction; Immunoassay*

Introduction

Domesticated representatives of the family camelidae are an important part of the national livestock populations of numerous countries. Any of the three known types of Brucella namely *B. abortus, B. melitensis and B. suis* may infect camels. The organism is readily transmitted between infected and non-infected camels. According to Mohammed [1] there is a general lack of information on brucellosis in camels despite serological evidence of Brucella infection with a varying incidence. Although reports appear fairly regularly in the literature concerning the incidence of brucellosis in camels, it is less clear how serious the disease is in nomadic Arabian camel herds. *Alton, Jones, Angus and Verger [2] stated that Incontrovertible evidence of Brucella infection is obtained by isolation and identification of the organisms. Since it is not always possible to isolate the causal organisms from infected camels, serological tests play a major role in the routine diagnosis of brucellosis.*

Although a number of tests are employed in the serological diagnosis of camel brucellosis, researchers are still looking for the perfect test, immunoassay which detects an infected animal early stage of the disease as well as at all subsequent stages. It is quite clear that the classical serology has historically played a very prominent role in the diagnosis of camel brucellosis. The agglutination tests, as simple as they may be seen, have been intensively studied with respect to their serological and diagnostic performance and a number of modifications have been introduced, driven by the need for greater diagnostic specificity. The application of new serological techniques to the diagnosis of camel brucellosis will not necessarily result in a markedly improved test from a diagnostic standpoint of view. It is necessary to consider new techniques in view of what recently learned from research developments. The vast majority of pervious research on camel brucellosis was based on serological testing as a tool for diagnosis. Wernery [3] stated that none of the serological brucellosis tests

are validated for use with camel sera. Suspicious serological test results to detect camel brucellosis were noticed by different researchers [4-8]. They concluded that the Tube Agglutination Test (TAT) has increased sensitivity to immunoglobulin M (IgM) rather than immunoglobulin G (IgG). Wernery and Wernery [9] solved the problem of unspecific reactions in the Tube Agglutination Test (TAT) by using a 5% solution of phenol sodium chloride. This solution known to increase the specificity of the Tube Agglutination Test (TAT) and at the same time decreased the cross- reactivity. Mohammed [1] found that Tube Agglutination Test (TAT) has an inferior ability in detecting positive and negative sera as well as prozone reactions.

The present investigation is an attempt to develop, optimize and standardize a diagnostic system for camel brucellosis. Specifically, the purpose of this research is:

a. To develop an optimal test which is easy to use, economical and can detect early infection as well as all subsequent stages of the disease.
b. To develop a diagnostic test with greater sensitivity and specificity for the diagnosis of camel brucellosis.
c. To evaluate responses of the prozne phenomenon to the developed diagnostic system in camel brucellosis.
d. To develop and evaluate a Buffered Tube Agglutination Antigen (BTAA) for the diagnosis of camel brucellosis.

Materials and methods

One hundred thirty two positive camel serum samples identified as such by the classical Tube Agglutination Test (TAT) were used in the present investigation. A further 568 negative samples in the classical Tube Agglutination Test (TAT) were also used during this research. Each serum was analyzed using both the classical Tube Agglutination Test (TAT) and the Buffered Tube Agglutination Test (BTAT).

Data about this investigation was attained through:

a. Testing the newly developed Buffered Tube Agglutination Test (BTAT) antigen.
b. Analysis of sensitivity and specificity of the developed Buffered Tube Agglutination Test (BTAT).
c. Evaluation of the responses of the prozone phenomenon.

Diluents

Phosphate-Buffered Saline

Normal saline (8.5g of sodium chloride dissolved in 1 liter of distilled water) adjusted to a pH of 6.4 by the addition of 6.67g of potassium dihydrogen phosphate (KH2 PO_4) and 3.00g of anhydrous disodium hydrogen phosphate (Na $2HPO_4$) was prepared.

Phenol-Saline Solution

A solution consisting of 0.85% of sodium chloride and 0.5% of phenol in distilled water was prepared.

Buffered Diluents

20g of sodium hydroxide (NaOH) were dissolved in 300ml of sterile 0.5% phenol saline. 90ml of concentrated lactic acid (85%) were added and mixed. Sufficient sterile phenol-saline was added to make 1 liter. The pH was adjusted to 3.64 - 3.65.

Antigens

The propagation of B. abortus for the production of Brucella antigen

Seed culture

An ampoule of a freeze dried seed culture (obtained from the National Veterinary Services Laboratories NVSL, Ames, Iowa, USA) was reconstituted with 2 ml of sterile distilled water and used to inoculate a number of potato agar slopes contained in tubes. These were incubated at 37°C for 48 hours and used to seed production cultures.

Production cultures

Seed material on a potato agar slope, was harvested by adding 10 ml of sterile phosphate buffered saline, pH 6.4. The tube was rolled between the palms of the hand until the Brucella was suspended. This suspension was added to 1800 ml of buffered saline, pH 6.4, contained in a 2 liter aspirator jar fitted with a hose and protective bell attachment.

Roux flasks of potato agar were inoculated by inserting the neck of each flask into the bell of the aspirator jar and delivering 5 ml of the seed suspension into each flask. Each roux flask was tilted of the backwards and forwards so that the suspension was uniformly distributed over the surface of the agar. The roux flasks were then incubated at 37°C for 72 hours with the medium uppermost in the flasks. After incubation the flasks were examined individually, and any one showing contamination or detachment of the agar were discarded. The mouth of the Roux flask was then flamed and the fluid contained in each flask was poured off into disinfectant. 50 ml of phenol-saline was added to each flask. The flasks were left for 30 minutes with medium towards the bottom, after which each flask was gently agitated until the Brucella were suspended. The pooled suspension was filtered through sterile absorbent cotton.

Processing the bacterial suspension

The Brucella cells were removed from the suspension by centrifugation in weighed tubes in a refrigerated centrifuge. The supernatant was discarded and the tubes were reweighed. The deposit was suspended in twice its volume of phenol-saline with agitation on a shaking machine. The Brucella cells were killed in a water bath at 95°C for 60 minutes. After cooling to room temperature, a sample of the suspension was inoculated on to a potato agar slope and into a tube of dextrose Andrade's broth. A smear was also made on a slide and stained by Gram's Method. The cultures were examined daily for seven days. Sterile and pure bacterial suspensions were used to prepare *B. abortus* antigens.

Preparation and standardization of the tube agglutination test antigens

The Tube Agglutination Test (TAT) antigen was prepared in the same way according to Mohammed [1] and according to Table 1 regarding the pH. The only exception when preparing this antigen is that the Buffered Tube Agglutination Test (BTAT) antigen was suspended in buffered diluent.

Preparation of the Tube Agglutination Test Antigen

A quantity of suspension containing a known weight of packed cells of B. abortus (80g) was poured in a Pyrex bottle and phenol-saline was added in ratio of 1:10 cells-saline dilution. The suspension was mixed by shaking and the then filtered through sterile absorbent cotton. Sufficient phenol-saline was passed through the filter to bring the volume up to 1825 ml. The suspension was stirred with a magnetic stirrer at room temperature for 2 hours, and then stored at 4°C for 16 hours.

The suspension was removed from the refrigerator and agitated on a magnetic stirrer for 24 hours. The packed cell volume (PCV) was then determined according to the procedure described by Schalm, Jain and Carroll [10]. The cell concentration and pH were adjusted as shown in Table 1.

Antigen	pH	Cell Concentration
Tube Agglutination test	7.00	4.5%
Buffered Tube Agglutination test	3.65	4.5%

Table 1: *The Tube Agglutination Test Antigen.*

Optimization of the Tube Agglutination Test

Technique of the test

The Tube Agglutination Test (TAT) antigens were diluted 1:100 before use. With a 0.2 ml pipette serum was withdrawn from the blood tube. In rack#1 the pipette was inserted to the bottom of the first tube. 0.08 ml of serum was delivered. 0.04 ml, 0.02 ml and 0.01 ml were placed in the second, third and fourth tubes. Using the same procedure, the same serum sample was distributed in the tubes of

the rack#2. The same procedure was repeated with the rest of a total of 700 camel serum samples. With an automatic pipette 2.0 ml of either diluted antigen namely Tube Agglutination Test (TAT) antigen and Buffered Tube Agglutination Test (BTAT) antigen in phenol-saline, were delivered into each tube. This resulted in dilutions of 1:25, 1:50, 1:100 and 1:200. The racks of tubes were gently shaken and placed in the incubator at 37°C for 48 hours.

Statistical Analysis

The difference between the classical Tube Agglutination Test (TAT) and the Buffered Tube Agglutination Test (BTAT) was assessed by chi-square McNemar's test. A level of P 0.05 was considered statistically significant. All analyses were conducted using SPSS software version 17.

Result and Discussion

Result

The tubes were observed against a dull black background with light coming from behind the tubes. A positive reaction (P) is the one in which the serum antigen mixture is clear and gentle shaking does not disturb the flocculi. An incomplete reaction (I) is the one in which the serum antigen mixture is partially clear and gentle shaking does not disturb the flocculi. A negative reaction (N) is the one in which the serum antigen mixture shows no sign of clearing and gentle shaking reveals no flocculi. Prozone phenomenon is a false negative response resulting from high antibody titer which interferes with formation of antigen antibody lattice necessary to visualize a positive flocculation test and it could be due to antibody excessor it may be blocking antibody or non-specific inhibitor in the serum.

Recording and Interpretation the Tube Agglutination Test Reactions

A positive reaction was recorded as positive (P), an incomplete reaction (I) and as negative (N). Titers obtained in the test were equal to International Unit (IU) Alton., *et al*. [2], for example, a serum giving 'P' at 1:25 contains 25 IU/ml.

Comparison of the classical Tube Agglutination Test (TAT) and the Buffered Tube Agglutination Test (BTAT)

To compare the classical Tube Agglutination Test (TAT) and the Buffered Tube Agglutination Test (BTAT), the chi-square McNemar's statistical was calculated (Table 2)

Test	X^2	P
BTAT vs. TAT	9.131	0.003

Table 2: *Chi-square McNemar's Test of Difference between BTAT and TAT for Diagnosis of Camel Brucellosis.*

On comparing the Buffered Tube Agglutination Test (BTAT) with the classical Tube Agglutination Test (TAT), a higher difference was obtained (X^2 McNemar's = 9.131), with 47 sera identified positive by the Buffered Tube Agglutination Test (BTAT) and were considered negative by the classical Tube Agglutination Test (TAT) due to presence of prozone reactions.

Discussion

This study is an attempt for the development, optimization and implementation of an immunoassay to be used in serological diagnosis of camel brucellosis. It seems that there is a general lack of data concerning the disease in camels despite serological evidence of brucellosis in these animals Mc Grane and Higgins, [12]. Variations have been noticed in the results of camel serology Wernery [3]. As a basis for solving this problem, it is necessary to optimize and standardize the immunoassays used in the serological diagnosis of camel brucellosis.

The principal objective of the serologic tests used in brucellosis control and eradication programs is to detect infected animals that may cause the disease to spread. Therefore, one of the most important drawbacks of the Classical Tube Agglutination Test (TAT) is the existence of false-negative reactors that appear as a result of the prozone phenomenon Mohammed [1]. In the present study, the prozone phenomenon produces 47 false negative agglutination reactions detected in the Tube Agglutination Test (TAT) and were found positive

when tested with the Buffered Tube Agglutination Test (BTAT) (Table 3). From these findings, the researcher would postulate that there is a tendency of camel sera to be associated with prozone phenomena. This confirms previous surveys associating the prozone phenomena to high titer sera [13,14]. For this reason, Mustafa [15] suggested that the agglutination test should be done using higher dilutions to overcome the problem of prozone reactions.

BTAT Results	Disease status		
	Positives	Negatives	Total
Positive	132	47	179
Negative	0	521	521
Total	132	568	700

Table 3: *Evaluation of Buffered Tube Agglutination Test (BTAT) for Diagnosis of Camel Brucellosis.*

The Tube Agglutination Test (TAT) seems to be the most used immunoassay in the diagnosis of brucellosis in camels. With respect to the sensitivity and specificity, it was found to suffer from poor diagnostic performance. To develop a Tube Agglutination test with greater sensitivity and specificity, it is important to understand the role of non-specific antibodies.

The examination of camel sera with the newly developed Buffered Tube Agglutination Test (BTAT) antigen showed that non-specific antibodies are not involved in the Buffered Tube Agglutination Test. This is probably due to the acidic buffer which inhibits immunologically non-specific agglutinins. This means that in addition to the specific IgM detected by the buffered antigen, the IgG_1 class antibodies are already present in the majority of camel sera tested with the Buffered Tube Agglutination Test (BTAT). In a previous study Jones [16] was able to demonstrate that bovine IgG_1 agglutinates very poorly except at an acid pH. In contrast, the classical Tube Agglutination Test (TAT) has casted serious doubts with respect to its efficacy in the serological diagnosis of camel brucellosis.

The classical Tube Agglutination Test (TAT) although was the most commonly used serological test for the diagnosis of camel brucellosis [17,18,19] was found to be less sensitive (detected only 132 positive camel sera) than the Buffered Tube Agglutination Test (BTAT) towards the detection of 179 positive camel serum samples (Table 3).

The work description in this investigation establishes that Buffered Tube Agglutination Test (BTAT) is capable of demonstrating the best analytical performance with respect to both sensitivity and specificity when compared to the classical Tube Agglutination Test (TAT). The sensitivity and specificity of the Buffered Tube Agglutination Test (BTAT) were high (73.74% and 91.73%, respectively) (Table 4).

Statistic	Value	95% Cl
Sensitivity	73.74%	66.66% - 80.03%
Specificity	91.73%	89.15% - 93.86%

Table 4: *Diagnostic Test Evaluation of Buffered Tube Agglutination Test (BTAT).*

It could be concluded that the Buffered Tube Agglutination Test (BTAT) demonstrates high analytical sensitivity and specificity for both IgM and for IgG_1. Wright and Nielsen [20], suggested that an immunoassay of high analytical sensitivity for IgG_1 alone may be offer a better compilation of diagnostic sensitivity and diagnostic specificity than the classical assays in current use.

Prozone and other blocking phenomena, particularly in high titer serum, are reported to cause false negative reactions Mohammed [1]. Interestingly, investigation of responses of prozone phenomena on the classical Tube Agglutination Test (TAT) also revealed that this test was found to be associated with high prevalence of prozone reactions (47/700) (Table 3). These findings were consistent with the results published by Mustafa [15] who reported that the Tube Agglutination Test (TAT) often fails leading to prozone phenomena.

Furthermore, the Buffered Tube Agglutination Test (BTAT) developed in this investigation, was found to be capable of eliminating the limitations of the classical Tube Agglutination Test (TAT) with respect to prozone phenomena (Table 3). The absence of prozone reactions, the high sensitivity and specificity values make the Buffered Tube Agglutination Test (BTAT) an excellent immunoassay for the routine diagnosis of camel brucellosis. It is also interesting to speculate what effect the newly developed Buffered Tube Agglutination Test (BTAT) will have on the future serological diagnosis of camel brucellosis.

Conclusion

The Buffered Tube Agglutination Test (BTAT) demonstrates a better combination of diagnostic sensitivity and diagnostic specificity than the classical Tube Agglutination Test (TAT) in current use. The high sensitivity and specificity values make the Buffered Tube Agglutination Test (BTAT) an excellent immunoassay for the routine diagnosis of camel brucellosis. On the other hand, the Buffered Tube Agglutination Test (BTAT) developed in the present investigation was found to solve the problem of prozone phenomenon.

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Volume 1 Issue 1 January 2016