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Serological Study of Camel Brucellosis in Elgadaref State, Eastern Sudan

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Abstract This work has been formulated to contribute to the enhancement of diagnostic capabilities by studying different serological techniques. The study area was chosen for their high camel populations, ELgadaref State. A total of 343 serum samples from different camel herds were collected. Serum samples were subjected to serological investigation; all samples were tested using three serological tests which were Rose Bengal Plate Test, competitive ELISA and Complement Fixation Test. Seroprevalence was (36.2%, 42.6 and 19.8%) with the RBT, CFT and cELISA respectively.

Keywords: *Brucella, RBT, cELISA, CFT, serology*

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1. Introduction

When thinking about conducting research in camel brucellosis, bearing in mind that brucellosis is endemic in Sudan and that Sudan have one of the biggest camel populations in the world, camels are highly susceptible to brucellosis caused by *Brucella melitensis* and *Brucella abortus*. Complexities can arise in diagnosis of camel brucellosis, especially as this disease causes only few clinical signs in contrast to its clinical course in cattle, sheep and goats. Because none of the commonly used serological tests can be perceived as a perfect test for Brucella diagnosis in camel and most serological tests used for camels have been directly transposed from either cattle or small ruminants without adequate validation, an incorrect diagnosis may occur when diagnosis is based on one serological test.

This study has been formulated to contribute to the enhancement of diagnostic capabilities by studying different serological methods which include RBPT, cELISA and CFT techniques. The area was chosen for its high camel populations, Elgadaref State.

2. Methodology

2.1. Sample Collection

343 Blood samples were taken from camels and processed as described by [1]. The skin over the jugular vein was rubbed with 70% alcohol and disinfected by the application of tincture of iodine. Then 7ml of blood was withdrawn using a labeled vacutainer. Samples were put in a wire basket under shade, before taken to laboratory with minimum possible shaking. These samples were kept overnight at 4°C for separation of sera. These sera were then separated from the whole blood by centrifugation, placed in sterile bijou bottles labeled and stored frozen for testing.

2.2. Serological Test

Three serological tests were conducted, these are, Rose Bengal Plate Test (RBPT), Enzyme-linked immunosorbent assay (c-ELISA) and Complement fixation test (CFT).

2.2.1. Rose Bengal Plate Test

The antigen used in the RBPT was obtained from the Veterinary Research Institute (VRI), Soba. The sera and the antigen were brought to room temperature before testing.

The test was done as described by [2] by dispensing 0.03ml of each serum to be tested to an enamel plate and equal amount of RBPT antigen was added to each serum sample and both were mixed together, rocked by hand for four minutes, after which the test was immediately read. Result was read as follows: -

Negative when there was no agglutination or clumping, or showing a pattern of dispersed particles without clumps.

Positive when there was agglutination, with moderate to large clumps.

Positive results to RBPT were classified into five categories according to [2].

Weak positive: When very weak fine agglutination occurred, this could be hardly seen by naked eyes.

Positive: when agglutination was fairly visible

Positive with ring formation: When the agglutination forming a ring.

Strong positive: where there was a granular agglutination

Very strong positive: where the agglutination was very rapid and large clumps occurred, leaving only clear fluid.

Positive and negative control sera were used to be sure the antigen working perfectly.

2.2.2. Enzyme-linked Immune Sorbent Assay (c-ELISA)

The test was carried out as described by Animal Health Veterinary laboratory Agency, U.K (kit manufacture).

- Kit content:

Plates: Plates pre-coated with B. melitensis LPS antigen.

Diluting buffer: Tablets of phosphate buffered saline (PBS)+ Phenol Red Indicator +Tween 20.

Wash solution: Na2HPO4 plus Tween 20

Conjugate: As supplied (Store at -20°C)

Chromogen: OPD tablets (toxic)

Substrate: urea hydrogen peroxide tablets

Stopping solution: citric acid

Control: positive serum and Negative serum

Equipment Required:

Microtiter plate reader with 450nm filter

Single and multichannel variable volume pipettes

Disposable tips for the above pipettes.

Reagent troughs for multichannel pipetting

10 litre container for wash fluid

4°C ± 3°C refrigerator

Rotary shaker, capable 160 Revs/Min (or a 37°C ± 3°C incubator)

Microliter plate shaker

Sterile distilled or deionized water

Bottles, tubes and beakers for storage of sera and reagents

Absorbent paper towels

Freezer for storage of conjugate.

Test procedure:

The conjugate solution was prepared immediately and diluted to working strength with a diluting buffer according to instructions on the ampoule label.

20µl of each test serum was added per well and columns 11 and 12 were left for controls.

20µl of the negative control was added to wells A11, A12, B11, B12, C11 and C12.

20µl of the positive control was added to wells F11, F12, G11, G12, H11 and H12.

The remaining wells (had no serum) were act as the conjugate controls.

Immediately 100µl of the prepared conjugate solution was dispensed. This gave a final serum dilution of 1/6.

The plate was then vigorously shaken (on the microtitre plate shaker) for 2 minutes in order to mix the serum and conjugate solution. The plate was covered with the lid and -incubated at room temperature (21°C ± 6°C) for 30 minutes on a rotary shaker, at 160 revs/min.

The contents of the plate were taken out and the plate was rinsed 5 times with washing solution and then thoroughly dried tapping on an absorbent paper towel.

The microtiter plate reader was switched on and allowed the unit to stabilize for 10 minutes.

Immediately before use the substrate and chromogen solution were prepared by dissolving one tablet of H2O2 in 12 ml of distilled water. When dissolved the OPD tablet was added and mixed thoroughly. This took a few minutes; the use of a magnetic stirrer greatly increased the speed with which it dissolved. 100µl of this solution was added to each well. (This solution was not stored).

The plate was left at room temperature for a minimum of 10 minutes and a maximum of 15 minutes.

The reaction was slowed by adding 100µl of stopping solution to each well.

Condensation from the bottom of the plate was recovered with an absorbent paper towel. Read plate at 450nm.

Test interpretation:

The lack of color development indicated that the sample tested was positive. A positive/negative cut-off was calculated as 60% of the mean of the optical density (OD) of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value was regarded as positive.

Evaluation of test results:

The test results of each plate were evaluated by checking the following values.

Binding Ratio = Mean of 6 negative control wells

Mean of 6 positive control wells

The binding ratio was found to be greater than 10.

The mean OD of six negative control wells was greater than 0.7.

The mean OD of six positive control wells was less than 0.1.

The mean OD of the four conjugate control wells was greater than 0.7.

Any plate test results which did not comply with the above values were rejected and samples were re-examined.

2.2.3. Complement fixation test

Principles

Test serum, in which the naturally occurring complement (C') was inactivated, mixed with standard antigens and Guinea Pig complement (GPC'). The GPC' is fixed in the reaction between the antigen and any antibody in the test serum. Absence of antibody leaves the added complement unfixed. The indicator system, consisting of sheep red blood cells sensitized with a haemolysin (specific antibody to sheep red blood cells), was added to the reaction well enables any residual complement to be detected and is visualized by the lysis of the sheep red blood cells. Absence of available complement and therefore presence of antibody in the test serum is visualized by the sheep red blood cells remaining intact. [5]

Pretest preparations:

Alsever's Dilution:

Glucosis	4.1 g
NaCl	0.84 g
Sodium citrate	1.6 g
Citrici acid (H3C6H5O7 x H2O)	0.11 g
Redestilata	2000 ml

Adjust pH to 6.1

Barbital (Veronal) Buffered Saline:

This was used as the standard diluent for the CFT. Following was the composition of Veronal Buffer (VB; pH 7.4; Sodium 5, 5, Diethyl Barbiturate: 3.75g; 5, 5,

Diethyl-Barbituric Acid: 5.75g; Magnesium Chloride: 1.68g; Calcium Chloride: 0.28g; Sodium Chloride: 85g).All the contents were dissolved in 500 ml of hot distilled water (80-90°C) for 10-15 mins.

The final volume was adjusted to 3000 ml. This stock buffer was stored in a refrigerator. For making the working dilution of the buffer, one part of the stock buffer was mixed with four parts of cold distilled water and the final pH was adjusted to 7.4.

Sheep erythrocytes:

Blood from a healthy sheep, negative for anti-Brucella antibodies, was collected under aseptic conditions into equal volume of Alsever's solution. It was mixed thoroughly and centrifuged at 12000 rpm for 10 mins. Thereafter, the supernatant was discarded along with the thin layer of white cells and the erythrocytes were washed twice, first with the PBS and then with the Veronal buffer by centrifugation at 5000 rpm for 10 mins. To make 3% suspension of sheep erythrocytes, 300 µl of erythrocytes were suspended in 9.7ml of Veronal buffer (VB). The Alsever's solution was autoclaved at 110°C and stored at 4°C.

Haemolysin:

Haemolysin (anti-sheep erythrocyte antibody raised in a rabbit) was prepared by intravenous (i/v) inoculation of 1% sheep RBC's in a healthy rabbit. The immunization for haemolysin was done for duration of 20 days as for the method described by [5]. The rabbit was bled by cardiac puncture and serum was separated. High titre serum was stored at 4°C in a refrigerator.

Complement:

Serum from a guinea pig used as the source of good quality complement, was obtained from Indian Veterinary Research Institute, Izatnagar.

Test procedure:

The undiluted test sera and appropriate working standards were inactivated for 30 minutes in a water bath at 58°C. One serum dilution was tested.

Using standard 96-well microtiter plates with round (U) bottoms, the technique was performed as follows:

Volumes of 25 µl of diluted inactivated test serum were placed in the well of the first, second and third rows. The first row was an anti-complementary control for each serum. Volumes of 25 µl of CFT buffer were added to the wells of the first row (anti-complementary controls) to compensate for lack of antigen.

Volumes of 25 µl of CFT buffer were added to all other wells except those of the second row.

Volumes of 25 µl of antigen, diluted to working strength, were added to each well except in the first row.

Volumes of 25 µl of complement, diluted to the number of units required, were added to each well.

Control wells containing diluent only, complement + diluent and antigen + complement + diluent, were set up to contain 75 µl total volume in each case.

A control serum that gives a minimum positive reaction was used in each set of tests to verify the sensitivity of test conditions.

The plates were incubated at 37°C for 30 minutes and a volume 25µl of sensitized SRBCs was added to each well. The plates were re-incubated at 37°C for 30 minutes.

The results were read after the plates have been centrifuged at 1000 g for 10 minutes at 4°C allow unlysed cells to settle. The degree of haemolysis was compared with standards corresponding to 0, 25, 50, 75 and 100% lysis. The absence of anti-complementary activity is checked for each serum in the first row.

The degree of haemolysis is compared with standards corresponding to 0, 25, 50, 75 and 100% lysis.

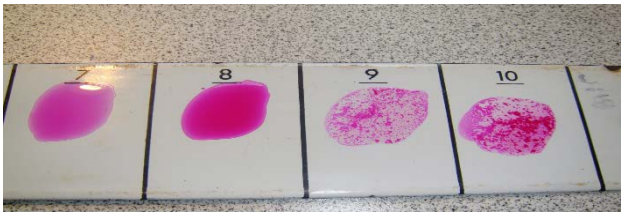

Figure 1. Rose Bengal Plate Test (RBPT): Showing different degrees of agglutination

Figure 2. Competitive Enzyme-linked immune sorbent assay (c-ELISA): showing positive and negative results in the presence of standard serum controls

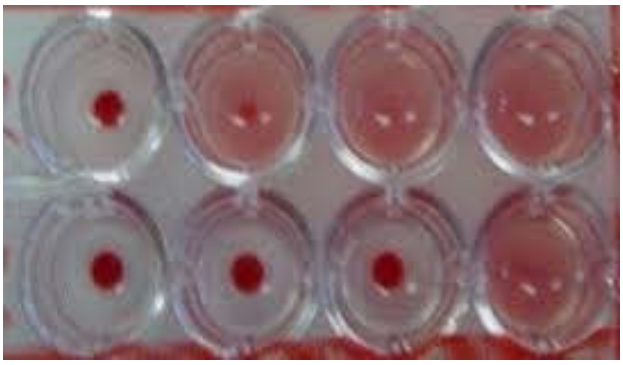

Figure 3. Complement fixation test (CFT): showing partial and complete hemolysis

3. Result

3.1. Positivity

The positivity of Brucellosis in Camel in Gedaref state was found to be 36.2%, 42.6% and 19.8% with RBPT, CFT and c-ELISA respectively; details are shown in the table and figure below.

Table 1. Percentage of positive sera by different test methods

Test	Positive	Negative	Total	% Positive
RBT	124	219	343	36.2
CFT	146	197	343	42.6
c-ELISA	68	275	343	19.8

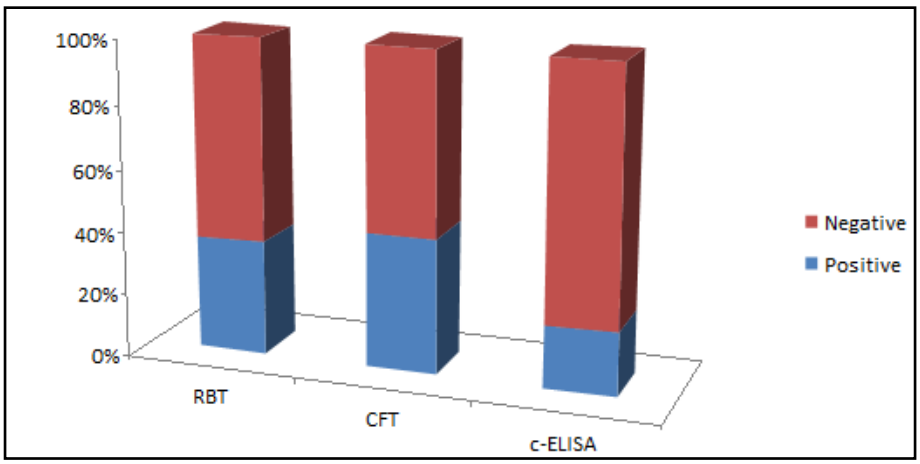

Figure 4. Positivity of brucellosis in Gadarif state

3.2. Performance of RBPT, CFT and cELISA in Gedaref

185 samples were found negative by all the three tests; 59 were found positive by all the three tests. While 124 samples were found positive by RBPT, 146 positives by CFT & 68 positive by c-ELISA.

219 negative with RBPT, 197 with CFT and 275 negative with c-ELISA.

185 samples out 343 were found negative with all the three tests, 2 out of the 34 samples found negative with RBPT were positive with both CFT & c-ELISA, while 7 of them were found positive with c-ELISA only and 25 of them were positive with CFT. 124 samples were found positive with RBPT, 59 of them were found positive with both c-ELISA and CFT, 60 positives with CFT and negative with c-ELISA. All c- ELISA. Positive samples were found positive with CFT.

12.3% of the samples found negative with RBPT were positive with CFT while 4% of the RBPT positive samples were found negative with CFT;

Table 2. Cross tabulation between RBPT and CFT

		CFT		Total
		Negative	Positive	
RBT Negative	Count	192	27	219
	%	87.7	12.3	100
RBT Positive	Count	5	119	124
	%	4.0	96.0	100
Total	Count	197	146	343
	%	57.4	42.6	100.0

The level of agreement between RBPT and CFT was 90.67%,the kappa statistics was 8 which indicates substantial agreement between CFT and RBPT. However, the agreement between RBPT and c-ELISA was moderate with Kappa statistics being 48. Also the agreement between CFT and c-ELISA is moderate with Kappa statistics 41.

23.6% of the samples found negative with ELISA were positive with RBT while 13.2% of the ELISA positive samples were found negative with RBT; Table below shows the cross tabulation of RBT and c-ELISA.

Table 3. Cross tabulation between RBT and c-ELISA

		RBT		Total
		Negative	Positive	
cELISA Negative	Count	210	65	275
	%	76.1	23.6	100
cELISA Positive	Count	9	59	68
	%	13.2	86.8	100
Total	Count	219	124	343
	%	63.8	36.2	100.0

30.9% of samples negative with ELISA were positive with CFT while 10.3 0f samples positive with ELISA were negative with CFT. Table below show the cross tabulation between c-ELISA and CFT.

Table 4. Cross tabulation between c-ELISA and CFT

		CFT		Total
		Negative	Positive	
cELISA Negative	Count	190	85	275
	%	69.1	30.9	100
cELISA Positive	Count	7	61	68
	%	10.3	89.7	100
Total	Count	197	146	343
	%	57.4	42.6	100.0

4. Discussion

Seroprevalence in the algadaref area was (36.2%, 42.6 & 19.8%) with the RBT, CFT and cELISA respectively. The results presented here are similar to those of other studies that found seroprevalence rates for brucellosis in Sudanese camels to range from 1.8% to 43.9%. Thus, [3] reported seroprevalence of 2%, 3.1% and 7.5% in 750 camels in central, western and eastern Sudan, respectively; found the prevalence of brucellosis to be 26.5% in camels in eastern Sudan; Yagoub et al, found 1.82% seroprevalence in 79 young camels, 6.95% in adult males and 13.77% in adult females of 1,502 camels in the same localities as studied here; [7] reported 7.76% of 1,314 camels to be seropositive in Darfur, western Sudan; and [6] found seroprevalence of 13.9% and 43.9% in camels in different localities in Sudan.

The performance of the serological tests in camels is affected by many factors (prevalence of the disease , cross

reaction with other organisms and serum storage and contamination).

In this study CFT was used for the second time in the field of camel serology in Sudan, but its result affected by anti complementary factors may be due to autolysis, hemolysis and/or contamination of the serum samples.

5. Conclusion

- The prevalence of Brucellosis in camel is high and poses threat to public health given the common practice of consuming raw camel milk and liver.
- The increase of the spread and prevalence of camel brucellosis due to epidemiological factors includes mixing of infected camels with healthy animals, mixed herding of different animal species, lack of brucellosis control measures and lack of knowledge of brucellosis by nomads.
- Epidemiological sero-surveillances along with studies on serological and bacteriological isolations are most required to elucidate the exact situation of the disease in the country.
- Continuous investigation of the disease in camels to reveal the various impacts of the disease: epidemiologically, economically and its public health threat. All these collectively will help in planning for applied operational control approaches in different parts of Sudan

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