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STUDY OF CAMEL BRUCELLOSIS USING RT-PCR FOR PREPARING CAMEL POSITIVE GOLD STANDARD SERA

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ABSTRACT: *This research is conducted in group of camels which kept in separated farm with Brucella history in Dubai Emirate, UAE. used different serological test (RBPT, MRBPT and C-ELISA), Milk Ring Test and Real time PCR for the same animals with time interval to evaluate the performance of these serological tests in field of camel brucellosis and compering their result with gold standard method (RT-PCR). We goal kept the serum obtained from animals Brucella DNA detected in their Milk as positive Gold Standard Sera for validation of different serological test using in field of camel brucellosis without enough validation.*

KEYWORDS: *Brucella, RBT, MRBT, cELISA, Serology, RT-PCR*

INTRODUCTION

When thinking about camel brucellosis, bearing in mind that brucellosis is new disease of camel, and that Dubai Emirate It is one of the largest venues for camel racing festivals in the world, has a big farm of milk camel industry. 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 serology alone. (1).

METHODOLOGY

Sample collection:

A total number of 45 serum samples were collected from racing camel kept in closed and separate farm has history of brucellosis as described by (2). to evaluate the different serological test accuracy (RBT and cELISA) and the immune response of camel to brucella infection with the time.

From these 45 animals, 15 animals are milking from them milk sample were collected and subjected to (MRT) to evaluate the milk antibodies in camel infected with brucella with time interval.

Milk samples were collected from the same animals for RT- PCR, in this study we considering the PCR as gold standard test in the difficulties of brucella culture

Serological test:
Three serological tests were conducted, these are, Rose Bengal Plate Test (RBPT), Enzyme-linked immunosorbent assay (c-ELISA) , Modified RBPT and MRT.

Rose Bengal plate test:
The antigen used in the RBPT was produced by symbiotic (Benga Test) commercial kit. The sera and the antigen were brought to room temperature before testing.The test was done as described by (3) 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 (3):
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.

Enzyme-linked immune sorbent assay (c-ELISA):
The test was carried out as described by ingezim brucella compact 2.0 (Multispecies), prod Ref: 10.BRU.K3. described by (7).

- **Kit content**:
Plates: 96 well micro titration plates divided in strips (12x8).
Wash solution: bottle with wash solution 25x concentrated.
Conjugate: vial containing specific conjugate ready to use.
Substrate: vial containing substrate (TMB) ready to use.
Stopping solution: vial containing stopping solution.
Control: positive serum and Negative serum

Equipment Required:
Microliter plate reader with 450nm filter
Single and multichannel variable volume pipettes
Disposable tips for the above pipettes.
Reagent troughs for multichannel pipetting
10-liter 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
Absorbent paper towels

Test procedure:
The kit has been designed to detect antibodies specific for LPS of brucella spp. Being able to detect a very low titters of antibodies in sera of different infected animal species. The techniques are based on the blocking enzyme immunoassay described below:

The sold phase is plate coated with purified LPS of brucella abortus. After adding the sample to well, if it contains specific antibodies against brucella, they will bind to the antigen absorbed on plate while if the sample does not contain specific antibodies they will not bind to the antigen. After we add a specific monoclonal antibody (conjugated with peroxidase) against LPS antigen coated to the plate, it will compete with the antibodies of the serum sample. If the serum samples contain specific antibodies, they will not permit the binding of the labeled Mab to the antigen whereas if it does not contain specific antibodies the Mab will bind to antigen on the plate. After washing the plate to eliminate all non-fixed materials, we can detect the presence or absence of labelled Mab by adding the substrate (TMB) that will develop a colorimetric reaction determined by optical density.

Milk Ring Test:
In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (>100 lactating cows), the sensitivity of the test becomes less reliable. False-positive reactions may occur in recently vaccinated cattle (less than 4 months) or in samples containing abnormal milk, such as colostrum or that due to mastitis. (4).

Test procedure
The test is performed by adding 30 µl of antigen to a 1 ml volume of whole milk that has been stored for at least 24 hours at 4c. The height of the milk column in the tube must be at least 25 mm. If bulk tank samples from large herds are to be examined, the volume of milk should be increased to 3 ml. The milk samples must not have been frozen, heated or subjected to violent shaking. The milk/antigen mixtures are incubated at 37c for 1 hour, together with positive and negative control samples. A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered positive as it might be significant, especially in large herds. The test is considered to be negative if the color of the underlying milk remains homogeneously dispersed in the milk column. If the milk at the bottom of the tube becomes gradually whitened, the result is regarded as inconclusive and the test should be repeated. {5}

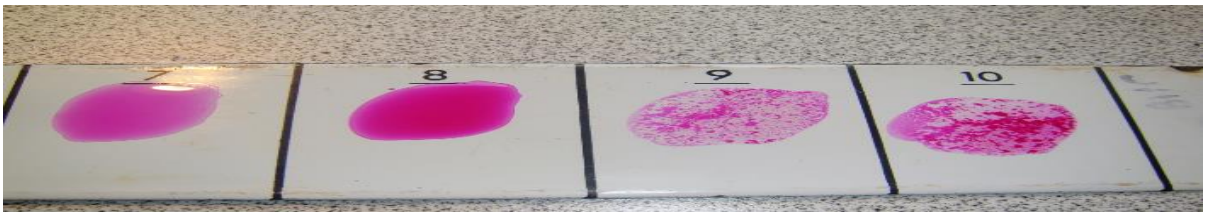


Fig 1: Rose Bengal Plate Test (RBPT): Showing different degrees of agglutination.


Fig 2: Milk Ring Test (MRT): Showing positive and negative result.

Real Time PCR (RT-PCR):

Real-time PCR (RT-PCR) provides means of detecting and quantifying DNA targets by monitoring PCR product accumulation during cycling as indicated by increased fluorescence.

In this study, the performances of a designed real-time PCR assay using TaqMan probes and targeting the insertion sequence IS711, for the detection of Brucella at genus level was used.

The real-time PCR assay was compared to previously described serological test.

The TaqMan Assay portfolion comprehensive set of products was used.

Gene-specific probe, primer sets and master mix were used.

The 7500 Fast Real-Time PCR System with specialized optical system enables easy and accurate calibration, Easy-to-use, spontaneous software.

The TaqMan probe–based chemistry, the gold standard in allelic discrimination and quantitative gene expression offering high sensitivity, specificity, and reproducibility

PCR reaction for detection of Brucella genome was targeting the IS711. (6) . PCR reaction was set using BioRad iScript TM One Step RT PCR using following protocol:

Prepared reaction mix was cycled and fluorescence was measured in Bio Rad IQ5 real time machine under following cycling conditions:

Cycle 1: 95°C - 10 min

Cycle 2: 95°C - 15 s

60°C - 1 min

Cycle 2 was repeated 40 times. Acquisition of fluorescent data was at the end of the annealing step (60°C - 1 min).

RESULT

Result of RBPT and c-ELISA Test:
All 45 serum samples were reacted positive to RBT antigen test with different strengths (+++, ++ and +). Except six animals reacted Negative in the two quarters time. See table (1) and (2).
All 45 serum samples were giving positive result with C-ELISA except 3 animals were negative in the two quarters time. See table (1) and (2).

Table 1: The result of RBPT and c-ELISA test in the first quarter of the year 2018:

Sample ID	RBT First Run	ELISA First Run
1	+++	Pos
2	++	Pos
3	++	Pos
4	_	Pos
5	+	Neg
6	++	Pos
7	+++	Pos
8	++	Pos
9	++	Pos
10	+++	Pos
11	+++	Pos
13	+++	Pos
14	+++	Pos
15	++	Pos
16	_	Pos
17	+++	Pos
18	++	Pos
20	+	Pos
21	+++	Pos
22	+	Pos

23	**+++**	**Pos**
24	**+++**	**Pos**
25	**_**	**Pos**
26	**+++**	**Neg**
27	**++**	**Pos**
28	**_**	**Pos**
29	**++**	**Neg**
30	**++**	**Pos**
31	**_**	**Pos**
32	**+++**	**Pos**
33	**+++**	**Pos**
34	**++**	**Pos**
35	**_**	**Pos**
36	**++**	**Pos**
37	**+**	**Pos**
38	**++**	**Pos**
39	**++**	**Pos**
40	**++**	**Pos**
41	**+**	**Pos**
42	**+**	**Pos**
43	**+++**	**Pos**
44	**+++**	**Pos**
45	**+**	**Pos**

Table 2: The result of RBPT and c-ELISA test in the second quarter of the year 2018:

Sample ID	RBT First Run	ELISA First Run
1	**+++**	**Pos**

2	**++**	**Pos**
3	**++**	**Pos**
4	**_**	**Pos**
5	**+**	**Neg**
6	**++**	**Pos**
7	**+++**	**Pos**
8	**++**	**Pos**
9	**++**	**Pos**
10	+++	**Pos**
11	+++	**Pos**
13	+++	**Pos**
14	+++	**Pos**
15	++	**Pos**
16	_	**Pos**
17	+++	**Pos**
18	++	**Pos**
20	+	**Pos**
21	+++	**Pos**
22	+	**Pos**
23	+++	**Pos**
24	+++	**Pos**
25	_	**Pos**
26	+++	**Neg**
27	++	**Pos**
28	_	**Pos**
29	++	**Neg**
30	++	**Pos**
31	_	**Pos**
32	+++	**Pos**

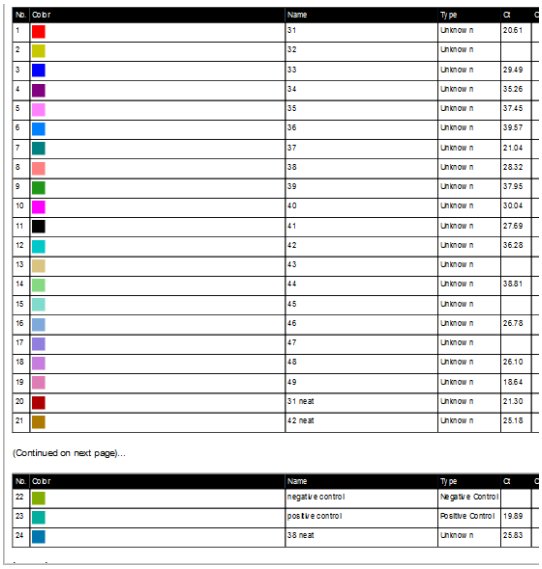
33	+++	**Pos**
34	++	**Pos**
35	_	**Pos**
36	++	**Pos**
37	+	**Pos**
38	++	**Pos**
39	++	**Pos**
40	++	**Pos**
41	+	**Pos**
42	+	**Pos**
43	+++	**Pos**
44	+++	**Pos**
45	+	**Pos**

Result of MRT Test: Out of 15 lactating camels, 9 milk samples were reacting positive for Milk Ring Test. See table (3).

Table 3: The result of MRT Test:

Sample ID	MRT result
31	Pos
32	Neg
33	pos
34	pos
35	Neg
36	pos
37	pos
38	pos
39	pos
40	Neg
41	Neg
42	Neg
43	pos
44	pos
45	Neg

Result of RT-PCR: 15 Milk samples in were run for RT-PCR, all samples were positive has CT value. Except 4 samples reacting Negative has No CT values. see fig (3).



No.	Color	Name	Type	Ct	C
1		31	Unknown	20.61	
2		32	Unknown		
3		33	Unknown	29.49	
4		34	Unknown	35.26	
5		35	Unknown	37.45	
6		36	Unknown	39.57	
7		37	Unknown	21.04	
8		38	Unknown	28.32	
9		39	Unknown	37.95	
10		40	Unknown	30.04	
11		41	Unknown	27.69	
12		42	Unknown	36.28	
13		43	Unknown		
14		44	Unknown	38.81	
15		45	Unknown		
16		46	Unknown	26.78	
17		47	Unknown		
18		48	Unknown	26.10	
19		49	Unknown	18.64	
20		31 neat	Unknown	21.30	
21		42 neat	Unknown	25.18	

(Continued on next page)...

No.	Color	Name	Type	Ct	C
22		negative control	Negative Control		
23		positive control	Positive Control	19.89	
24		38 neat	Unknown	25.83	

Fig 3: Real Time PCR (RT-PCR): Showing positive and negative result.

DISCUSSION

Unequivocal diagnosis of brucellosis can be made only by the isolation and identification of Brucella organism from abortion materials (fetal stomach contents and cotyledons), milk and vaginal discharges (8), but it is not always practical and possible, and bacterial culture results are often negative for infected animals (9). Therefore; it is often necessary to resort to serological tests to identify the specific antibodies in the presence of Brucella antigens (10).in this study we used RBT, MRBT, C-ELISA and MRT and the result shown that, RBPT is sensitive and reliabletest for screening and C-ELISA is most practicable confirmatory test for camel brucellosis,however there is 3 samples reacted negative with RBT 1:1 concentration and gave positive result with C- ELISA but when tested with MRBT 1:3 concentrations it reacted positive (+).

- Out of 15 dairy camels milk tested with RT- PCR, there is two samples positive with RT-PCR and C-ELISA and negative for RBT 1:1, but it gives positive reactions with modified RBT 1:2. thee is 3 samples positive with C-ELISA and RBT gave Negative CT values with RT-PCR. 12 samples gave the same result in the three test. These 12 animals reacted positive for all tests we can use their serum as gold standard sera for future validation of different serological test in UAE.

CONCLUSION

- We recommend Modified RBPT for screening of camel brucellosis to increase the sensitivity, comparing with RBPT, C-ELISA and RT-PCR.

- The engezim competitive ELISA is very sensitive test for detection of antibodies against brucella infection in camel.
- RT-PCR is acting as gold standard method in this study due to complexities of brucella isolation, it gave good result, however the diagnostic performance of RT-PCR method depend on sample type used. Considering the intermittent extraction of brucella with the milk, decreased sensitivity is expected, and this has been confirmed in this study as well.

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