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EVALUATION OF INDIRECT ELISA TEST IN DIAGNOSIS OF BRUCELLOSIS IN CAMELS

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SUMMARY

A serological survey was carried out on 365 camels (Camelus dromedaries, one humped camel) in Saudi Arabia. Out of these 250 were imported from the Sudan, 80 from the Northern Abattoir and 35 blood samples were collected from local markets in Jeddah, Saudi Arabia. Examination of these samples with Standard tube agglutination test (SAT), Rose Bengal plate test (RBPT), Buffered acidified plate antigen test (BAPAT), and Rivanol test (Riv. T) was done and their positive results were (6.85%), (9.59%), (12.02%), and (7.67%) respectively.

Application of the indirect enzyme linked immunosorbant assay (iELISA) on the same camel sera using anti-bovine, anti-ovine, and anti-goat conjugates revealed positive results of (11.5%), (7.4%), and (6.8%) respectively. The technique of ELISA with anti-bovine conjugate showed excellent agreement with BAPAT, as a sensitive test, with Kappa (K) values of 0.947 and good agreement with Riv. T, as a specific test, with K values of 0.78. The ELISA with anti-bovine conjugate was proved to be sensitive as well as specific test for examining camels for brucellosis. Anti-bovine conjugate in ELISA correlated very well with camel sera than the anti-ovine or the anti-goat conjugates.

Two Brucella isolates were obtained from five aborted foeti. While no Brucella isolates were obtained from 35 camel milk samples. The isolated strains proved to be Brucella melitensis biovar 3.

INTRODUCTION

The camel (Camelus dromedaries, One-humped camel, Dromedary) is an important livestock species uniquely adapted to hot and arid environments. It can thrive and flourish under tough and arid circumstances in the desert and nomadic

areas, where it can survive for up to 20 days without any water while cattle can survive without water for only four days. Camel, is known also as a desert ship, is a highly beneficial animal, since; it produces milk, meat, hair, and hides. It serves for riding, as a beast burden and as a draft animal for agriculture and short-distance transport (Schwartz and Dioli, 1992). Camels are also known by their high resistance to different pathogenic infective agents, however; they can contract brucellosis through direct and indirect contacts. Recently, camels became the subject of intensive and systematic interest in particularly with increasing the development potential of dry sandy regions in Afreica and Asia.

Brucellosis threatens the reproduction of this animal species. Whereas, the disease is characterized by abortion, metritis, infertility, retention of placenta and stillbirths in she-camels and hygroma of knee, orchitis and epidydimitis in male camels. Camels can be infected with any member of the Genus Brucella. In Africa, Brucella abortus and melitensis are the causative agents of the disease in camels, while in Asia, B. melitensis is the main species that infects camels (Higgens, 1986).

Diagnosis of brucellosis depends mainly on detection of antibodies by different serological tests, although isolation of the causative organism is laborious and time consuming, but it provide a definitive diagnosis. Besides, attempts for isolation of the organism from camel tissues and milk have met with difficulties and variable success (Higgens, 1986). Different conventional serological tests were employed for detection of the disease in camels, nevertheless; the application of recent and advanced techniques for diagnosis of brucellosis in camels is not properly applied. Among these tests is the enzyme linked immunosorbant assay (ELISA), which was designed primarily for detection of bovine brucellosis. The lack of anti-camel conjugate is the main reason to hamper the application of this test in diagnosis of camel brucellosis.

In the current study, it was intended to employ the indirect enzyme linked immuno sorbant assay (ELISA) on camel sera using different anti-species conjugates (anti-bovine, anti-ovine and anti-goat conjugates) in a trial to locate the most proper anti-species conjugate which reacted and related better to camel antibodies without affecting the sensitivity and specificity of the test. This was carried out in comparison with different serological tests, which applied on the same camel serum samples. In the meantime, trials for isolation of the causative organism were carried out from camels milk and tissues whenever, possible. Identification and biotyping of the isolated strains of Brucella species were also carried out.

MATERIAL AND METHODS

MATERIAL:

Samples:

1.1 Blood Samples:

A total of three hundred and sixty five blood samples were collected from camels. 250 out of them were collected from Sudanese camels, imported from the Sudan to Kingdom of Saudi Arabia through Jeddah Islamic Port (JIP), 80 samples were collected from the Northern Abattoir of Jeddah and the remaining 35 camel blood samples were collected from local camel market from Jeddah suburbs.

1.2 Milk Samples:

Thirty-five camel milk samples were collected from local camel market in Jeddah. Where 20 ml of individual milk samples were aseptically collected from each she-camel in sterile screw capped bottles, and divided into two portions for carrying out Milk Ring Test (MRT) and for isolation of Brucella organisms.

1.3 Aborted Foeti:

Five-aborted foeti were collected from local camel market and abattoir of Jeddah.

2.Antigens used for conventional serological tests:

Whole-cell antigens of the Rose Bengal, Buffer Acidified plate, Rivanol, Milk Ring, and Standard Tube Agglutination tests were obtained from the Veterinary serum and vaccine Research Institute, Abbassya, Cairo, Egypt.

3.Biological reagents and chemicals used in indirect ELISA technique:

A number of 365 serum samples of imported (Sudanese) and local camels were prepared for iELISA test and kept at - 20 °C until used. The indirect ELISA technique was automated to detect the presence of IgG.

3.1.Buffers:

Washing, diluting, coating and blocking buffers were prepared according to Alton et al. (1988).

3.2.Brucella abortus crude lipopolysaccharide (LPS) antigen:

Smooth lipo-polysaccharides (LPS) antigen was extracted from freeze-dried, heat killed B. abortus strain 1119 by the hot water/hot phenol method according to Stemshorn (1979). The antigen was standardized, titerated and diluted 1/100 to obtain the optimal concentration.

3.3.Conjugates:

Rabbit polyclonal antibody specific for both heavy and light (H+L) chains of bovine IgG conjugated with horseradish peroxidase (HRP) was obtained from ICN, ICN Biomedical Inc. Costa Mesa, California, 92626 USA.

Anti-ovine and anti-goat conjugates with HRP were obtained from Zgmed Lab. Inc., San Francisco USA.

4. Substrate:

An OPD (O-phenylenediamine) substrate was used in the protocol. A tablet of the OPD was dissolved in 10 ml of de-ionized distilled water (DDW). 0.1 M solution of hydrogen peroxide (H_2O_2) of Gilbert Lab., France, was prepared shortly before use by diluting 0.5 ml of 30% (8.8 M) solution with 43.5 ml of distilled water.

3.5. ELISA plates: It was 96 flat bottom wells of Maxisorp, Nunc, Inter. Med. Denmark.

3.6.Reader: The ELISA reader was Titer Tek Multiscan-plus.

Media and supplements:

Brucella base medium was used for isolation, identification and typing of Brucella isolates. It was obtained from Oxoid Limited, Basingstoke, Hampshire, England.

Brucella selective supplements containing different antibiotics including, Bacitracin, Polymyxine B-sulphate, Nalidixic acid and Nistatin were obtained from Oxoid, Unipath LTD, Basingstoke, Hampshire, England.

Methods:

Serological tests:

Standard tube agglutination test (SAT) was applied in this study using the European assay according to Alton et al. (1988). While Rose Bengal Plate test (RBPT) was done according to Morgan et al. (1969). Buffered acidified Plate Antigen test (BAPAT), Rivanol test (RIV. T) and Milk Ring test (MRT) were carried out according to Alton et al. (1988).

Indirect Enzyme Linked Immuno-Sorbant Assay (iELISA) was carried out according to Alton et al, (1988). The reading time after addition of the substrate was 15 minutes and at 405 nm interference filter. All the other steps of serum dilutions, washings, amounts of antigen, conjugate and substrate and time of incubation were the same as stated in Alton et al. (1988).

2. Bacteriological examinations:

Direct culture examination of the stomach contents aspirated from the fourth stomach of the aborted foeti was done under complete aseptic conditions.

Milk samples were centrifuged for 10 minutes at 3000 (rpm); the sediment and cream mixture was cultured onto Brucella base media containing different antibiotics of the selective supplements. The cultured plates were incubated at 37 oC and the carbon dioxide tension was adjusted to 10 %.Cultures were routinely examined on the third and fourth days and again after 10 days before being discarded as negative.

Typing of Brucella isolates was done according to CO_2 requirement, H_2S production, urease test, growth in the presence of dyes (thionin and basic fuchsin 20 ug/ml), reaction with monospecific sera (A & M), and lysis by Tbilizi and Iz phages according to the protocol of Alton et al. (1988)

RESULTS and DISCUSSION

The usefulness of a diagnostic test depends on the accuracy of test results, which in turn depends on the specificity and sensitivity of a test. Specificity is the ability of the test to correctly detect the negative cases and the sensitivity is the ability to correctly identify the positive cases.

The results of different conventional tests (Table 1) carried out on 365 camel sera revealed that Buffered acidified plate antigen test (BAPAT) detected the highest number of reactors (12.05%) followed by Rose Bengal plate test - RBPT- (9.59%), Rivanol test -Riv.T- (7.67%), and finally the Standard tube agglutination test - SAT- (6.85%). Similar results were obtained by other workers in Egypt (Nada, 1984; Salem et al 1987; and Atwa, 1997). The elevated sensitivity of the BAPAT, over other serological tests and RBPT in particular, may be attributed to the final pH of a serum:antigen mixture of 4.02 ± 0.04 (Alton et al. 1988). This pH enables some of the IgM, besides; IgG1, IgG2, and IgA to share in the reactions (Wright and Nielsen, 1990). In addition, the amounts of serum (antibodies) used in the BAPAT (0.08 ml) is greater than the amounts of serum used in the RBPT (0.03 ml). On the other hand, the relatively acidic pH of the RBPT (3.65) permits lesser amounts of IgM to share in the reaction, since; this class of immunoglobline is known to be acid labile (Allan et al. 1976).

Application of Rivanol test on the same camel sera revealed that only 7.76 % of the examined sera were positive reactors. This relatively low incidence achieved by the Riv. T may be attributed to the fact that, Rivanol solution (2-ethoxy, 6.9. diaminoacridine lactate) precipitates IgM present in serum samples before carrying out the test. Thus, this test detects mainly the presence of specific IgG (Pietz and Cowart, 1980; and Angus and Barton, 1984). Therefore, any reaction develops in this test is considered as a conclusive results for positive cases (Alton et al., 1988).

From the aforementioned results, Riv. T was chosen as the standard specific test and any serological outcomes of indirect Enzyme immunosorbant assay (iELISA) technique were measured on its results. As well as, BAPAT was also selected as the standard sensitive test and any serological outcomes were measured on its results.

Finally, SAT detected the lowest number of reactor animals (6.85 %) if compared with the other serological tests. This indicates that SAT is inferior to other tests in specificity and sensitivity. Furthermore, this test gave unsatisfactory results because it fails to detect many animals in the incubation stage of the disease, as well as many in the chronic phase as stated in the manual of the OIE Standard Commission, OIE (1996).

The same camel serum samples were examined by the indirect (iELISA) using anti-bovine, anti-ovine, and anti-goat conjugates, respectively

(Table 2). It was evident that iELISA with anti-bovine conjugate detected the highest percent of reactors (11.5%). Application of the same test using anti-ovine conjugate detected (7.4%) while the iELISA using anti-goat conjugate detected only (6.8%). It is very clear that application of the iELISA using anti-bovine conjugate gave better results than that obtained by the same test using anti-ovine and anti-goat conjugates. Since, the iELISA with anti-ovine conjugate failed to detect one animal, which was positive to the Riv. T (specific test). Application of the same test using anti-goat conjugate failed to detect three cases, which were positive to the Riv. T. On the other hand, all samples positive to the same test with anti-bovine conjugate were positive in the Riv. T. So, the agreement was made between iELISA with anti-bovine conjugate and each of the BAPAT, as the sensitive test and Riv. T, as the specific test.

The comparison between iELISA with polyclonal anti-bovine IgG conjugate and BAPAT with Kappa values showed an agreement more than 0.9 (excellent agreement). While, the K values between the same test and Riv. T was over 0.7 which indicates a good agreement (Tables 3, 4). This finding indicated that the sensitivity of iELISA using anti-bovine conjugate is similar to the BAPAT and its specificity is comparable to Riv. T on camel sera. It also indicated that the higher sensitivity of the ELISA is not on the expense of its specificity. The obtained results referred to a close relatedness between the polyclonal anti-bovine conjugate and camel sera. This finding was in harmony with that obtained by Jacques et al. (1998). However, they obtained their results after examining sheep sera using anti-ovine and anti-bovine conjugates. They added that there is no significant differences as regards to the specificity, lower limit of detection and the estimated sensitivity of the two iELISAs. The application of the iELISA with anti-bovine conjugate on camel sera in this study and the successful application of iELISA on sheep sera using anti-bovine conjugate in other study (Jacques et al., 1998) could be a corner stone for the use of one ELISA protocol for detection of brucellosis in all ruminants based on using one species conjugate (Anti-bovine conjugate).

Trials for isolation of the causative agent were carried out on 35 milk samples and five aborted foeti. Only two Brucella strains were isolated from the aborted foeti, while no organisms were isolated from milk samples. The isolated strains were typed and found to be B. melitensis biovar 3 (Table 5). Isolation of B. melitensis from camel tissues was also carried out in Saudi Arabia by Radwan et al. (1992) and Radwan et al. (1995). This finding indicates that infected sheep and goat flocks play a major role in transmission of the disease to camels. In Saudi Arabia, a public health problems frequently occurred, due to the local custom of drinking raw camel milk within the country, leading to high percent of human brucellosis (Kiel and Khan, 1989). Particularly, B. melitensis is considered as t he most

Table (1):Examination of different Camel serum samples for Brucellosis using conventional serological tests.

locality	Total No. of Examined Sera	SAT		RBPT		BAPAT		RIV.T	
		+ve*	-ve	+ve	-ve	+ve	-ve	+ve**	-ve
Imported Sudanese Camels	250	13	237	19	231	23	227	14	236
Local Camels, Abbatoir	80	11	69	14	66	18	62	12	68
Local Camels, market	35	1	34	2	33	3	32	2	33
Total No.	365	25	340	35	330	44	321	28	337
%		6.85	93.15	9.59	90.41	12.05	87.95	7.67	92.33

SAT: Standard Tube Agglutination Test

RBPT: Rose Bengal Plate Test

BAPAT: Buffered Acidified Plate Antigen Test Riv. T: Rivanol Test

* Positive SAT samples are those with titers of 1/40 (80 IU) and above, lower titers considered negative

**Positive Riv. T samples are those with dilutions of 1/25 and above.

Table (2): Examination of Camel serum samples for detection of Brucella antibodies using indirect ELISA technique with anti-bovine, anti-ovine and anti-goat conjugates.

locality	Examined Sera	ELISA with Anti-bovine conj		ELISA with Anti-ovine conj		ELISA with Anti-goat conj	
		+ve*	-ve	+ve	-ve	+ve	-ve
Imported Sudanese Camels	250	22	228	13	237	12	238
Local Camels, Abbatoir	80	17	63	12	68	11	69
Local Camels, market	35	3	32	2	33	2	33
Total No.	365	42	323	27	338	25	340
%		11.5	88.5	7.4	92.6	6.8	93.2

*Positive samples are those with 20 ELISA units (EU) or more.

Table (3): Intertest comparison between iELISA with anti-bovin conjugate and BAPAT on Camel serum samples.

		ELISA with Anti-bovine conj	
		Positive	Negative
BAPAT	Positive	41 (a)	3 (b)
	Negative	1 (c)	320 (d)

$$\text{Kappa (K)} = \frac{N(a+d) - (a+b)(a+c) - (c+d)(b+d)}{N^2 (a+b)(a+c) - (c+d)(b+d)} \qquad K = 0.947$$

K < 0.5 = Poor agreement

0.5 ≤ K 0.7 = Moderate agreement

K ≥ 0.7 = Good to excellent agreement

K of the table = 0.947

Table (4): Intertest comparison between iELISA with anti-bovine conjugate and Riv. T on Camel serum samples.

		ELISA with Anti-bovine conj	
		Positive	**Negative**
Riv.T	**Positive**	28 (a)	- (b)
	Negative	14 (c)	323 (d)

$$\text{Kappa (K)} = \frac{N(a+d) - (a+b)(a+c) - (c+d)(b+d)}{N^2 (a+b)(a+c) - (c+d)(b+d)} \qquad K = 0.78$$

K < 0.5 = Poor agreement

0.5 ≤ K 0.7 = Moderate agreement

K ≥ 0.7 = Good to excellent agreement

K of the table = 0.947

Table (5): Serological profile of Brucella positive culture samples.

Type of samples	Number of samples	Serological Results Of She-camels				ELISA*			Culture Results
		SAT	RBPT	BAPAT	RIV.T	Anti bovine	Anti ovine	Anti goat	
Aborted foeti	1	1/40	+ve	+ve	1/50	39.1	24.8	23.2	+**
	2	1/80	+ve	+ve	1/100	87.7	35.9	31.7	+**
	3	1/40	+ve	+ve	1/50	67.8	39.1	34.3	-ve
	4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* Positive readings are those with 20 ELISA units (EU) or above.

** The isolated strains were typed and they were Brucella melitensis biovar 3.

pathogenic and virulent strain to man among Genus Brucella (Alton et al. 1988). Man can also contract the disease from camels through direct contact with infected discharges of aborted foeti or through ingestion of infected milk or milk products (Kiel and Khan, 1989 and Fayed, 1992).

However, the low recovery rate of Brucella organisms from sero-positive camels in this study and in other previous studies (Nada, 1984; Salem et al, 1987; and Atwa, 1997) requires further investigations to assess the reasons.

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