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Seroprevalence of *Brucella* among Camels in Upper Egypt

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Abstract

The objective of this work was to determine the seroprevalence of brucellosis among camel obtained from El Shalateen area, Red Sea governorate. Identification of *Brucella* isolates, from naturally infected slaughtered camel, using bacteriological and molecular methods to identify the prevalent field strain in camels. This investigation was carried out on 470 camels during the period from July 2020 to June 2021 in El Shalateen area, Red Sea governorate, Egypt using Modified Rose Bengal plate test (mRBPT), Immunochromatographic Assay (ICA) and further confirmation by complement fixation test (CFT) for evaluation of the seroprevalence of camel brucellosis and characterization of *Brucella* microorganism on bacteriological and molecular basis. The results revealed that the seroprevalence was 10.9%, 8.9%, and 8.5% using mRBPT, ICA and CFT, respectively. The results revealed that there is a highly significant association between brucellosis diagnosed by mRBPT and CFT and the age of the camel. The highest percentage of seropositivity was recorded in older camels > 6y, (p-value < 0.0001). While brucellosis by ICA showed a significant relationship with age ($P < 0.05$). But there was a non-significant association between brucellosis diagnosed by mRBPT, ICA, and CFT and the sex of the camel, ($P > 0.05$). The results also revealed that the sensitivity was 100% for mRBPT and ICA While the specificity was 97.44% and 99.53% for mRBPT and ICA, respectively using CFT as the gold standard test. *Brucella* isolation was 6.7% isolated from costocervical and precrural lymph nodes, liver spleen, and testicles which were identified biochemically as *Brucella melitensis* biovar 3. DNA extraction and PCR amplification from isolates revealed 5 out of 5 isolates with a molecular size of 282 bp identified as *Br. melitensis* DNA. In conclusion, ICA can be used as a rapid screening and confirmatory test for the diagnosis of camel brucellosis. Besides, CFT is still a gold standard test because of its balance between sensitivity and specificity.

KEYWORDS
Camel, *Brucella*, Serology, Bacteriology, PCR.

INTRODUCTION

Brucellosis is a highly contagious zoonotic disease caused by a facultative intracellular Gram-negative coccobacillus known as *Brucella* and affects domestic and wild mammals. Camelids are susceptible to *Br. melitensis*, *Br. abortus* and *Br. ovis* (Gwida *et al*., 2012 and Gutema and Tesfaye, 2019). Camel brucellosis causes abortions, stillbirths, infertility, orchitis, and epididymitis, it is a zoonotic disease that can spread and cause disease to humans especially those in contact with infected animals and those consuming milk or dairy products usually manufactured using traditional methods (Benkirane, 2006). It can be transmitted between animals by direct contact, contaminated environment, conception products, body fluids, contaminated feed, water, and raw milk. Also transmitted to humans by the inhalation of aerosolized bacteria, contact with contaminated tissues, and consumption of contaminated raw dairy products and meat from domestic livestock (water buffalo, goats, sheep, cattle, pigs, and camels) (Wissam *et al*., 2019).

The highest prevalence is found when camels are reared with infected small ruminants. The seroprevalence of camelid brucellosis varied between 0.4% in Chad to 37.5% in Sudan (Wernery, 2014). The Middle East wars, inadequate preventive measures and control programs, the uncontrolled animal transportation, age, sex, management, and husbandry practices increase the risk factors for brucellosis (Gutema and Tesfaye, 2019).

Diagnosis of brucellosis in camel is based on serological techniques, bacteriological isolation, and PCR. There are many serological-based tests such as the Rose Bengal Plat test (RBPT), Buffered Acidified Plat Antigen test (BAPAT), Complement Fixation test (CFT), and Immunochromatographic assay (ICA) which are considered the most practical methods to screen and confirm the diagnosis of this disease. Serological methods are not always sensitive or specific and many false-positive reactors may occur due to cross-reactivity with other antigens (Kim *et al*., 2007). So, it was recommended to depend on more than one serological test to detect all positive reactors (Radulescu *et al*., 2007). Serological diagnosis is based on testing sera by a screening test of high sensitivity, followed by a confirmatory test of high specificity (Nielsen and Yu, 2010).

ICA is a simple version of ELISA for the qualitative detection of antigen-specific antibodies in serum. It allows the detection of specific IgM as well as specific IgG antibodies and a high sensitivity is assured for all stages of the disease (Nielsen and Yu, 2010). It can detect certain antibodies or antigens in a variety of body fluids including saliva, blood, urine or cerebrospinal fluid (Dug-

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dale, 2009). The test is considered ideal for developing countries and rural areas where the disease is common and laboratory support is not routinely available (Dey *et al*., 2006).

Bacterial culture and isolation are still the gold standard method for diagnosis of Brucellosis, but it needs high-security laboratory facilities (biological containment level 3), highly experienced personnel, takes a long time for results and it is considered a hazardous procedure (Nielsen and Yu 2010),

Several assays based on polymerase chain reaction (PCR) have been developed (Wernery 2014; Wissam *et al*., 2019). PCR can be used for the detection of *Brucella* species in body fluid, tissues, and blood obtained from infected livestock and human patients as well as to differentiate between species and biovar (Casalinuovo *et al*., 2016). The aim of this study was to evaluate the seroprevalence of camel brucellosis in Egypt and the characterization of *Brucella* microorganism on the bacteriological and molecular basis.

MATERIALS AND METHODS

Ethical approval

The animal handling and procedures were carried out according to the guidelines of ethical committees of the Faculty of Veterinary Medicine, Zagazig University, and Animal Health Research Institute, Dokki, Egypt.

Animals

A total of 470 camels of both sexes (78 females and 392 males) with different ages were used in this study during the period from July 2020 to June 2021. They were obtained from El Shalateen area, Red Sea governorate, Egypt; these animals were not previously vaccinated against brucellosis.

Samples collection

Blood samples

10 ml blood samples were collected from the jugular veins of all animals under aseptic conditions. Serum samples were prepared and kept frozen (-20°C), till analysis.

Samples for *Brucella* Isolation

Lymph nodes (n=30) were collected from seropositive slaughtered male camel including costocervical and precrural lymph nodes, liver (n=15), spleen (n=15), and testicles (n=15) for isolation, identification, and typing of *Brucella* organisms according to Alton *et al*. (1988).

Serological tests

Modified Rose Bengal plate test (mRBPT): The test was carried out according to Blasco *et al*. (1994).

Complement fixation test: The test was done as described by Alton *et al*. (1988) using warm fixation technique.

Immunochromatographic Assay ICA (*Brucella* Antibody Ab Rapid Test) according to Bronsvoort *et al*. (2009).

Bacteriological examination

Tissue specimens were cultured on tryptose soy agar medium with selective antibiotic supplement (Ewalt *et al*., 1983), (Oxoid) according to Alton *et al*. (1988). Plates were incubated at 37°C in 10% Co_2 incubator, then examined daily for 10 days for *Brucella* growth. Isolates were identified as *Brucella* according to the methods described by Alton *et al*. (1988).

DNA extraction from Brucella cultures

Few colonies were harvested and suspended in 200 µl of sterile, DNase, RNase-free deionized water. Colonies were killed by the addition of 67% methanol and 33% saline mixture then washed with normal saline several times by centrifugation at 6000 rpm for 10 min until bacteria sediment completely clear and directly suspended in distilled water at an optical density of 0.15 to 0.209 at 600 nm (approximately 10^9 cells per ml) or stored at -20oC until extraction of DNA.

Polymerase chain reaction (PCR)

PCR amplification was performed according to the method of Mullis and Faloona (1987). A typical reaction mixture contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 (wt/vol) triton X-100, 0.2 mg of bovine serum albumin (fraction IV; Sigma) per ml, and 10 mM tris-HCl (pH 8.5). Each reaction mixture was supplemented with 100 mM of each of the four deoxyribonucleotides, 100 ng of sample DNA, 10 pM of each oligonucleotide primer (F-5`TGGAGGTCAGAAATGAAC3`, R-5` 3` GAGTGCGAAACGAG) and 0.5U of Taq polymerase (Promega).

Statistical analysis

Numerical data were described as mean ± SE and count data were expressed as percentages. Data of gene expression was analyzed by one-way ANOVA after screening the normality of the data by Shapiro Wilk test. Dennett's test was run to test the significance between each of the diseased and control groups. A chi-square test for association was performed to assess the relationship between brucellosis and each age and sex of camels. One-sample chi-square was run to test the significance of percentages of different seropositives for different dilutions. The level of significance was set as $p < 0.05$. The data were analyzed by SPSS version 25 (Armonk, NY: IBM Corp) and Graph Pad Prism 8.0.2 (GraphPad Software, Inc).

RESULTS AND DISCUSSION

The seroprevalence of camel brucellosis using mRBPT, ICA, and CFT was 10.9%, 8.9%, and 8.5%, respectively (Table 1). The seroprevalence comes in accordance with that estimated in Egypt by Sayed *et al*. (2017) who stated that the percentages were 12.90%, and 11.50% using RBPT and CFT respectively. Ibrahim *et al*. (2020) reported a percentage of 10% and 9% using RBPT and CFT, respectively. However, this result differs from that obtained by Petros and Geremu (2018) who recorded 3.6% and 3.1% using RBPT and CFT, respectively. And Hika *et al*. (2022) found the percentage was 2% using CFT.

Infections obtained in this study and those obtained by other authors in different localities of Egypt may be attributed to various factors such as the climatic changes, the years during which the studies were performed, the areas from which the animals were examined, and the different serological tests used confirmed by bacterial isolation or not. Also, following hygienic measures, prevention, and control, vaccinal program, and test and slaughter policy. Employing of complement fixation test (CFT) in this study revealed lower differences between the percentages of *Brucella*

reactors than mRBPT. The test gave negative results in some serum samples that were identified as reactors in other tests, such reactions may be regarded as false positive reactions by mRBPT, which may be attributed to the presence of some Gram-negative bacteria (*E. coli*, *Salmonella dublin*, *Yersinia enterocolitica* O:9, and *Pasteurella tularensis*) which share *Brucella* in its antigenicity and thus cross-react with the used antigen. The complement fixation test detects primarily IgG1 and the presence of IgG1 correlated with the state of actual infection even if present in small amounts.

From Table 2, CFT results revealed that 40 out of 470 were positive as 14(1/4), 6(1/8), 6(1/16), 4(1/32), 3(1/64) and 7(1/128) with total seropositive 40/470 (8.5%). The highest percentage was at 1/128 titration 7(1.5%) indicating the high infection and high specificity of CFT which pick up the true infection. This agreed with that revealed by Khoudair (2004).

The results revealed that the sensitivity was 100% for mRBPT and ICA, while the specificity was 97.44% and 99.53% for mRBPT and ICA, respectively using CFT as gold standard test (Table 3). Our obtained results were similar to those reported by Abebe *et al.* (2014) and Nour *et al.* (2017). But these results disagreed with Eisa *et al.* (2013) who found that the sensitivity of RBPT and ICA was 87.75% and 92.15%, and with Wissam *et al.*, (2019) who reported that the sensitivity of RBPT was 68.95%. In our opinion, the results of the sensitivity and specificity clarified the importance of using a series of serological tests in the diagnosis of brucellosis and avoiding relying on the use of a single test or a few numbers of tests. The obtained results indicated the suitability of using mRBPT for screening and ICA for screening and confirmation purposes. CFT is still a superior test as it gave the higher balance of sensitivity and specificity than other tests used.

The age-level seroprevalence stated in this study revealed that there was highly significant association between brucellosis diagnosed by mRBPT and CFT and the age of the camel (Table 4). The highest percent of seropositivity was recorded in older camels which is consistent with other studies (Asaad *et al.*, 2020; Ibrahim *et al.*, 2020). This finding may be attributed to the long duration of animal exposure to the infection. Other studies reported that the seroprevalence was higher among young camels than the old ones (Sayed *et al.*, 2017; Nayel *et al.*, 2020).

The prevalence among males was non-significantly higher than among females (Table 5). This result agreed with recent studies (Kebede 2016; Petros and Geremu 2018) and disagreed with Chimedtseren *et al.* (2018) and Hika *et al.* (2022).

Bacterial culture plays an important role in confirming the presence of disease and it is essential for antimicrobial susceptibility, biotyping, and molecular characterization which provides valuable epidemiological information to know the sources of infection in outbreak scenarios and the strain diversity in endemic regions (Alvarez *et al.*, 2011). From Table 6, bacteriological isolation and identification revealed 5 isolates out of 75 (6.7%) tissue samples obtained from 15 seropositive slaughtered camel including 2/30 (6.7%) lymph nodes, 0/3 (0%) liver, 1/15 (6.7%) spleen and 2/15 (13.3%) testicles. Typing of isolates according to Alton *et al.* (1988) resulted in the finding that *Brucella melitensis* biovar 3 is the serotype that existed in the examined animals, similar findings were reported by many authors (Hosein *et al.*, 2016: Sayed *et al.*, 2017) who isolated *Brucella melitensis* biovar 3 from different tissue samples of camel and recorded that *Brucella melitensis* biovar 3 was the prevalent strain in Egypt.

The low recovery rates of *Brucella* from different samples obtained from seropositive camel by using traditional methods of isolation need the use of more advanced tools like PCR. PCR assay can simultaneously detect and differentiate between *Br. abortus* and *Br. melitensis* at the same time and by one reaction (Tuba *et al.*, 2012).

The obtained *Brucella* isolates from naturally infected slaughtered camel were examined with multiplex conventional PCR for detection and identification of *Br. abortus* and *Br. melitensis*. The obtained results revealed that 5 out of 5 isolates with molecular size 282 bp were identified as *Br. melitensis*, as shown in Figure 1. The obtained results agreed with Bricker and Halling (1994) and Khoudair (2004) who stated that PCR is considered as an accurate rapid tool and saves time for routine diagnosis of brucellosis and the total percentages of detected strains using PCR was 100.0% which typed as *Br. melitensis* biovar 3, the authors added that all culture positive samples were positive to PCR.

Table 1. Seroprevalence of brucellosis in camel by using mRBPT, ICA and CFT.

Total Examined No.	mRBPT			ICA			CFT		
	-ve	+ve	% of +ve	-ve	+ve	% of +ve	-ve	+ve	% of +ve
470	419	51	10.9	428	42	8.9	430	40	8.5

-ve: Negative; +ve: Positive

Table 2. Seropositive and titration of camel samples using complement fixation test.

	No. of examined serum samples	Results of complement fixation test at the following dilution												Percentage of positive samples	
		4-Jan	%	8-Jan	%	16-Jan	%	Jan-32	%	Jan-64	%	1/128	%	No.	%
Total	470	14	2.98	6	1.3	6	1.3	4	0.85	3	0.64	7	1.5	40	8.5

1/4: Suspect. from 1/8 till 1/128: Positive.

Table 3. Area under the curve, sensitivity, specificity, PPV, NPV, and accuracy for assessing performance of ICA, and mRBPT considering CFT as gold standard.

	ICA	mRBPT
AUC (95% C.I)	0.998 (0.98 – 1.00)	0.987 (0.97 – 0.99)
Sensitivity (95% C.I)	100 (91.19 - 100)	100 (91.19 - 100)
Specificity (95% C.I)	99.53(98.33 – 99.94)	97.44 (95.47 – 98.72)
PPV (95% C.I)	95.24(83.38 – 98.76)	78.43 (66.99 – 86.70)
NPV (95% C.I)	100	100
Accuracy (95% C.I)	99.57 (98.47 – 99.95)	97.66 (95.85 – 98.83)

CI: Confidence interval; PPV: Positive predictive values; NPV: Negative predicted values.

Table 4. Seroprevalence and (correlation) association between brucellosis diagnosed by ICA, mRBPT, and CFT test results and age of camel.

Age	No.	mRBPT			P-value	ICA			P-value	CFT			P-value
		-ve	+ve	%		-ve	+ve	%		-ve	+ve	%	
1-4 y	347	323	24	6.9		324	23	6.6		325	22	6.3	
4-6 y	73	57	16	21.9	0.0003***	62	11	15.1	0.01*	63	10	13.7	0.005***
> 6 y	50	39	11	22		42	8	16		42	8	16	
Total	470	419	51	10.9		428	42	8.9		430	40	8.5	

***highly significant difference $p < 0.0001$; * significant difference $p < 0.05$

Table 5. Seroprevalence and (correlation) association between brucellosis screened by mRBPT, ICA, and CFT test results and sex of camel.

Sex	Total No.	mRBPT			p. value	ICA			p. value	CFT			p. value
		-ve	+ve	%		-ve	+ve	%		-ve	+ve	%	
Male	392	351	41	10.5	$> 0.05^{NS}$	362	36	9.2	$> 0.05^{NS}$	358	34	8.7	$> 0.05^{NS}$
Female	78	68	10	12.8		72	6	7.7		72	6	7.7	

NS: non-significant difference.

Table 6. Isolation and Identification of *Brucella* spp in camel.

Type of tissue	Camel		
	No.	+ve	%
Lymph nodes	30	2	6.7
Liver	15	0	0
Spleen	15	1	6.7
Testicles	15	2	13.3
Total No. of isolates	75	5	6.7

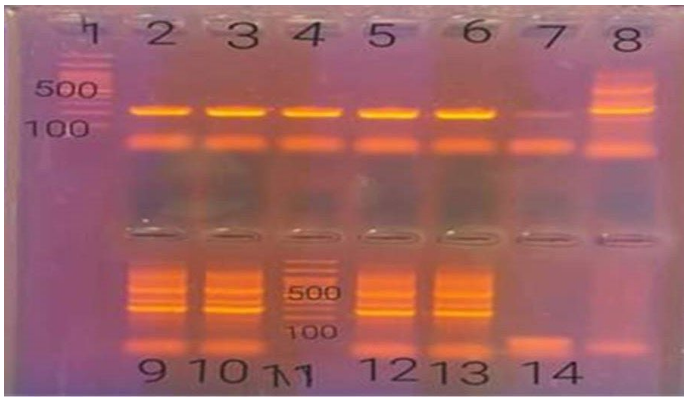

Fig. 1. Identification of isolated colony from tissues of seropositive slaughtered camel using Conventional PCR. Agarose gel electrophoresis of PCR amplified. The figure shows a single band at 223 bp DNA detecting general *Brucella* and the lower part of the figure shows the 282 bp DNA detection of *Brucella melitensis*. Lane 1: Marker 100 bp, Lane 2 to Lane 6: Samples of positive *Brucella*, Lane 8: Sample negative *Brucella* Lane 9, 10: positive *Brucella melitensis* band at 282bp, Lane 11: Marker 100bp, Lane 12, 13: sample positive *Brucella melitensis*, Lane 14: negative *Brucella melitensis*.

PCR is the more reliable, accurate technique in comparison with serological tests and tissue cultures, results can be obtained within less than 24 hours, and it is able to differentiate vaccinated from naturally infected camel (Casalinuovo *et al*., 2016).

CONCLUSION

The seroprevalences of camel brucellosis are 10.9%, 8.9%, and 8.5% using mRBPT, ICA and CFT, respectively. ICA can be used as a rapid screening and confirmatory test for diagnosis of camel brucellosis because it is easily performed as well as its high sensitivity and specificity. CFT is still a gold standard test because of its balance between sensitivity and specificity besides the good correlation of its results with bacteriological isolation. PCR is an important tool for the diagnosis and identification of *Brucella* spp. *Brucella melitensis* biovar 3 is the predominant strain in Upper Egypt.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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