



Original Research Article

# Diagnostic Efficiency of Different Serological Tests and Real time PCR for Detecting Brucella Infection in Camels' Sera

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**ABSTRACT**

Evaluation of the real-time PCR, rose bengal test (RBT), competitive ELISA, and complement fixation test (CFT) was done on 335 camels sera. Real-time PCR, classified 335 camel serum samples to 268 (80%) as positive and 67 (20%) as negative. Real-time PCR, using species specific primers, distinguished 94/104 serum samples due to *B. abortus*, 4/104 samples due to *B. melitensis* and 6/104 due to mixed infection. The results of serological tests revealed that modified mRBT75 using 75 µl of serum, detected the highest number of positive samples 271 (80.9%), while 262 (78.2%), 257 (76.7%), 253 (75.5%) and 245 (73.1%) samples were found to be positive for brucellosis using CFT, cELISA, mRBT50, and RBT25, respectively. Compared to other serological tests, the CFT proved to have the best results in the criteria of test validations, namely; specificity (88%), PPV (96.9%), NPV (80.8%), PLR (7.9), NLR (0.06) and DOR (133.8). The Kappa (K) statistic agreements values between real-time PCR and rose bengal (RBT25), modified (mRBT50), (mRBT75), cELISA and CFT was 0.562 (± 0.053), 0.613 (± 0.052), 0.725 (± 0.048), 0.710 (± 0.047) and 0.801 (± 0.041), respectively. The authors recommend the use of real-time PCR on camel sera to confirm the disease.

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**1. Introduction**

Brucellosis is an infectious disease caused by the bacteria of the genus *Brucella*. These bacteria are primarily passed among animals, and they cause disease in many different vertebrates as well as sea mammals (Godfroid et al., 2005). Various *Brucella* species infect sheep, goats, cattle, deer, elk, pigs, dogs, and several other animals including camelids. Brucellosis has been reported in the six camel species, particularly when came into contact with large and

small ruminants infected with *B. abortus* or *B. melitensis* (OIE, 2016). In man the disease is known as ‘undulant fever’ or ‘Malta fever’ which is a serious public health problem. Human brucellosis remains one of the most common zoonotic diseases worldwide, with more than 500,000 new cases annually (FAO /WHO, 1986). The prevalence of brucellosis in the animal reservoirs determines the incidence of human cases (Pappas and Papadimitriou, 2007; Von Hieber D., 2010).

In Egypt, brucellosis was first reported in camels (Zaki, 1948), since then camel brucellosis was recorded by many authors with variable incidence; 10.3% by Hamada et al (1963), 2% El-Nahas (1964), 8.3% Fayed et al (1982), 7.9% Nada (1990), and 10.7% Barsoum et al (1995).

Camels known as “dessert ship” are well adapted to severe environmental conditions, and it is the only domestic species that able to survive, and reproduce in harsh climatic conditions. Camelidae are bred for production of meat, milk, hair, and hide, besides; carrying and transporting heavy loads. The total number of dromedary camels in the world are under reported however it is estimated to be about 24 million, 20% of them are distributed in Asia and 80% in Africa. Somalia alone owned 7 million dromedary camels that constitute 30% of the world's camel population. Somalia and Sudan have the largest populations, with some 70 percent of the African camel herd (Al-Juboori and Baker, 2012).

In Egypt Camel population has always been underestimated as 120,000 in 2005 (Sads, 2009). Egypt imports large numbers of camels mainly from the Sudan, Somaliland and Ethiopia in a trial to achieve food security in animal protein. The majority of camels are imported to Egypt through walking in caravans from Somaliland and Sudan to Egypt and gathered in local camel market (Birqash) in Imbaba, Giza. Other camels are imported by shipping through the sea ports to Egypt. The Arabian Gulf countries (GCC) import camels from Somaliland in large numbers for meat production and some of these imported camels are used in athletic racing tournaments.

The quarantine regulations for investigating imported camels in Egypt and GCC countries are fragile and in the best conditions only 10 % from imported camel shipments are tested for brucellosis mainly by conventional RBT. In addition, many camels are introduced to Egypt without passing through quarantine stations by smuggling through the wide land border between Sudan and Egypt which extend up to 1000 km in length. Brucellosis is recorded in camels in each of Sudan, Somaliland and Ethiopia. Accordingly, imported camels jeopardize and complicate the brucellosis situation in Egypt and GCC countries, and impede any national programs to control the disease in farm animals. On the other hand, Brucellosis in camels has a great impact on public health. Particularly, the custom of drinking raw camels milk is prevailing particularly in nomadic

regions in Middle East countries, as it is believed to have an aphrodisiac effect and a cure many human illness (Hamdy and Amin, 2002).

Diagnosis of brucellosis in different animal species depends largely on isolation of the causative agent, or detection of the antibodies through a battery of serological tests with varying levels of sensitivities and specificities. Nevertheless, direct detection of this pathogen through bacterial isolation from contaminated samples is the golden standard of diagnosis, yet; it is difficult, less sensitive, laborious, and time consuming since it takes several days to weeks for proper identification of this pathogen (Alton et al., 1988; OIE, 2016).

Most Brucellosis diagnostic serological tests were primarily standardized and directly designed for testing cattle sera. There are national, regional and international demands for validation of conventional serological tests if used for detecting brucellosis in different animal species other than cattle (OIE, 2016). Furthermore, camels are proved to possess a unique profile of heavy-chain antibodies of the immunoglobulin G sub-classes (IgG2 and IgG3) as they do not incorporate light chains. The heavy-chain antibodies constitute approximately 75% of the IgG in camel sera (Daley et al., 2005). The exclusive nature of immunoglobulins in camels substantiates the necessity for validation and/or modifications of serological tests to increase their efficacy in detecting brucellosis in camelids.

The rose bengal (RBT) or card test is a simple spot agglutination test using antigen stained with rose bengal and buffered to a low pH,  $3.65 \pm 0.05$  (Morgan et al., 1969). Rose bengal test is a simple, fast, reliable with high sensitivity and is considered by the OIE as one of the prescribed tests for international trade. It has been used for several decades, proving to be successful for eradicating bovine brucellosis in some countries, and is one of the official tests currently used in the EU for the eradication of *B. melitensis* infection in small ruminants (Garin-Bastuji et al., 1998). In order to increase the sensitivity of RBT in small ruminants and to minimize the discrepancies with the CFT result, three volumes of serum and one volume of antigen, approximately 75  $\mu$ l of serum and 25  $\mu$ l of antigen are used and the test is termed the modified mRBT. (Blasco et al., 1994; Ferreira et al., 2003).

Complement fixation test is the most prominent test to detect antibodies in different animal species including camels. CFT is considered as being the

most sensitive and specific test for brucellosis because complement fixing antibodies remain in the serum for longer periods than SAT antibodies (Tserendash and Shumilov, 1970; Waghela et al., 1978). The CFT is of high specificity and used frequently to confirm *Brucella* infection in different animal species including camels. CFT has been and is a widely used as a confirmatory test in control/eradication programs.

Competitive enzyme immunoassays are more widely used for the diagnosis of brucellosis in most mammalian species through incorporating SLPS passively immobilized in 96 well polystyrene plates. The test based on competition between a monoclonal antibody specific for a common epitope of OPS and test serum. By selecting a monoclonal antibody with slightly higher affinity for the antigen than most of the vaccinal/cross-reacting antibody but with lower affinity than most antibodies arising from infection, thus the most cross-reacting antibodies could be eliminated in the majority of cases.

The polymerase chain reaction, (PCR) as a diagnostic tool in brucellosis is advancing and will be soon at the point of replacing actual bacterial isolation. It is rapid, safe and cost effective. The PCR, including the real-time format, provides an additional means of detection and identification of *Brucella* spp. (Bricker and Halling, 1994; Bricker et al., 2003; Yu and Nielsen, 2010). The major advantages of real-time PCR are that it can be performed in a very short time, does not require electrophoretic analysis and overcomes the cross reaction with gram-negative bacteria.

Several studies demonstrated the presence of *Brucella* DNA in serum and this was attributed to the release of nucleic acids in serum due to breakdown of the organisms during bacteremia. Recently, Wareth et al. (2015) detected *Brucella* genomic DNA by real-time PCR in the serum of infected cattle, sheep and goats in Egypt. And they found that real-time PCR is more sensitive and specific compared with serological tests.

The first species-specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker and Halling (1994). The assay, named AMOS-PCR, was based on the polymorphism arising from species specific localization of the insertion sequence IS711 in the *Brucella* chromosome, and comprised five oligonucleotide primers that can identify *B. abortus*, biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis*. In previous study done on camels we used AMOS

PCR for the diagnosis of brucellosis in camels' milk samples where only one milk sample gave PCR-positive result out of 12 samples tested negative by conventional culture methods (Hamdy and Amin, 2002). It is based on the observations that the repetitive genetic element IS711 is unique to *Brucella* species, and for most species at least one copy of the element occurs at a unique species- or biovar -specific chromosomal locus (Halling et al., 1993). The unique locations of these elements are the basis of the diagnostic assay employed in this study.

Detection of brucellosis in camel sera by PCR has been described by Alshaikh et al. (2007) in Saudi Arabia. Serum is considered the preferred clinical specimen for diagnosis of human brucellosis by PCR (Zerva et al., 2001). In camels, serum real-time PCR was not validated, but had a high diagnostic sensitivity, as it was able to detect as little as 23 fg of *Brucella* DNA per reaction, with a probability of 95% (Gwida et al., 2011).

In this study, real time PCR based on detection of the repetitive genetic element IS711 as unique to *Brucella* species was applied on positive and negative camel sera to estimate its diagnostic efficiency. So the aim of this study was directed to compare the diagnostic performance of mRBT at three consecutive concentrations of serum (25µl, 50 µl & 75 µl), cELISA and CFT in camels, real-time PCR for detecting *Brucella* DNA in camel sera and to identify on species level the causative agent of brucellosis in short time using real-time PCR. These objectives will enable recommendation of the proper validated test and/or tests to diagnose brucellosis in camelids and to suggest effective control program to prevent the transmission of the disease from camels to different animal species and to minimize the risk of human exposure.

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## 2. Materials and methods

### 2.1. Samples

In the period between March 2014 and March 2015, approximately 6570 blood samples were collected from male camels gathered from Somaliland and Eastern Ethiopia and placed in Berbera Quarantine station ready for exportation to kingdom of Saudi Arabia (KSA) and Egypt. All camel samples were tested by modified mRBT (using 75 µl of serum and 25 µl of antigen) and 418 samples found to be reactors with apparent prevalence of 6.36%. Out of them, 268

reactor samples were selected for the study. In addition, 67 negative serum samples were collected from governmental camel research farm, free from brucellosis, located in Matrouh region (North East of Egypt). A total of 335 camel sera were subjected to the employed serological tests.

## 2.2. Serological Study:

All camel serum samples were tested by standard and modified RBT in three consecutive concentrations of serum. The antigen to sera ratio; 1:1, 1:2, and 1:3, using fixed quantity of antigen and 3 different quantities of serum as follow; 25  $\mu$ l, 50  $\mu$ l, and 75  $\mu$ l respectively. Antigen used for RBT, was supplied from CZ Vetrinaaria, S.A. (Pontevedra) – Spain. The antigen was standardized to give a positive reaction with a 1/45 dilution but negative against the 1/55 dilution. The test was conducted as described in the Terrestrial Manual of Standards for Diagnostic Tests and Vaccines (Alton et al., 1988; OIE, 2016).

Standard *Brucella abortus* complement fixation test antigen a product of AHVLA, UK was used. The antigen was titrated and the final dilution was 1: 10 in veronal buffer diluent. Positive and negative control sera are the national reference sera standardized according to OIE. Positive control sera contain 595 International CFT Units (ICFTU) per milliliter for CFT was used.

Guinea pig complement and Hemolysin (rabbit anti-sheep RBCs) are products of Lilli Dale, UK, were titrated and 3% of sheep RBCs were used and standardized in the CFT. The test was performed according to (Alton et al., 1988). Any serum showing a value  $\geq 20$  ICFTU per milliliter was considered positive.

The cELISA was done and results were interpreted according to the instructions of the manufactures using Svanovir™ *Brucella*-Ab cELISA kit (Svanovia Biotech AB Uppsala, Sweden). The kit was validated according to the kit instructions, the validation guidelines of the OIE (OIE, 2016) and (ISO/IEC 17025:2005). The positive cutoff point was calculated as 0.586 nm. Additionally, the percent inhibition (PI) was calculated from the formula:

$$PI = 100 - [(Mean OD \text{ samples} \times 100) / (Mean OD \text{ Conjugate control})].$$

## 2.3. Real-time PCR:

Real-time PCR assay run in this study, exploited the polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome. Identity was determined by the size of the product amplified from primers hybridizing at various distances from the element to *B. abortus* and *B. melitensis*.

### 2.3.1. DNA extraction.

DNA extraction from serum samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200  $\mu$ l of the serum sample suspension was incubated with 10  $\mu$ l of proteinase K and 200  $\mu$ l of lysis buffer and incubated at 56°C for 10 minutes. After incubation, 200  $\mu$ l of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100  $\mu$ l of elution buffer provided in the kit. Purity and concentration of DNA was determined photometrically using a Nano Drop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

### 2.3.2. Oligonucleotide Primers.

Primers used were supplied from biobasic (Canada) and are listed in Table (1).

### 2.3.3. SYBR green real-time PCR amplification:

Primers were utilized in a 25-  $\mu$ l reaction containing 12.5  $\mu$ l of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.5  $\mu$ l of each primer of 50 p.mol concentration, 5.5  $\mu$ l of nuclease-free water, and 6  $\mu$ l of DNA template. Negative template controls that contained 6  $\mu$ l of water instead of DNA and positive controls that contained DNA of *Brucella* (*B. abortus* strain 544 and *B. melitensis* strain 16 M) were included in each run to detect any amplicon contamination or

**Table (1): Primers and Real-time PCR amplification**

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
					Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
1S711	<b>Brucella genus</b>	<b>IR1</b> GGC-GTG-TCT-GCA-TTC- AAC-G	839	94°C 15 min.	94°C 15 sec.	55°C 30 sec.	72°C 45 sec.	94°C 1 min.	55°C 1 min.	94°C 1 min.
		<b>IR2</b> GGC-TTG-TCT-GCA-TTC- AAG-G								
	<b>B. abortus</b>	<b>1S711-specificPrimer</b> TGC-CGA-TCA-CTT-AAG- GGC-CTT-CAT	498	94°C 15 min.	94°C 15 sec.	55°C 30 sec.	72°C 30 sec.	94°C 1 min.	55°C 1 min.	94°C 1 min.
		<b>B. abortus-specific Primer</b> GAC-GAA-CGG-AAT-TTT- TCC-AAT-CCC								
	<b>B. melitensis</b>	<b>1S711-specificPrimer</b> TGC-CGA-TCA-CTT-AAG- GGC-CTT-CAT	731	94°C 15 min.	94°C 15 sec.	55°C 30 sec.	72°C 45 sec.	94°C 1 min.	55°C 1 min.	94°C 1 min.
		<b>B. melitensis-specific Primer</b> AAA-TCG-CGT-CCT-TGC- TGG-TCT-GA								

amplification failure. The real-time PCR assay was performed in a Stratagene MX3005P (Stratagene, La Jolla, Canada) real time PCR machine with the following running conditions:

The extracted DNA from the positive samples were examined with the *Brucella* IS711 species specific real-time PCRs for *B. abortus* and *B. melitensis* using the primers listed in the table (1) as described previously (Bricker and Halling, 1994). Optimist obtain dissociation curve achieved by reaction condition of 1 minute at 94°C (Secondary denaturation), 1 minute at 55°C (Annealing) and 1 minute at 94°C (Final denaturation). The samples scored positive (positive amplification of DNA to a limit that exceeded the threshold line) by the

### 3. Results

The real-time PCR in this study was considered to be the “gold standard” for diagnoses of brucellosis in camel sera. In the current study, 335 camel samples tested by real-time PCR targeting the DNA of *Brucella* genus, classified 268 (80%) as true positive and 67 (20%) true negative (Table 2 & Fig. 2).

The employed serological tests carried out in this study on 335 camel sera revealed that mRBT<sub>75</sub> detected the highest number of positive samples 271 (80.9%), followed by real-time PCR 268 (80 %), while 262 (78.2%), 257 (76.7%), 253 (75.5%) and 245 (73.1%) samples were found to be positive for brucellosis with CFT, cELISA, mRB<sub>50</sub>, and sRB respectively (Table 2). The agreements between real-time PCR and standard rose Bengal (sRBT), modified (mRB<sub>50</sub>), (mRB<sub>75</sub>), cELISA and CFT was 0.562 ( $\pm$  0.053), 0.613 ( $\pm$  0.052), 0.725 ( $\pm$  0.048), 0.710 ( $\pm$  0.047) and 0.801 ( $\pm$  0.041), respectively (Table 3). The sensitivity of sRBT, mRBT<sub>50</sub>, mRBT<sub>75</sub>, cELISA, and CFT was, 85.8, 88.8, 95.1, 91.8, and 94.8%, respectively. While the specificity of the same tests, was 77.61, 77.61, 76.1, 83.6, and 88%, respectively. The positive predictive value (PPV) of sRBT, mRBT<sub>50</sub>, mRBT<sub>75</sub>, cELISA, and CFT was 93.8, 94, 94.1, 95.7, and 96.9%, respectively. While, the negative predictive value of the same order of the employed tests was 57.7, 63.4, 79.7, 71.8, and 80.8% respectively (Table 2).

instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values. Analysis of the SYBR green real-time PCR results. Amplification curves and cycle threshold (ct) values were determined by the Stratagene MX3005P software.

#### 2.4. Statistical analysis

Data analyses were carried out using a statistical software program (SPSS for Windows, Version 21.01, IBM, SPSS Inc., Chicago, USA). The agreement between different serological tests was calculated using Kappa analysis. As no gold standard was available we selected the real-time PCR as the “gold standard” which classify tested animals as true positive and true negative.

Real-time PCR detected 6 (1.8%) samples which were negative by all serological tests. Only one sample (0.3%) found to be positive by CFT and negative by all other tests. Modified RBT<sub>75</sub> detected one sample (0.3%) that found to be negative by other tests (Table 4).

The Receiver Operating Characteristic (ROC) curve for different cut-off points of both the true positive rate (Sensitivity) and the false positive rate (100-Specificity) of employed tests is demonstrated in fig. (1). Considering the real-time PCR as gold standard, ROC curve expressing the sensitivity (true positive rate) versus the false positive rate were plotted for all serologic tests. Data were obtained from ROC curves including the area under the curve (AUC) representing accuracy, the best positive cutoff points and the equivalent true positive/ false positive rates according to Hanley and McNeil (1983). A test with perfect discrimination (no overlap in the two distributions) has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). Therefore the closer the ROC curve is to the upper left corner, the higher the overall accuracy of the test (Zweig and Campbell, 1993). Based on the ROC curve result displayed in Table (2) and Fig. (1) it is evident that CFT is the most accurate test compared with other serological tests.

**Table (2): Validation of the different serological tests done on camel sera in comparison with real-time PCR as a gold standard**

		rtPCR		Relative Se %	Relative Sp. %	PPV	NPV	PLR	NLR	DOR	Accuracy (AUC)* ± SE
		Positive n(268)	Negative n(67)								
RBT <sub>25</sub>	Positive n(245)	230	15	85.8%	77.61%	93.8%	57.7%	3.8	0.18	20.9	0.817 (0.0278)
	Negative n(90)	38	52								
RBT <sub>50</sub>	Positive n(253)	238	15	88.8%	77.61%	94%	63.4%	3.97	0.14	27.5	0.832 (0.0274)
	Negative n(82)	30	52								
RBT <sub>75</sub>	Positive n(271)	255	16	95.1%	76.1%	94.1%	79.7%	3.9	0.06	62.5	0.856 (0.0271)
	Negative n(64)	13	51								
cELISA	Positive n(257)	246	11	91.8%	83.6%	95.7%	71.8%	5.6	0.1	56.9	0.877 (0.0243)
	Negative n(78)	22	56								
CFT	Positive n(262)	254	8	94.8%	88%	96.9%	80.8%	7.9	0.06	133.8	0.914 (0.0211)
	Negative n(73)	14	59								

Sample size: 335, Positive group: 268 (80.00%), Negative group: 67 (20.00%) Se. = Relative Sensitivity, Sp. = Relative Specificity, PPV = Positive Predictive Value, NPV = Negative Predictive Value, PLR = Positive Likelihood Ratio, NLR = Negative Likelihood Ratio, DOR = diagnostic odds ratio, AUC: Area under curves.

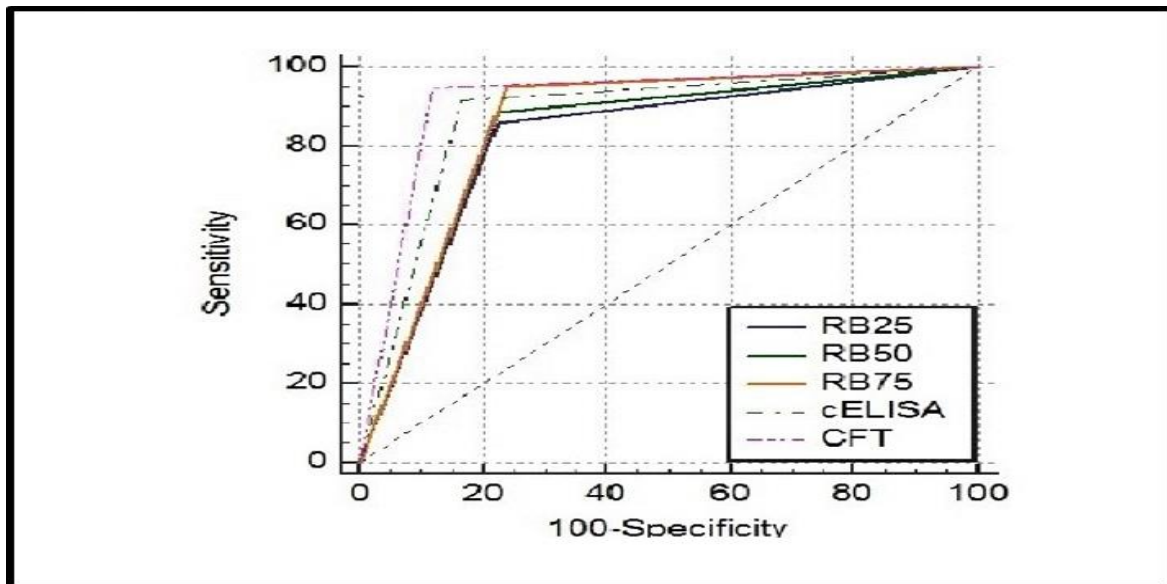
\*Confidence interval: 95% CI, Binomial exact, (DeLong et al., 1988).

**Table (3): Correlation and Agreement between real-time PCR as a Gold Standard and serological tests done on camel sera.**

Serological Test of 335 samples	Status and Numbers	Real-time PCR		Agreement, k value (± SE)
		Positive n(268)	Negative n(67)	
RBT <sub>25</sub>	Positive, n = 245	230	15	0.562 (Moderate) ± 0.053
	Negative, n = 90	38	52	
RBT <sub>50</sub>	Positive, n = 253	238	15	0.613 (Good) ± 0.052
	Negative, n = 82	30	52	
RBT <sub>75</sub>	Positive, n = 271	255	16	0.725 (Good) ± 0.048
	Negative, n = 64	13	51	
cELISA	Positive, n = 257	246	11	0.710 (Good) ± 0.047
	Negative, n = 78	22	56	
CFT	Positive, n = 262	254	8	0.801 (Very Good) ± 0.041
	Negative, n = 73	14	59	

**Table (4). The trend and correlation of positive samples per test used for detection of brucellosis in camel sera**

Samples	rtPCR	Positive samples				
		CFT	cELISA	RB75	RB50	RB25
220	220	220	220	220	220	220
13	13	13	13	13	0	0
6	6	0	0	0	0	0
7	7	7	0	7	7	7
7	7	7	7	7	7	0
2	2	2	2	0	0	0
3	3	3	3	0	0	0
3	3	0	0	3	3	3
3	3	2	0	3	0	0
1	1	0	0	1	0	0
1	1	0	0	0	1	0
1	1	0	1	0	0	0
1	1	0	0	1	0	0
268	268	254	246	255	238	230
	0	0	0	3	3	3
	0	0	5	5	5	5
	0	6	6	6	6	6
	0	1	0	0	0	0
	0	1	0	1	1	1
	0	0	0	1	0	0
<b>Total Positive samples</b>	<b>268</b>	<b>262</b>	<b>257</b>	<b>271</b>	<b>253</b>	<b>245</b>



**Figure (1). Diagnostic performance of serological tests on camel sera using real-time PCR as a “gold standard” expressed as receiver operating characteristics (ROC) curves as a validation tool.**



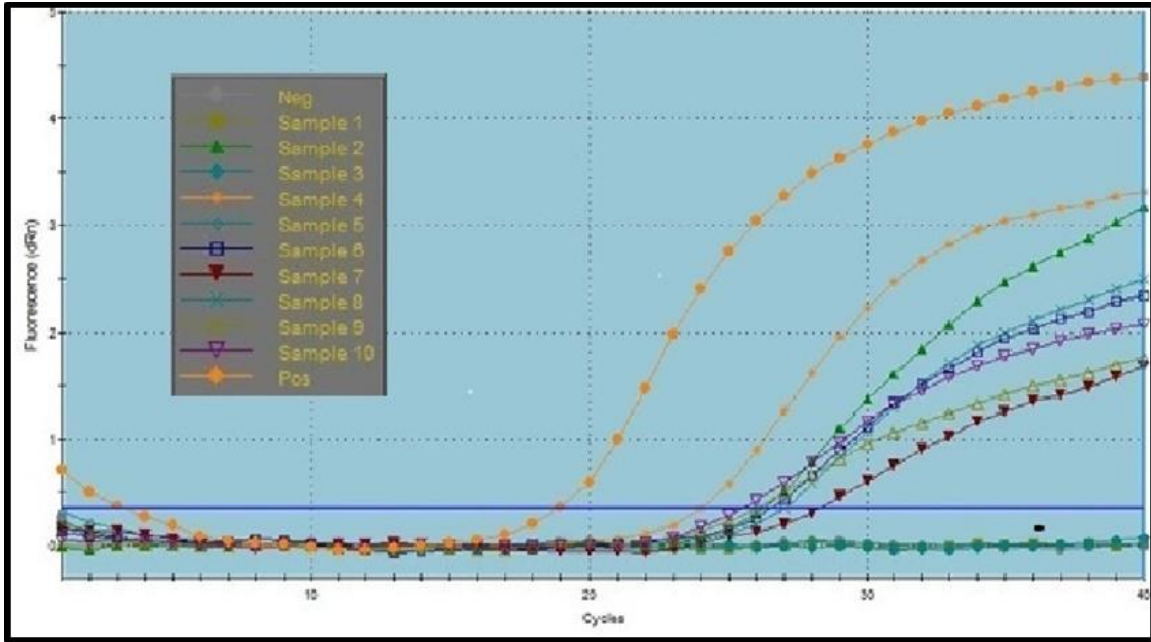


Figure (2). Real-time PCR amplification curves of the *Brucella* genus, showing - Positive control (upper curve); - Negative control (lower curve); - Samples numbers: 2, 4, 6, 7, 8, 9, 10) Positive samples; - Samples numbers: 1, 3, 5) Negative samples

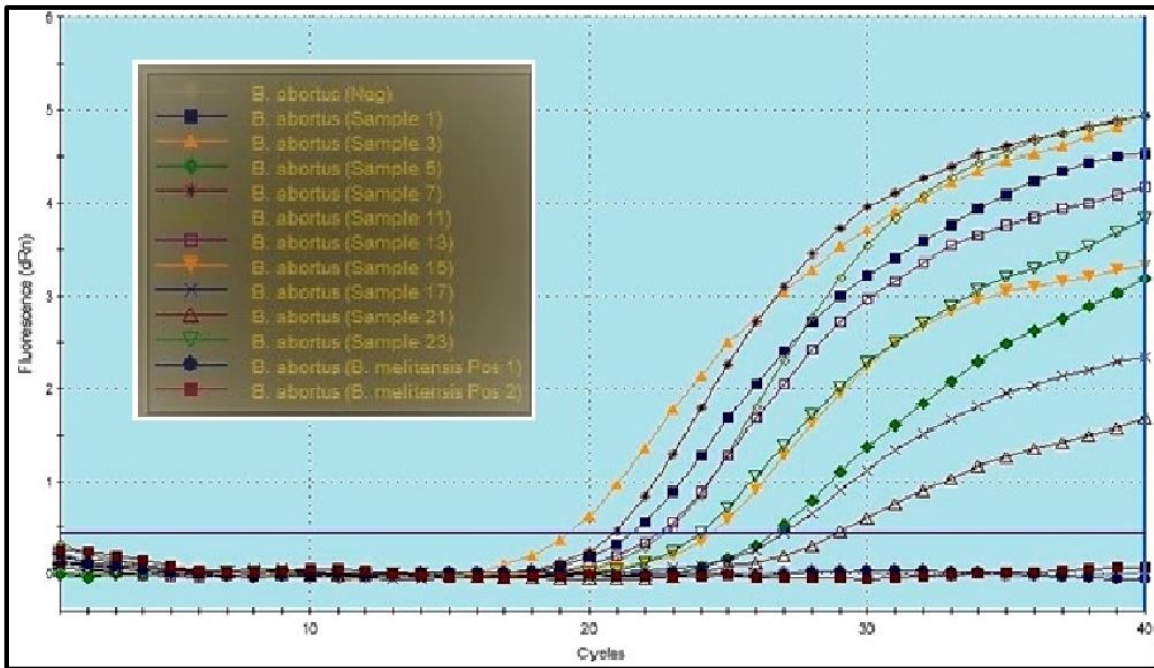
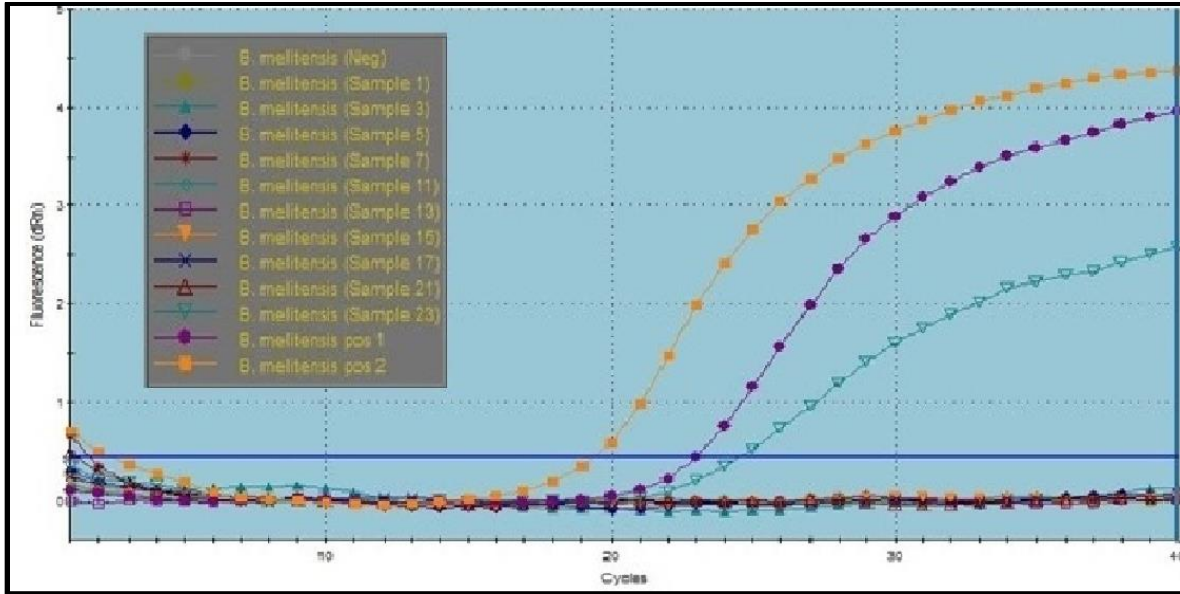


Figure (3). Real-time PCR species specific for *B. abortus* specific primer, amplification curves showing - Negative control (lower curve) - Samples numbers: (1, 3, 5, 7, 11, 13, 15, 17, 21, 23) Positive samples -, (*B. melitensis* 1 & *B. melitensis* 2) Negative control.



**Figure (4). Real-time PCR species specific using *B. melitensis* specific primer, amplification curves showing -Negative control (lower curve) -Sample number (23) Positive sample –Samples numbers (1, 3, 5, 7, 11, 13, 15, 17, 21) Negative Samples –(*B. melitensis* 1 & 2) Positive control.**

#### 4. Discussion:

A total of 6570 serum samples collected from male camels prior to exportation from Somaliland to Egypt and Arabian Gulf countries were tested by mRBT<sub>75</sub> and 418 samples found to be reactors with apparent prevalence of 6.36%. A previous sero-prevalence of camel brucellosis in Somaliland carried out during July and November 2008 using RBT and i-ELISA revealed that the prevalence rate was 3.9% and 3.1% respectively (Ghanem et al., 2009). The high prevalence rate of brucellosis in camels in Somaliland in current study (6.36%) compared with the previous study (3.9%) may be attributed to the fact that no control programs have been applied yet in Somaliland to control the disease in camels during the period between 2009 to 2015, besides; this study was carried out on large number of camels (6570) where the other study was done only on 1246 camels.

In the current study, real-time PCR carried out on camel sera succeeded to identify 268 camels positive for brucellosis and all negative camel samples (n = 67) gave negative result by the test, with sensitivity and specificity of 100%. Detection of the Brucella DNA in 268 camel sera as detected by real-time PCR was considered as a golden mark referring to the exposure of these camels to Brucella organisms.

Although camels appear to be very susceptible to Brucella infection, isolation of Brucella organisms from camel samples is rare and difficult (Hamdy and Amin 2002; Wernery et al., 2007; Ghorbani, et al., 2013). On the contrary, Brucella DNA is proved to be persistent in sera and can be detected at any time by any of PCR techniques. In human Brucella DNA proved to persist in serum, for weeks to months after completion of the standard treatment regimen (Maas et al., 2007; Zerva et al., 2001). The proportion of individuals with *B. melitensis* DNA was significantly higher for symptomatic non-focal-disease patients than for asymptomatic subjects. Therefore, PCR appears to be a useful method for identifying chronic brucellosis patients (Castan˜o and Solera, 2009). On the other hand, the persistence of Brucella DNA in camel sera is hypothesized to the unique immunoglobulins configuration of the fully active antibodies that are naturally devoid of light chains. The absence of the light-chain variable domain in camel sera is compensated for by extended complementarily-determining regions (CDR) that provide an adequate antigen-binding surface and demonstrate affinities comparable to those of conventional antibodies (Conrath et al., 2003). The presence of the heavy-chains in camel sera was proved to have a killing effect on some of Gram

positive and Gram negative bacteria (Szynol et al., 2004). These heavy chain immunoglobulins in blood play a role in killing *Brucella* organisms during phases of bacteremia and release of *Brucella* DNA in camel sera (Castano and Solera, 2009; Takele et al., 2009). In addition, the detection of *Brucella* DNA in serum persist in infected camel sera for long periods and the real-time PCR had been proved to detect up to 23 fg of *Brucella* DNA fragment in camel sera (Gwida et al., 2011). However, the exact time of appearance of the *Brucella* DNA from the day of infection, and the exact time for its persistence in camel sera should be investigated in future study.

The presence of the *Brucella* DNA in camel sera indicates that these animals were exposed to *Brucella* infection at any part of their lives. However, animals having *Brucella* DNA in their blood represent a threat to other animal species as well as to public health as they may be asymptomatic carriers and shedders and should be excluded from any breeding activities. The presence of *Brucella* DNA justifies the removal of animals from the herd according to OIE guidelines.

The results presented here indicate that IS711 real-time PCR assay is a specific and sensitive tool for detection of *Brucella* spp. infections in dromedary camels. For this reason, we propose the employment of IS711 real-time PCR as a complementary tool in brucellosis screening programs and for confirmation of diagnosis in doubtful cases. The real-time PCR assay targeting IS711 presented an identical or a greater sensitivity than the two other bcp31 and per target genes (Bounaadja et al., 2009). It has been demonstrated that real-time PCR applied to serum samples in camels was more sensitive than other methods of diagnosis (Khamesipour et al., 2015). The major advantages of real-time PCR are that it can be performed in a very short time, with no requirement for electrophoretic analysis, and overcome cross-reaction with gram-negative bacteria.

The humoral immune response of camels exposed to *Brucella* infection has not been validated. The International regulations adopted by OIE recommended the use of bovine serologic tests for the diagnosis of brucellosis in other domestic and wild animals including camels provided that validation of every test should be carried out. Diagnostic accuracy is the ability of the test to discriminate between true positive and true negative animals. Different parameters of diagnostic accuracy of the test are applied in this study, including, sensitivity, specificity, predictive values, likelihood ratios, the

area under the ROC curve, and diagnostic odds ratio (Irwig et al., 2002).

Out of the 335 tested camel sera, 220 (65.67%) were positive by real-time PCR as well as employed serological tests and 50 (14.93%) were negative by all tests. It is evident from the obtained results that real-time PCR and employed serological tests detected 270/335 (80.6%) of reactor camel serum samples (Table, 4). Real-time PCR detected 6 (1.8%) samples which were negative by all serological tests. This result could be attributed to the high sensitivity of real-time PCR that could detect 23 fg of *Brucella* DNA (Gwida et al., 2011).

Real-time PCR carried out in camel sera classified 94/104 (90.4%) samples due to *B. abortus*-DNA, 4/104 (3.8%) samples with *B. melitensis* DNA and 6/104 (5.8%) samples with mixed *abortus* and *melitensis* DNA (Fig. 3 & 4). Previous studies illustrated that *B. abortus* and *B. melitensis* constitute the majority of *Brucella* species that are frequently detected in clinical specimens of diseased camels (Khamesipour et al., 2015). On the other hand, another study done in UAE on imported camels from Sudan revealed that only *B. abortus* DNA was detected in camel sera by real-time PCR (Gwida et al., 2011). In Somalia *Brucella abortus* isolated from infected cattle (Wernery et al., 1976 and Andreani et al., 1982), while *B. melitensis* have been isolated from infected human patient (Wheat et al., 1995). *Brucella melitensis* were isolated 26 times from a total of 100 milk samples from seropositive Saudi Arabian dromedaries (Radwan et al., 1992). A comprehensive culture study done on 300 slaughtered dromedaries in Iran revealed that *B. melitensis* biovars 1 and 3 were isolated from lymph nodes in 1% (3/300) of the camels, (Zowghi and Ebadi 1988). The authors are of the opinion that *B. abortus* and *B. melitensis* infections in the dromedaries originated from neighboring large and small ruminants.

Among the employed immunoassays, the CFT scored the highest level of specificity (88%). However; CFT gave positive results for 8 samples that were negative by real-time PCR and these samples were from animals free from brucellosis (Tables, 2 & 4). This finding may be attributed to false positive reaction. This finding is in agreement with what reported by Sunaga et al., (1983) who reported that five dromedaries imported into Japan had positive complement fixation (CFT) and slow agglutination reactions. The animals were immediately slaughtered and no *brucella* organisms

were isolated; however, *Yersinia enterocolitica*, serotype 09 was identified. It is known that false-positive (unspecific) reactions with other gram negative species of bacteria may occur (Bisping and Amtsberg, 1988).

On the other hand, CFT gave negative results for 14 samples that were positive by real-time PCR. This may be ascribed to the fact that the sensitivity of the employed serological tests, including CFT, is varying depending on the stage of infection. It was observed that in the latent stage of infection in camels and during a period of two years, about 5% of the camels had fluctuating titers from positive to negative to positive and about 20% of the serologically positive camels turned negative with RBT and cELISA (Von Hieber, 2010). In addition, it has been proved that the pathogens can conceal themselves in camels, most probably in lymph nodes, and do not produce detectable antibodies in those intracellular hiding places (Wernery et al., 2007).

The modified rose bengal test (mRBT<sub>75</sub>) using 75 µl of camel serum was superior in sensitivity (95.1%) to standard RBT (85.8%) using 25 µl of serum or mRRB<sub>50</sub> (88.8%). The high sensitivity of the mRB<sub>75</sub> due to increasing the amount of antibodies in serum samples sharing in the reaction was not on the expense of the specificity, as it scored 76.1%, compared with specificity of the sRBT and mRBT<sub>50</sub> as they scored 77.61% for both tests.

The RBT in its conventional method using equal quantities of antigen and sera in a ratio of (1:1) is proved to be the least test to detect the disease in camels, as, it scored (85.8 %) in sensitivity. The test gave the lowest sensitivity among the employed serological tests, and thus the test loses one of its major advantages as a screening test. Even doubling the quantity of serum in mRBT<sub>50</sub> does not increase the sensitivity of the test to a significant levels as the test scored (88.8%) in sensitivity. The World Animal Health Organization (OIE, 2016) recommended the modifications adopted by Blasco et al. (1994) to use mRBT in testing sheep and goats (OIE, 2016). The

## 5. Conclusion

Camels can be an important reservoir for transmission of brucellosis to other animal species and humans and have to be included in national programs for control and eradication of brucellosis in endemic countries including Egypt and GCC countries as well as in the exporting countries with the large camel populations, namely; the Sudan,

modification done on RBT in testing sheep and goats by using 75 µl of serum and 25 µl of antigen increased the sensitivity of test. In our opinion, the mRBT<sub>75</sub> is the proper test that can be very helpful as a screening test to start with for testing camels for brucellosis, and we recommend its use in quarantine stations and in survey programs to detect the disease in camels.

Competitive ELISA detected positive camel samples 246/268 and negative camel samples 56/67 with sensitivity of 91.8% and specificity of 83.6%. The cELISA failed to detect 22 samples which were positive by real-time PCR. Similar finding was obtained on 103 testing camel sera and the obtained relative sensitivity and specificity was (89.5%) and (83.3%) respectively (Sayour et al., 2015). The cELISA demonstrated the lower sensitivity when compared to mRBT<sub>75</sub>, and CFT. While, the specificity of the cELISA was (83.6%) and it was superior to the mRBT<sub>75</sub> (76.1%) but inferior to the CFT (88%). In other study cELISA detected 616 positive camel serum samples out of 828 (74.4%) (Gwida et al., 2011), and this result was attributed to the fact that cELISA was specially standardized to work with bovine sera. It has to be stressed that sensitivity and specificity may vary considerably if another cELISA kit will be tried (Gwida et al., 2011).

Compared to other serological tests employed in this study, the CFT proved to have the best result in the criteria of test validations, namely; specificity (88%), PPV (96.9%), NPV (80.8%), PLR (7.9), NLR (0.06) and DOR (133.8). The mRB<sub>75</sub> showed the highest relative sensitivity (95.1%) but on the account of the specificity (76.1%). This result was in accord with that obtained by several studies (Waghela et al., 1978, Gwida et al., 2011, Sayour et al., 2015). However, CFT is considered by other authors as being the most sensitive and specific test for brucellosis in camels for the reason that, CFT antibodies remain in the serum for longer periods than SAT antibodies (Waghela et al., 1978). In addition, it was determined that the CFT was four times more sensitive than the SAT (Shumilov, 1974).

Somaliland and Ethiopia. RBT in its conventional method using equal quantities of serum and antigen should not be used to screen camels for brucellosis as it proved to be of lower sensitivity. The use of mRBT<sub>75</sub> as a screening test is recommended in quarantine stations and in survey programs. Real-time PCR based on targeting IS711 element, is the test of choice to identify camels infected with brucellosis. In cases of budgetary issues due to the cost of the real-

time PCR, alternatively, CFT is the test of choice to confirm the disease status in suspected camels. For the control purposes, we suggest the castration of reactor male camels to prevent the transmission of the disease through genital intercourse and to prevent reactor camels from breeding activities.

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