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ORIGINAL PAPER

Identification of *Brucella melitensis* from camel's blood by vitek2 and real time polymerase chain reaction

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ABSTRACT

Introduction and aim. Brucellosis is a zoonotic disease. Experimental clinical and laboratory diagnosis is still facing problems in identifying the organism. The present study will diagnose a *Brucella* infection in camel blood in Qatar using serological assays. Isolation and identification were performed on a camel blood sample. Brucella in bacterial isolates was determined by real-time polymerase chain reaction (RT-PCR) as a gold standard test.

Material and methods. A total of 220 samples, 200 random serum samples, and 20 EDTA blood samples were selected among the above-mentioned random samples, and 20 serum samples from camel handlers were collected from Al Shahaniya province, Qatar. The Rose Bengal test (RBT), buffered antigen plate agglutination test (BAPAT), and enzyme linked immunosorbent assay (cELISA) for the monoclonal antibody in serum samples were performed using commercially available kits. For the molecular detection of *Brucella*, conventional PCR and real-time PCR (GPS kit) were used for the genus-specific insertion sequence IS711. *Brucella melitensis* (MICROBOSS Hightech GmbH kit) was used to identify subspecies.

Results. The results identified by vitek2 compact (30%) showed *B. melitensis* in 6 samples out of 20 isolates. Both conventional (66.67%) and RT-PCR (83.33%) analyses supported this, demonstrating the presence of *Brucella*. These tests also showed that *Brucella* species were present in Rose Bengal 182/200 (91%), BAPAT 182/200 (91%), and cELISA (90%) 180/200 in camel serum.

Conclusion. To conclude, the prevalence of brucellosis in dromedary camels is higher in this region, and as a matter of urgency, measures should be taken to control the disease.

Keywords. Brucella melitensis, brucellosis, camels, conventional PCR, RT-PCR, serological assays

Introduction

Brucellosis is a global zoonotic infection. *Brucella* species infect sheep, goats, cattle, deer, elk, pigs, dogs, camels, and the environment as well as humans.[1] People and camels get brucellosis from gram-negative bacteria in the genus *Brucella* when they come into contact with large or small ruminants that are infected with *Brucella abortus* or *Brucella melitensis*.[2,3] Brucellosis is propagated not only to people in contact with infected animals but also polluted products; the portal entry into the body takes many routes, for example, raw milk into the digestive tract, intact skin, mucous membranes, and through respiration. *B. melitensis* is the foremost cause of human brucellosis (94% of cases); B. abortus

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is significantly less common (6%), indicating that it is a significant pathogen for humans worldwide.[4,5] *Brucella* species are facultative intracellular gram-negative bacteria that cause brucellosis, which is depicted by abortion in camels and undulant fever, arthritis, endocarditis, and meningitis in humans.[6] Human brucellosis is most widespread in countries reported for *Brucella*, and in people who arrive back from indigenous areas.[7-9] There are no vaccines against brucellosis for humans.[10] Although brucellosis infection is of considerable significance in animals, the ailment in humans is less acknowledged, despite information that it is related to considerable and prolonged morbidity.[11,12] The almost asymptomatic epidemiology of the disease requires the necessary and precise exploration of the species, which is the core demand to reach the objective.

Brucella are special coccobacilli that can live inside cells and prefer placental trophoblasts. They are also known to infect the rough endoplasmic reticulum and reticuloendothelial tissues in the spleen, liver, and blood vessels, which is a major public health concern.[13-17] Numerous Brucella species, some of which are OIE-registered, such as those that affect cattle (*B. abortus*), sheep, goats, camels (*B. melitensis*), and swine (*B. suis*), are known to infect various animal species.

Serological tests are the fundamental brucellosis screening tests. Standard methods of brucellosis testing are laborious and tedious, pose a risk of infection, and can generate discordant results. Culturing brucellosis is challenging. The Rose Bengal test (RBT), the buffered antigen plate agglutination test (BAPAT), and enzyme-linked immunosorbent assays (cELISA) are used to detect Brucella species. To overcome the genetic diversity of *Brucella* species, molecular methods are used to diagnose brucellosis; hence, polymerase chain reaction techniques are adapted.[13,18,19]

Aim

This study aimed to conduct serological screening, isolation, and identification of *Brucella* species in camels' blood. To detect *Brucella* subspecies in bacterial DNA by real-time PCR as a confirmatory test.

Material and methods

Collection of samples

A total of 220 samples of dromedary camels, 200 camel serum samples, and 20 serum samples from camel handlers were randomly collected from different farms located in the Al Shahaniya province, Qatar. None of the animals tested had been administered with any vaccine; collected samples were used for the agglutination of Brucella antibodies. Twenty EDTA blood samples of seropositive camels were used for isolation techniques, and the positive isolates were subjected to IS711 RT-PCR analysis.

Serological assays

Rose Bengal test

5 mL of blood was collected aseptically from the jugular vein of camels and subjected to centrifugation to separate the serum at 5000 rpm for 5 min. On a white porcelain plate, 50 µL of serum and 20 µL of RBT reagent were mixed gently and spread out to about 2 cm in diameter. The plate was then moved around gently on a rocker (Boeco, Germany) for 4 minutes and viewed under light to see if the substances mixed. A visible precipitation was recorded as positive. Control negative and control positive were included in the tests.[20]

Buffered antigen plate agglutination test

Buffered antigen plate agglutination test (BAPAT) was done according to Mahmoud et al., adding 30 µL reagent with 80 µL serum and agitated gently for 8 min at intervals to test the agglutination reaction.[21] Agglutination was considered positive, indicating the presence of specific antibodies to Brucella. To check the sensitivity of the reaction, all positive samples were repeated with different dilutions and examined.

Detection of Brucella using cELISA

cELISA was performed for all the serum samples collected using the Svanova kit (Svanova-Sweden) in the DS2ELISA machine (automated) as per the given protocol. The same tests were performed for 20 human serum samples as well. The optical density was read at 450 nm in automated DS2ELISA. The status of the sample is determined as ≥30% positive or <30% negative.

Isolation of bacteria

Twenty EDTA blood samples were collected aseptically from the jugular veins of positive camels in Oxoid signal blood culture bottles and sent to the lab in an ice box.[22] Culture systems were incubated anaerobically at 37 °C, and the cultures that showed growth within 3 days were incubated both aerobically and anaerobically and examined daily for a week by allowing the blood broth mixture to flow over. The loop of growth is streaked on Tryptic soy agar and Brucella agar and incubated for 3–14 days. It was found to have a smooth, yellow, honeycomb-like colony structure both in aerobic and anaerobic incubation (5% CO_2).

A small colony was used for gram-staining and found to be gram-negative, pink-coloured cocci. Then the colony was subcultured on TSA, MacConkey, and sheep blood agar enriched with 5% horse serum plates to ensure bacterial growth before considering it negative. The isolates recovered were identified according to the conventional method prescribed by the vitek 2 compact automated system (bioMérieux, Marcy-l'Étoile, France). The isolate was subcultured on TSA plates and incubated at 37°C. After growth, it was stored at 4°C until used for identification.

Identification of Gram-negative bacteria

A small colony was used for Gram staining and viewed under an Olympus microscope. Pink-coloured cocci were found to be gram-negative bacteria. The Vitek 2 compact GN card, which contains 64 biochemical tests, was used to identify organisms, grade the isolates from acceptable to excellent identification, and give the details of the biochemical tests.[23]

Preparation of bacterial inoculum

The Densicheck was standardized first with (0, 0.5, 2.0, and 3.0 mL) and the blank set with 3 mL of saline. According to the kit, a suspension of each isolate was made by mixing the bacterial colonies in 0.45% NaCl saline at pH 5 and standardizing it with Densicheck (BioMérieux, Marcy-l'Étoile, France) at a level of 0.5–0.63 McFarland in an opacity tube. The time taken for preparation and card filling must be less than 30 minutes.[24] The card was filled, sealed, and inserted into the reader, which is subjected to kinetic colorimetric measurement. The results were obtained after 8 to 10 hours for gram-negative bacteria.

Bacterial DNA extraction

Based on the identification of the organism as *B. melitensis* by the vitek 2 compact, DNA was extracted from the isolate using a three-step approach (heat shock: suspend the isolate in 200 µl of phosphate-buffered saline (PBS) and centrifuged three times at 10000 rpm for 10 min (wash), heat inactivation was done at 100°C for 10 min, and it was cooled immediately). Then 30 µL of DNA was extracted. Qiagen DNA mini kit (Hilden, Germany), followed by Kingfisher Duo Prime (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to references. DNA quantification was done by a Quawell UV-VIS spectrophotometer (Q5000) and a Nanodrop 8 spectrophotometer (NDE2200281, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Real-time PCR and conventional PCR assay

The RT-PCR reaction was performed in triplicate in 96-well 0.1 µL plates (qPCR 96-well plates, Micro Amp TM, Applied Biosystem). Bacterial DNA was analyzed by RT-PCR with the IS711 primer probe. Amplification of the Brucella DNA genus was in Bayeta et al.[25] Using (forward: GCTTGAAGCTTGCGGACAGT) and (reverse: GGCCTACCGCTGCGAAT), probe (5'-6-FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA 3') (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total mix volume was 15 µL/sample containing: Master mix 3 µL (Applied Biosystems, Waltham, Massachusetts, USA), 0.3 µL for each forward and reverse primer, 0.1 µL of the labelled probe, and 3.5 µL DNA and water to make up the total volume. Quant-studio 7flex RT-PCR (Applied Biosystems, Waltham, Massachusetts, USA) is used with the following thermal profile: The reaction mixture was initially incubated for 5 minutes at 95°C. Amplification was performed for 40 cycles at 95 °C for 30 s, followed by annealing and extension at 60 °C for 1 min. A GPS kit (Genetic PCR Solutions-Dtec-QPCR Test, Lot 002600320217, Spain) was used to analyze the same DNA samples twice, once with diluted DNA and once without diluted DNA. Subspecies were done using the MicroBOSS Hightech GmbH kit, and optimisation was done repeatedly, which confirmed the amplification curve for *B.melitensis* at Ct 21.8 to be positive. Samples exhibiting sigmoid curves below 35 threshold cycles (CTs) were considered positive, and negative controls of Brucella were included in each run to detect any contamination or amplification failure. The instrument automatically set the threshold and additionally confirmed electrophoresis.

The conventional PCR reaction was performed in duplicate in microtiter plates using a verity 96-well thermal cycler system with the following run conditions: Following 40 cycles of 72°C for 32 s, 54°C for 30 s, and the 4°C hold stage previously mentioned for typing, there will be 1 cycle of 95°C for 10 min, 1 cycle of 95°C for 15 min.[17] The total reaction mixture volume is 25 µL, containing 12.5 µl of TaqManTM Universal Master Mix (Applied Biosystems, Waltham, Massachusetts, USA). 1 µL of each primer, 2 µL of bacterial DNA as a template, and nuclease-free water sum up to a total reaction volume of 25 µL. 1.5% Agarose gel electrophoresis served to verify the PCR product. 3 µL loading buffer (Invitrogen), 15 µL PCR product, ladder 5 µL with 50 bp ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA), adjust the mode to 100 V and time 30 min. visualized in UV trans illuminator (spectroline). The Amplicon size was matched with the ladder.

Ethics approval

Experiment has been approved by administration of Tharb camel Hospital.

Results

The serological test results were obtained from 220 serum samples: 200 camel serum and 20 camel handlers serum. Out of 200 serum samples, 182 were strong positives; the RBT was found to be strong visibly; mild agglutination (18 weak positives) was confirmed as a weak positive under the agglutination viewer; and the camel handlers serum (20 suspected mortal samples) was found to be negative in all the tests. Among 182 seropositive samples, 20 were selected for blood culture (EDTA blood sample) of camels who had a previous history of brucellosis. When the samples were examined for brucellosis using the RBT, 182 samples (91%) out of a total of 200 tested strong positive for the presence of *Brucella spp*., and 18 samples were weak positive. When cELISA was performed to detect the IgM antibodies against *Brucella spp.* Out of 180 (91%) samples, positive results are shown in Table 1.

Table 1. Total number of positive results per serological test and real-time PCR used for the detection of brucellosis in camels*

Tests	Total no. of samples	Sample type	Positive	Negative	Suspected	%	
						Positive	Negative
RBT	200	Camel sera	182	0	18	91	0
RBT	20	Human sera	0	20	0	0	100
BAPAT	200	Camel sera	182	0	18	91	0
BAPAT	20	Human sera	0	20	0	0	100
ELISA	200	Camel sera	180	0	18	90	0
ELISA	20	Human sera	0	20	0	0	100
Blood culture	20	Camel blood	6	14	0	30	70
RT-PCR IS711	6	Camel blood	5	1	0	83.33	16.67
RT-PCR GPS	6	Camel blood	2	4	0	33.33	66.67
Conventional-PCR IS711	6	Camel blood	4	2	0	66.67	33.33

* RBT – Rose Bengal test, BAPAT – buffered antigen plate test, ELISA – enzyme immunosorbent assay, RT-PCR – real-time polymerase chain reaction, PCR – polymerase chain reaction

Table 2. Number of positive results per isolate for the detection of brucellosis in blood samples of camels

Sample no.	Sample Type	Incubation Period	Gram stain	Media/Agar used
1	Blood	5-days aerobic	*Gram-negative bacteria*	TSA/MC
2	Blood	3-5-days aerobic/ anaerobic	*Brucella melitensis*	TSA/MC
3	Blood	5-days aerobic	*Brucella melitensis*	TSA/MC
4	Blood	5-days anaerobic	*Brucella melitensis*	TSA/MC
5	Blood	4-days aerobic/ anaerobic	*Brucella melitensis*	TSA/MC
6 (repeat sample)	Blood	4-days aerobic/ anaerobic	*Brucella melitensis*	TSA/MC

Table 1 depicts the findings of the RBT, BAPAT, ELISA, RT-PCR, and conventional PCR assays. The issues of RT-PCR analysis show the presence of Brucella species in 6 samples (83.33%) out of 20 samples. The results of serological assays showed the presence of Brucella species. Similar to RBT (91%), ELISA (90%), and BAPAT assay (91%) in the camels' samples. The growth in the oxoid signal culture system depicts the presence of the organism, which was dressed on different agar plates and incubated. After 3–7 days, colony growth was tested with gramme stain. The positive results of bacterial isolates are illustrated in Table 2. Bacterial DNA was uprooted, quantified, and analyzed. The results of RT-PCR analysis using the IS711 manual show the manifestation of Brucella species in 5 samples (83.33%) out of a total of 6 bacterial DNAs (Fig. 1).

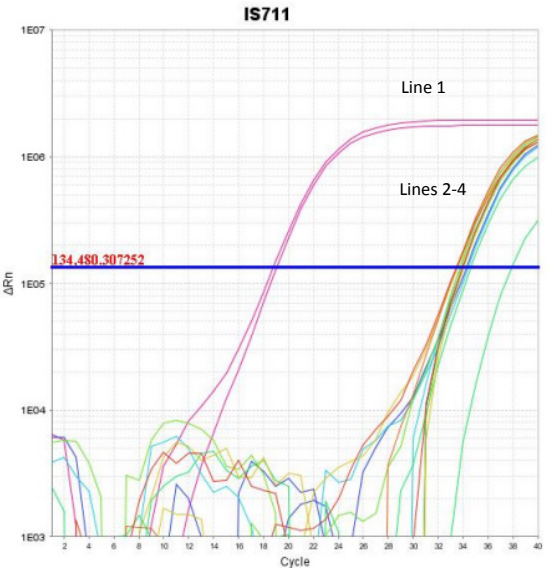


Fig. 1. IS711 Real-time PCR amplification; Ct 21.8 (Line 1 – control positive and lines 2-4 – bacterial DNA)

Table 3 reveals the presence of Brucella species in the insulate with other organisms, which was re-cultured to yield the pure isolate. The presence of Brucella species in (3%) was linked by RT-PCR analysis (Fig. 2). 182 samples (91%) out of the total 200 samples show the presence of Brucella *spp.*

Table 3. Presence of *Brucella spp.* and other organism in the isolate*

Serial no.	Identified	Gram-positive	Gram-negative	Serology
1	*Brucella melitenisis*	NIL	No other Gram-negative	RBT (182) = +++
2	NIL	NIL	NIL	RBT (18) = ++
3	NIL	NIL	NIL	
4	*Brucella melitenisis*	NIL	*Spingomonas paucimobils*	BAPAT (182) = + +++
5	NIL	NIL	*Burkholdaria gladioli*	BAPAT (18) = ++
6	*Brucella melitenisis*	NIL	*Aeromonas salmonicidas, Oligella ureolytica*	Cellist (180) = +++
7	*Brucella melitenisis*	NIL	No other gram-negative	cELISA (20) = N
8	*Brucella melitenisis*	NIL	NIL	
9	*Brucella melitenisis*	Staphylococcus cohini	NIL	

* NIL – no organism found, RBT – Rose Bengal test, BAPAT – buffered antigen plate test, ELISA – enzyme immunosorbent assay

According to the Rose Bengal test, 182 samples (91%) showed the presence of *Brucella* species. By BAPAT assay, 180 samples (90%) reveal the presence of

Brucella species. by ELISA. The results of colony growth on colourful agar plates are depicted in Figure 3, which shows isolates used for bacterial DNA isolation.

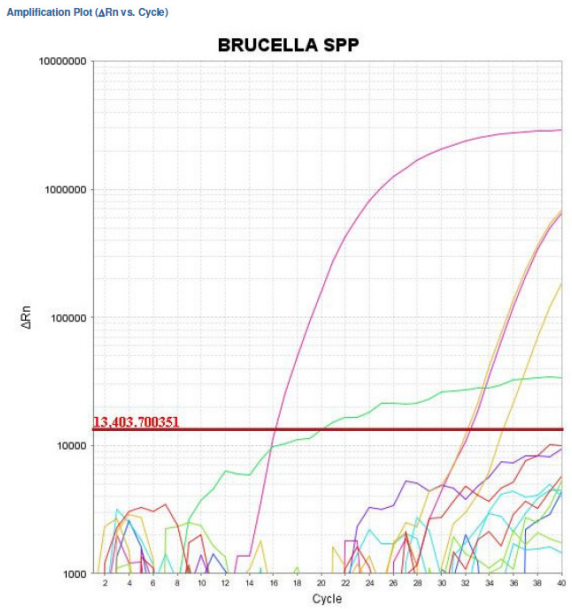


Fig. 2. Real-time PCR amplification curves of the *Brucella* spp. in the bacterial DNA of camels

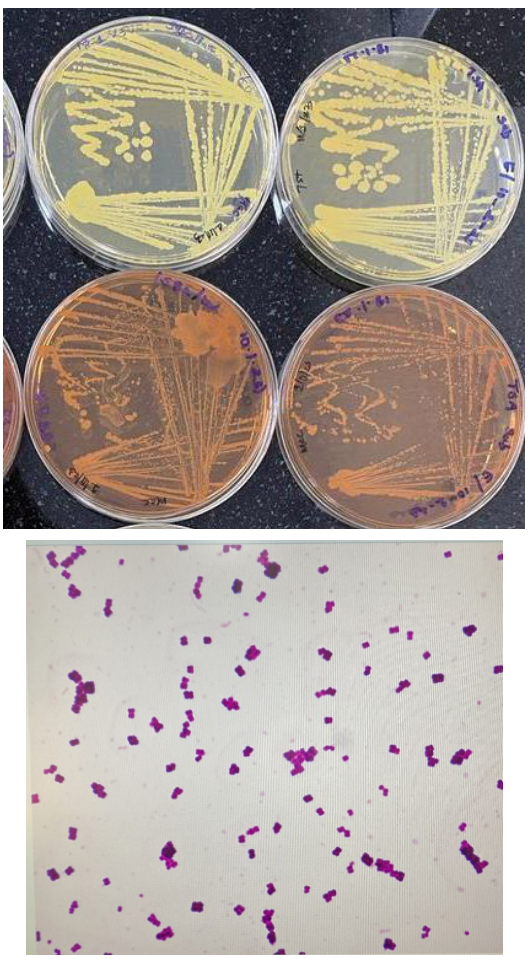


Fig. 3. *Brucella* colony growth in different plates

The bacterial DNA samples that gave a positive result with the IS711 manual were used to identify the subspecies. The sub-species was identified using MicroBoss for *B. melitensis*. Optimisation was done, and the amplification curve for *B. melitensis* was attained at Ct=21.8 and control positive curve was obtained at Ct=23.02. This indicates the presence of *Brucella* species and sub species in the given bacterial DNA.

The PCR product was confirmed by 1.5% agarose gel electrophoresis loaded into the gel, and the amplicon was matched with the ladder. The issues were delved into independently, and RT-PCR yielded positive results (Fig. 4). A repeated analysis was done with the tackle. In this trial, BMEII0466 with the following sequence garbling external membrane protein was used both conventionally and by RT-PCR (Fig. 5)

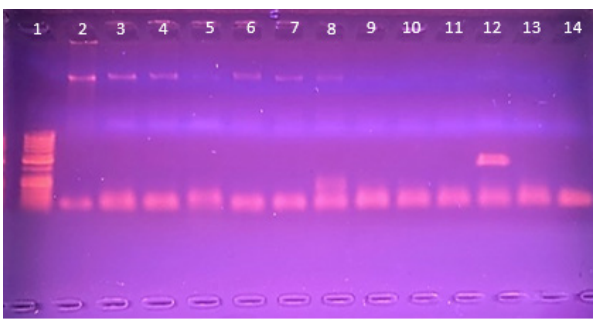
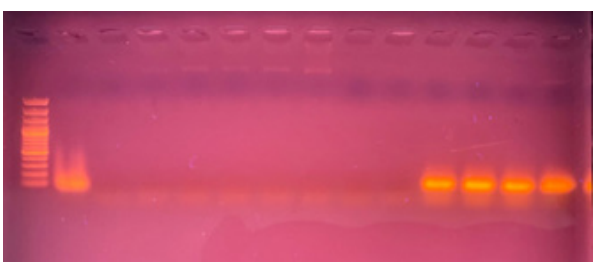


Fig. 4. Agarose gel electrophoresis of PCR products

Result of real-time PCR on *Brucella* isolates. Lane 1, 50bp plus DNA ladder (Invitrogen); lines 2 to 13 are *Brucella* spp. isolates; line 14, reference positive control.



BMEII0466	5′-cy5- CCTCGGCATGGCCCGCAA-BHQ-2-3′
BMEII0466(f)	TCGCATCGGCAGTTTCAA
BMEII0466(r)	CCAGCTTTTGGCCTTTTCC

Fig. 5. Number of positive results conventional and RT-PCR used for the detection of *B. melitensis* [BMEII0466] ref

Discussion

Brucellosis is a neglected transmittable disease affecting all domestic and wild animals and humans with important economic and public health importance.[26,27] Dependable identification of the applicable *Brucella* species is challenging with any individual approach and available serology.[28] The camel handlers lab personnel might be asymptotic without any affiliated symptoms

when being a carrier. There are few studies available on the outcomes of asymptomatic brucellosis and most of them are case reports.[29] However, we still hope to give a clinical reference for opinion and treatment to strengthen active webbing and surveillance.[30] Webbing and identification of serological tests is the top tool for effective epidemiological analysis.[31]

Our findings demonstrated that the highest prevalence of *Brucella* in camel serum and 182 samples showed that the existence of *Brucella* (91%) out of 200 samples. The BAPAT assay, 182 positive and 18 suspected samples, confirmed the presence of *Brucella* (91%). The ELISA assay, 180 camel sera and 18 suspected samples, demonstrated the presence of *Brucella* (90%). The existence of *Brucella* DNA as showed by the RT-PCR was regarded as evidence for the potential risks for the consumers who utilizes products of *Brucella* infected camels. The prevalence of brucellosis is comparatively high in imported camels.[7]

In peer reviewed papers that describe the prevalence of bovine brucellosis in animals, only data are available on perceptivity and particularity of the serological tests in Egypt. The asemantic infection leads to revocation in female camels, orchitis and epididymitis with frequent sterility in males, because of the localization of *Brucella* within reproductive organs.[32,33] These issues reveal an advanced circumstance of brucellosis among the camels in this region. Indeed though detailed reports on camel brucellosis aren't reported yet from Qatar, advanced prevalence of the complaint in camels is reported from the neighboring regions. A previous report discusses 15 camels that were set up to be infected with *Brucella* in Jordan.[34] Whereas, in another study conducted in Sudan revealed that 40 tested samples were positive for *Brucella* when 2,000 camel serum and milk samples were examined.[35] Bacterial infections are transmitted to humans from other sources where antibiotics are used for various purposes and should cause emerging resistant strains.[36,37] Detection of brucellosis in cattle and humans relies on the ways employed for discovery and identification of the pathogens; subspecies, due to inheritable diversity and analogous to other contagious and non-infectious conditions of brucellosis in camels, are asymptomatic and require individual ways to be estimated. The results indicate that the blood samples of camels shows the presence of *Brucella* species for brucellosis.[38] Molecular methods like PCR are often applied to detect brucellosis.[39] In this discussion, the RT-PCR test detected *Brucella*. Followed by the tract of results of RT-PCR in aborted camels as verified and reported by Al-Majali et al.[40]

Numerous researches were published on the detection of *Brucella* by PCR, both from pure culture and from field samples mostly of cattle origin.[41-44] Diagnostic techniques are important for control and extinction of brucellosis; isolation provides the specific diagnosis and it is considered to be the gold standard method.[45] The demerits of the culture technique is that it is tedious, hazardous, and lacks sensitivity and specificity. The serological tests are the tools used for detection of *Brucella* infection.[46] The RT-PCR assay is a valued diagnostic tool when culture fails or serological results are indecisive in brucellosis detection[47] Intermediated screening must be done.

The complications and death due to infection cause economic and social damage. It is a main public threat, in developing and low income countries due to human and camel relations. This disease spreads to the local community via the habit of consuming milk and meat and there is a lack of awareness regarding brucellosis among nomadic people, which reduces the rate of reproduction by causing fetal loss in pregnant women, abortion in camel, still births, infertility, and swollen testes. This affects the sale value of camels, marketing of milk, meat and wool. Since camel racing is the main entertainment of the Middle East, it will affect the economic status of the countries. Therefore the uncontrolled spread of disease affecting animals, humans and environment needs effective molecular diagnostic tools.

Conclusion

The present study was concluded by serological tests and cELISA. The blood samples of seropositive camels were subjected to the isolation technique, followed by the identification of *Brucella*. The presence of bacterial DNA was confirmed for *Brucella* species and subspecies by gold-standard tests like RT-PCR. The prevalence of *Brucella* in camels is higher in this region, and drastic steps must be taken to control the spread of disease from camel to camel as well as from camels to humans. Further studies are required to identify *Brucella* species by sequencing.

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Declarations

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Author contributions

Conceptualization, K.M. and M.R.; Methodology, K.M; Software, U.S.; Validation, S.S, S.M. and K.M.; Formal Analysis, K.M.; Investigation, S.M.; Resources, S.M.M.; Data Curation, U.S.; Writing – Original Draft Preparation, K.M.; Writing – Review & Editing, U.S.;

Visualization,S.M.M .; Supervision, M.R.; Project Administration, K.M.

Conflicts of interest
No conflicts of interest.

Data availability
All the data in this study is completely incorporated in the manuscript.

Ethics approval
Experiment has been approved by administration of Tharb camel Hospital.

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