The Pharma Innovation Journal 2021; SP-10(10): 81-86

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(10): 81-86

www.thepharmajournal.com
Received: 04-08-2021
Accepted: 06-09-2021

Neharika Saxena
Department of Veterinary Public Health, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Rajni Joshi
Department of Veterinary Public Health, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Salauddin Qureshi
Division of Biological Standardization, IVRI, Izatnagar, Bareilly, Uttar Pradesh, India

Corresponding Author
Neharika Saxena
Department of Veterinary Public Health, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

A comparison of rose Bengal plate test and polymerase chain reaction in diagnosis of brucellosis in humans in contact with camels

Neharika Saxena, Rajni Joshi and Salauddin Qureshi

Abstract
Camel is reared for its milk and meat and is used for transportation by nomadic and rural populations in Africa and Asia including India. Brucellosis is an important zoonotic disease. In camels, this is caused by *Brucella melitensis* and *Brucella abortus*, which are pathogenic to man. Brucellosis can be readily transmitted from camels to humans by raw milk or its products. A large number of cases of human Brucellosis due to consumption of raw camel milk and meat have been reported from various countries worldwide. We had earlier found the seroprevalence of Brucellosis in camels in and around Bikaner. Therefore, we wanted to assess the public health significance of camel Brucellosis by testing humans in contact with camels.

Blood samples from 188 humans (109 males and 79 females) from Bikaner and nearby villages were analyzed by Rose Bengal Plate Test (RBPT). Out of 188 sera tested, 17 (4 females and 13 males) were positive by RBPT. However, none of the 26 humans samples (17 positive and 9 negative by RBPT) tested was *Brucella* positive by Polymerase Chain Reaction (PCR). This suggests that Brucellosis is prevalent in humans in this region in terms of antibody detection by serological methods. However, detection of DNA of *Brucella* organisms in blood by PCR may not be advised for regular screening for Brucellosis since there is intermittent bacteremia in Brucellosis and *Brucella* DNA may not be detectable continuously throughout the course of the disease. The OIE has approved RBPT, but not PCR for screening of Brucellosis.

Keywords: *Brucella*, brucellosis, camel brucellosis, human brucellosis, RBPT, PCR

Introduction
Brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella* that affects several different species of domestic animals and man. It is spread across the Mediterranean region of Europe, the Middle East, North and East Africa, Central and South Asia, and Central and South America [1]. Animals that are mostly infected by this bacterium include cattle, goats, sheep, dogs, pigs and camels among others. Humans act as an accidental host to this disease while animals are its natural reservoirs [2]. *Brucella abortus* and *B. melitensis* affect both camels and humans.

Brucellosis is transmitted to people by coming in contact with animals that are infected or their contaminated products. *Brucella* can gain entry into the body through many routes. For example, the digestive tract, intact skin, mucous membranes and via lungs. *Brucellae* are facultative intracellular coccobacilli that have a predilection towards placental trophoblasts and for the rough endoplasmic reticulum [3]. It displays a predilection towards reticulo-endothelial tissues like spleen, liver and blood vascular system once inside the blood [4].

Brucellosis has emerged as a problem of serious public health concern worldwide [5]. It has also been listed as an occupational disease. The people at risk include Veterinarians, animal handlers, meat-packing plant employees, slaughter house workers and lab workers [6]. In man, this disease manifests various symptoms like undulating fever, profuse sweating, headache, anorexia, joint pain and spondylitis [7]. In animals, it leads to abortion, stillbirth, epididymitis, orchitis and placentitis [8]. Infection is transmitted to humans from infected animals mainly by direct or indirect contact with animals, and consumption of unpasteurized milk, contaminated meat and milk products.

Bikaner is a city in the northwest of the state of Rajasthan, India. It is located 330 kilometres northwest of the state capital, Jaipur. Bikaner city is the administrative headquarters of Bikaner district and Bikaner division. It's located on Latitude 28.027138 and Longitude 73.302155. Bikaner has a population of about 650,000 people.

The present study was undertaken to determine the status of Brucellosis in humans in Bikaner district and some adjoining villages of Rajasthan state of India employing the common serodiagnostic method – Rose Bengal Plate Test (RBPT). Whole blood DNA of the humans positive by RBPT along with some negative samples were also analyzed by multiplex PCR employing Bruce Ladder.

Materials and Methods

Blood samples

The guidelines of Institutional Ethics Committee of RAJUVAS, Bikaner were followed throughout the study. Blood samples were collected from humans in contact with camels from Bikaner city and some nearby villages. Sera from 188 humans were included in the serological study. Of these, 109 were males and 79 females. These included animal owners, veterinarian and laboratory staff. The samples were collected from Bikaner city and nearby villages. All the serum samples were subjected to analysis with RBPT. DNA samples from whole blood of 26 humans (17 RBPT positive and 9 negative) were subjected to PCR analysis.

Rose Bengal Plate Test (RBPT)

Equal volumes (10 µl each) of RBPT coloured antigen (Punjab Veterinary Vaccine Institute, Ludhiana) and test serum were mixed on a clean glass slide [9] with the help of a sterilized toothpick. The slide was observed for 4 min. Formation of clumps was considered a positive test while the absence of clear clumps was considered a negative reaction.

PCR Assay

Bruce Ladder PCR was employed to amplify the *Brucella* DNA from whole blood samples of humans for molecular diagnosis of Brucellosis.

0.5x TBE Working Buffer: 10x TBE: 5ml; DW: To make the volume up to 100ml.

1.5 g of agarose was dissolved in 100 ml of working buffer (0.5x TBE) and RedSafe (InfoBio) was added @10 µl per 100ml of 0.5 TBE buffer.

Lysis Buffer: 100mM KCl, 20mM TrisHCl (pH8.3), 5mM $MgCl_2$, 0.2mg of gelatin per ml and 0.9% polysorbate 20.

Genomic DNA was extracted from whole blood samples of RBPT positive cases by using Genomic DNA isolation kit (Qaigen). 100 µl whole blood sample was mixed with 100 µl lysis buffer. Proteinase K was added to a final concentration of 60 µg/ml and the mixture was incubated for 60 mins at 55 °C. Proteinase K was inactivated by heating the mixture to 95 °C for 10 min followed by centrifugation at 12000 ×g for 10 min at 4 °C. The supernatant was collected in a fresh centrifuge tube to which 0.1 volume of sodium acetate (3M) and 0.6 volume of isopropanol were added. The contents were mixed gently and kept on ice for 1 hour and then centrifuged at 8000 g for 10 min. the pellet was washed with 70% alcohol twice and dried at 37 °C. Finally the pellet was suspended in 20-40 µl of Tris EDTA buffer and stored at -20 °C till further use.

Bruce-ladder identification was based on the numbers and sizes of six products amplified by PCR[10]. Six primers were used with PCR master mix (TAKARA) in 25 µl reaction (Table 1). Each primer (forward and reverse) was used at the rate of 10 picomoles per reaction. The composition of reaction mixture was as per Table 2. The PCR reactions were carried out in a Thermal Cycler (BioRad) with the cycling conditions given in Table 3.

Agarose gels premixed with RedSafe examined under UV rays using the Gel Documentation System to visualize the amplified products. The amplicon size and concentrations were determined by comparing with the ladder which was run with the samples.

Table 1: Oligonucleotides used in Bruce-ladder multiplex PCR assay

Primers	Sequence (5'–3')	Amplicon size (bp)	Gene	Reference
BMEI0535f	GCG CAT TCT TCG GTT ATGAA	450	bp26	Lopez-Goni *et al*., 2008
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA			
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1071	omp31	
BMEII0844r	GCG TCC AGT TGT TGT TGA TG			
BMEI1436f	ACG CAG ACG ACC TTC GGT AT	794	Polysaccharide deacetylase	
BMEI1435r	TTT ATC CAT CGC CCT GTC AC			
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587	eryC	
BMEII0428r	AAT GAC TTC ACG GTC GTT CG			
BR0953f	GGA ACA CTA CGC CAC CTT GT	272	ABC transporter binding protein	
BR0953r	GAT GGA GCA AAC GCT GAA G			
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218	rpsL	
BMEI0752r	GAT GTG GTA ACG CAC ACC AA			

Table 2: Composition of the PCR reaction mixture

Component	Volume / reaction (µL)
Master Mix (Takara)	12.5
Forward Primer (10 pMoles)	0.75
Reverse Primer (10 pMoles)	0.75
DNA sample	10
Distilled water	1
Total	25

Table 3: PCR cycling conditions

Stages	Steps	Temperature (ºC)	Duration	No. of cycles
I	Initial Denaturation	95	7 min	1
II	Denaturation	95	35 sec	25
	Annealing	64	45 sec	
	Extension	72	3 min	
III	Final Extension	72	6 min	1

Analysis of PCR products

Agarose gels were examined under UV rays using the Gel Documentation System to visualize the amplified products. The amplicon size and concentrations were determined by comparing with the molecular ladder which was run along with the samples. PCR amplified products were visualized using Agarose Gel Electrophoresis. Agarose gel (1%) was prepared using 1x Tris Acetate EDTA (TAE). Addition of Ethidium bromide (Genei™) was done to agarose at a concentration of 5µg/100ml after cooling it to 56^0C. TAE (1x) was used as casting and running buffer. 3 µl of amplified DNA was loaded using 1.5 µl of 100 bp ladder (GeneRuler 100 bp DNA Ladder-0.1 µg/µl, Thermo Scientific) was used as marker. Mini gel electrophoresis assembly (Midi submarine gel system, Genexy scientific Pvt. Ltd.), using power supply for 80 minutes was used to carry out Electrophoresis at 6.5 V/cm of gel in 1x TAE running buffer for 80 minutes. The amplified product was detected with the help of ethidium bromide in agarose gel electrophoresis. The UV transilluminator (Benchtop UV Transilluminator-BioDoc-It Imaging System, UVP) was used to visualize and automatically photograph the bands which were stored for further use.

Results and Discussion

Analysis of human sera by RBPT

A total of 17 human samples were positive and 171 samples were negative by RBPT (Fig. 1, Table 4). In our present study, prevalence in humans by RBPT was found to be 9.04%. Among the positive humans, 76.5% were males and 23.5% females, respectively.

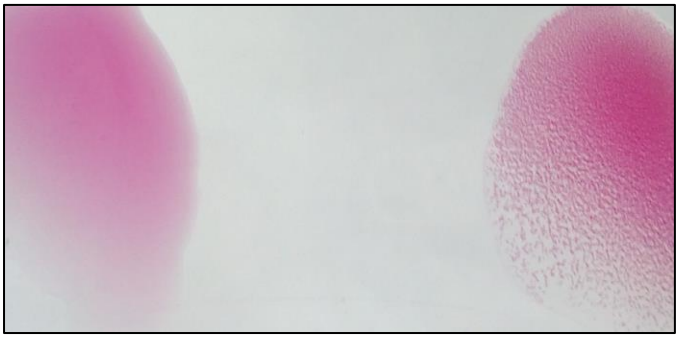

Fig 1: RBPT testing of sera for Brucellosis Left: Brucellosis negative serum; Right: Brucellosis positive serum

The seroprevalence of brucellosis was estimated[11] in 1085 veterinary professionals (366 veterinary officers and 719 paraveterinary personnel) engaged in animal husbandry practices in ten districts of Rajasthan State (India), the sera were screened by Rose Bengal test (RBT) and then positive samples were subjected to tube agglutination test (TAT) for antibody titration. An overall 3.68% seroprevalence was recorded in veterinary professionals with 3.00% in veterinary officers and 4.03% in paraveterinary personnel.

There was an outbreak of polyarthritis among 48 people in Kanvari village in Churu district of Rajasthan. Out of these, 91.6% of the people were diagnosed positive for Brucellosis [12]. A study [13] was conducted in Bikaner and 175 people (of which 155 were from villages) were tested for Brucellosis. Of the infected, two were veterinarians. Amongst the risk factors elucidated, the history of ingestion of raw milk ranked the highest (86.86%) and animal contact, whether occupational (62.28%) or household (16%), ranked behind.

A study [14] was conducted in western Rajasthan in which 350 susceptible people (Veterinarians, slaughter house workers and milk vendors) were tested with Stained Febrile Antigen test and ELISA and their clinical history was recorded. It was found that 42% of meat handlers, 28% of veterinarians and milkmen with 13% of Pyrexia of Unknown Origin (PUO) cases and 4% of normal healthy people tested positive for Brucellosis. They opined that people in animal contact are much more at risk of contracting Brucellosis than their counterparts who are not. It was reported [15] that 8.5 per cent of the employees of an organized dairy farm at Karnal had *Brucella* agglutinin with a titre of 80IU and above. In Pune 611 serum samples received for VDRL and 46 serum samples for Widal, were tested for the presence of *Brucella* agglutinins [16]. They found *Brucella* agglutinins in 133 (21.8%) of the sera received for VDRL and 19 (3.1%) of the sera received for Widal.

In a study conducted in India[17] over a period of 5 years, a total of 3,532 patients of pyrexia of unknown origin were subjected to Wright's tube agglutination test for brucellosis. Out of the 3,532 patients tested, 28 (0.8%) were found seropositive for brucellosis. Males outnumbered females by a ratio of 3:1. In a study conducted in Uttaranchal[18] a total of 352 human serum samples were screened for brucellosis and found a prevalence rate of 4.97 per cent in samples that included specimens from persons occupationally exposed to animals. In a study from Kerala [19] the seropositivity for brucellosis was reported as 1.6 per cent. A prevalence of 2.45 per cent was observed among the general population and 1.14 per cent among the veterinary students. A prevalence of 17.39 per cent was recorded among field veterinarians.

Our study has yielded data that shows the frequency is much higher than the national country level figures, comparable to few states like Kerala, Uttaranchal and Haryana but far less than the earlier studies conducted in Rajasthan. It may indicate that the prevalence of Brucellosis has a gradually declining trend. In a study entitled, "Brucellosis in high risk group individuals" 618 individuals were tested [20]. The disease prevalence was at 30.92 per cent in veterinary assistants, 41.23 % in veterinary inspectors, 12.37% in veterinary officers, 6.18% in veterinary supervisors, 6.18 % in group D workers, 2.06 per cent in shepherds and 1.03 per cent in butchers, respectively.

The findings of our present study suggest that Brucellosis is a problem of concern in humans who are in contact with Brucellosis affected camels in and around Bikaner district of Rajasthan state of India. Based on serological analyses of 188 human serum samples obtained from different individuals, it

has transpired that camel brucellosis is a problem of serious public health concern in and around Bikaner district of Rajasthan state of India. This is important because this disease is zoonotic and currently there is no vaccine or cure for humans Brucellosis.

Table 4: Human sera positive by RBPT

S. n.	Case no.	Age (yrs)	Sex	RBPT
1	HB1	30	M	+
2	HB5	30	M	+
3	HB8	5	M	+
4	HB11	6	M	+
5	HB57	34	F	+
6	HB72	25	F	+
7	HB73	9.5	M	+
8	HB82	3	F	+
9	HB83	1	M	+
10	HB84	1.5	M	+
11	HW4	27	M	+
12	HO11	23	F	+
13	HO15	22	M	+
14	HO21	29	M	+
15	HO24	17	M	+
16	HO27	35	M	+
17	HO29	35	M	+

PCR Analysis of human whole blood DNA samples

PCR using DNA from *B. abortus* strains (positive controls) amplified five fragments of 1682, 794, 587, 450, and 152 bp in size; with *B. melitensis* DNA, an additional 1071 bp fragment was amplified. However, none of the 26 samples from RBPT positive or negative humans analyzed was positive for *Brucella* by the Bruce Ladder PCR in our study (Table 5, Figs. 2 & 3).

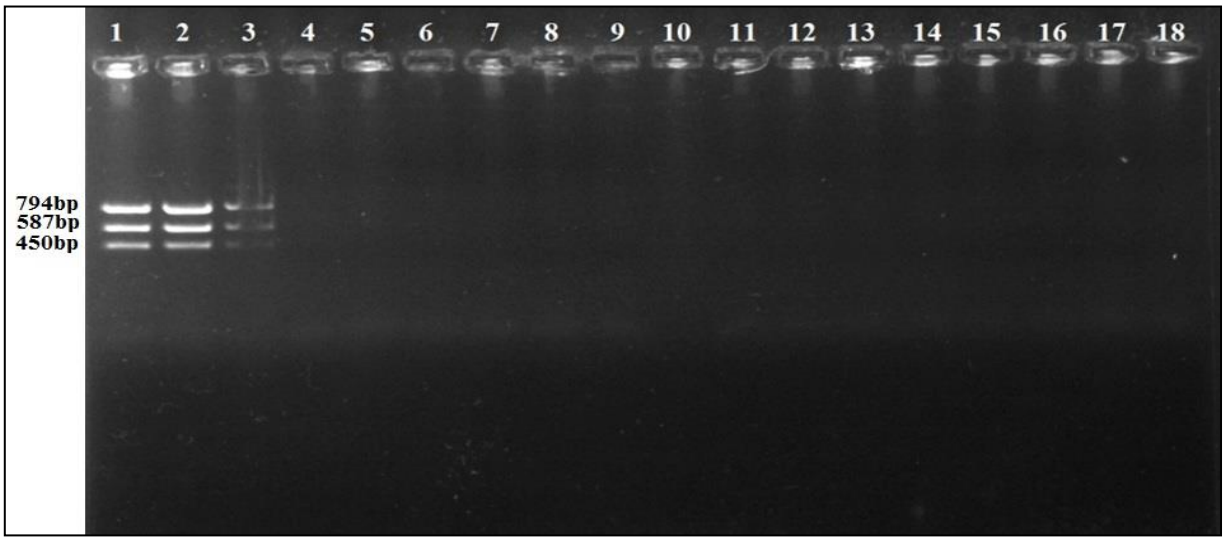

Fig 2: Bruce ladder PCR of human whole blood DNA samples (1 to 13). Lanes 1, 2 & 3 contain positive controls.

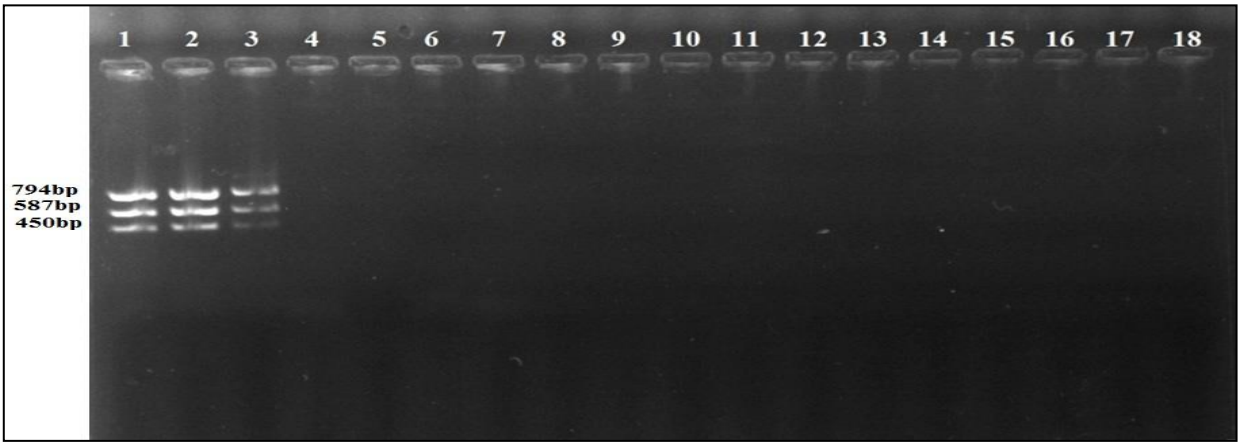

Fig 3: Bruce ladder PCR of human whole blood DNA samples (14 to 26) Lanes 1, 2 & 3 contain positive controls.

Table 5: Human blood samples analyzed by PCR

S. no.	Sample no.	Age (years)	Sex	RBPT	PCR	Location
1	HB75	30	F	Negative	Negative	Bikaner
2	HB17	10	M	Negative	Negative	Bikaner
3	HB84	1.5	M	Positive	Negative	Bikaner
4	HB83	1	M	Positive	Negative	Bikaner
5	HB1	30	M	Positive	Negative	Bikaner
6	HB11	6	M	Positive	Negative	Bikaner
7	HB26	20	M	Negative	Negative	Bikaner
8	HB5	30	M	Positive	Negative	Bikaner
9	HB8	5	M	Positive	Negative	Bikaner
10	HB74	44	M	Negative	Negative	Bikaner
11	HB15	10	M	Negative	Negative	Bikaner
12	HB16	10	M	Negative	Negative	Bikaner
13	HB24	10	M	Negative	Negative	Bikaner
14	HB82	3	F	Positive	Negative	Bikaner
15	HB57	34	F	Positive	Negative	Bikaner
16	HB73	9.5	M	Positive	Negative	Bikaner
17	HB19	25	M	Negative	Negative	Bikaner
18	HB72	25	F	Positive	Negative	Bikaner
19	HB22	10	M	Negative	Negative	Bikaner
26	HW4	27	M	Positive	Negative	Gadwala
20	HO21	29	M	Positive	Negative	Gadola
21	HO24	17	M	Positive	Negative	Gadola
22	HO29	35	M	Positive	Negative	Gadola
23	HO27	35	M	Positive	Negative	Gadola
24	HO11	23	F	Positive	Negative	Gadola
25	HO15	22	M	Positive	Negative	Gadola

In our present study, none of the whole blood DNA samples of 25 camels and 26 humans tested were positive by PCR. A possible reason of seropositivity of cases negative by PCR in our present study could be elimination of the pathogen from the body by the immune system. The antibody is known to persist in blood long after the removal of the pathogen from the body. Alternatively, the initial bacterial load after infection may be just enough to induce antibody response by antigenic stimulation but not adequate enough to cause a full blown disease along with detectable bacteremia. Thus, the quantum of bacterial load and the magnitude of individual immune response may be the factors responsible for incoherence between the results of serological and molecular assays.

A study [21] was carried out on 200 samples from cattle suspected of Brucellosis to ascertain the effectiveness and suitability of PCR and commercially available ELISA kits. In PCR, only 3 out the 200 sample showed a band of size 905bp, typical of *Brucella* species. Other samples failed to show positive reaction by PCR. In comparison, ELISA could detect 75 out of the 200 samples as positive. They concluded that ELISA is a better confirmatory test than PCR for screening animals for Brucellosis.

In Brucellosis, bacteremia is not persistent throughout the course of the disease. There are waves of bacteremia alternated by phases of latency when the microorganisms hide inside the phagocytes in the reticuloendothelial system. This is manifested in the form of intermittent fever. If blood is drawn from the infected individual during the latent phase, it may not contain bacteria and hence no microbial DNA can be detected in this sample by molecular techniques even if the microorganisms are present in the body. On the other hand, a single antigenic exposure of the immune system may induce detectable antibodies against the pathogen for a considerable period of time.

PCR is a very sensitive technique [22]. It does not cross react with closely reactive bacteria except *Orchobacterium anthropi*, the closest known relative of *Brucella* [23]. An evaluation of Bruce-ladder multiplex PCR assay was performed in seven laboratories using 625 Brucella strains from different animal and geographical origins. This test could differentiate in a single step all of the classical Brucella species, including the S19, RB51, and Rev.1 vaccine strains. Although this PCR assay cannot differentiate among biovars from the same species, Bruce-ladder has been found to be species specific and all the strains and biovars from the same *Brucella* species have been shown to give the same profile. A major advantage of the Bruce-ladder PCR assay over other multiplex PCR tests is that it can identify and differentiate all of the Brucella species and the vaccine strains in the same test. Bruce-ladder PCR can be a useful tool for the rapid identification of *Brucella* strains of animal or human origin (Lopez-Goni *et al*., 2008).

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