Prevalence Of Human Brucellosis Among High Risk, Symptomatic Cases In Anand District Of Gujarat-India

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Abstracts: Background and Objectives**:** Brucellosis is a bacterial zoonosis causing high economic losses worldwide. Because of nonspecific and chronic symptoms, diagnosis requires high clinical suspicion and laboratory confirmation. Prevalence of brucellosis in India, more so in Anand district, is not well studied. This study was conducted as a pilot to find prevalence of human brucellosis among high risk symptomatic cases along with the clinical profile and associated risk factors. Methodology**:** This is a cross-sectional descriptive study conducted in Anand district of Gujarat, India. Samples from 50 high risk symptomatic cases were subjected to serology, blood culture and genome detection by conventional Polymerase Chain Reaction (PCR) using B4/B5 primers, for detection of brucella infection. Data was analyzed using Epi Info software (version: 3.5.1). Results**:** We detected sero-prevalence of 14% and genome detection of 16% with no isolation in blood culture. Among them four (8%) were positive by serology and PCR both. Titers in seropositive cases ranged from 1:40 to 1:160. Fever was the most common clinical complain among sero-positive (71.4%) and PCR positive (62.5%) cases. History of direct contact with cattle was present in all serology and PCR positive patients. Consumption of unpasteurized milk or milk products (86%), consumption of uncooked or partially cooked meat (28.5%) and working as veterinarian (14.3%) were other risk factors identified. Conclusions**:** Thus we found brucellosis to be a significant health problem. A larger Indian study with follow up of positive cases, comparative study for standardization of various diagnostic modalities and response to therapy is strongly needed. [Singh S NJIRM 2015; 6(5):22-27]

Key Words: human brucellosis, high risk individuals, prevalence.

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Introduction: Human brucellosis is world's most widespread bacterial zoonosis with multisystem involvement that may present with a broad spectrum of clinical manifestations[1]. It is caused by Brucella spp., a facultative intracellular bacteria, capable of evading host defense mechanisms. Out of six classical species, three i.e Brucella melitensis, Brucella abortus, Brucella suis have been the common cause of human disease[1,2,3]. Although rarely life-threatening, it can be a severely debilitating disease with its resultant impact on the economy. [1,2]

The epidemiology of brucellosis is complex and it changes from time to time. Worldwide, reported incidence of human brucellosis in disease endemic areas varies widely, from <0.01 to >200 per 100,000 population.[4,5] In a recent systematic review to assess global burden of human brucellosis, the rate have ranged from 0.02-0.08 in USA to 52.29- 268.8 cases per 100,000 person years in semi-rural areas of Iraq.[6] The true incidence of human brucellosis however, is unknown for most countries including India, which may be 25 times higher than the reported incidence due to misdiagnosis and under-reporting.[3,4,7] The problem is compounded by the absence of national surveillance programs, diagnostic facilities and reliable data in endemic areas.[5] Indian subcontinent is endemic and in India, direct contact of man and animal is common, due to cattle rearing and occupational group like veterinarian and dairy worker along with consumption of unpasteurized milk or milk products [8].

Anand, the milk city of India, is likely to have significant burden of human brucellosis due extensive network of milk cooperative and cattle rearing population. Previous studies have shown highly variable but significant prevalence of Brucellosis in animals (8-40%) as well as in human beings (15%).[9]

The aim of this study is to find prevalence of human brucellosis among high risk symptomatic cases in Gujarat and the most suitable diagnostic test.

Material and Methods: This is a cross-sectional descriptive study duly approved by institutional Human Research Ethics Committee. The study was

conducted from 2011 to 2012 at a tertiary care, teaching hospital located in rural part of Gujarat.

Study population was randomly distributed in six locations in and around Anand district of Gujarat. Each area was visited by a team of three members (two investigators and one phlebotomist) and arranged meeting with the villagers to orient them about the work. Out of people who gathered, persons of any age or sex, who were having any symptoms suggestive of brucellosis (fever, chills, nocturnal sweating, headache, joint pain, back pain, body ache, difficulty in breathing, decreased appetite, fatigue or weakness or malaise, GIT disturbances, sleep disturbance) with presence of risk of having brucellosis (direct contact with animals, veterinarian, dairy worker, slaughter house workers, butcher, unpasteurized milk or milk product consumer, uncooked or partially cooked meat consumer) were included in this study after taking written consent. Individuals who satisfied the above criterion but had confirmed diagnosis of other illness were excluded from the study.

Complete history of all patients was taken in pre prepared proforma. Blood samples were collected using all aseptic precautions as per Standard Operative Procedure Manual. Culture samples were immediately transferred to Public health department, veterinary college and kept in CO_2 (10%) incubator at 37˚C. Plain tube were taken to the Microbiology laboratory, for Standard Tube Agglutination Test (SAT). The EDTA sample were kept at -20˚C until processed for PCR.

SAT was performed for detection of IgG antibodies against Brucella abortus and Brucella melitensis, using a commercially available serological kits (Tulip Diagnostics) Brucel-A, Brucel-M containing standardized, stained, smooth specific antigen of Brucella spp. Serum dilutions ranging from 1:20 to 1:1280 were tested. For blood culture biphasic media (himedia) was used and incubated for a maximum duration of 35 days. The subcultured plates were incubated at 37˚C (CO_2) for seven days for isolation of organism.

Standard control strain of Brucella abortus and Brucella melitensis (from Indian Veterinary Research Institute, Izatnagar, India) were used in both biphasic media and Brucella agar plates.

The DNA from the human blood sample was extracted by method described by Leal- Klevezas et al and a set of genus-specific primers for Brucella, B4 (Forward with 5 -- 3' sequence of TGGCTCGGTTGCCAATATCAA) /B5 (Reverse with 5 -- 3' sequence of CGCGCTTGCCTTTCAGGTCTG) with a product length of 223bp (synthesized by Eurofins Genomics India Pvt Ltd, Bangalore, India) was used for PCR amplification.[10,11]

PCR was performed in Applied Biosystems (ABI) GeneAmp Thermal Cycler 2720. PCR for each sample was repeated to find the reproducibility. Visualization of PCR products was performed by agarose gel electrophoresis. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (SynGene, Gene Genius BioImaging System, UK).

For genome detection by PCR standard Brucella abortus and Brucella mellitensis strains obtained from the Indian Veterinary Research Institute, Izatnagar, India were used as positive controls. Autoclaved, nuclease-free distilled water (MBI Fermentas) was run as a negative control during every batch of PCR reactions.

Data were recorded and counted using Microsoft Excel and analyzed using Epi Info software (version: 3.5.1).

Results: Distribution of 50 high risk symptomatic individuals from different locations of Anand district was variable. Overall seropositivity for Brucella antibody by SAT was 14% (seven out of 50) and Brucella species specific genome detection by PCR from blood samples was 16% (eight out of 50) with no positive blood culture.

Titres in seropositive cases ranged from 1:40 to 1:160. Table 1 shows distribution of titres and species distribution of Brucella spp.

Table 1: Distribution of antibody titres among seropositive patients (n=07)

Serology	Titre			Total
	1:40	1:80	1:160	
B. abortus	00	01	00 (0%)	01

	(0%)	(12.5%)		(12.5%)
B. melitensis	02 (25%)	04 (50%)	01 (12.5%)	07 (87.5%)
Total	02 (25%)	05 (62.5%)	01 (12.5%)	08* (100%)

*one patient was positive for both *B abortus* and *B. melitensis*

Male to female ratio in the study was 2:3. Among 20 male patients , serology and PCR positivity was two (10%) and one (5%) respectively and this was five (16.7%) and seven (23.3%) in 30 female patients. The difference was not statistically significant as corelation between gender with serology and PCR was not less than 0.05 (P = 0.8 and 0.18 respecticely)

Majority of patients i.e. 38 (76%), were between age group of 31 to 60 years. Among them highest number i.e. 15 (30%) were from the age group of 41 to 50 years. In table 2, age group wise distribution of seropositive and PCR positive results are mentioned.

Table 2: Distribution of serology and PCR positive patients according to age group (n=50)

Age group (In years)	Positive		Total no of patients in the age group
	Serology n(%)	PCR n(%)	
21-30	01(20%)	00(00%)	05
31-40	00(00%)	03(25%)	12
41-50	02(13.3%)	01(6.7%)	15
51-60	02(18.2%)	03(27.3%)	11
61-70	02(40%)	01(20%)	05
71-80	00(00%)	00(00%)	02
Total	7(14%)	8(16%)	50

Maximum serology and PCR positivity was found in the age group of 61-70 years i.e. 40% (two out of five) and 51-60 years i.e. 27.3% (three out of eight) respectively.
Fever was the main clinical presentation among seropositive (71.4%) and PCR positive (62.5%) patients. Nocturnal sweating, headache, backache and bodyache were also common presentation. Distribution of various clinical manifestations is shown in table 3

Table 3: Clinical distribution of patients among seropositive patients (n=07), PCR positive patients (n=08) and positive by both methods (n=04)

Symptoms	Serology Positive (%)	PCR Positive (%)	Positive by Both (%)
Fever	71.4	62.5	50
Chills	14.3	12.5	00
Nocturnal sweating	71.4	25	50
Headache	57	75	50
Joint pain	43	37.5	50
Back pain	57	75	75
Body ache	57	50	75
Difficulty in breathing	28.5	37.5	25
Decreased appetite	14.3	12.5	00
Fatigue	14.3	37.5	25
GIT disturbances	57	37.5	25
Sleep disturbance	28.5	37.5	25

History of direct contact with cattle was present in all serology and PCR positive patients. Other risk factor that were present were consumption of unpasteurized milk or milk products (86%), consumption of uncooked or partially cooked meat (28.5%) and veterinarian (14.3%) by profession.

Discussion: Anand district with its large population involved in dairy industry and cattle farming has a prevalence of 14-16% of human brucellosis as was found in previous and this study.[9] In a study by Sharma and Savalia in Gujarat, seroprevalence of 16.35% was found with 14.7% seropositivity in patients with pyrexia of unknown origin (PUO).[12] Earlier study conducted in Gujarat by Panjarathinam and Jhala, found 8.5% prevalence among human cases.[13] Various studies from other parts of India show seroprevalence ranging from 0.8% to 9.94% in patients with PUO.[13-17] Kadri et

al. have reported minimum sero-prevalence of 0.8% among PUO case from Kashmir.[17] Thus seroprevalence of human brucellosis varies widely in different region of India. Various factors like age, occupation, immunity, endemicity, prevalence among animals, duration of exposure, healthcare facilities available and suspicion among clinician may be responsible for wide difference in seroprevalence in India.[3,7]

Various investigators have used different titres for diagnosis of human brucellosis and it has been noticed that SAT titres of < 1:160 cannot always be disregarded without follow up.[1] Brucella antibody titre in our study ranged from 1:40 to 1:160. Only one case (12.5%) had titre more than 1:80 and rest seven cases (87.5%) had titre less than or equal to 1:80. These were similar to finding by Seyed Mohammad et al., who found 12.5% of seropositive individuals having titre higher then 1:80 and 82.5% had titre lower than 1:80.[18] Appannanavar et al., had also considered low titre of 1:80 significant in there seroprevalence study among PUO patients.[14] Low titre are commonly present in human cases of chronic brucellosis and can't be ignored.[4,7]

Other methods of antibody detection must be adopted as false negative and positive serology by SAT is possible due to prozone phenomenon, presence of incomplete antibodies and cross reacting antibodies to certain organisms.[19]

Culture is considered gold standard for human brucellosis, but shows low sensitivity (10 to 30%) depending upon stage of disease.[20,21] Alsayed and Monem et al. had found sensitivity of culture to be 57% in acute cases and only 13% in relapse or chronic cases.[21] In our study all seropositive symptomatic patients were considered to be chronic cases due to history for more than one year with no culture isolation.

Baddour and Alkhalifa, evaluated three primer pairs for detection of Brucella genus DNA in peripheral blood samples of confirmed brucellosis case.[11] Maximum cases were detected by using B4/B5 primer (98% sensitivity) followed by JPF/JPR primers (88.4% sensitivity) and F4/F2 primers (53.1% sensitivity) with 100% specificity. The limit of detection was significantly high in B4/B5 primers i.e. $7x10^2$ cfu/ml.[11] In our study we found eight (16%) patients positive for Brucella specific DNA by conventional PCR method by B4/B5 primers from blood samples.

None of our patients were positive by culture method while some were detected positive by either serology (n=7, 14%) or PCR (n=8, 16%) or both (n=4, 8%). Similar results were obtained by Elfaki et al., where three out of 25(12%) patients were negative by SAT and culture method but were positive by PCR. In the same study 48% patients were positive by SAT and PCR methods but negative by culture.[22]

Discrepancies among serology by SAT, culture and PCR have also been demonstrated by quantitative real time PCR (Q-PCR) in the diagnosis and follow up of patients. Casteno et al found 48.6% known cases of brucellosis positive by PCR but none was positive by blood culture.[23] Among the PCR positive patients, three were negative by both SAT and Coombs serological methods. Four patients were negative by SAT but showed significant titre by Coombs test.[23]

We found that 85.7% of seropositive high risk individuals were in age group of 31 to 70 years. Mukhtar et al. had also found 71% of seropositive patients among same age group. In most of studies seroprevalence and positive cases were found in adults' only.[24] In most of the studies, male has been found to be more affected by brucellosis than female, may be because of higher chances of occupational exposure. In contrast to this in our study male to female ratio was 2:5 & 1:7 among seropositive and PCR positive individuals respectively. But there was no statistical significance ($P > 0.05$) between gender and serology or PCR in our study.

Fever has been reported as the most common presentation seen in 78-100% cases.[1,3,7] Other manifestations like headache, back pain, anorexia, body ache, arthralgia, night sweats and fatigue are found in 40-80% cases.[1,4,14] In our study the common clinical presentation was fever (71.4%), while headache, back pain, anorexia, body-ache were present in 57%, 43%, 14.3%, 46% respectively.

Direct contact with animal, consumption of unpasteurized milk or milk products and raw or partially cooked meat are the proven risk factors for human brucellosis.[1-3,7] Occupational exposure among veterinarians, laboratory personals, and abattoir workers can also lead to human brucellosis. In our study all serology and PCR positive patients were having history of direct contact with cattle. Among seropositive patients one was veterinarian. All PCR positive and 86% of seropositive patients were having history of unpasteurized milk or milk product consumption. History of raw or partially cooked meat consumption was also present in 28.5% serology positive and 12.5% PCR positive patients.

Conclusion: To conclude we found human brucellosis as a public health problem with prevalence of 14% by serology and 16% by PCR among high risk symptomatic individuals of Anand district. But the results cannot be generalized keeping in mind the small sample size and lack of follow-up of positive cases. A case control study with follow up must be conducted along with comparative studies of different diagnostic tests like serology, culture and PCR to find out sensitivity and specificity in Indian population.

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