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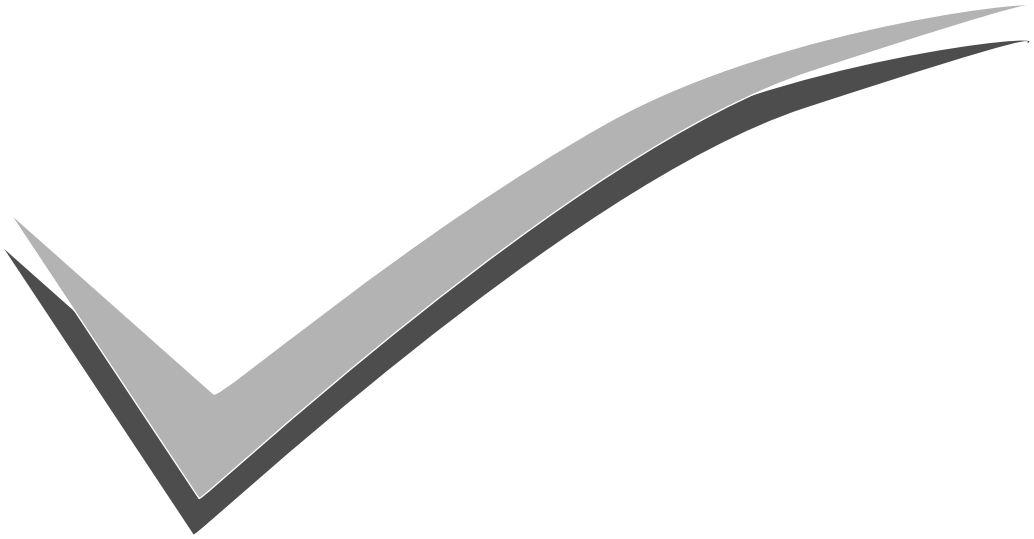

BRUCEL - A/M

Slide/tube test for detection of antibodies to Brucella Abortus/Melitensis

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Low incidence and the prevalence of brucellosis among patients with pyrexia of unknown origin based on real-time polymerase chain reaction, enzyme-linked immunosorbent assay and standard agglutination test results in Puducherry, South India

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Abstract

CONTEXT: Brucellosis is a zoonotic disease and an important differential to be considered in patients with pyrexia of unknown origin (PUO). The laboratory diagnosis of brucellosis has always been affected by various factors such as the slow growth of the organism and cross-reacting antibodies. Hence, a diagnostic test with high sensitivity and specificity is the key for the accurate and rapid diagnosis. **AIM:** The study aimed to evaluate the role of real-time polymerase chain reaction (PCR) in the rapid diagnosis of human brucellosis from direct serum samples of patients with PUO. **MATERIALS AND METHODS:** An observational study was conducted from October 2014 to September 2016 at a tertiary care hospital in Puducherry where 138 blood samples were obtained from the patients with PUO. Serum separated from each sample was tested for brucellosis using Cobas 480 Z real-time PCR system. Simultaneously, the samples were also subject to blood culture, standard agglutination test (SAT) and enzyme-linked immunosorbent assay (ELISA) (IgG and IgM) for brucellosis. **RESULTS:** All the samples were found to be negative for brucellosis using real-time PCR. Blood culture also did not yield any growth of *Brucella spp*. Among the serological tests, all the samples were negative by SAT, whereas two were positive for IgM and four for IgG anti-*Brucella* antibodies using ELISA. **CONCLUSION:** Brucellosis is not a common cause of PUO among patients attending this hospital since all the samples were negative by highly sensitive and specific tests such as real-time PCR and blood culture. This study highlights the limitations of serological tests such as ELISA in the accurate diagnosis of brucellosis.

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Full Text

Introduction

Brucellosis is usually a disease affecting animals and is an important health problem worldwide. It is highly endemic in most parts of the Mediterranean littoral, Arabian Peninsula, South America and Central Asia. The prevalence rate of human brucellosis in certain countries has been reported to be more than 10/100,000, but still the true prevalence in most of the countries is unknown due to deficiencies in the diagnosis and reporting.[1] Brucellosis is also an important health problem in India. As 80% of the Indian population live in the rural and suburban areas, the risk of acquiring this zoonotic infection is high due to close contact with animals. Brucellosis in humans is mostly occupational but can also be transmitted by the ingestion of infected milk and milk products. Infected individuals have diverse clinical features, the most common presentation being an acute febrile illness.[2] Various studies have also shown that the prevalence of brucellosis ranges between 0.8% and 6.8% among patients with PUO.[3],[4],[5] The diagnosis of brucellosis is often challenging because of diverse clinical presentations and mostly rely on the laboratory diagnosis. Routine microbiological diagnosis such as the isolation of bacteria in culture and serological tests has major limitations, and many polymerase chain reactions (PCRs)-based methods introduced recently have shown to overcome these limitations.[6]

Only a very few studies based on PCR are done on direct patient samples. This study intends to evaluate the role of real-time PCR in the rapid diagnosis of brucellosis along with other methodologies such as culture, standard agglutination test (SAT) and enzyme-linked immunosorbent assay (ELISA) on serum samples of patients with pyrexia of unknown origin (PUO).

Materials and Methods

The present study was conducted following approval from the Institutional Ethics Committee, and informed written consent was obtained from all the participants of the study. This is an observational study conducted at a tertiary care hospital in Puducherry which included 138 patients with PUO admitted in the hospital over a study period of two years from October 2014 to September 2016. Patients with definitive diagnostic evidence for other infectious etiologies such as malaria, filaria, typhoid, typhus, dengue, influenza, viral hepatitis and others were excluded from the study. The aim of the study was to assess the role of real-time PCR in the rapid diagnosis of human brucellosis from the direct serum samples of patients with PUO. The secondary objective was to compare the results of real-time PCR with other methodologies such as culture, SAT and ELISA. Sample collection – Under sterile aseptic precautions, 10–20 ml of venous blood was collected and one portion (10ml) was used for culture and the serum separated from the remaining sample was used for serological tests and real-time PCR. For real-time PCR, serum samples were stored at −20°C until further analysis. Blood culture – About 10 ml of venous blood was inoculated into the BACTEC bottles and loaded into the BACTEC 9120 system (BD). Samples negative after one week of incubation were further incubated at 37°C for one more week and blind subcultures were performed onto blood agar and MacConkey agar. SAT – Tube method of the SAT was performed following the manufacturer's instructions using a ready-made antigenic suspension, BRUCEL-A containing killed, stained and standardised smooth cell antigens of Brucella abortus procured from Tulip diagnostics private limited, Goa, India. ELISA – separate ELISA kits detecting IgM antibodies and IgG antibodies against Brucella antigens were procured from NovaTec Immunodiagnostica GmbH, Germany. The principle and procedure of both the kits were essentially the same. The test was performed according to the manufacturer's instructions. Real-time PCR – For the detection of Brucella spp., we targeted a 207 bp fragment conserved region of bcsp 31 gene, encoding a 31 kDa immunogenic membrane protein of B. abortus. This region is specific to the Brucella genus and is present in all biovars. DNA was extracted from the serum using high-pure PCR template preparation kit supplied by Roche diagnostics GmbH, Germany. All steps of the DNA extraction were performed according to the manufacturer's instructions. This TaqMan probe-based assay was done using primers and probes procured from TIB MOLBIOL, Berlin, Germany. The primers used for amplification were as per Queipo-Ortuno et al. as follows:[7] Forward primer (5“-3”) – GGCTCGGTTGCCAATATCAAT, Reverse primer – (5“-3”) GTCTGCGACCGATTTGATGT and Probe (5“FAM-3”BBQ) ATCAAG TCGGGCGCTCTGGAGT. Real-time PCR was performed using the Cobas 480 Z System, Roche Molecular Diagnostics, Germany. Each 20 µl reaction volume included 10 µl of 2x master mix and 2 µl of template DNA. Primers and probe (8 µl) were used at a final concentration of 0.6 µm and 0.2 µm, respectively.

Kit provided positive and negative controls as well as internal controls were used for all the diagnostic tests. For real-time PCR, the bcsp 31 gene from B. abortus S19 strain was used as internal positive control.

Results

A total of 138 patients with PUO were included in this study. The majority of the participants belonged to 21–30 years' (21.7%) group and 41–50 years' (21.7%) group. The mean age was 33 years, and the male-to-female ratio was 1.3: 1. Most of them (39.1%) were farmers by occupation [Graph 1]. While most of the patients (81.9%) did not have any identifiable risk factors, few (18.1%) had at least one identifiable risk factor for brucellosis [Table 1]. SAT: at a diagnostic titre of 1 in 160 dilutions, all the samples were negative for brucellosis by SAT. ELISA: totally six samples were positive for anti-Brucella antibodies by ELISA. IgM anti-Brucella

antibodies were positive in two patients and IgG anti-Brucella antibodies were positive in four patients and none were positive for both. Both the patients positive by IgM ELISA were male and had no identifiable risk factors for brucellosis. Among the four patients who were positive for IgG anti-Brucella antibodies, three were male, one was female and two were farmers by occupation. Blood culture – blood culture using the BACTEC 9120 system did not detect any growth even after two weeks of incubation. Real-time PCR – all the serum samples were found to be negative for bcsp 31.[INLINE:1] {Table 1}

One patient tested positive by IgG ELISA and also had an antibody titre of 1 in 80 dilutions by SAT. He was a farmer rearing cattle for more than 25 years and a repeat sample from the patient did not show any rise in antibody titres. The results of the various diagnostic tests are depicted in [Graph 2].[INLINE:2]

Discussion

Blood culture of all the 138 samples did not yield any growth of Brucella even after employing an extended incubation period of two weeks duration. Although previous studies have shown that the overall sensitivity of blood culture varies hugely between 53% and 90%, few studies have shown a superior sensitivity of around 95% for automated detection methods such as BACTEC 9000 series.[8]

At a diagnostic titre of 1 in 160 dilutions, none of the samples were positive for brucellosis by SAT. Studies have shown a sensitivity of 95.6% and specificity of 100.0% for SAT in culture-positive patients.[9] The fact that SAT is a good sensitive test for brucellosis is also highlighted in many other studies.[10],[11]

In this study, two patients were positive by IgM ELISA and four patients were positive by IgG ELISA. None of the patients were positive by both IgG and IgM ELISA. Patients positive by ELISA were negative by other tests such as the SAT, blood culture and real time-PCR. One patient had a titre of 1 in 80 dilutions by SAT who was also positive for anti-Brucella IgG antibodies by ELISA, but blood culture and real-time PCR were negative. The patient was a farmer who was raising cattle for more than 25 years. A repeat sample from the patient, however, did not show any rise in antibody titre in SAT. Repeated subclinical exposures resulting in seroconversion could be a possibility in this patient. The sensitivity and specificity of ELISA have been reported variably in different studies. While few studies show good sensitivity and specificity for both IgM and IgG ELISA (95% and 100% for IgM ELISA and 99% and 84% for IgG ELISA, respectively), few other studies show conflicting results unfavouring ELISA to be a superior test than SAT.[12],[13] Moreover, CDC's Council of State and Territorial Epidemiologists has stated that the case definition for laboratory diagnosis for Brucella does not include non-agglutination based tests such as ELISA as it was associated with false-positive reporting of brucellosis.[14]

All 138 samples were also negative for Brucella species in this study. Previous studies have already established that PCR is a very sensitive test for the diagnosis of brucellosis and even very low levels of DNA can be detected.[15],[16] A high sensitivity and specificity varying between 88%–93.5% and 98.4%–100%, respectively, have also been described for Taqman-based real time-PCR assays for brucellosis.[7],[17]

Conclusion

The objective to establish real-time PCR as a rapid diagnostic tool for the diagnosis of brucellosis from the serum sample of patients could not be achieved since all the samples tested were negative for brucellosis by real-time PCR. Nevertheless, the negative results in real-time PCR along with negative results in specific tests such as blood culture suggest that brucellosis is not a common cause of PUO among patients attending this hospital. As the positive results in ELISA did not correlate with the results of blood culture and real-time PCR, it can also be emphasised that the treatment of brucellosis based on the results of serological tests alone should be discouraged and a combination of tests has to be done.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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ORIGINAL ARTICLE ASIAN JOURNAL OF MEDICAL SCIENCES

Seroprevalence of brucellosis among the high-risk population in Ujjain district, Madhya Pradesh

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ABSTRACT

Background: Brucellosis is the most common zoonotic disease caused by Gram-negative coccobacillus belonging to genus Brucella. It is a recognized public health problem in developing countries including India. **Aims and Objectives:** The aims of these study were to determine the seroprevalance of brucellosis in population having occupation dealing with animals and thus are in close contact of animals. **Materials and Methods:** The study was conducted in semi-urban areas of central India. Blood samples were collected from personnel working in slaughter houses, meat shops, and veterinarians and their close contact and who are willing to participate in study. A total 102 samples collected randomly from butchers (n = 20), veterinarians (n = 29), and animal handlers (n = 53) and were tested for *Brucella abortus* and *Brucella melitensis* by a commercial kit which allows the detection of both complete (IgG and IGM) and incomplete antibodies. **Results:** A total 102 subjects were included in the study and overall prevalence of brucellosis among high-risk group was found to be 2.9%. One veterinarian doctor was also found positive for both *B. melitensis* and *B. abortus*. Highest prevalence of brucellosis was found in veterinarians (6.8%) followed by animal handlers (1.8%), and none of the butcher was tested positive for any of the *Brucella* antibody. **Conclusion:** The present study screened all possible known high-risk groups for brucellosis and revealed that veterinarians have high chances of getting the infection. Occupation-related disease like brucellosis needs regular surveillance and integration into control and prevention program at a local and national level.

Key words: Brucellosis; High risk; Prevalence; India

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INTRODUCTION

Brucellosis is the most common zoonotic disease caused by Gram-negative Coccobacillus belonging to genus *Brucella* that leads to considerable morbidity and loss of man-day across the globe and thus perpetuates poverty. It is a recognized public health problem in low- and middle-income countries including India.[1] A high prevalence in certain geographic areas is well recognized, although largely underestimated.[1] More than 500,000 new cases are reported each year, and according to the World Health Organization, this figure underestimates the magnitude of the problem.[2]

Epidemiological evidence shows that in India, brucellosis is present in different species in farm animals including cattle, goat, camel, horses, and pigs.[3] *Brucella melitensis* and *Brucella abortus* are of major concern in India. Brucellosis is almost invariably transmitted to men from infected

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domestic animals. Milk and milk products are common sources of infection. The raw milk, clotted cream, and unevenly heated milk can harbor live *Brucella* organisms.[4] Seropositivity among animal handlers, veterinarians, and dairy workers has also been documented from India.[4-7] Due to the deceptive nature of clinical signs and symptoms of the disease, it may be easily misdiagnosed or diagnosed as pyrexia of unknown reason. The infection may remain subclinical or in latent form among the high-risk persons, thereby underestimating the true incidence of brucellosis. In spite of high prevalence of animal brucellosis, the human disease has not been much studied.

The laboratory diagnosis of Brucellosis is again challenging as the culture sensitivity is very poor especially in chronic cases. The IgG antibodies in chronic disease can be detected by the 2 Mercaptoethanol (2ME) test and the Comb's test. STAT titers of 1:160 and above are diagnostic, but the cutoff limit of antibody titer should be considered according to the herd immunity of the community and a local base line cutoff titer should be determined and addressed. Furthermore, immunocapture agglutination assay can detect the IgG antibodies with higher sensitivity and specificity.[8]

Ujjain has a population of 6.18 million with semi-urban and rural population, most of which is prone to handle animals for various reasons. We have studied the seroprevalence of brucellosis among the high-risk population as butchers, veterinarians, and animal handlers, and their contacts and to correlate the seroprevalance with the sociodemographic information of the study subjects.

Aims and objectives

The aim of these study to determine the seroprevalance of brucellosis in population having occupation dealing with animals and thus are in close contact of animals.

MATERIALS AND METHODS

The study was approved (no. 50/2016) by ethical committee of R. D. Gardi Medical College, Ujjain. The written informed consent was taken from the participants by visiting the butcher houses and their shops, veterinarians, and animal handlers.

The study was conducted in rural areas of Ujjain, Madhya Pradesh. Personnel working in slaughter houses, meat shops, and veterinarians and their close contact and who are willing to participate in study were contacted and interview for their sociodemographic characteristics and information related to risk of acquiring *Brucella* infection. The data were collected in the predesigned pro forma. Participants were simultaneously educated regarding the risk of transmission and measures to prevent brucellosis. Persons who were not involved in animal handling and no potential risk for brucellosis as evident from history, were excluded from the study.

5 mL of blood was collected with all sterile precautions in plain and EDTA vials using vacutainers. Blood was properly labeled and transported to the Central Clinical laboratory of RD Gardi Medical College in a cold box within 12 h of collection. After separating serum by centrifuging at 6000 g for 15 min, it was kept in refrigerator on (2–8°C) until tested. The serum samples were tested using commercially available kit for *B. abortus (Brucella* CAPTVircell Span supply by Merill diagnostics, India) which works on principle of single step immunocapture assay to detect both complete and incomplete antibodies. Simultaneously, the serum was also tested for *B. melitensis* (BRUCEL A/M, Tulip Diagnostics India) a tube agglutination test to detect total, IgG, and IGM antibodies. The serum agglutination test and microtiter plate test use phenol-killed whole S-cells to detects antibodies against S-LPS antigen of *Brucella*. Seropositive participants were referred to CR Gardi Hospital associated with R.D. Gardi Medical College for further clinical evaluation and management.

All statical analysis was done by SPSS software version 23 for presentation of different variable applied and describe by Chi-square test, it was divided in seronegative and seropositive group for all risk factors. Then, titer of $\geq$ 1:160 was considered positive for presumptive identification of Brucellosis in high-risk subjects.[4,6]

RESULTS

Blood samples of 102 high-risk persons including butchers (n=20), veterinarians (n=29) (including doctors and supporting staff) animal handlers, and their close contacts (n=53) were tested in the study. All participants were tested for both *B. abortus* and *B. melitensis*. Overall, the prevalence of brucellosis among high-risk group in our study was found to be 03 (2.9%) (Table 1).

Brucella CAPT, microtiter variant of tube agglutination done for *B. abortus,* was found positive in 03/102 (2.95%) participants, while one participant was found positive for *B melitensis* antibodies. One veterinarian doctor was positive for both *B. melitensis* and *B. abortus*. Highest prevalence of brucellosis was found in veterinarians 02/29 (6.8%) followed by 01/53 (1.88%) in animal handlers and none of the butcher was found positive for significant titer of *Brucella* antibody (1:160).

Table 1: Results of serological tests for brucellosis (n=102) (titer≥1:160)

Category	Number	*B. Abortus (N=3)*	*B. Melitensis* (%)	Both (%)
Butchers	20	-	-	-
Veterinarians	29	02 (6.8)	01 (2.6)	01 (2.6)
Animal handlers	53	01 (1.88)	-	-
Total	102	03 (2.95)	01 (0.98)	01 (0.98)

B. Abortus: Brucella Abortus, B. Melitensis: Brucella Melitensis

The age ranged from 18–72 years; mean age 32.36±11.91 years; of whom n=70 (67%) were male and n=32 (33%) were female (Table 2). Most (n=59) of the participants have attained graduation and approximately 26.4% were Muslims having non-vegetarian diet (50%). Hand hygiene was found poor as most (86%) of the participants were washing their hands with only water and higher positives 6.6% of participants were not using gloves while handling animals or animal products which were statically significant (P=0.04).

History of animal handling was present in 38% for 11–20 years, in 25% for <10 years, and 5% reported to have a duration of job >41 years. Forty-four participants (43.1%) were performing animal handling for >7 h in a day (Table 3), of which 6.81% participants were found seropositive with statistical significance of P=0.04 (Table 3), 58 (56.8%) were involved in their job for <7 h in a day.

Table 2: Sociodemographic characteristics, participants (n=102) included in this study

Variable	Butcher (n=20)	Veterinarian (n=29)	Animal Handler (n=53)
Gender			
Male	20	23	27
Female	00	06	26
Religion			
Hindu	00	27	23
Muslim	20	02	06
Diet			
Vegetarian	00	16	35
Non-Vegetarian	20	13	18
Education Status			
Illiterate	11	06	26
Literate	09	23	27
Use of Gloves			
Yes	17	12	28
No	03	17	25
Hand Washing			
Water	18	20	50
Soap	02	09	03
Hand Wipe			
Same Napkin	19	02	41
Different Napkin	01	27	12
Injury on Hand			
Present	01	02	02
Absent	19	27	51

DISCUSSION

We, to the best of our knowledge, are first to screen and determine the prevalence of brucellosis in the high-risk population from semi-urban areas in western Madhya Pradesh. Brucellosis is one of the most common zoonotic diseases in the world. Certain occupations are considered carrying high risk such as abattoir workers, veterinarians, butchers, meat inspectors, and farmers.[9]

Our study noted 2.9% of prevalence of Brucellosis among high-risk group as was noted previously by Yohannes and Gill.[10,11] A study by Agasthya et al.,[2] revealed that the prevalence of Brucellosis was 6.18% in veterinary supervisors, 2.06% in shepherds, and 1.03% in butchers. Similar study conducted by Thakur and Thapliyal revealed a prevalence rate of 4.9% in samples obtained from persons exposed to animals.[12] The prevalence of brucellosis has been widely reported in different regions such as in Orissa (6.8%)[13] and in Andhra Pradesh (11.51%)[14] which are much higher than present study findings. We have not detected any butcher with significant titter of *Brucella* antibody this may be due to awareness of brucellosis in butchers.[2] A study from Nigeria confirmed the endemic brucellosis, especially bovine brucellosis among slaughtered cattle at the abattoir, hence making it a source of occupational hazard to workers who were directly involved in the processing of meat fromanimals.[15]

We found that the female participation was less (n=32) which may be due to less number of females participating in the potential hazardous activities such as slaughtering and handling animals. All seropositive cases were male. Worldwide, brucellosis is more likely to occur in males rather than in females, as seen in various.[14,16-18] This is, however, in contrast with a study by Hussein et al., who reported relatively higher incidence among females.[19] Thus, the possible for higher prevalence of Brucellosis in male can be due to higher exposure to potential risk factors, though both are equally susceptible for acquiring the infection as gender does not influence the immune response to *Brucella*.[20]

We found that only 14% of study participants use soap to wash their hands which are lower than a study conducted by Ismayilova et al., where 38% of high-risk population was washing hands after handling animals.[21] Against the presumption that the risk of infection should be higher in

Table 3: Multivariate analysis of risk factors in relation to sera-reactivity

	Seronegative (<1:160)		Seropositive (>1:160)		Chi-square	P value
	Count	Row, n (%)	Count	Row n (%)		
Education						
Literate	53	100	0	0	3.34	0.06
Illiterarate	46	93.8	3	6.12		
Activity						
No hand wash	54	96.4	2	3.54	0.173	0.67
Hand wash	45	97.8	1	2.17		
Gloves						
No gloves	42	93.33	3	6.66	3.91	0.04
Gloves	57	100	0			
Working hour						
≤7 h	58	100	0	0	4.074	0.04
>7 h	41	93.18	3	6.81		
Drying hand						
No	73	97.3	2	2.66	0.075	0.78
Yes	26	96.2	1	3.70		
Injury on hand						
No	95	98.9	1	1.11	20.62	0.00
Yes	4	66.6	2	33.3		

population who have higher duration (in years) of exposure of handling animals, but we have not found any correlation between the duration of exposure and the number of individuals who tested positive and studies have shown that seropositivity decreases in population who have worked for over >40 years.[22] This might be due to the reason that with increased duration of experience the individual must be careful while handling animals. Furthermore, in our population level of academic education did not influence *brucella* seropositivity, similar finding were reported by Yohannes and Gill.[10]

We have included equal number of vegetarian and non-vegetarian population in our study, but we have not found any association of eating habits with the seroprevalance.[10] We did not found that any association between any medical illness such as diabetes, hypertension or any chronic illness, and long-lasting fever during past 6 months in contrast seropositivity for brucellosis was observed among pyrexia of unknown origin cases, animal handlers, and dairy workers in Goa, India.[5] The asymptomatic infections are detectable by serological tests, especially IgG antibodies. The incidence of human brucellosis (321 cases annually) in the study by Kumar et al.,[6] has shown that it is a serious disease present in the population. In India, the prevalence of animal brucellosis has been well studied.

As brucellosis is a zoonotic diseases and it mainly transmitted through contact of disease animal or their products. Prevention is dependent upon increasing public awareness through health education programs and safe livestock practices. Active cooperation between health and veterinary services should be promoted. This study will help in prevention of brucellosis as high-risk group were screened and the participants were educated for safe livestock handling to take precautions during occupational activities and hand hygiene. Such type of small studies can give an idea of local soreprevalence of Brucellosis and with the help of this guidelines can be formulated for screening for high-risk individuals by easily available standard tube agglutination or ELISA tests.

Limitations of the study

The study has some limitations such as though we have screened the known high-risk groups with immune capture agglutination assay (Brucella Capt) which detects both agglutinin and incomplete antibodies, but the serological tests are not specific though good for screening the population, the positives result should be confirmed by other tests like culture, polymerase chain reaction based technique. Large scale study would be better alternates for detection of brucellosis in future.

CONCLUSION

The present study revealed that veterinarians have high prevalence of brucellosis among high-risk groups and to deal with such occupation-related disease as brucellosis regular surveillance of the disease needs to be integrated into control and prevention program at a local and national level, knowledge of risk factors is vital in control and prevention of brucellosis.

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HS, VD, DS, MP- Were responsible for the conceptualizing the study design. **RP, AB, HS, MP**-were involved in microbiological work and analysis. **VK-** Helped with statistical analysis and in preparation of first draft of the manuscript. **SRB-** Provided assistance for data collection. All authors contributed in the development of final MS and approved it.

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