

Original Research Article

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Seroprevalence Studies of Brucellosis among Human using Different Serological Tests

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ABSTRACT

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A study was conducted to determine the prevalence of Brucellosis in humans in and around border areas of Jammu, J&K, India using different serological tests viz., RBPT, STAT and I-ELISA. A total of 75 sera samples from humans were tested by RBPT, STAT and I-ELISA. In humans, overall prevalence of 1.33% was recorded. Age-wise 36-50 yr persons while sex-wise males had higher prevalence.

Introduction

Brucella has always been of zoonotic importance all though the history and clinical conditions synonymous with brucellosis have been described since the time of Roman Empire (Godfroid *et al.*, 2005). Cocco-like forms resembling *Brucella* have been detected in carbonized cheese from the Roman era (Capasso, 2002). But, it was in 1887, when Sir David Bruce isolated the organism from the spleen of a British soldier who died from the Mediterranean fever in Malta and named it as *Micrococcus melitensis*.

Later, it was renamed *Brucella melitensis* in the honor of Sir David Bruce (Godfroid *et al.*, 2005).

Among all species of *Brucella*, *B. melitensis* is considered to be the most pathogenic to humans (Benkirane, 2006) causing maximum number (up to 90%) of human cases (Corbel, 1997) followed by *B. abortus* and *B. suis*. Brucellosis is an occupational direct anthroozoonosis. Public health significance of brucellosis is not only because of its status

as one of the world's major zoonoses but also because it causes a serious reduction in availability of quality food, especially the animal proteins. It also poses a serious threat to livestock economy by causing abortion, loss of offspring, infertility and reduction in milk yield.

Most cases in humans are caused by contact with infected animals or animal products, such as unpasteurized milk and cheese prepared from infected unpasteurized milk (Papaps *et al.*, 2005).

Thus, due to paucity of epidemiological data of humans brucellosis in and around border areas of Jammu region and unavailability of simple and sensitive method for diagnosis of humans and goat brucellosis, this study has been proposed with the objective of studying the prevalence of brucellosis in humans in and around border areas of Jammu.

Materials and Methods

A total of 75 human sera samples were collected from in and around border areas of Jammu region. The distribution of collected samples as per the area and species has been shown in table 1. In humans, the samples were collected from occupationally exposed groups *viz.*, veterinarians and patients with history of pyrexia of unknown origin (PUO), arthralgia etc. along with their age and sex particulars (Tables 1 and 2). All samples were subjected to RBPT, STAT and I- ELISA tests.

In humans, blood collection was done using sterilized disposable syringes (5ml) and serum separated by same procedure as adopted in animals. The serum samples collected were aliquoted and stored at -20°C till used.

The RBPT was performed according to the method described by Alton *et al.*, (1975). The Rose Bengal Antigen was procured from Biological Products (BP) Division, IVRI.

Before the test, both, serum and antigen were allowed to come at room temperature. Then, the test was performed by mixing 30 µl each of serum and antigen on a glass plate. With continuous shaking, the plates were looked for any appearance of agglutination. Appearance of agglutination within 4 min of mixing of reagents was taken as positive while absence of agglutination was recorded as negative result.

The test was performed in clean glass tubes (14 mm x 100 mm) according to the method described by Alton *et al.*, (1975). The contents of tube were thoroughly mixed followed by incubation at 37°C for 18 hours. After incubation, the tubes were kept at room temperature for ½ an hour followed by interpretation of results. A titre of ≥40IU was taken as positive in goat while ≥80IU was the cutoff in human.

The ELISA was performed as per the method standardized by Singh (2008), using S-LPS extracted from *B. abortus* S 99.

Smooth LPS was extracted from heat-killed cells of *B. abortus*, by the hot water/hot phenol method as described by OIE with minor modifications (2008a)

For the extraction, 5g of lyophilized cells of *B. abortus* strain 99 was suspended in 170 ml of distilled water (DW) and heated to 66°C. An equal volume of phenol (90%; v/v) in DW, also heated to 66°C, was added and the solution was stirred continuously for 20 min. It was then cooled to 4°C and centrifuged at 12,000g for 20 min at 4°C. The phenol phase (bottom layer) was recovered and filtered through Whatman #1 to which three volumes of chilled methanol reagent was added. It was mixed thoroughly and left to precipitate at 4°C for 2 h. The precipitate was recovered by centrifugation at 12,000x g at 4°C and re-suspended in the 80 ml of DW and centrifuged at 6,000g for 20 min. The pellet

was re-suspended in 80 ml of DW and stirred at 4°C overnight. The solution was then centrifuged at 10,000g for 15 min at 4°C and the supernatant was decanted. Another 80 ml of DW was added to the pellet, which was then stirred for 1 h and centrifuged as before.

The two supernatants were pooled, filtered through membrane filter (0.3µm), and 50-100µg each of ribonuclease, deoxyribonuclease and proteinase K were added. This mixture was incubated for 18 h at 20°C. It was re-precipitated with methanol and re-suspended as above in 2 ml of DW. The solution was dialyzed extensively against DW until free of phenol.

The resultant antigen was lyophilized, weighed and resuspended in DW to give 1mg LPS/ ml. This was finally freeze dried in 1 ml volume and stored at 4°C for future use. The cut-off values for I-ELISA were determined in terms of PP value (percent positivity); and in case of goats it was 58 and in humans is 67. The serum samples of goats and humans having PP value > 58 and >67 respectively, were recorded as positive. In the test proper the positive and negative controls along with a conjugate control were always included in each microtitre plate.

Anti-human conjugate was procured from Bangalore Genei.

The relative sensitivity and relative specificity of the test was calculated using the method described by Mcdiarmid and Hellstrom (1987).

Relative Sensitivity (%) =

$$\frac{\text{Serum samples positive to both test compared and standard test}}{\text{Serum samples positive to standard test}} \times 100$$

Relative Specificity (%) =

$$\frac{\text{Serum samples negative to both test compared and standard test}}{\text{Serum samples negative to standard test}} \times 100$$

The kappa value, odd's ratio, accuracy, predictive value and likelihoodratio were calculated using JavaStat-2-way Contingency Table Analysis at 95% confidence interval.

Results and Discussion

In the present study, a total of 75 sera samples originated from veterinarians / animal handlers and the patients with history of PUO, arthralgia, joint pain etc. The distribution of collected samples as per the area and species has been shown in table 1.

All the samples were subjected to RBPT, STAT and I-ELISA. Upon analysis of 75 human serum samples, an overall prevalence of 1.33% (Tables 3 and 4) was obtained whereas 2 (2.66%), 1(1.33%) and 4 (5.33%) samples were found positive to RBPT, STAT and I-ELISA, respectively, in individual test (Table 5).

In case of humans, out of three age groups of 20-35, 36-50 and 51-65 yr, 36-50yr persons recorded highest prevalence with the values as 3.33%, 0.0% and 10.0% for RBPT, STAT and I-ELISA, respectively (Table 6).

Conversely, in humans, males observed higher prevalence over females and none of the female samples was detected positive by any test (Table 7).

Odds ratio which depicts risk factor for acquiring the disease was found to be most susceptible among the 36-50 year age group individuals were found most susceptible (Table 8).

The results obtained in different serological tests viz., RBPT, STAT and I-ELISA were analyzed statistically in terms of (a) relative sensitivity (b) relative specificity (c) accuracy, (d) positive predictive value, (e) negative predictive value, (f) positive likelihood ratio, (g) negative likelihood ratio, (h) Kappa values and by analyzing the presence of anti-*Brucella* antibodies in different tests combinations. The relative sensitivities and specificities of the tests were calculated using the method described by Mcdiarmid and Hellstrom (1987) (Tables 9–11).

As I-ELISA are the prescribed test for international trade (OIE Manual, 2009 a, b) in human the statistical analysis was done by taking I-ELISA as standard. The same pattern was followed in human as well.

Upon screening of human (75) sera samples by RBPT, STAT and I-ELISA, the results obtained were compiled in different tests combinations as shown in table 5 and 11.

In humans, out of 75 samples tested, 2 samples were exclusively positive to I-ELISA. One sample positive to both RBPT and I-ELISA was observed while none of the samples were positive exclusively in RBPT and STAT. Interestingly, one sample was found positive in all the three tests (Table 5).

In humans (n=75), RBPT recorded 50% sensitivity with 100% specificity, 100% positive predictive value, 97.26% negative predictive value and infinite positive likelihood ratio. Similarly, STAT recorded 25% sensitivity with 100% specificity, 100% positive predictive value, 95.94% negative predictive value and infinite positive likelihood ratio. However, negative likelihood ratio was observed higher in STAT than RBPT (Table 9).

In humans (n=75), RBPT (97.33%) was having high accuracy value than STAT (96%) using I-ELISA as standard (Table 10).

In the present study the 75 human sera samples were examined and revealed an overall prevalence of 1.33% which was lower than that of Sharma (2010) in Jammu who found overall prevalence of 3.12% in humans. The prevalence in humans was also lower than that of Sharma *et al.*, (2016) who found 4.96% in Jammu, higher to that of 0.8% reported by Kadri *et al.*, (2000) in Kashmir.

Test wise, 2 (2.66%), 1(1.33%) and 4 (5.33%) samples were found positive to RBPT, STAT and I-ELISA, respectively. The values are significantly lower than that of Pathak *et al.*, (2014) in Goa who observed 4.25%, 3.54% and 6.02% seroprevalence by RBPT, STAT and I-ELISA respectively and also to Sharma (2010) in Jammu who found 3.12%, 0.00% and 21.80%, samples were positive to RBPT, STAT and I-ELISA, respectively.

The results were also lower than that of Sharma *et al.*, (2016) who found 9.91%, 9.09% and 16.52% samples were found positive to individual tests viz., RBPT, STAT and I-ELISA, respectively

The age wise analysis of data in humans, the highest prevalence was observed in the 36-50 yr age group (3.33, 0.0, and 10.0% by RBPT, STAT and I-ELISA, respectively which could be due to their higher occupational exposure to animal rearing and managerial practices.

The results are differing from that of Sharma (2010), the highest prevalence was observed in the 31-40 yr age group (8.30, 0.0, and 50.00% by RBPT, STAT and I-ELISA, respectively) and also to Sharma *et al.*, (2016) who found higher prevalence in 20-35 yr age group.

Table.1 Age-wise distribution of human serum samples

Species (No. of samples)	Age-group (yr)	No. of samples
Human (75)	20-35	20
	36-50	30
	51-65	25

Table.2 Sex-wise distribution of human serum samples

Species (No. of samples)	Sex	No. of samples
Human (75)	Male	45
	Female	30

Table.3 Sero-prevalence of brucellosis in humans

Species (No. of samples)	RBPT Positive (%)	STAT Positive (%)	I-ELISA Positive (%)
Human (75)	2 (2.66)	1 (1.33)	4 (5.33)

Table.4 Overall sero-prevalence of brucellosis in humans

Species	Samples examined	Samples positive	% positive
Human	75	1	1.33

Table.5 Sero-prevalence of brucellosis among humans (n=75) in different places of border areas of Jammu as detected by RBPT, STAT, I-ELISA

TESTS PLACE (Number of Samples)	RBPT		STAT		I-ELISA	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Vijaypur, Samba (10)	0 (0.00)	10 (100.0)	0 (0.00)	10 (100.0)	0 (0.00)	10 (100.0)
RS Pura, Jammu (10)	0 (0.00)	10 (100.0)	0 (0.00)	10 (100.0)	0 (0.00)	10 (100.0)
Kanachak, Akhnoor (10)	1 (10.0)	9 (90.00)	1 (10.0)	9 (90.00)	1 (10.0)	9 (90.00)
Veterinary Professionals/Animal Handlers (10)	1 (10.0)	9 (90.00)	0 (0.00)	10 (100.0)	1 (10.0)	9 (90.00)
Community Health Centre RS Pura (35)	0 (0.00)	35 (100.0)	0 (0.00)	35 (100.0)	2 (5.71)	33 (94.28)
TOTAL (75)	2 (1.71)	73 (97.33)	1 (1.33)	74 (98.66)	4 (5.33)	71 (94.66)

Table.6 Age-wise sero-prevalence of brucellosis in humans

Age Group	No. of persons	RBPT +ve (%)	STAT +ve (%)	I-ELISA +ve (%)
20-35	20	0 (0.0)	0 (0.0)	0 (0.0)
36-50	30	1 (3.33)	0 (0.0)	3 (10.0)
51-65	25	1 (4.0)	1 (4.0)	1 (4.0)

Table.7 Sex-wise sero-prevalence of brucellosis in humans

Sex (No. of samples)	RBPT +ve (%)	STAT +ve (%)	I-ELISA +ve (%)
Male (60)	2 (3.33)	1 (1.66)	4 (6.66)
Female (15)	-	-	-

Table.8 Age wise Odds ratio in humans

Age(yr)	20-35	36-50	51-65
20-35	-	0.00 95% CI=0.00-3.43 P- value=0.27	0.00 95% CI=0.00-22.56 P- value=1.0
36-50	-	-	2.67 95% CI=0.22-71.39 P- value=0.62
51-65	-	-	-

Using I-ELISA as standard

Table.9 Statistical analysis of RBPT and STAT taking I-ELISA as standard in humans (n=75)

Test	Kappa Value	Relative sensitivity (%)	Relative specificity (%)	+ve predictive value (%)	-ve predictive value (%)	+ve likelihood ratio	-ve likelihood ratio
RBPT	0.65	50	100	100	97.26	Inf	0.50
STAT	0.39	25	100	100	95.94	Inf	0.75

Table.10 Accuracy of RBPT and STAT taking I-ELISA as standard in humans (n=75)

Test	Accuracy (%)
RBPT	97.33
STAT	96

Table.11 Presence of anti-Brucella antibodies in different serological test combinations in humans (n=75)

Test	1	2	3	4	5	6	7	8
RBPT	-	+	-	-	+	-	+	+
STAT	-	-	+	-	+	+	-	+
I-ELISA	-	-	-	+	-	+	+	+
Total (75)	71	0	0	2	0	0	1	1

Sex wise studies revealed the prevalence was higher in males (3.33, 1.66 and 6.66% by RBPT, STAT and I-ELISA, respectively) as compared to females (0.0 % by all 3 tests). This is in accordance with Sharma (2010) who reported higher prevalence in males (4.00, 0.00 and 28.0 % by RBPT, STAT and I-ELISA, respectively) as compared to females (0.0 % by all 3 tests) and also to Sharma *et al.*, (2016) who also found higher prevalence in males. However, in present study, the difference in the sero-positivity between males and females is statistically not significant which may be due to less number of female samples in occupationally-exposed group.

In the present study, on testing 75 samples of humans by RBPT, STAT and I-ELISA, the maximum number of samples was found positive by I-ELISA, followed by RBPT and least by STAT in human. The results of these tests were compiled in different tests combinations and upon analysis of these combinations, 8 samples of goats and 2 of humans were found exclusively positive to I-ELISA. This high sero-positivity exclusively to I-ELISA could only be best ascribed in its (I-ELISA) nature being a primary binding assay which can detect 1/100th of the antibodies to those detected by secondary binding assay *i.e.*, CFT (Tizard,1982). Moreover, the many epitopes of S-LPS (antigen used in I-ELISA in present study) make it a highly sensitive test in brucellosis serology. Further, the above finding was supported by the work of Cargill *et al.*, (1985) who revealed that some culture positive animals were negative in RBPT while positive by I-ELISA.

Conversely, not a single sample exclusively positive to RBPT was detected in, both, goats and human beings. The sensitivity of RBPT was 20% in goats while 50% in humans with high specificity values. These findings can only be best supported by the fact that RBPT is an oversensitive test for diagnosis in individual animals especially those vaccinated (MacMillan, 1990) and false negative reactions may occur due to prozone phenomenon (Nielsen, 2002). Further, in field conditions the sensitivity of RBPT is known to vary from antigen to antigen preparation as reported by Blasco *et al.*, (1994) who reported significant differences in sensitivity of RBPT antigens from different manufacturing sources. Similarly, Stryszak (1986) also reported RBPT to be a less sensitive and observed the sensitivity to be increased when the amount of RBPT antigen was reduced to half. In India, too, there are number of reports regarding lower sensitivity of RBPT (Singh, 2009; Sharma *et al.*, 2015) *viz.*, Rajkhowa *et al.*, (2005) observed it to be 33.3% during investigation in mithuns. Nevertheless, I-ELISA was observed to be a more sensitive test over RBPT and STAT and should be applied on a large scale to evaluate it for screening purposes for diagnosis of brucellosis in the country. In conclusion, in comparison to RBPT and STAT, I-ELISA was found to be most sensitive and detected maximum number of sera samples in human beings.

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