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Analysis of Sera from Cattle Suspected for Brucellosis by Agglutination Tests and ELISA

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ABSTRACT

Brucellosis is a major bacterial zoonosis of global importance. Bovine brucellosis is endemic in all states of India. However, the exact extent of the disease occurrence in the unorganized herds is not known. The present study was undertaken for serological diagnosis of brucellosis in cattle from a local gaushala (cow shelter) employing common agglutination tests RBPT, STAT and MAT and ELISA. Out of 56 samples from cattle from the Gaushala, 37.5% samples by RBPT, 64.28% each by STAT and MAT and 49.23% samples by ELISA, respectively were found to be positive for anti-Brucella antibodies. Among the 29 suspected serum samples from the field cattle, samples found to be positive for anti-Brucella antibodies were 100% by RBPT, 79.31% by MAT and 89.65% by ELISA, respectively. Titers of anti-Brucella antibodies in the sera of cattle from Gaushala were found to be 1.747$\pm$0.45, 1.935$\pm$0.62 and 0.943$\pm$0.46 by STAT, MAT and ELISA, respectively, while those in cattle from the field were found to be 1.577$\pm$0.60 and 0.783$\pm$0.59 by MAT and ELISA, respectively.

Introduction

Brucellosis is a major bacterial zoonosis of global importance. It occurs worldwide but is much controlled in developed countries by routine screening of domestic animals and vaccination program. Clinical disease is still common in Middle East, Asia, Africa, South and Central America, the Mediterranean Basin, and the Caribbean. About 500, 000 cases of human brucellosis are estimated to occur worldwide every year. It causes heavy economic loss to the animal industry through abortion, delayed conception, and temporary or permanent infertility in the affected animals (Kollannur *et al.,* 2007). In Punjab, overall 17.7% prevalence of brucellosis was reported in cattle and buffaloes (Jain *et al.,* 2013; Pandeya *et al.,* 2013). Brucellosis in animals is clinically characterized by late-term abortions and retention of placenta in females and orchitis and epididymitis in males, with excretion of organisms in semen, uterine discharges, and in milk (Jain *et al.,* 2013; Godfroid *et al.,* 2013). Once infected, the animal may continue to shed bacteria and remains a source of infection to others for long period (Pandeya *et al.,* 2013). Antibodies induced by vaccination interfere in serological diagnosis of brucellosis. Bovine brucellosis is

endemic in all states of India. In India, the occurrence of brucellosis is to the extent of 10% in the marginal herds and 50% in organized farms, and the socio-economic impact of the disease was estimated to run over Rs. 500 crores annually. However, little data are available in the published literature on antibody titers due to natural infection in cattle in unorganized herds, gaushalas etc. from different regions of India. The present study was, therefore, undertaken for serological analysis of brucellosis in cattle at a gaushala.

Materials and Methods

Collection of samples

Blood samples from cattle were collected from the gaushala, Ludhiana, Punjab. About 10 ml of blood was collected aseptically from the jugular vein of the animal. Serum was collected by centrifuging the clotted blood at 3000 rpm for 15 minutes.

Rose Bengal Plate Agglutination 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 (Morgan *et al.,* 1978) with the help of a sterilized toothpick. The slide was observed for 4 min for the formation of clumps. The formation of clumps was considered a positive test while the absence of clear clumps was considered a negative reaction.

Standard Tube Agglutination Test (STAT)

The scheme of dilution of sera followed for STAT is depicted in Table 1. Eight agglutination tubes were placed in a rack. 0.8 ml of 0.5% carbol saline was added to the first tube of the series. 0.5 ml of carbol saline was added to all the other tubes except in tube no. 06, 07 and 08 which contained 1.25, 1.50 and 1.75 ml of carbol saline respectively.

0.2 ml serum was added to the first tube and the contents were mixed. 0.5ml of *Brucella abortus* plain antigen (Punjab Veterinary Vaccine Institute, Ludhiana) was then added to tube numbers 1 to 9, giving a final dilution of 1: 10, 1: 20, 1: 40 and so on. To the tubes 10, 11 and 12, which were kept as controls, 0.75, 0.50 and 0.25ml respectively of *Brucella abortus* plain antigen was added. These tubes were incubated at 37° C for 24 hours and the results were read.

The results were compared with the antigen control tube showing 50% agglutination. The highest serum dilution showing 50% agglutination was taken as the end point for the titre serum. A titre of 1:40 or above was considered positive.

Microtitre Plate Agglutination Test (MAT)

MAT was performed as per the standard method (Williams and Whittemore, 1971).

Test protocol

Serum samples were serially diluted two fold in a final volume of 100 µl in 96 well U-bottom microtitre plate (Tarsons).

Equal volume of 100 µl *Brucella abortus* plain antigen (Punjab Veterinary Vaccine Institute, Ludhiana) was added to each well. Negative control well containing 100 µl of sterilized Normal Saline Solution and 100 µl of the antigen was also kept.

The plate was covered with a lid and incubated at 37°C for 24 hr followed by incubation at 4°C for 1 hour.

The formation of matt signified agglutination while button formation was indicative of a

negative reaction. Titres (log_{10} values) were recorded as the reciprocal of the highest dilution of the serum giving at least 50percent agglutination.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum samples of cattle were tested using Idexx Brucellosis Serum ELISA test kit.

Principle

Microtiter plates are supplied precoated with inactivated antigen. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for *B. abortus* binds to the antigen in the wells and forms an antigen/ antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidase- labeled anti ruminant IgG Conjugate is added, which binds to the ruminant antibodies complexed with the *B. abortus* antigen. Unbound Conjugate is removed by washing and the TMB substrate is added to the wells. The degree of the colour that develops (optical density measured at 450nm) is directly proportional to the amount of antibody specific for *B. abortus* present in the sample. The diagnostic relevance of the result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from wells containing the Positive Control.

Test procedure

All reagents must be allowed to come to 18-26 ^{o}C before use. Reagents should be mixed by gentle swirling or vortexing.

Dispense 90 µl Wash Solution into each well of the microtiter plate.

Add 10 µl of the undiluted serum samples and Controls into the appropriate wells of the microtiter plate. Final dilution = 1:10. Mix the contents within each well by gently shaking the microtiter plate briefly (a microtiter plate shaker can be used).

Cover the microtiter plate with a lid and incubate for 60 minutes at 37 ^{o}C in a humid chamber.

Wash each well with approximately 300 µl Wash Solution three times. Aspirate liquid contents of all wells after each wash. Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.

Dispense 100 µl Conjugate into each well.

Cover and incubate the microtiter plate for 60 minutes at 37 ^{o}C in a humid chamber.

Wash each well and aspirate the liquid contents of all wells after each well.

Dispense 100 µl of TMB substrate into each well.

Incubate the Substrate at 18-26°C for 15 minutes.

Stop the colour reaction by adding 100 µl Stop solution per well. The Stop Solution should be dispensed in the same order and at the same speed as the substrate.

Read the results using photometer at a wavelength of 450 nm.

To validate the assay, the optical density (OD) of the Positive Control should not exceed 2.000 and the OD of the Negative Control should not exceed 0.5000. The difference between the Positive and the Negative Control must be greater than or equal to 0.3000.

The plates should be read within two hours after the addition of stop solution.

Calculations

The OD of duplicates was averaged.

The OD of the Positive Control (PCx) and the OD of the samples were corrected by subtracting the OD of the Negative Control (NCx).

Positive Control corrected OD: PCx- NCx

Sample corrected OD: Sample A450- NCx

S/P ratio for each sample: S/P% = 100 x (Sample A 450- NCx / PCx- NCx)

Interpretation of results

S/P ratio less than 80% - Negative
S/P greater than or equal to 80% - Positive

Results and Discussion

Out of 56 suspected samples from cattle from the Gaushala, samples found to be positive for anti-Brucella antibodies were 21 (37.5%) by RBPT, 36 (64.28%) each by STAT and MAT and 32 (49.23%) by ELISA, respectively (Tables 2 and 3). Among the 29 suspected serum samples from the field cattle, samples found to be positive for anti-Brucella antibodies were 29 (100%) by RBPT, 23 (79.31%) by MAT and 26 (89.65%) by ELISA, respectively (Tables 4 and 5). Titers of anti-Brucella antibodies in the sera of cattle from Gaushala were found to be 1.747 ± 0.45, 1.935 ± 0.62 and 0.943 ± 0.46 by STAT, MAT and ELISA, respectively (Table 6). Titers in the sera of cattle from the field were found to be 1.577 ± 0.60 and 0.783 ± 0.59 by MAT and ELISA, respectively (Table 7).

There was no significant difference between mean titers by STAT and MAT of Gaushala cattle and between mean MAT titers as well as between the ELISA titers of the two groups of animals, respectively.

Even though a number of antigenic components of *Brucella* have been characterized, the antigen that dominates the antibody response is the lipopolysaccharide (LPS). Numerous outer and inner membrane, cytoplasmic, and periplasmic protein antigens have also been characterized.

Some are recognized by the immune system during infection and are potentially useful in diagnostic tests. The L7/L12 ribosomal proteins are important in stimulating cell-mediated responses (Oliveira and Splitter, 1994).

Immune response of host to *Brucella* infection is mediated through both humoral and cell-mediated immunity (Skendros and Boura, 2013). The role of humoral immunity against intracellular bacterial infections is limited and not protective. Antibody-mediated opsonization by Igs (IgM, IgG1, IgG2a, and IgG3) enhances phagocytic uptake of bacteria, limiting the level of initial infection with *Brucella* but has little effect on the intracellular course of *Brucella* infection (Bellaire *et al.,* 2005; Baldwin and Goenka, 2006).

As evident from our present data, infected animals had high titers of antibodies. However, the high titers do not indicate whether these are protective antibodies due to vaccine or acute response to infection in the absence of a differentiation of infected from vaccinated animals (DIVA) assay or whether they are of any relevance to prognosis.

Table.1 Scheme of dilution of sera for STAT

Tube No.	Carbol saline (ml)	Test serum(ml)	*B. abortus* plain antigen (ml)	Final dilution
1	0.8	0.2	0.5	1:10
2	0.5	Serial dilution was performed after thorough mixing.0.5ml of the contents was transferred from tube no.1 to the next tube up to tube no. 5. Finally 0.5 ml of the contents was discarded from tube no.5.	0.5	1:20
3	0.5		0.5	1:40
4	0.5		0.5	1:80
5	0.5		0.5	1:160
6	1.25		0.75	
7	1.50		0.50	
8	1.75		0.25	

Controls:
Tube No. 6- 25% agglutination
Tube No. 7- 50% agglutination
Tube No. 8- 75% agglutination

Table.2 Sera from cattle analysed for anti-Brucella antibodies by four serological tests

S. No.	Sample number	RBPT	STAT	MAT	ELISA
1	BS 1	N	N	N	N
2	BS 2	N	N	N	N
3	BS 3	N	N	N	N
4	BS 4	P	P	P	P
5	BS 5	N	N	N	N
6	BS 6	N	N	N	N
7	BS 7	N	N	N	N
8	BS 8	N	N	P	N
9	BS 9	N	N	P	N
10	BS 10	N	N	P	N
11	BS 11	P	P	P	P
12	BS 12	P	P	P	N
13	BS 13	P	P	P	P
14	BS 14	P	P	P	P

15	BS 15	N	P	N	N
16	BS 16	P	P	P	P
17	BS 17	N	P	N	P
18	BS 18	N	P	P	N
19	BS 19	N	N	N	N
20	BS 20	N	N	N	N
21	BS 21	N	P	P	N
22	BS 22	N	N	P	N
23	BS 23	N	P	P	P
24	BS 24	N	N	N	N
25	BS 25	P	P	P	N
26	BS 26	P	P	P	P
27	BS 27	N	P	P	P
28	BS 28	P	P	P	P
29	BS 29	P	P	P	P
30	BS 30	N	P	N	P
31	BS 31	N	N	N	N
32	BS 32	N	P	P	P
33	BS 33	P	P	P	P
34	BS 34	P	P	P	P
35	BS 35	N	P	P	P
36	BS 36	N	N	N	N
37	BS 37	N	P	N	P
38	BS 38	P	P	P	P
39	BS 39	P	P	P	P
40	BS 40	P	P	P	P
41	BS 41	N	N	P	P
42	BS 42	N	P	P	P
43	BS 43	P	P	P	P
44	BS 44	N	N	N	N
45	BS 45	P	P	P	P
46	BS 46	P	P	P	P
47	BS 47	N	N	N	N
48	BS 48	N	N	N	N
49	BS 49	N	P	P	P
50	BS 50	N	P	P	P
51	BS 51	P	P	P	P
52	BS 52	N	P	P	P
53	BS 53	N	P	N	P
54	BS 54	N	N	N	N
55	BS 55	P	P	P	P
56	BS 56	P	P	P	P

Table.3 Percentage of positive samples from Gaushala by RBPT, STAT, MAT and ELISA

Serological test	Number of samples		Percent positive
	Positive	Negative	
RBPT	21	35	37.5
STAT	36	20	64.28
MAT	36	20	64.28
ELISA	32	24	49.23

Table.4 Sera from field cattle positive for Brucellosis by RBPT, MAT and ELISA

S.no.	Animal no.	RBPT	MAT	ELISA
1	FS 3	P	P	P
2	FS 4	P	P	P
3	FS 5	P	P	P
4	FS 10	P	P	P
5	FS 15	P	P	P
6	FS19	P	P	P
7	S 1	P	P	N
8	S 2	P	N	P
9	S 3	P	N	N
10	S 4	P	P	P
11	S 6	P	P	P
12	S 7	P	N	N
13	S 12	P	P	P
14	S 17	P	P	P
15	S 18	P	P	P
16	S 19	P	P	P
17	S 20	P	P	P
18	S 24	P	P	P
19	S 33	P	P	P
20	S 34	P	N	P
21	S 38	P	P	P
22	S 39	P	P	P
23	S 40	P	P	P
24	S 43	P	P	P
25	S 44	P	N	P
26	S 47	P	P	P
27	S 49	P	N	P
28	S 51	P	P	P
29	S 53	P	P	P

Table.5 Percentage of positive samples from field cattle by RBPT, MAT and ELISA

Serological test	Number of samples		Percent positive
	Positive	Negative	
RBPT	29	0	100
MAT	23	6	79.31
ELISA	26	3	89.65

Table.6 Titers of anti-Brucella antibodies in sera of cattle from Gaushala

S. No.	Sample number	STAT	MAT	ELISA
1	BS 1	1.3010	1.0000	0.0684
2	BS 2	1.3010	1.0000	0.4764
3	BS 3	1.3010	1.3010	0.0758
4	BS 4	2.2041	2.5051	1.3222
5	BS 5	1.0000	1.0000	0.8646
6	BS 6	1.3010	1.3010	0.0736
7	BS 7	1.0000	1.0000	0.7809
8	BS 8	1.3010	2.5051	0.6319
9	BS 9	1.3010	2.2041	0.1001
10	BS 10	1.3010	2.5051	0.0776
11	BS 11	2.2041	2.5051	1.1153
12	BS 12	2.2041	2.5051	1.0032
13	BS 13	2.2041	2.5051	1.0361
14	BS 14	2.2041	2.5051	1.7271
15	BS 15	1.6020	1.3010	0.8589
16	BS 16	2.2041	2.5051	1.4086
17	BS 17	2.2041	1.3010	1.0518
18	BS 18	2.2041	2.5051	0.3805
19	BS 19	1.3010	1.0000	0.0979
20	BS 20	1.0000	1.0000	0.7397
21	BS 21	1.9030	2.5051	0.7407
22	BS 22	1.3010	2.5051	0.8679
23	BS 23	1.9030	2.5051	1.1306
24	BS 24	1.0000	1.0000	0.1505
25	BS 25	2.2041	2.5051	0.9017
26	BS 26	2.2041	2.5051	1.184
27	BS 27	1.9030	2.5051	1.154
28	BS 28	2.2041	2.5051	1.587
29	BS 29	2.2041	2.2041	0.954
30	BS 30	1.6020	1.3010	0.888
31	BS 31	1.3010	1.0000	0.344
32	BS 32	1.6020	2.2041	0.912
33	BS 33	2.2041	2.2041	1.394
34	BS 34	2.2041	2.5051	1.547
35	BS 35	1.9030	1.9030	1.315
36	BS 36	1.3010	1.3010	0.813
37	BS 37	1.9030	1.3010	1.129
38	BS 38	2.2041	2.5051	1.462
39	BS 39	2.2041	2.5051	1.447
40	BS 40	2.2041	2.5051	1.077
41	BS 41	1.3010	1.6020	1.295
42	BS 42	1.9030	1.9030	1.411
43	BS 43	2.2041	2.5051	1.557
44	BS 44	1.0000	1.0000	0.050
45	BS 45	2.2041	2.5051	1.383
46	BS 46	2.2041	2.5051	1.480
47	BS 47	1.0000	1.0000	0.619
48	BS 48	1.3010	1.3010	0.860
49	BS 49	1.6020	1.9030	1.106
50	BS 50	1.9030	2.2041	1.300
51	BS 51	2.2041	2.5051	1.196
52	BS 52	1.9030	1.9030	1.257
53	BS 53	1.6020	1.3010	1.273
54	BS 54	1.0000	1.3010	0.571
55	BS 55	2.2041	2.5051	1.158
56	BS 56	2.2041	2.5051	1.416
Mean ± SD		**1.747±0.45**	**1.935±0.62**	**0.943±0.46**

Table.7 Anti-Brucella antibody titers in sera of field cattle by MAT and ELISA

S. no.	Sample no.	MAT	ELISA
1	FS 1	1.0000	0.063
2	FS 2	1.3010	0.419
3	FS 3	2.5051	1.104
4	FS 4	1.9030	1.213
5	FS 5	2.5051	1.238
6	FS 6	1.3010	0.246
7	FS 7	1.6020	0.061
8	FS 8	1.3010	1.103
9	FS 9	1.0000	0.053
10	FS 10	1.6020	1.344
11	FS 11	1.0000	0.054
12	FS 12	1.0000	0.058
13	FS 13	1.0000	0.055
14	FS 14	1.3010	0.059
15	FS 15	2.5051	1.420
16	FS 16	2.2041	0.115
17	FS 17	1.6020	0.061
18	FS 18	2.5051	0.076
19	FS19	2.5051	1.537
20	FS 20	2.5051	0.126
21	S 1	1.6020	0.105
22	S 2	1.3010	1.354
23	S 3	1.0000	0.218
24	S 4	2.2041	1.664
25	S 5	1.3010	0.3252
26	S 6	2.2041	1.360
27	S 7	1.3010	0.862
28	S 8	1.6020	0.099
29	S 9	1.0000	0.186
30	S 10	1.0000	0.4178
31	S 11	1.6020	0.160
32	S 12	2.5051	1.444
33	S 13	1.3010	1.324
34	S 14	1.0000	0.4317
35	S 15	1.0000	0.416
36	S 16	1.6020	0.5603
37	S 17	2.5051	1.688
38	S 18	2.2041	1.607
39	S 19	2.5051	1.536
40	S 20	2.5051	1.5642

41	S 21	1.0000	0.065
42	S 22	1.0000	1.076
43	S 23	1.0000	0.0715
44	S 24	2.5051	1.420
45	S 25	1.0000	0.4512
46	S 26	1.0000	1.2029
47	S 27	1.0000	1.1594
48	S 28	1.3010	1.297
49	S 29	1.3010	0.6211
50	S 30	1.0000	0.319
51	S 31	1.3010	1.088
52	S 32	1.0000	0.128
53	S 33	2.5051	1.558
54	S 34	1.3010	1.612
55	S 35	1.0000	1.158
56	S 36	1.0000	0.065
57	S 37	1.0000	0.525
58	S 38	1.6020	1.498
59	S 39	1.9030	0.978
60	S 40	1.6020	1.304
61	S 41	1.0000	1.006
62	S 42	1.0000	0.056
63	S 43	2.2041	1.252
64	S 44	1.3010	0.981
65	S 45	1.0000	0.106
66	S 46	1.0000	0.697
67	S 47	2.5051	1.292
68	S 48	1.3010	0.062
69	S 49	1.0000	1.371
70	S 50	2.2041	1.518
71	S 51	2.5051	1.458
72	S 52	1.0000	0.055
73	S 53	2.5051	1.489
74	S 54	2.5051	1.324
Mean ± SD		**1.577±0.60**	**0.783±0.59**

The different titers observed in the same animals by agglutination assays (STAT, MAT, and IHA) and iELISA can be reconciled with the fact that these assays target antigens of different nature, i.e., agglutination assays are directed toward particulate antigens, whereas ELISA detects immune response to soluble antigens. ELISA is generally used to detect IgG antibodies (Long *et al.,* 1986). In brucellosis, specific IgM antibodies dominate during the acute phase of the disease (Smits *et al.,* 2003). Specific IgG antibodies are present in the serum of patients at later stages of the illness and in the serum of relapsing patients (Ariza *et al.,* 1992). ELISA is used to discriminate between the presence of specific IgM and IgG antibodies

and to roughly assess the stage of illness (Smits and Kadri, 2005).

In many countries, STAT is the routine diagnostic test for human and animal brucellosis. It has been reported (Hassanain and Ahmed, 2012) that STAT has a greater accuracy than that of the RBPT (93.3% and 76.6%, respectively). Ina study (Sareyyupoglu *et al.,* 2010), *Brucella* antibodies were investigated in bovine sera by RBPT, serum agglutination test, MAT, and 2-mercaptoethanol MAT, and MAT was determined as a fast, reliable, and economic test. On evaluation of canine brucellosis by MAT, it was shown (Kimura *et al.,* 2008) that MAT was more sensitive, simpler to perform, and easier than tube agglutination test. A study (Versilova *et al.,* 1974) has shown that the use of sheep erythrocytes sensitized with a specific LPS antigen in the IHA test provided a specific method, which is more sensitive than the agglutination test.

In a study by (Ghodasara *et al.,* 2010), STAT and iELISA were compared for detection of *Brucella* antibodies in cows and buffaloes. The seropositivity was found highest by iELISA (25%) followed by STAT (14.45%). iELISA, RBPT, MAT, and PCR were evaluated (Malik *et al.,* 2013) for diagnosis of brucellosis in buffaloes, and it was concluded that iELISA detected more samples as positive among these tests.

Among the suspected serum samples from cattle from the Gaushala, 37.5% samples by RBPT, 64.28% each by STAT and MAT and 49.23% samples by ELISA, respectively were found to be positive for anti-Brucella antibodies. Among the serum samples from the field cattle, samples found to be positive for anti-Brucella antibodies were 100% by RBPT, 79.31% by MAT and 89.65% by ELISA, respectively. Titers in the sera of cattle from Gaushala were 1.747 ± 0.45, 1.935 ± 0.62 and 0.943 ± 0.46 by STAT, MAT and ELISA, respectively. While those in cattle from the field were 1.577 ± 0.60 and 0.783 ± 0.59 by MAT and ELISA, respectively.

Conflict of interest

The authors declare that they have no competing interests.

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