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Spatial Distribution of Brucellosis in Small Ruminants of India using Indigenously Developed ELISA Kit

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India is a rich niche of ovine germplasm with diverse indigenous and cross breeds. Brucellosis is most contagious, often regionally neglected disease of small ruminants in the country. The current spatial prevalence of brucellosis in small ruminants of the country was not known. A total of 8904 samples [sheep (n1)-4868, goat (n2)-4036] from different states of the country were randomly collected and tested by indigenously developed iELISA kit. True prevalence of disease was found to be 5.5% (95% CI: 4.6-6.3%) and 2.3% (95% CI: 1.5-3.1), in sheep and goat, respectively. The study conclusively unraveled the epidemiological overview of disease at both state and national level. This information is useful for prioritizing regions for vaccination, designing control strategies and improvisation of clinical surveillance system.

Key words: sheep, goat, brucellosis, iELISA, India, spatial analysis, random sampling.

Small ruminants are socioeconomically important livestock species, ubiquitously reared as primary source of animal food and more than five million households in the country are engaged in rearing of small ruminants. India is a rich niche of ovine germplasm, accounts for more than 6.8% and 20% of world sheep and goat population respectively with diverse cross and indigenous breeds[1, 2]. One of the major contagious endemic bacterial diseases of small ruminants in the country is brucellosis and it is characterized by loss of productivity, abortion in the fourth or fifth month of gestation, stillbirths and reproductive failures[3]. *B. melitensis*-member of genus *Brucella* is the most predominant pathogen of infection in small ruminants and it has been estimated that economic loss per animal due to *B. meliteniss* infection in sheep and goats is INR 2122 and 1818 [4] respectively. Increasing demand for meat products, trade, free grazing and movement with frequent mixing of flocks of sheep and goats is contributing to high prevalence and wide distribution of brucellosis in these animals[5]. The disease is also of zoonotic importance as it transmits to animal owners and consumers either through direct contact with infected animals or consumption of contaminated milk and meat products respectively[6, 7, 8]. Hence, early detection, segregation of infected animals and removal from the flock are important to control the disease in these animals and transmission to human[9].

A major challenge in control of brucellosis is clinical diagnosis which cannot be generalized to all age, sex, species and breed of animals and disease confirmation requires laboratory diagnosis[10, 11]. Confirmative diagnosis of brucellosis requires isolation of the causal agent which is highly hazardous and failure to isolate

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pathogen is a frequent occurrence[12]. PCR based assays have been used in many laboratories but they cannot be considered for routine diagnosis and surveillance[13]. Rose bengal plate test (RBPT) is most widely used test but often compounded with false positive results due to low specificity[14, 15]. In recent years, enzyme linked immunosorbent assay (ELISA) is widely used for screening antibody in serum and milk of small ruminants[16, 17, 18, 19].

In India, seroprevalence of brucellosis has been recorded in more than 0.2million bovine population using indigenously developed AB_ELISA kit in the Institute (Avidin-Biotin ELISA) [20]. In continuation of our efforts in the development of diagnostics for brucellosis, indirect ELISA kit for small ruminant brucellosis has been standardized, validated, patented and commercially made available from the Institute. In the present study, random samples of small ruminants from different states of the country were screened by iELISA kit to document prevalence of brucellosis at national level. This will aid planners for formulation of control strategies.

MATERIALS AND METHODS

Sample frame and source

A total of 8094 samples [sheep (n1) - 4868, goat (n2) - 4036] from different states of the country were sourced through All India Coordinated Research Project (AICRP) units during the study period from 2006-2014 by multistage random sampling approach. Approximately 10mL of blood sample was collected from the jugular vein of each animal using vaccutainers without EDTA (Becton Dickson, UK). Samples were labeled using codes indicating the district and state. The clotted blood in the tubes were centrifuged at 3000g for 20min to obtain clear serum and transported on ice to the Institute. The samples were bar coded and stored at -20^0 C until tested.

Serodiagnosis by iELISA

All the serum samples were analyzed by iELISA. First, smooth Lipopolysaccharide (sLPS) antigen from standard strain *B. abortus* 99 was extracted as per the OIE protocol [21]. The polysorp microtiter plates (Nunc, Germany) were coated with 1:300 dilution of sLPS antigen at 100 µl per well (10ng/well) in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C for overnight. Antigen coated plates were washed three times with PBST wash buffer pH 7.2 (phosphate buffered saline containing 0.05 % tween 20). Test and control sera diluted in PBST blocking buffer (1:100) containing 2% bovine gelatin was added to respective wells (100 µl) of the plates in duplicates (test sera) and quadruplicate (control sera) and incubated at 37°C for 1hr. The plates were then washed as mentioned earlier. The anti sheep and goat horse raddish peroxidase (HRP) conjugate (Pierce, Germany), diluted 1:8000 in PBST buffer were added to all the wells (100 µl) and incubated for 1hr at 37°C on orbital shaker (300 rpm/min). After washing, freshly prepared o-Phenylenediamine dihydrochloride (OPD), (Sigma, Germany) solution containing 5mg OPD tablet in 12.5 ml of distilled water and 50 µl of 3% H_2O_2 was added and kept for color development for 10 min. Enzyme-substrate reaction was stopped by adding 1M H_2SO_4 (50 µl) and color development in the form of the optical density (OD) was read at 492 nm using an ELISA microplate reader (Bio-Rad). Percent positivity (PP) values which are used for the diagnostic interpretations are calculated as follows:

$$\text{Percent positivity (PP)} = \frac{\text{Average OD value of test serum}}{\text{Median OD value of strong positive control}} \times 100$$

The cut-off values established for diagnosis was decided after thorough screening and validation of assay[22]. Any sample of PP value below 54% is taken as negative, more than 54% as strong positive and sample with only 54% PP are recommended for retesting for confirmation.

Statistical analysis

The data was analysed using IBM SPSS software Version 22 (India). By using chi square test, significance of difference was determined and value of $p< 0.05$ was considered statistically significant in analysis of species wise prevalence. From apparent prevalence, true prevalence was calculated at 95% confidence interval (CI) using true prevalence program of the Survey tool box in which sensitivity and specificity of diagnostic test used and sample size were taken into consideration. This calculation was done as described by Rogan and Gladen (1978) [23].

RESULTS

Out of 8904 serum samples of small ruminants, 661 samples were positive by iELISA with apparent prevalence of 7.4 % (95% CI 6.9 – 8.0) and true prevalence of 4.1% (95% CI 3.5 -4.6). Species wise analysis revealed that out of 4868 sheep and 4036 goat samples, 425 (8.7% ; 95% CI 8.0-9.6) and 236 (5.8%; 95% CI 5.2-6.6) were positive, respectively. Statistically significant higher seropositivity was observed among sheep population than goats (P value <0.001).

State wise disease prevalence in sheep showed that seroprevalence varied from 0 - 28.6% among different states of the country (Table-1 and Fig 1) and overall true prevalence of disease in sheep population of the country was 5.5% (4.6-6.3%). Highest true prevalence was recorded in Bihar (28.6%; 95% CI 19.7-37.5) followed by Madhya Pradesh (11.9%; 95% CI 9.5 – 14.3) and Andhra Pradesh (8.1%; 95%CI 4.8-11.5). The lowest true prevalence was recorded in West Bengal, Uttar Pradesh and Kerala.

Similarly, in goat, true prevalence varied from 0 to 9.1% in 12 states and overall true prevalence of disease in goat population of the country was 2.3% (1.5 – 3.1).

Table 1. State wise seroprevalence of brucellosis in sheep population of India

Sl. No	State	Total	Positives	Apparent prevalence (95% CI)	True prevalence (95% CI)
1	Bihar	120	36	30.0 (22.5-38.7)	28.6 (19.7-37.5)
2	Madhya Pradesh	997	146	14.6 (12.6-17.0)	11.9 (9.5-14.3)
3	Andhra Pradesh	403	45	11.2 (8.5-14.6)	8.1 (4.8-11.5)
4	Rajasthan	358	34	9.5 (6.9-13.0)	6.3 (3.0-9.6)
5	Manipur	225	17	7.6 (4.8-11.8)	4.2 (4.0-8.0)
6	Karnataka	186	13	7.0(4.1-11.6)	3.6 (0-7.6)
7	Gujarat	1310	86	6.6 (5.3-8.0)	3.1(1.7-4.6)
8	Maharashtra	299	18	6.0(3.8-9.3)	2.5 (0-5.5)
9	Odisha	280	15	5.4 (3.3-8.6)	1.8 (0-4.7)
10	Tamil Nadu	403	13	3.2 (1.9-5.4)	0 (0-1.4)
11	Kerala	50	1	2.0 (0.4-10.5)	0 (0-2.4)
12	Uttar Pradesh	118	1	0.8 (0.1-4.6)	-
13	West Bengal	119	0	0 (0-3.1)	-
	Total	4868	425	8.7 (8.0-9.6)	5.5 (4.6-6.3)

Table 2. State wise seroprevalence of brucellosis in goat population of India

Sl. No	State	Total	Positives	Apparent prevalence (95% CI)	True prevalence (95% CI)
1	Andhra Pradesh	380	46	12.1 (9.2-15.8)	9.1 (5.6-12.7)
2	Manipur	125	15	12.0 (7.4-18.9)	9.0 (2.8-15.2)
3	Maharashtra	186	15	8.1 (4.9-12.9)	4.7 (0.5-9.0)
4	Rajasthan	207	15	7.2 (4.4 - 11.6)	3.9 (0 - 7.7)
5	Karnataka	807	61	7.6 (5.9-9.6)	4.2 (2.2-6.2)
6	Madhya Pradesh	725	40	5.5 (4.1-7.4)	2.0 (2.0-3.8)
7	Tamil Nadu	194	9	4.6 (2.5-8.6)	1.0 (0-4.2)
8	Punjab	48	2	4.2 (1.2-14.0)	0.5 (0-6.7)
9	Gujarat	888	32	3.6 (2.6-5.0)	0 (0-1.2)
10	Odisha	342	1	0.3 (0.1-1.6)	-
11	Kerala	47	0	0 (0-7.6)	-
12	Assam	87	0	0 (0-4.2)	-
	Total	4036	236	5.8 (5.2-6.6)	2.3(1.5-3.1)

Highest seroprevalence was recorded in Andhra Pradesh (9.1%; 95% CI 5.6 -12.7) followed by Manipur (9.0%; 95% CI 2.8-15.2) and Maharashtra (4.7%; 95% CI 0.5-9.0) and lowest in Assam, Kerala and Odisha (Table- 2 and Fig 2).

DISCUSSION

Brucellosis is an economically important disease of small ruminants and the disease in goats is usually caused by *B. melitensis* and less frequently by *B. abortus* and in sheep, rough strain of *Brucella, B. ovis* is less frequently involved *in* addition to *B. melitensis* and *B. abortus* [24]. There is lone report of *B.ovis* seroprevalence in sheep from India[25] and the reason for poor reporting is due to lack of diagnostic antigens and kits which can detect antibodies produced in response to *B.ovis* infection. However, several studies have reported *B. melitensis* infection in sheep and goats[20, 17, 26, 16, 27, 28]. In many of these studies, brucellosis has been diagnosed by either RBPT or SAT or in-house standardized ELISA for limited number of samples or imported ELISA kits. *Brucella* detection assays for goats and sheep are nearly the same as that of cattle because of the considerable genetic similarity between smooth strains of *Brucella i.e. B. melitensis* and *B. abortus*[29]. The O-chain structure of *B. melitensis* and *B. abortus* species are made up of overlapping epitopes and the practical implication is that the single antigen can be used for the diagnosis of brucellosis caused by smooth strains of *Brucella*[30] *Brucellae* sLPS is the main virulence factor which evinces strong immune responses and widely used for preparation of various diagnostic assays[30]. Based on above facts, in the present study, sLPS antigen from *B. abortus* S99 is conveniently used for the ELISA kit development. To avoid all the variabilities with respect to the strain, quality and preparation of antigen, standard OIE described antigen extraction and purification protocols were followed to suit small batches of antigen extraction.

Among 4036 goats and 4868 sheep sera samples screened by iELISA kit, 236 (5.8%) in goats and 425 (8.7%) in sheep were positive for anti-B*rucella* antibodies. Highest seroprevalence by iELISA has been recorded in goats of Andhra Pradesh and Manipur. Similarly, Bihar, Madhya Pradesh and Andhra Pradesh states showed highest seroprevalence of disease in sheep. In India, the largest National seroprevalence survey confirmed the overall incidence of 7.9% in 6305 sheep and 2.20% in 3849 goats from different states in the country by RBPT and SAT[20]. This is continuation of surveillance studies in small ruminant brucellosis in similar lines that of bovine brucellosis by using indigenously developed ELISA kit. The overall apparent prevalence by highly sensitive assay indicated 8.7% and 5.8% in

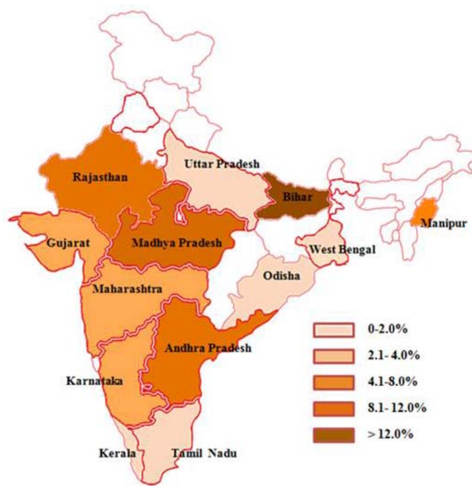

Fig.1. State wise seroprevalence of brucellosis in sheep population of india

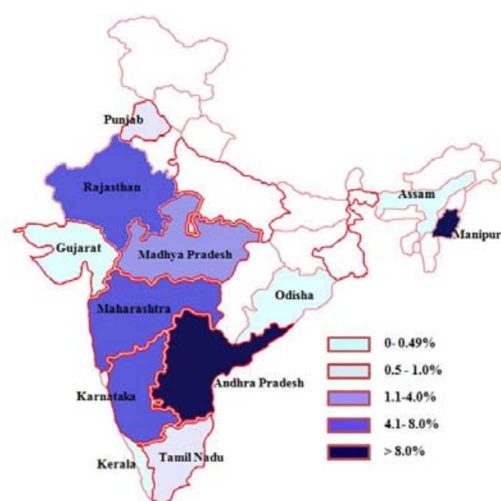

Fig.2. State wise seroprevalence of brucellosis in goat population of india

sheep and goats, respectively in the country. This marginal increasing disease trend especially in goats from 2.2% in the year 2002 to 5.8% in the present study is attributed to lack of vaccination and awareness program.

State wise disease prevalence in sheep showed highest true prevalence in Bihar (28.6%) followed by and Madhya Pradesh (11.9%) and Andhra Pradesh (8.1%). Similarly, in goats, highest seroprevalence was recorded in Andhra Pradesh (9.1%) followed by Manipur (9.0%), and Maharashtra (4.7%). The disease is quite alarming in Andhra Pradesh state which showed highest seroprevalence in both sheep and goats (11.9% and 9.0%). Seropositivity of 6% to 50% in organized goat farms of Andhra Pradesh with the history of abortion[31] has been reported. In another study of non- random sampling, 32.73% of seropositivity in sheep and goats of Rajasthan has been reported. The higher prevalence of brucellosis in small ruminants of Rajasthan is largely due to sharing of common grazing lands and intermixing of flocks during severe summer months.

There are several seroprevalence studies in both sheep and goat population from Uttar Pradesh. These earlier reports recorded seropositivity ranging from 4.7 - 21.23% in sheep [26, 33, 34] and 5.26 – 30.44% in goats [16, 26, 34]. Seropositivity of 11.50% and 10.46% were recorded in slaughtered goats and sheep, respectively [35, 36]. Most of these reports are based on purposive sampling and thus the results cannot be generalized to overall state or national status. Punjab state in the country is considered hot spot for brucellosis[37] and in small ruminants, seroprevalence of 16.06% and 15.33% in sheep and goats, respectively[27] and 7.20% and 5.30% in sheep and goats, respectively [38] have been reported. We have received meager goat samples (48) from Punjab and could not estimate the prevalence in this important region. All these studies clearly indicate that disease has been well recorded over the years in many geographical regions and now the time has come to seriously consider for control of the disease to safe guard small ruminants and public too.

Among southern states, seropositivity up to 24% and 25% in goats and sheep, recorded respectively in Tamil Nadu[39] and 7.8% seroprevalence in small ruminants of Karnataka[17] has been reported. Comparatively low seroprevalence of brucellosis in few states of India (Odisha and Kerala) should not be ignored because intermixing of flocks during summer and winter months and free trade between states within the country facilitate transmission of the disease to lower prevalent areas within no time.

Knowledge of seroprevalenec and spatio-temporal distribution of the disease is of paramount importance in strict surveillance and to strengthen the disease control program. This information is decisive in prioritizing the geographical regions for vaccination and implementation of other control strategies. With this study, some top 5 states can be prioritized (Bihar, Andhra Pradesh, Madhya Pradesh, Manipur and Rajasthan) for strict surveillance studies and to initiate control programs. Documentation of the spatial prevalence of the disease in the country and selection of suitable diagnostic assay are very challenging because of huge population of small ruminants. Brucellosis surveillance may be adopted in different ways viz., slaughter surveillance, on farm surveillance, livestock market surveillance, enhanced passive surveillance etc. The present study tries to provide spatial prevalence of brucellosis and to stress the need for continuous surveillance. Further strict random sample procurement drive from all other remaining states of the country to know the status of disease in small ruminants will facilitate disease mapping.

For any new test to be accepted for the diagnosis of brucellosis, it would be required to perform better than the existing tests in use. Here, iELISA kit with 95.66% and 96.33%, diagnostic sensitivity and specificity appears to be promising feature of the newly developed assay. So for, 35 kits were sold to the other organizations at nominal cost. The kit is not only generating revenue to the institute but also saving one fifth exchequer for other organization by avoiding importation of the kits. Hence iELISA kit for sero-screening of small ruminant brucellosis is recommended for large scale sero-prevalence of brucellosis throughout the country.

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