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A Study on the Epidemiology of Bovine Brucellosis in Punjab (India) Using Milk-ELISA

H. K. AULAKH, P. K. PATIL, S. SHARMA, H. KUMAR, V. MAHAJAN, K. S. SANDHU

Department of Epidemiology and Preventive Veterinary Medicine, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

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Abstract

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Bovine brucellosis, caused by *Brucella abortus*, is a serious zoonotic disease manifested by reproductive disorders resulting in huge economic losses to dairy farmers. A random survey was conducted to study the epidemiology of brucellosis in Punjab (India) using sampling software Survey Toolbox. Two-stage sampling procedure was adopted; in the first step, villages were selected randomly from sampling frame of all the villages of Punjab followed by selection of owners, and animals in individual farms were identified using random sampling. In all, 32 villages were selected and then 345 animals (approximately 5%) were sampled from these villages. The milk samples collected were screened for brucella antibodies employing ELISA test. The overall apparent prevalence of brucellosis was found to be 18.26% (true prevalence - 17.68%). The prevalence in the central zone of the state was significantly higher, viz. 23.2% (chi square = 11.34, $p < 0.01$) compared to 14.2% in the sub-mountainous zone and 5.8% in the arid irrigated zone. The disease prevalence was found to be non-significantly higher (chi square 1.029, $p = 0.310$) in cattle (20.67%) compared to buffaloes (16.41%) and increased with age (chi square = 8.572, $p < 0.05$) in both species. There was significant association between disease and abortion (chi square = 22.322, $p < 0.01$) and maximum abortion cases due to brucellosis were found in > 6 month of gestation (95.7%). The disease was significantly associated with the retention of placenta (chi square = 8.477, $p < 0.01$), however there was no significant relationship of the disease with repeat breeding (chi square = 0.044, $p = 0.834$). The results of the study suggested that the accurate epidemiological scenario of the disease may be obtained by employing multistage sampling procedures using milk-based ELISA.

Cattle, buffaloes, brucellosis, epidemiology, milk ELISA

Bovine brucellosis, caused by *Brucella abortus*, is a serious zoonotic disease manifested by reproductive disorders such as abortions, infertility, retention of placenta, stillbirth and calf loss in animals, and results in huge economic losses to dairy farmers (Radostits et al. 2000; Singh et al. 2002). Various studies have been conducted in India to establish the prevalence of the disease in bovines (Sandhu et al. 2001; Singh et al. 2004ab; Sharma et al. 2007) but these studies were based mainly on non-random sampling techniques. Hence the information gathered from such surveys cannot be extrapolated to apply to state or national bovine populations. Moreover, these studies were based on detection of antibodies in serum using conventional serological tests. The milk ring test is probably the most widely used test for the screening and monitoring of brucellosis in dairy cattle (Alton et al. 1988), but its sensitivity and specificity is questionable (Huber and Nicoletti 1986). In the last decade, several indirect enzyme-linked immunosorbent assays (ELISA) have been developed and successfully tested on milk samples (Romero et al. 1995; Vanzini et al. 2001). The present study was designed to assess the epidemiology of the disease in the bovines of Punjab (India) using milk ELISA employing a multistage sampling procedure.

Address for correspondence:
P. K. Patil
Department of Epidemiology and Preventive Veterinary Medicine
College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004
India

Tel: 91-161-2414030 (work)
Fax: 91-161-2400822
E-mail: pkpatilvet@yahoo.com
http://www.vfu.cz/acta-vet/actavet.htm

Materials and Methods

Study area

Punjab is the north-western state of India bordering with Pakistan on the west and situated between the 29.30° N to 32.32° N latitude and 73.55° E to 76.50° E longitude. Punjab state is broadly divided into 3 agro-climatic zones, viz. Sub Mountain Region (annual rainfall 800–900 mm), Central Plain Region (annual rainfall 500–800 mm) and Southern-Western Region (< 400 mm). Approximately 70% of human population live in villages and agriculture is the main occupation. India has 200 million cattle, 76 million buffaloes, 110 million goats and 46 million sheep, of which Punjab has 2.64 million cattle, 6.17 million buffaloes, 0.41 million goats and 0.44 million sheep. The estimates of milk, egg and wool production in 2003–2004 were 839.1 million kilograms, 3068 million numbers and 0.554 million kilograms, respectively (www.husbandrypunjab.org).

Selection of villages and animals

Villages form the basic geographic and administrative units in the state. To draw a simple random sample from the population of animals, two-stage sampling procedure was adopted. In the first step, villages were selected followed by selection of owners and animals and then animals were identified from the individual farm. A computerized list of the villages of the state was used as sampling frame and villages were selected (n = 32) from the whole state using simple random sampling, without replacement, using the 'Random Village' program of Survey Toolbox (Cameron 1999). The selected village was visited to prepare the sampling frame of all the farmers of the village having dairy animals. The animals were selected either using random number tables at the spot or in the laboratory using the 'Random Animal' program of the Survey Toolbox. Once the list of the owners and animals to be selected was ready, the farm of the owner was visited. As animals were not individually marked, the owner was asked to call out loud each animal. The animals were given temporary identification number based on the order of calling by the owner. Then the animal with a temporary number corresponding to the selected random number was identified. The total bovine population in these villages was 6980, of which 345 animals (approximately 5%) belonging to various owners were randomly selected. The 150 cattle selected were mostly Holstein-Friesian crossbred, whereas 195 buffaloes selected were mostly of the Murrah breed.

Processing of Milk samples

About 20–30 ml milk sample was collected from each selected animal without addition of any preservative. Milk samples were kept on ice while transported and stored at -20 °C until use.

ELISA

The samples collected were subjected to milk-based ELISA (VMRD, Inc-Pullman, WA, USA). Milk samples were tested undiluted after removal of the fat layer following centrifugation of milk samples. Briefly, 100 µl of samples and controls were added to antigen-coated plate and incubated at 25 °C for 30 min. The plate was washed four times, and 100 µl of diluted (1X) antibody-peroxidase conjugate was added to each well and incubated at 25 °C for 30 min. The plate was washed again, and 100 µl of substrate solution was added to each well, and incubated at 25 °C for 10 min. Then 100 µl of stop solution was added to each well, and the optical density (OD) was measured at 620 nm. The mean OD of negative controls for each plate should be < 0.50 and that of positive controls should be between 0.60–1.80. For interpretation of the test results, SP value was calculated:

$$\text{SP (\%)} = \frac{\text{(OD value of the sample - OD value of negative control)}}{\text{(mean OD of positive control - OD value of negative control)}} \times 100$$

Where:
SP = Sample /positive control ratio
Sample OD = OD value of sample
Mean NC = Mean OD value of negative control
Mean PC = Mean OD value of positive control
The samples producing SP ratio of < 25 were considered negative and with value > 25 were positive.

Statistical analysis

True prevalence was calculated at 95% confidence interval (CI) using the 'True Prevalence' program of the Survey Toolbox, in which sensitivity, specificity and sample size were taken into consideration (Cameron 1999). A standardized questionnaire for animals sampled at the farms was filled in at the time of blood collection. The data were analyzed using Epi Info 2002 (Dean et al. 2002) and SPSS (Statistical Package for Social Sciences) for Window version 11.0.1©SPSS Inc. USA computer software programs.

Results and Discussion

The milk samples from 345 randomly selected animals were analyzed with ELISA, which revealed prevalence of 20.67% and 16.41% in cattle and buffaloes, respectively; the overall apparent prevalence of brucellosis was 18.26%. Given the sensitivity and specificity of ELISA at 100 and 99.3%, respectively, true prevalence was calculated to be 17.68%

(95% CI 15.64–19.73). The prevalence in the present study is slightly lower compared to 22.5% reported by Gumber et al. (2004) employing milk ELISA on bulk milk samples collected from four districts of Punjab. Earlier studies have estimated the disease in the state from as low as 7.54% to as high as 18.07% (Sandhu et al. 2001; Singh et al. 2004ab; Sharma et al. 2007). The variation could be due to different survey techniques employed by various workers. While the whole population was taken as target population in this survey, earlier studies were either confined to some selected farms or conveniently selected samples. The primitive Rose Bengal plate test has been used for analysis of samples in most previous surveys as compared to the highly sensitive and specific ELISA in the present study. The results of indirect ELISA (SP ratio) were plotted against the number of infected and uninfected animals (Fig. 1). Among the samples considered negative for antibodies to brucella, the significant number of animals had a SP ratio of less than 0 (61.3%), followed by 0–10 (27.7%) and 10–25 (11.0%). Alternatively, among the samples diagnosed positive for brucella antibodies (SP ratio ≥ 25), no significant peak was observed. The proportionate distribution of animals above the SP ratio of 25 clearly indicates that once the animal becomes infected, the antibody titre in the animal continues to increase till it reaches the SP ratio of 150.

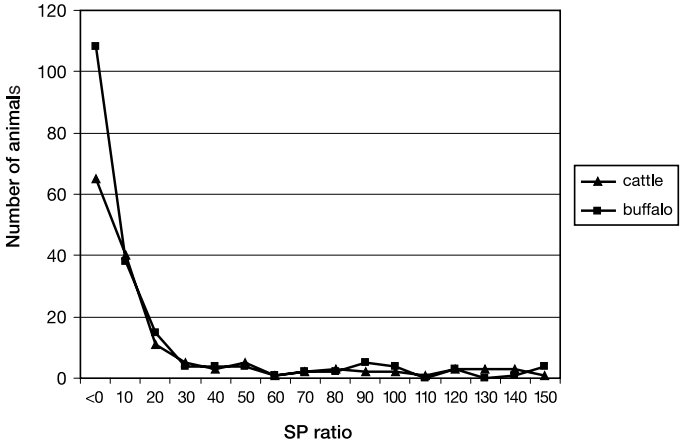

Fig. 1. Identification of uninfected versus infected animals in the bovine population

The prevalence in various districts had a significant variation. High prevalence was recorded in the districts of Moga, Ludhiana, Amritsar and Kapurthala (Fig. 2). All these districts fall within the central zone, which has significantly higher, viz. 23.2% (chi square = 11.34, $p < 0.01$) prevalence in comparison to 14.2% in the sub-mountainous zone and 5.8% in the arid irrigated zone. The central zone is the most fertile and has large-sized dairy farms; as a result, maximum sales and purchases of animals occur in this zone of the state. Cow slaughter is banned in the state, hence the dairy farmers mostly sell brucella-positive animals in cattle markets and the disease gets transmitted to another farm. Furthermore, farmers usually do not screen the animals against brucellosis prior to purchase, consequently the introduction of a single infected animal at the farm leads to a storm of abortions in the herd. Earlier studies have also reported higher prevalence of the disease at organized dairy farms (Singh et al. 2004a).

Various host factors associated with the disease were studied so as to help in the formulation of a control programme. Species-wise (Table 1) disease prevalence was found to be non-significantly higher (chi square 1.029, $p = 0.310$) in cattle (20.67%) as compared

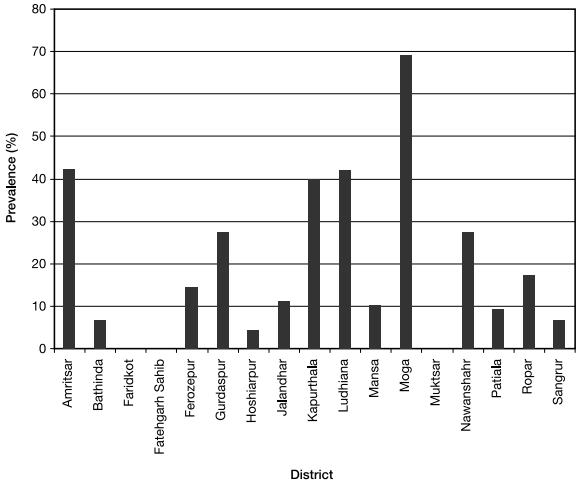

Fig. 2. Prevalence of brucellosis in various districts of Punjab

Table 1. Species-wise prevalence of brucellosis

Species	Total	Positive	% Prevalence
Cattle	150	31	20.67
Buffalo	195	32	16.41
Total	345	63	18.26

Chi square - uncorrected	1.029 (P = 0.310)
Chi square - Mantel-Haenszel	1.026 (P = 0.311)
Chi square - corrected (Yates)	0.764 (P = 0.382)
Odds ratio	1.327 (95% CI = 0.770-2.286)
Risk ratio	1.259 (95% CI = 0.807-1.961)

to buffaloes (16.41%). Risk of disease was found to be higher in cattle (1.3 times) in comparison to buffaloes as revealed by the risk ratio and odds ratio. Similarly to the present results, most of the studies conducted earlier have also reported higher prevalence of the disease in cattle as compared to buffaloes (Sharma et al. 1979, 2003; Chatterjee et al. 1986), whereas in contrast, higher disease prevalence in buffaloes in comparison to cattle was reported by some authors (Sharma and Saini 1995; Dhand et al. 2005). Thus, for any control program to be successful, both species will have to be

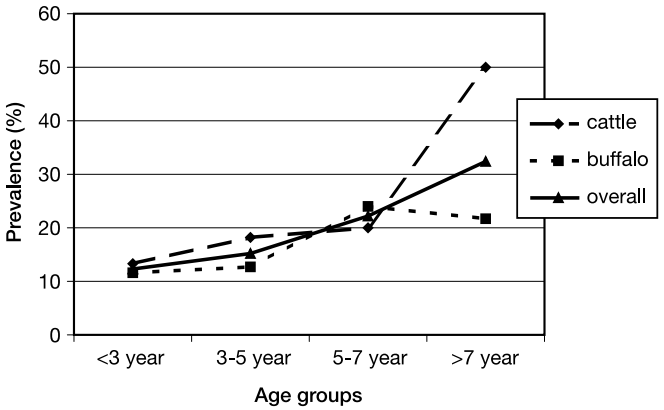

Fig. 3. Age-wise prevalence of bovine brucellosis

Table 2. Association of brucellosis with abortion, retention of placenta (ROP) and repeat breeding

History	Species	Total cases	Disease positive	Prevalence
Abortion	Cattle	31	13	41.93%
	Buffalo	26	10	38.46%
Total abortion cases		57	23	40.35%
No abortion history		288	40	13.89%
$\chi 2 = 22.322$, $p = < 0.01$, Odds ratio = 4.19, Relative risk = 2.19				
ROP	Cattle	9	5	55.5%
	Buffalo	6	2	33.3%
Total ROP cases	15	7	46.7%	
No ROP history	330	56	16.97%	
$\chi 2 = 8.477$, $p = < 0.01$, Odds ratio = 4.28, Relative risk = 2.75				
Repeat	Cattle	23	2	8.69%
breeding	Buffalo	18	5	27.77%
Total repeat breeding cases		41	7	17.07%
No repeat breeding history		304	56	18.42%
$\chi 2 = 0.044$, $p = 0.834$				

included in the programme. The slight variation in results can also be attributed to species susceptibility, as cattle, especially the crossbred cattle having exotic germplasm, are more susceptible to stress conditions than buffaloes. Genetic differences in cattle and buffalo may be another reason for low prevalence in buffaloes.

Other host factor studies were the age groups of animals. In the present study, animals were grouped into four age groups, viz. < 3 years, 3–5 years, 5–7 years and > 7 years and the prevalence of brucellosis in different categories was found to be 12.3, 15.2, 22.2 and 32.4%, respectively. It was found that disease prevalence increased with age in both species (Fig. 3). In animals of the age group > 7 year, significantly higher prevalence of disease (chi square = 8.572, $p < 0.05$) was recorded. Lower prevalence of brucellosis in young ones could be attributed to resistance of sexually immature cattle to infection, which become susceptible to disease with age (Paul 1980), or passive immunization of calves through colostrum of their infected dams. Similar results had been reported by various authors (McDermott et al. 1987a,b; Ahmad and Munir 1995; Silva et al. 2000). Although susceptibility to disease increases with age, it seems to be more commonly associated with sexual maturity than age (Radostits et al. 2000).

Brucellosis is reported to cause abortion, retention of placenta, repeat breeding, infertility and prolonged intercalving period due to early embryonic deaths (Roberts 1999). Out of 345 animals screened (Table 2), 57 animals had a history of abortion and of these, 23 animals (40.35%) were positive for brucellosis. There was significant association between disease and abortion (chi square = 22.322, $p < 0.01$) and the risk of abortion was 4.19 times higher in animals with brucellosis than without brucellosis. Similarly to the present study, Saini et al. (1992) reported 38.18 and 41.8% abortions in cattle and buffaloes due to brucellosis, respectively. The results are also in alignment with studies conducted by various authors (Jha et al. 1993; Srinivasa et al. 1999; Sandhu et al. 2001). The study confirms brucellosis to be the major aetiological agent of abortion in farm animals in the state. Out of 23 abortion cases due to brucellosis, maximum cases of abortion were found in > 6 months of gestation (95.7%), only 4.34% in 3–6 months of gestation and none of the positive animals had history of abortion in 1–3 months of gestation. The higher incidence of abortion in the third trimester is due to the uterine environment conducive for the multiplication of the bacteria, which in turn causes foetal death and abortion by invading placental cotyledons and destroying villi. During the survey, retention of the placenta was noticed in 15 animals and of these, 7 animals were found positive for brucellosis. There

was significant association between disease and retention of the placenta (chi square = 8.477, $p < 0.01$) and the risk of placental retention was 4.28 times higher in animals with brucellosis than without brucellosis. However, the number of animals with a history of retention of the placenta was too small to arrive at a meaningful conclusion. In the present survey, 41 animals had a history of repeat breeding and out of these, 7 animals (17.07%) were found positive to the disease. There was no significant relationship between repeat breeding and brucellosis (chi square = 0.044, $p = 0.834$) in the present study. The present findings are in agreement with Bachh et al. (1988) who reported brucellosis to be higher in cattle with a history of abortion (89%) as compared to those with a history of returns to service (67%). In another study (Rahman et al. 1997), prevalence of brucellosis was the highest in the buffaloes with retained placenta (7.1%) and the lowest in repeat breeder buffaloes (1.2%).

The results of this study show that brucellosis is widespread in cattle and buffaloes of Punjab (India). In India, cow slaughter is banned; as a result, either the dairy farmer has to keep the infected cattle at the farm, or these cattle are left on the roads spreading the disease to healthy cattle. Therefore, the only alternative to control and eradicate the disease from the healthy population is a statutory mass vaccination programme.

Epidemiologická studie bovinní brucelózy v Pandžábu (Indie) s využitím mléčného ELISA testu

Bovinní brucelóza, způsobovaná *Brucella abortus*, je významná zoonóza, které se projevuje poruchami reprodukce, což způsobuje farmářům závažné ekonomické ztráty. Náhodný průzkum s využitím testovacího software Survey Toolbox byl proveden za účelem provedení epidemiologické studie brucelózy v Pandžábu (Indie). Byl využit postup dvojitého výběru vzorků. V první fázi byli ze všech vesnic v Pandžábu náhodně vybráni farmáři a následně byli vybráni majitelé a zvířata z jednotlivých farem, taktéž pomocí systematického náhodného výběru. Celkem bylo vybráno 32 vesnic, z kterých bylo 345 zvířat testováno (cca 5 %). Získané vzorky mléka byly testovány na přítomnost protilátek proti brucele pomocí ELISA testu. Relativní prevalence brucelózy byla 18,26 % (skutečná prevalence – 17,68 %). Prevalence v centrální oblasti státu byla významně vyšší, tj. 23,2 % ($\chi^2 = 11,34$, $p < 0,01$) ve srovnání s 14,2 % v podhůří a 5,8 % v suchých zavlažovaných oblastech. Prevalence výskytu brucelózy byla nevýznamně vyšší ($\chi^2 = 1,029$, $p = 0,310$) u skotu (20,67 %) ve srovnání s buvoly (16,41 %). U obou druhů vzrůstala s věkem ($\chi^2 = 8,572$, $p < 0,05$). Byla zjištěna významná souvislost mezi onemocněním a aborty ($\chi^2 = 22,322$, $p < 0,01$). Nejvyšší počet abortů kvůli brucelóze byl pozorován ve více než 6 měsících gestace (95,7 %). Toto onemocnění statisticky významně souviselo s retencí placenty ($\chi^2 = 8,477$, $p < 0,01$), ale nebyla zjištěna významná souvislost mezi onemocněním a zhoršeným zabřezáváním ($\chi^2 = 0,044$, $p = 0,834$). Výsledky studie naznačují, že přesná epidemiologická situace může být zjišťována s využitím vícestupňového testování za pomoci mléčného ELISA testu.

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