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A study on the epidemiology of brucellosis in Punjab (India) using Survey Toolbox

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Summary

A random survey was conducted to study the epidemiology of brucellosis in Punjab (India), using the 'Survey Toolbox' sampling software. A two-stage sampling procedure was adopted: in the first stage, villages were selected, and in the second the selection of animals was made. In all, 52 villages were selected randomly from a sampling frame of all the villages of Punjab. The total number of animals in these villages was 18,644, out of which 973 animals (approximately 5%) belonging to various owners were randomly selected. Serum samples collected from the animals were screened for *Brucella* antibodies by an avidin-biotin enzyme-linked immunosorbent assay, which showed the apparent overall prevalence of brucellosis to be 12.09% (true prevalence, 11.23%). The prevalence varied from a low of 0% to a high of 24.3% in various districts. Higher variance (0.08) was noted within villages than between different villages (0.03). The prevalence rates among buffaloes and cattle were 13.4% and 9.9%, respectively. The seroprevalence of brucellosis was found to be significantly higher (chi square = 24.50, $p < 0.001$) in animals with a history of abortion (33.87%) than in those without such a history (11.63%).

Keywords

Avidin-biotin enzyme-linked immunosorbent assay – Bovine brucellosis – Epidemiology – India – Punjab – Risk factor – Survey Toolbox.

Introduction

Brucellosis is a serious zoonotic disease that causes abortions, infertility, retention of placenta, stillbirth and calf loss in animals, and results in huge economic losses to dairy farmers (1, 14). Various surveys have been conducted in India to establish the prevalence and risk factors associated with the disease (7, 8, 13, 15) in bovines but all these surveys appear to have used primitive techniques – mostly the purposive selection method or convenient sampling methods, which are non-random selection approaches. Hence the information gathered from these surveys cannot be extrapolated to apply to state or national bovine populations. The present study was therefore designed to assess the epidemiology of the disease in cattle and buffalo (*Bos bubalis*) in Punjab (India) using the 'Survey Toolbox' random sampling software (2). This study appears to be the first of its kind in India that has used such software and whose results can be projected at the state and national level. On the basis of the results obtained, programmes can be developed to control or eradicate the disease.

Materials and methods

Villages form the basic geographic and administrative units in the state of Punjab. Most of the farms in the villages are small; the animals are in close contact and tend to mingle freely while grazing or drinking water. The animals, which are reared using similar husbandry techniques, are thus exposed to the same infectious diseases. For the purposes of this survey, all the animals in the village were therefore considered to belong to one large herd, although owned by many different people. To draw a simple random sample from the population of animals in the village was difficult, as there were so many different owners, and no sampling frame was available. In addition, animals were not individually identified. To overcome these problems, a two-stage sampling procedure was adopted: villages were selected in the first stage, and in the second Survey Toolbox was used to select animals (2). A total of 52 villages were selected by one of the research institutes of the Indian Council of Agricultural Research in Bangalore, namely, the Project Directorate on Animal Disease Monitoring and Surveillance (PD-ADMAS). The selection was made by means of simple random sampling, without replacement and without stratification, while animals were selected via probability proportional to size sampling.

Avidin-biotin serum enzyme-linked immunosorbent assay technique

The enzyme-linked immunosorbent assay (ELISA) was performed with an avidin-biotin (AB) ELISA kit procured from the PD-ADMAS, and the protocol provided by the developers was followed precisely. The kit had been calibrated by the developers, using the indirect brucellosis ELISA kit of the International Atomic Energy Agency as a reference. The cut-off percentage positivity value was established by the PD-ADMAS, using brucellosis-positive and brucellosis-negative serum samples confirmed by means of the indirect ELISA kit. The overall specificity and sensitivity of AB ELISA have been reported to be 98.8% and 98.2%, respectively (12). The kit has also been validated for use in the field by six laboratories in India.

Briefly, for antigen coating, the required volume of working dilution of smooth lipopolysaccharide (S-LPS) antigen was prepared in coating buffer (carbonate and bi-carbonate buffer), by adding 4 µl of S-LPS stock solution per ml of coating buffer. A total of 100 µl of working dilution of S-LPS was dispensed into all 96 wells of a micro-plate. After overnight incubation at 4°C, the plate was washed three times with wash buffer, after which control and test serum samples (1:100 dilution of dilution buffer) were added. Two immunoconjugates, biotin-anti-bovine IgG and avidin-horseradish peroxidase working solution, were added at a dilution rate of 2.5 µl and 4 µl, respectively, per ml of dilution buffer. This was followed by addition of substrate (H_2O_2) at a dilution rate of 4 µl per ml of chromogen solution. The reaction was stopped by adding 1% H_2SO_4 and results were read at 492 nm using an ELISA reader.

Positive and negative controls were provided with the ELISA kit. A plate was selected for analysis only if its strong positive control optical density (OD) ranged between 0.5 and 1.0 OD, as recommended by the kit manufacturers. Then the median absorbance of four strong positive control wells was calculated. Percentage positivities (PP) of test sera and control were calculated as:

$$PP = \frac{\text{OD of test well}}{\text{median OD of strong positive wells}} \times 100.$$

PP values equal to or greater than 28% in the test wells were considered positive, as recommended by the kit manufacturers.

Statistical analysis

The data was analysed using the following software:

– Win Episcope 2.0 (software developed by a group of researchers in the Netherlands, the United Kingdom and Spain, namely, I. De Blas, C. Ortega, K. Frankena, J. Noordhuizen & M. Thrusfield)

– Epi Info 2002 (4)

– SPSS (Statistical Package for Social Sciences) for Windows version 11.0.1© (SPSS Inc.).

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. In the analysis, only the apparent prevalences were compared. Correlation and regression values were calculated using the Epi info 2002 program. A chi square test at 95% level of significance was used to compare categorical variables: districts, species, history of abortion and month of abortion.

Chi square test compares the actual observed frequencies in the sample with the expected frequencies if there were no relationship between the variables. This test is based on the assumption that the sample frequencies are normally distributed around the expected value, and for it to be true the expected frequencies of all cells of the table should be large (preferably ≥ 5) and no cell in the table should have a frequency of zero. In the case of the 'district' variable, the expected frequency of one cell was less than one and of six others was less than five; therefore, some districts that were in close geographic proximity were collapsed together so as

to make the chi square test valid. Similarly, the expected frequencies of the 'month of abortion' variable were less than one in three cells, and less than five in ten cells; adjacent months were therefore collapsed together to form three categories representing three trimesters of pregnancy. The continuous variable 'age' was converted into a categorical variable, as animals were categorised into five groups and data were analysed using the chi square test. Comparisons within various categories of a variable were made on the basis of the contribution of each cell to the chi square test statistic. Each cell contributes a little less than one degree of freedom (DF) to the overall DF, so if the contribution for a particular cell exceeded four, the category was considered to be significantly different from the others. The odds ratio and risk ratio were calculated by means of standard epidemiological procedures to find out the strength of association of various factors.

Results

A total of 52 villages were selected randomly from a sampling frame of all the villages of Punjab. In all there were 18,644 animals in these villages, out of which 973 animals (approximately 5%) belonging to various owners were selected at random. Serum samples from these animals were analysed with AB ELISA, which showed the apparent overall prevalence of brucellosis to be 12.09% (CI = 10.33-13.85). Given the sensitivity and specificity of the ELISA (12) at 98.2% and 98.8%, respectively, the true prevalence was calculated to be 11.23% (CI = 10.18-12.26).

The seroprevalence of brucellosis varied significantly (chi square = 63.37, $p < 0.001$) from a low of 0% to a high of 24.3% in various districts of the state.

The study also investigated whether the prevalence varied with the bovine population in different districts. For this purpose, a correlation and regression analysis was made of the data about the prevalence of disease in various districts in relation to the bovine population in these districts. The results indicated weak (though positive) correlation (0.27) of the disease with bovine population, while regression statistics indicated non-significant association ($F = 0.07$; $P = 0.8$).

Village-wise prevalence of the disease also varied from 0% to 63.2%, with variance ranging from 0 to 0.27 in various villages. Higher variance was found within individual villages (0.08) than between different villages (0.03). It was hypothesised that the seroprevalence of brucellosis may have some relationship to the size of the bovine population (both cow and buffalo populations) of the village. However, no such association that was statistically significant could be found.

Various host factors associated with the disease were studied so as to help in formulating a control programme. Species-wise, seroprevalence in cattle was found to be 9.9% and in buffaloes to be 13.4%; however, this difference was not statistically significant (chi square = 2.796, $p = 0.095$).

Another host factor studied was the age of animals. It was found that disease prevalence varied with age, with the lowest prevalence in the age group of 'up to two years' (0.6%), increasing to 11.8%, 12.4% and 15.8% in animals of age groups 'two to four years', 'four to six years' and 'six to eight years' respectively. The seroprevalence was 13.6% in animals older than eight years. Considering the complete data, the prevalence was significantly different in different age groups (chi square = 9.8, $p = 0.04$), with the maximum contribution towards significance coming from the age group of 'up to two years'. However, if this young age group is removed from the data, the difference in the prevalence in the remaining groups becomes non-significant (chi square = 1.82, $p = 0.6$)

Other risk factors were the history and month of abortion. For analysis of these factors, the age group of up to two years was removed from the data to avoid bias as there are no chances of abortion in animals below two years of age (this fact was also verified from the data). Of the remaining 810 animals (of more than two years of age), 62 animals (7.65%) were found to have a history of abortion. The seroprevalence of brucellosis was found to be significantly higher (chi square = 24.50, $p < 0.001$) in animals with a history of abortion (33.87%) than in those without such a history (11.63%). The animals with history of abortion were 3.89 times more likely to be seropositive than those without such history.

In terms of the month of abortion, the largest number of abortions (25.80%) occurred in the sixth month of pregnancy. For analysis by chi square test, the 'month of abortion data' were collapsed to form three categories, containing animals aborted in the first (9.67%), second (53.22%) and third (37.09%) trimester of pregnancy. The seroprevalence of brucellosis was 16.67%, 27.27% and 33.34%, respectively, in animals with histories of abortion in the first, second and third trimester of pregnancy. However, these differences were found to be statistically non-significant (chi square = 3.43, $p = 0.18$).

Discussion

A high prevalence (11.23%) of brucellosis was detected in Punjab (India) in the random survey described in this paper. Earlier studies (7, 8, 13, 15) have given varying estimates of the prevalence of disease in the state, ranging from a low of 7.54% to a high of 26.6%. The differences

among these studies could be due to the different survey techniques employed by various researchers. While the whole of the bovine population was taken as the target population in this survey, earlier studies were either confined to selected farms or analysed conveniently selected samples. Also the Rose Bengal plate test was used for analysis of samples in most of the previous surveys rather than the highly sensitive and specific AB ELISA in the present study. Other researchers (11, 16, 17) have also noted the variation in seroprevalence that was found when different tests were used.

The results reported here are generally in agreement with a broadly based survey (6) conducted in the state using a milk-based ELISA technique, in which prevalence was found to be 8.48%. The high prevalence found in the present study is of great significance as the disease causes huge economic losses. Abortions caused by the organism are reported to cause a loss of Rs. 5,098.5 per animal, which is almost half of the value of the animal (14). In addition brucellosis causes infertility and records show (6) that 51.31% of all cases of infertility in the area under study are due to this disease. It also has public health significance, as disease can be transmitted to humans such as farm workers from infected animals. A study has shown that the risk of humans on a farm being infected is higher when brucellosis is more prevalent among the animals (unpublished data). In humans the disease causes very painful symptoms, including headaches, body aches, shivering and orchitis, as well as causing loss of labour. Therefore, urgent steps are needed for its control in the state.

In terms of districts, high prevalence was noted in Gurdaspur, Amritsar and Hoshiarpur. This higher rate could be due to a greater number of movements of animals in these districts. However, agroecological and climatological factors may also favour the perpetuation of the disease in these districts, as they are located in only two agro-climatic regions of Punjab: the Central Plain region and Undulating Plain region. Further independent studies are needed in these districts to identify risk factors.

Earlier studies (10) have reported that the bovine population on farms is an important risk factor for the prevalence of brucellosis. As in this study, villages rather than farms were taken as units, so prevalence was expected to be higher in villages with larger herds of cattle. However, contrary to this assumption, this study found that the prevalence of disease had no significant relation to the village bovine population or the bovine population in the district. The authors could not find reasons for higher intra-village variance as compared with inter-village variance. Further studies are needed to elucidate the reasons.

Two host factors were studied in the survey: the species and age groups of animals. No significant differences could be found in the seroprevalence of disease in the two different species studied, cattle and buffalo. The results show that buffaloes are as prone to disease as cattle, if not more so, contrary to the earlier notion that buffaloes are more resistant. Results of earlier research (5) give credence to the results of this study. Thus, for any control programme to be successful, both species will have to be included in the programme.

The age of animals was categorised to perform the chi square test. The seroprevalence was found to be significantly lower in animals of less than two years of age. (A lower prevalence of brucellosis in young animals had also been reported earlier [9].) The reason may be that with the passage of time animals are more likely to be exposed to the bacteria and contract the disease. Overall seroprevalence was found to vary significantly in different age groups, but if data on animals up to two years of age are removed from the database, no significant differences can be detected in seroprevalence in the other age groups. This indicates that the overall significance is actually due to a very low seroprevalence in the young age group and not to real differences between age groups.

Of the animals of reproductive age (excluding animals aged less than two years to avoid bias), 7.65% had a history of abortion, and of these 33.87% were seropositive. The seroprevalence was significantly higher in animals with histories of abortion than in those without such histories, indicating that brucellosis is a major cause of abortions in the region. Other diseases and conditions are prevalent in the region under study and account for the other 66.13% of abortions. Further investigations are needed to explore those diseases and conditions so that holistic control programmes for all abortion-causing diseases can be designed. Animals with histories of abortion in different trimesters of pregnancy were compared, but no significant differences could be found in the seroprevalence of the disease, indicating that abortions can occur in any trimester of the pregnancy.

Conclusions

A high prevalence of brucellosis was detected in Punjab; therefore, urgent steps are needed for its control. The prevalence of disease had no significant relation to the size of bovine populations in either individual villages or districts. Young animals had a low seroprevalence of the disease. The prevalence was not significantly different in cattle and buffalo.

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Étude sur l'épidémiologie de la brucellose dans le Punjab (Inde) en utilisant le logiciel Survey Toolbox

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Résumé

Une étude aléatoire a été effectuée sur l'épidémiologie de la brucellose au Punjab (Inde) en utilisant le logiciel d'échantillonnage « Survey Toolbox ». On a adopté un protocole d'échantillonnage en deux étapes : la première étape a consisté à sélectionner les villages, la seconde, les animaux. En tout, 52 villages ont été sélectionnés de façon aléatoire dans un cadre d'échantillonnage regroupant tous les villages du Punjab. Sur les 18 644 animaux que comptait l'ensemble de ces villages, 973 animaux (environ 5 %) appartenant à divers propriétaires ont été sélectionnés aléatoirement. Les échantillons de sérum prélevés sur les animaux ont été soumis à une recherche d'anticorps dirigés contre *Brucella* à l'aide d'une épreuve immuno-enzymatique (ELISA) à l'avidine-biotine, qui a mis en évidence une prévalence globale apparente de la brucellose de 12,09 % (prévalence réelle, 11,23 %). La prévalence variait entre 0 % et un maximum de 24,3 % selon les districts. On a constaté une variance plus élevée (0,08) à l'intérieur des villages qu'entre eux (0,03). Le taux de prévalence chez les buffles et les bovins était respectivement de 13,4 % et 9,9 %. La séroprévalence de la brucellose était significativement plus élevée (khi carré = 24,50, $p < 0,001$) chez les animaux ayant avorté (33,87 %) que chez les autres (11,63 %).

Mots clés

Brucellose bovine – Épidémiologie – Épreuve immuno-enzymatique à l'avidine-biotine – Facteur de risque – Inde – Punjab – Survey Toolbox.

Estudio sobre la epidemiología de la brucelosis en el Punjab (India) con 'Survey Toolbox'

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Resumen

Los autores describen un análisis por muestreo aleatorio destinado a estudiar la epidemiología de la brucelosis en el Punjab (India), para el cual se utilizó un programa informático de muestreo denominado 'Survey Toolbox' [Módulo de análisis por muestreo]. Se adoptó un protocolo de muestreo en dos fases: en la primera se seleccionaban los pueblos, y en la segunda los animales que se iban a someter a prueba. A partir de un marco de muestreo compuesto por todos los pueblos del Punjab, se seleccionaron aleatoriamente 52 pueblos. En ellos había un total de 18.644 animales, de entre los cuales se seleccionaron al azar 973 (aproximadamente un 5%) pertenecientes a diversos propietarios. Tras extraer las pertinentes muestras séricas, se sometieron éstas a un ensayo inmunoenzimático (ELISA) con avidina-biotina para detectar anticuerpos antibrucélicos. A partir de los resultados se calculó una prevalencia general aparente del 12,09% (prevalencia real: 11,23%). En varios distritos, este parámetro oscilaba entre el 0% y el 24,3%. Se obtuvo una varianza mayor dentro de los pueblos (0,08) que entre pueblos distintos (0,03). Las tasas de prevalencia en búfalos y bovinos resultaron de 13,4% y 9,9% respectivamente. Se observó también que la seroprevalencia de brucelosis en los ejemplares con antecedentes de aborto era significativamente mayor (33,87%) (ji cuadrado = 24,50; $p < 0,001$) que en los demás animales (11,63%).

Palabras clave

Brucelosis bovina – Ensayo inmunoenzimático con avidina-biotina – Epidemiología – Factor de riesgo – India – Punjab – Survey Toolbox.

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