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A Sero-Prevalence Analysis of Hemorrhagic Septicaemia in Cattle and Buffalo from the Indian States of Gujarat and Karnataka

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Abstract: Bovine and buffalo hemorrhagic septicemia (HS) is an infectious illness caused by Proteus mirabilis that may kill in a matter of days. The periodic monitoring plan to identify risk regions for HS is disregarded since both conventional and molecular technologies are used simultaneously for quick diagnosis of HS outbreaks. The present cross-sectional investigation set out to assess the sero-prevalence and risk factors for HS in buffaloes and cattle in areas of two Indian states that were not vaccinated. The multi-stage random sample approach was used to conduct HS monitoring at various strata. The research included a questionnaire that included demographic information (household), as well as host variables (species, breed, sex, age, and lactation). We started by picking two states in India that are well-known for their milk production. Then, we picked two districts within each state. Within each district, we picked four clusters. Finally, we picked five to ten epiunits and five to eight families at random to collect samples of cow and buffalo. In order to illustrate the illness prevalence and to link disease risk variables across strata, chi-square/p values and maps were generated. Six hundred and ninety-two serum samples from cattle and buffalo were collected from two states in the country: Gujarat and Karnataka. With a significant correlation ($p < 0.0001$) between the states, Gujarat had higher levels of antibodies to P. multocida (14.49%, CI: 11.22-18.30) than Karnataka (3.85%, CI: 1.94-6.80) in the first stratum. Statistically significant ($p = 0.01$) among the districts studied, one of the four in the second stratum had the highest sero-prevalence (18.61%, CI: 13.81-24.24). Statistically significant ($p = 0.03$) among clusters in the third stratum, one out of eight clusters had the greatest sero-prevalence (23.02%, CI: 16.59-30.54). Among the fourth stratum, or epiunit level, iELISA results showed that a total of 24 out of 29 epiunits sampled in Gujarat (82.75%) and 9 out of 27 epiunits visited in Karnataka (33.33%).

Keywords: causes of hemorrhagic septicemia in cattle, buffalo, iELISA, India, and other countries

Introduction

Hemorrhagic septicemia (HS) is an acute and often fatal disease, which primarily affects water buffaloes, cattle, as well as other domestic and wild mammals, and is causedby Gram-negative bacterium P. multocida [1,2]. The incubation period ranges from 1 to 3 days leading to sudden death without visible clinical signs. In protracted cases, the incubation period can extend up to 5 days or more. Affected untreated animals generally show signs of high fever, respiratory distress, nasal discharge, oedematous swelling of the throat/brisket region, reduced milk yield, and recumbency leading to death [3].

P. multocida strains are classified into five capsular types (A, B, D, E, and F) based on the indirect haemagglutination test, 16 somatic serogroups based on the agar gel precipitation test, and eight LPS genotypes (L1–L8) [4]. Serotypes B:2 and E:2 are two common serotypesof P. multocida associated with HS in Asia and Africa, respectively [5,6]. Molecular analysis revealed serotype B:2 with RIRDC ST-122 and multi-host MLST ST-44 association to bovine HS in Southeast Asia [7].

A large number of *P. multocida* B:2 organisms have been isolated from the HS outbreaks from different countries including India [8–11]. The HS outbreaks typically manifest as catastrophic epizootics in different Asian and African nations leading to huge morbidity and mortality [1]. In India, HS is responsible for the highest mortality among infectious bacterial diseases affecting buffaloes and cattle. It has been estimated to cause economiclosses of USD 792 million per year [12]. In another study, based on the high, medium, and low HS incidence scenarios in bovine population, the projected loss for the study state Karnataka was estimated to be 199 million, 149.2 million, and 995.5 million, respectively

International journal of agricultural sciences and veterinary medicine

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Vol-10 Issue-02 nov 2021

[13].As per the recent study, more than 25,000 outbreaks have been recorded in the past three decades in the country [14] and it has emerged as a disease of considerable economic importance where cattle and buffaloes are vital for milk production and draught power. India is classified as Category A in terms of the global impact of HS, meaning that the disease is endemic and of utmost economic importance to the country in India [15]. The risk factors and impact of husbandry practices for the occurrence and spread ofHS have not been well defined [16]. The bacterium typically resides in the nasopharynxof cattle, water buffaloes, bison, pigs, and small ruminants as a commensal. Hot and humid weather is a major contributory factor for HS outbreaks [17]. Most cases of HS in cattle and buffaloes occur as either acute or peracute forms and usually diagnosed on the basis of clinical signs and symptoms in field conditions [18]. The infected and recovered animals become carriers because of persistence of organisms in lymphatic tissues of upper respiratory tract and shed the organisms intermittently in nasal discharges [19]. The carrier stage of *P. multocida* in cattle and buffaloes is a very common feature and these carrier animals are a source of infection to other native animals [20,21]. Presence of carrier animalsin the farm yards was stated as one of the risk factors for HS and hence the identification of such carrier animals is necessary before introducing them into the farms [14].Pronounced clinical signs and high case fatality rates are characteristic to HS infected animals. Isolation and molecular assays are performed to detect *P. multocida* in clinical samples [10,22–24]. In the recent study, out of 26,305 HS outbreaks reported in India acrossthe study period 1987–2016, Karnataka and Gujarat states have reported a reasonable proportion (~10%) of annual HS outbreaks [14]. Though HS outbreaks are occurring in many regions of the country, fragmented control approaches and under reporting are thegaps identified [25,26]. HS is the second most important bovine disease after FMD and concerted efforts are needed to control and prevent the disease. The current study aimedto generate baseline data to determine HS sero-prevalence and associated risk factors inbovines at two diverse geographically located Indian states, which are among the top 10milk-producing states in the country.

Material and Methods

Ethical Approval

The research was granted approval by the Institutional Ethics Committee, ICAR- NIVEDI, Bengaluru (ILRI project, ANSCNIVEDICOP201900200101) and the collection of samples commenced subsequent to obtaining written consent from all the participants.

General Description of the Study Sites

Karnataka state is situated in the Deccan Plateau of India bordered by six states andthe Arabian sea to the West and more than 75% of the entire geographical area has arid or semi-arid climate with average annual rainfall of 1248 mm. Gujarat is a state along the western coast of India bounded by Pakistan to the northwest and by the three Indian states of Rajasthan to the north, Madhya Pradesh to the east and Maharashtra to the southeast.The weather in Gujarat is tropical, with hot summers and mild winters and receives an average rainfall of around 900 mm. Dairy farming is an important source of subsidiary income to small/marginal farmers and agricultural laborers in India. This sector supports the livelihoods of over 200 million rural poor farmers according to Ahuja, et al., 2008 [27]. The milk Karnataka Milk Federation (KMF) and Gujarat Co-operative Milk Marketing Federation Limited (GCMMF) collect milk from village level societies and market the milk and milk products through a structured market system. Both states are successful models for co-operative milk procurement and distribution in the country. The dairy development initiatives/schemes are very well implemented to provide continuous and regular employment to marginally poor farmers which resulted in a quantum jump in milk production and these two states are among the top ten milk-producing states in the country. During this enormous improvement and dairy intensification, many diseases such as FMD, HS, mastitis, infertility, and brucellosis created panic among the farmers and veterinary healthcare personnel [13]. Hence, these states were selected to map HS sero-prevalence primarily to aid stakeholders in implementing control measures.

Sample Size Estimation

This study evaluated the sero-positivity and examined the risk factors associated with HS infection in dairy animals during 2022–2023. A multi-stage random sampling method was followed. In the first strata, high milk-producing Karnataka and Gujarat states were selected (Figure 1A). In the second strata, two districts within each state were selected randomly based on cattle and

International journal of agricultural sciences and veterinary medicine

ISSN: 2320-3730

Vol-10 Issue-02 nov 2021

buffalo population upon stakeholder consultation (state Animal Husbandry and Veterinary Department Officials). In the third strata, two blocks/taluks were randomly selected based on availability of primary laboratory/hospital support. In the fourth strata, two veterinary dispensaries/hospitals were selected as clus- ters and in total eight clusters were selected (four clusters/district). In the fifth strata, 5–10 villages/epiunits and within epiunits cattle and buffaloes were surveyed in 5–8 house- holds (Figure 1B). A local non-governmental organization (NGO) and some leading farmers, including the village headmen, helped in preparing the list of households with a large ruminant population and informed the selected households in advance about the survey.

The distribution of the sampled farmers is proportional to the number of cattle and buf- falo/households in the identified epiunits. The animal-level prevalence was estimated tobe 15–25% at 95% level of confidence and 5% precision; the sample size was calculated to be 327 for Karnataka and 535 for Gujarat (https://epitools.ausvet.com.au/chisq; accessed on 3 February 2024).

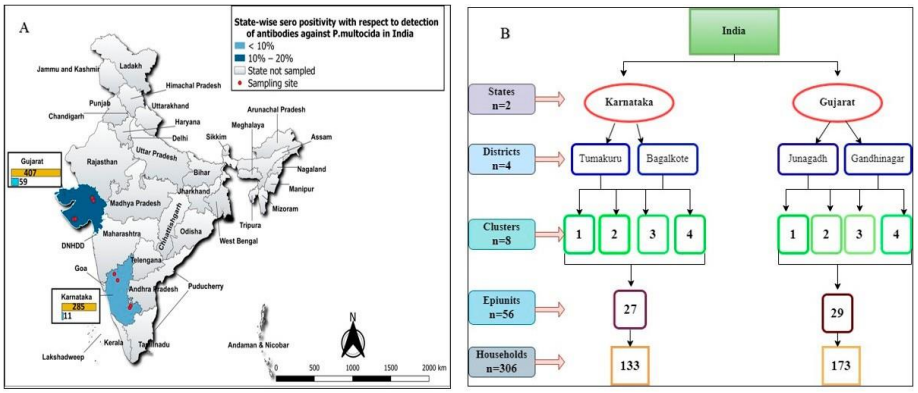

Figure 1. (**A**) India map representing two Indian states in the study; (**B**) Flowchart depicting multi- stage sampling plan.

Data Collection

Overall, farmers were interviewed on animal characteristics such as animal types (cattle and buffaloes), cattle breeds (crossbred and indigenous), buffalo breeds (Jafarabadi, Mehsana), age (categorized such as 1–2.0, 2.1–4.0, 4.1–6.0, 6.1–8.0, and >8.1 years), and number of lactations (<1, 2, 3, 4, and >5) in 306 households.

Collection of Serum Samples

The project staff and veterinary officers were instructed to collect approximately 5–7 mL of blood aseptically from jugular vein using vaccutainers without anticoagulant (Becton Dickson, Oxford, UK). Separated serum samples from blood clots were transported to ICAR-NIVEDI, Bengaluru, India on ice at a temperature of 2–8 °C. Serum samples received in the institute were centrifuged at 500 g for 3–5 min and separated clear sera was stored at 20 °C until tested. A total of 692 dairy animals from four districts, within which eight clusters consisted of 56 epiunits owned by 306 households from two states, were included in the scope of this study.

Laboratory Procedures

Serological analysis was conducted using the indirect enzyme-linked immunosorbent assay (iELISA) method (Bioassay Technology Laboratory, Jiaxing, Zhejiang Province, China). The IELISA protocol was executed in accordance with the guidelines provided by the manufacturer, adhering to the prescribed test protocol and calculation methods. The values were calculated by comparing the sample well with controls, where the average OD of positive 1.00 and the average OD of negative 0.10. A cutoff value of the average negative value plus 0.15 was tabulated as per the manufacturer's recommendation. A sample withan OD value less and greater than the cutoff value were deemed negative and positive, respectively. Sensitivity and specificity of the test were not defined in the kit to estimate apparent and true prevalences in the assayed samples.

Statistical Analysis

Information from the questionnaire was digitized into a Microsoft Excel spreadsheet(Microsoft Corporation) and serological results were interpreted as sero-negative = 0 orsero-positive = 1. The positive animals in epiunits, clusters, and districts within the stateswere identified. To

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Vol-10 Issue-02 nov 2021

correlate the degree of association between potential risk factors, the chi-square/p value was calculated using Ausvet epitool with a 95% confidence interval (https://epitools.ausvet.com.au/chisq; Accessed on 3 February 2024). The maps were prepared using QGIS software version 3.43.

Results

Sero-Prevalence of Hemorrhagic Septicemia (HS) at Different Strata

A total of 692 cattle and buffalo serum samples were sourced from two Indian states located distantly viz., southern Karnataka and western Gujarat. Out of the 285 samples collected from Karnataka, 259 samples were from cattle and the remaining 26 samples from buffaloes. Similarly, out of the 407 samples collected from Gujarat, 232 samples were from cattle and the remaining 175 from buffaloes. Sero-prevalence of antibodies to *P. multocida* was high in Gujarat (14.49%, CI: 11.22–18.30) compared to Karnataka state (3.85%, CI: 1.94–6.80) and a significant (p < 0.0001) association to HS was observed between these top two milk-producing states (Table 1). An investigation into HS sero-prevalence across four districts revealed the highest sero-prevalence in Junagadh district of Gujarat state (18.61%, CI: 13.81–24.24) and between the districts statistical significance (p = 0.01) was evident. However, this was not true with the districts visited in Karnataka (Figure 2A,B). Similarly, high sero-positivity was noted in Junagadh cluster among the four clusters of Gujarat state (23.02%, CI: 16.59–30.54) at statistical significance (p = 0.03).

Mehsana	0/26	14/136	14/162 (8.64)		
Total	0/26 (CI: 0–13.23)		22/175 (12.57%, CI: 8.05–18.41)	22/201 (10.95%, CI: 6.99–16.10)	
X^2 /p value		–		0.20/0.66	3.41/0.065
Lactation		1	0/69	26/147	26/216 (12.04)
		2	4/85	10/81	14/166 (8.43)
		3	3/73	7/78	10/151 (6.62)
		4	2/26	7/62	9/88 (10.23)
		>5	2/32	9/39	11/71 (15.49)
		Total	11/285 (3.85%, CI: 1.94–6.80)	59/407 (14.49%, CI: 11.22–18.30)	70/692 (10.12%, CI: 7.97–12.61)
X^2 /p value		4.47/0.35		6.26/0.18	5.68/0.22

– represents no sample; * significant at p < 0.001.

Under the age category, the highest number of samples [233 (33.67%)] belonged to the 2–4 years age group followed by 221 samples (31.94%) in the 4–6 years age group and 32 samples (4.62%) were from the 1–2 years age group. Similarly, lactation wise, the highest proportion of samples was from the first lactating animals, accounting for 31.12%.

Conversely, the lowest number of samples was from animals with over five lactations, comprising 71 samples (10.26%). In the age category, the highest sero-prevalence was in theage group of 1–2 years (18.75%) and similarly, in the first lactating animals (12.04%). Out ofthe 306 households (HH), 56 HH had vaccinated against FMD and only one farmer had vaccinated against HS. In the HS vaccinated farm, all tested animals were sero-negative.

Discussion

HS has emerged as the second most frequently reported bacterial disease during theperiods of 1990–2000 and 2000–2010 after FMD, causing the highest number of fatalitiesamong large ruminants [13]. In this study, sero-prevalence of HS in cattle and buffalo wasconducted at 306 HH comprising 692 animals from two districts each in Karnataka andGujarat states to evaluate HS burden in these high milk-producing regions of the country.

The disease has a brief incubation period of 12–14 h, approximately 30 h and 46–80 h for subcutaneous infection, oral infection, and natural exposure, respectively [28]. Clinical courses of per-acute and acute cases were 4–12 h and 2–3 days, respectively [29]. Initialsymptoms include high fever, loss of appetite, followed by increased respiration rate, nasal discharge, salivation, submandibular oedema, and finally recumbency [30]. In suchsituations, serological diagnostic assays are of little value. However, the antibody based iELISA test system is used as a screening test for the detection of *P. multocida* antibodies in serum of infected/carrier/recovered animals. Using

International journal of agricultural sciences and veterinary medicine

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Vol-10 Issue-02 nov 2021

ELISA, HS sero-prevalence in dairy cows was reported from India and Thailand [25,31]. The sensitivity and specificity valuesobtained from the ELISA using a coating antigen from *P. multocida* B:2 via heat extraction were higher than that of IHA test [32]. Hence, in the present study, we employed ELISA assay to record HS prevalence. Based on serology, the overall sero-prevalence of HS was found to be 10.12% in two states visited, which is much higher than the reported study from organized farms in other parts of the country [25]. Sero-prevalence of antibodies to *P. multocida* was high in Gujarat (14.49%), compared to Karnataka state (3.85%) and a significant (p < 0.0001) association to HS was observedbetween these states. Investigation into the HS prevalence across districts revealed sig- nificantly higher (p = 0.01) sero-prevalence in the top milk-producing district (Junagadh)of Gujarat state and in all four clusters visited in the Gujarat state. This clearly indicated wide spread prevalence of the disease in the state. This might be attributed to the lack of vaccination efforts to protect animals against HS and there is a need to revisit both activeand passive HS surveillance at cluster, epiunit, and household level in an area. It was interesting to note that in a household, all four animals were sero-positive for HS and some households had three and or two HS positive animals. This represents transmission of the disease within the households which may eventually lead to outbreaks in the absenceof vaccination.

Higher HS sero-prevalence was recorded in indigenous cattle than crossbred cattle in Gujarat state and this may be due to the high number of indigenous cattle sampled in the study (p = 0009). Also, higher sero-prevalence is attributed to semi-intensive grazing of indigenous cattle unlike the relatively controlled movement in intensive system of rearing practiced for the crossbred animals. Similarly, significantly highest (p = 0.065) sero- prevalence was recorded in indigenous buffalo breed Jafarabadi than the crossbred Mehsana (crossbred between Murrah and Surti buffalo breeds). In general, increased susceptibility to HS outbreaks was observed in crossbred animals as they are more susceptible to heat stress during hot and humid climates [32,33]. In the present study, sero-prevalence in buffaloes was found to be non-significantly higher than cattle (p = 0.75) but in general, buffaloes are more susceptible to HS [26,32].

Similarly, a study reported high risk of HS morbidity and mortality in small farms and these small holding farms are common at epiunit level (usually maintaining 3–5 animals)as noted in the present study and reported elsewhere [13]. Hence, HS vaccination is equally important for both cattle and buffaloes.

Sero-prevalence was found to be higher in young compared to other age group animals.There is a continuous decrease in sero-prevalence as the age group of animals increasedfrom 2–4 to 2–8 years at non-significant level. A study reported susceptible age group forHS is between 6 months and 2 years [1]. Similarly, sero-prevalence was recorded to be non- significantly higher in first lactating animals compared to other lactations. Both lactationand age of the animals were very well correlated with each other in the current study.

P. multocida produce a short duration of immunity in animals up to 4–6 months and the presence of HS antibodies indicates recent infection. As per the farmers and veterinarians feedback, there were no HS-related deaths since the last two months in the regions surveyed.Higher sero-prevalence report in the current study indicates that cattle and buffaloes may be harboring *P. multocida*. These carrier animals may act as a potent source of infection to naïve animals during environmental or transportation stress [28]. Some limitations ofthe study are that we could not source samples as per the sampling target owing to the lumpy skin disease outbreak during 2021–2023 in India, inaccessibility of the region/s, and the limited logistic support to reach regions was yet another limitation. Isolation of *P.multocida* is a gold standard method for diagnosis of HS, but isolation is tedious and timeconsuming for a large number samples; hence, antibody detection by ELISA is preferred for surveillance of HS.

The HS disease course is very short and the case fatality rate typically approaches 100% unless the animal is treated very early. Young animals are mainly affected in endemic regions and outbreaks are particularly common during the rainy season in unvaccinatedanimals. In such situations, locating the endemic areas for vaccination is helpful for preventing the outbreaks [16,34–36]. Every year, HS outbreaks have been reported in Gujarat and Karnataka states and hence compulsory vaccination against HS due to *P.*

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Vol-10 Issue-02 nov 2021

multocida B:2 is a first and foremost step to prevent the annual outbreaks in bovines. The present study on HS sero-prevalence correlates well with the 10% reported occurrence of HS outbreaks among overall reported outbreaks in the country.

Conclusions

The current study documents a very high sero-prevalence rate of HS disease in Gujarat state compared to Karnataka in cattle and buffaloes. Similarly, within states at different strata, a high sero-prevalence rate for the disease was recorded in few clusters, epiunits, andhouseholds. Disease in young animals, indigenous breeds, and buffaloes highlights disease risk for compliance of control measures. The findings underscore regional variations in disease burden, necessitating tailored disease management strategies such as vaccination of the young and heifers and strict surveillance measures for the control of HS in farms inthe rom the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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surveyed regions.

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Informed Consent Statement: All farmers gave their written consent to participate in the study.

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