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Comparison of indirect haemagglutination test and enzyme-linked immunosorbent assay for detection of serum antibodies to *Pasteurella multocida* in bovines

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ABSTRACT

Haemorrhagic septicaemia (HS) is an acute and fatal disease of cattle and buffaloes caused by *Pasteurella multocida* serotype B:2. The present study was undertaken to detect the antibodies to *P. multocida* by indirect haemagglutination assay (IHA) and indirect enzyme-linked immunosorbent assay (I-ELISA) and their results were compared. Serum samples (1,010) were collected from Assam, Karnataka, Odisha, Punjab, Tamil Nadu, Uttarakhand and Uttar Pradesh of India. An overall seroprevalence of 5.74 and 18.61% was observed by IHA and I-ELISA, respectively. Breed-wise, Hallikar (14.28%) and Khillari (50%) showed highest seroprevalence by IHA and I-ELISA, respectively. Sex and age-wise, there were no significant differences in seroprevalence rates between male and female and different age groups by both tests. Source-wise, the I-ELISA detected the maximum seroprevalence (74.60%) in Cattle Breeding Farm, Hosur, Tamil Nadu while the IHA detected 21.62% in Sona Farm, Udhamsingh Nagar, Uttarakhand. The I-ELISA had a relative sensitivity and specificity of 94.83 and 86.03%, respectively in comparison to IHA. Concordance value showed an agreement in results of 86.53% between IHA and ELISA, which was further corroborated by the results of kappa statistics indicating a moderate agreement between these 2 tests.

Key words: Haemorrhagic septicaemia, IHA, I-ELISA, *Pasteurella multocida* serotype B:2

Haemorrhagic septicaemia (HS), an acute and fatal septicaemic disease of cattle and buffalo with a high morbidity and mortality in India (Verma and Jaiswal 1997), is caused mostly by *Pasteurella multocida* serotype B:2 and characterized by oedematous swelling of head, neck and swollen haemorrhagic lymph nodes (Carter and De Alwis 1989). Buffaloes were found more susceptible than cattle while, young and adult animals were found more susceptible than older animals (De Alwis *et al.* 1990). India has large population of cattle and buffalo and HS accounts for a heavy mortality in these animals resulting in losses of more than 200 million rupees annually in India (Singh *et al*. 2008). On the basis of distribution of the HS in the Asian continent, India has been kept in category A countries (FAO-WHO-OIE 1994). The vaccination of bovines against HS is common in India. The present study was undertaken to detect the antibodies to *P. multocida* by indirect haemagglutination assay (IHA) and indirect enzyme-linked immunosorbent assay (I-ELISA) and their results were compared.

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MATERIALS AND METHODS

Collection of serum samples: About 5 ml blood from each of the 1,010 bovine were collected in aseptic condition using labeled sterile disposable syringes from 12 sources situated in the state of Asom, Karnataka, Odisha, Punjab, Tamil Nadu, Uttarakhand and Uttar Pradesh of India. Most of these animals were vaccinated in June and July and blood was collected between August and February. Serum was separated and stored at –20°C in small aliquots till use.

Antigens: Pasteurella multocida serotype B:2 (strain P52) culture obtained from *Pasteurella* Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar, was tested for purity by colony and biochemical characterization and was used to prepare antigens for IHA and I-ELISA.

Heat extract antigen (HEA): A heat extract antigen was prepared for IHA as per Sawada *et al.* (1982). A 12–18 h casein sucrose yeast (CSY) broth culture of *P. multocida* serotype B:2 was heavily seeded on to CSY agar plates and incubated for 24 h at 37°C. The growth was harvested by washing the plates using 2–3ml of 0.3% formalinized normal saline solution (NSS). The suspension was centrifuged at 8,000 rpm for 30 min at 4°C. The supernatant was discarded and the turbidity was adjusted to opacity tube 6. The cell suspension was then heated for 30 min at 56°C in a water

bath. The heated suspension was centrifuged at 8,000 rpm for 30 min at 4°C and the supernatant was collected and stored at –20°C.

Boiled extract antigen (BEA): A 12–18 h broth culture of *P. multocida* serotype B:2 was seeded on dextrose sucrose agar (DSA) in standard petri plates and incubated at 37°C for 18–24 h. The cells grown on heavily seeded DSA plate were harvested in phosphate-buffered saline (PBS, pH 7.4). This suspension was heated in a boiling water bath for 1h and then centrifuged at 8,000 rpm for 15 min. The supernatant fluid was collected, which was designated as boiled extract antigen (BEA) and stored at –20°C. This was used as antigen in I-ELISA.

Immunohemical characterization of antigens: The protein concentration of HEA and BEA was estimated as per Lowry *et al.* (1951). The SDS-PAGE and western blotting of antigens were carried out as per Laemmli (1970) and Towbin *et al.* (1979), respectively.

Serological tests

Indirect haemagglutination assay (IHA)

Coating of sheep erythrocytes (sensitization): Fresh sheep blood was collected in equal volume of Elsevier's solution, centrifuged at 2,000 rpm for 5 min and washed 3 times in NSS by centrifugation. The supernatant was discarded and the packed cells collected. One volume of packed erythrocytes was mixed with 15 volumes of antigen and placed at 37°C for 30 min, with frequent shaking. The cell suspension was then centrifuged at 2,000 rpm for 5 min, supernatant was discarded and the sensitized cells were washed 3 times in physiological saline by centrifugation and resuspended in NSS to get 1% suspension.

Test procedure: The test was performed in 96 well round-bottom microtitre plates. First of all, 90µl of normal saline was added to all wells of the first column and 50µl in all other wells. Then 10µl inactivated serum samples in all wells of the first column were added and mixed properly. Thereafter 50µl was transferred to second well and serial dilution was made up to 10^{th} well of the row, after that 50µl was discarded. The 11^{th} and 12^{th} well of the row was left as controls. Sensitized sheep red blood cells (SRBC) was added @50µl to each well except antigen control. In antigen control a 50µl of unsensitized sheep red blood cells (URBC) was added. The plates were incubated at 37°C for 2 h and observations were recorded. Thereafter, the plates were kept at 4°C overnight shaked lightly, allowed to resettle and read again. Samples which had titre equal to or more than 1:40 was considered positive (Gelagay *et al.* 2004).

Indirect enzyme-linked immunosorbent assay (I-ELISA): ELISA was carried out as described by Klaassen *et al*. (1985) with slight modifications. The 96-wells flat bottom ELISA plates were coated with boiled extract antigen appropriately diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6) at a concentration of 1.2µg/100µl/well and plates were incubated at 4°C overnight. At the end of incubation, the plates were washed with PBS-T (PBS with 0.5% Tween) for 3 times and dried by tapping against the filter paper pad. Then 200µl PBS-T containing 1% bovine serum albumin (BSA) added to each well to block unreacted sites in the wells and incubated for 1 h at 37°C. After incubation, the plates were washed 3 times with PBS-T and dried by tapping against the filter paper pad. Test serum samples (100µl) as well as positive and negative control serum in 1:200 dilutions in PBS were added in duplicate wells in plates. The plates were incubated for 1 h at 37°C and then washed 3 times with PBS-T. The 100µl of rabbit anti-bovine HRPO conjugate diluted in 1:10,000 in PBS-T was added to each well. The plates were incubated for 1 h at 37°C. After incubation, the plates were washed 3 times with PBS-T. The substrate solution was prepared in citrate buffer (0.1M, pH 4.6) by adding OPD (6mg/ 12ml) and H_2O_2 (30%) @ 0.05% and added immediately to all the wells in 100µl volume. The plates were incubated at 37°C for 20 min for development of colour reaction. Stopping solution (4N H_2SO_4) 100µl in volume to each well was added to stop the reaction. The plates were read at 490 nm in ELISA reader along with controls (positive, negative, conjugate and substrate control). In order to establish a positive cut-off optical density (OD) value, 15 known negative calf's serum samples (confirmed by slide agglutination test) were tested and the cut-off value was calculated by following formula:

$$\text{Cut-off OD value} = \text{Mean ELISA OD of negative sera} + (2.5 \times \text{Standard Deviation})$$

Comparison of results of IHA and I-ELISA: The IHA was considered as standard and results of I-ELISA were compared by calculating relative sensitivity, relative specificity, accuracy (McDiarmid and Hellstrom 1987), predictive value of positive and negative test results, kappa statistics (Thrusfield 2005) and concordance percent (Perrin and Sureau 1987).

RESULTS AND DISCUSSION

The overall seroprevalence of HS by I-ELISA and IHA was 18.61% and 5.74%, respectively. Therefore, I-ELISA was found to be more sensitive test than IHA. Similar views were expressed by many workers (Solano *et al.* 1983, Opuda-Asibo *et al.* 1985, Kawamoto *et al.* 1994, Singh 2009). Seleim *et al.* (2003) reported that ELISA was recently adapted for diagnosis and monitoring of *P. multocida* infection and has significant advantages over the indirect hemagglutination assay. It is most probable that the sensitivity of the serological test is dependent on the *P. multocida* antigen used. In present study, the HEA was used in IHA while BEA in I-ELISA, which had protein concentration of 3.5 and 1.9mg/ml, respectively. IHA had employed a crude capsular extract which is coated on fixed sheep RBC and may therefore, not

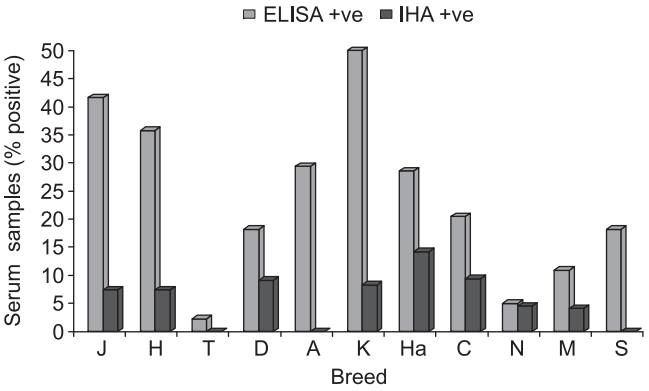

Fig. 1. Breed-wise seroprevalence of *P. multocida* in bovines. Breed: J, Jersey; H, Holstein Friesian; T, Tharparkar; D, Deoni; A, Amritmahal; K, Khillari; Ha, Hallikar; C, Crossbred; N, Non-descript; M, Murrah; S, Surathi.

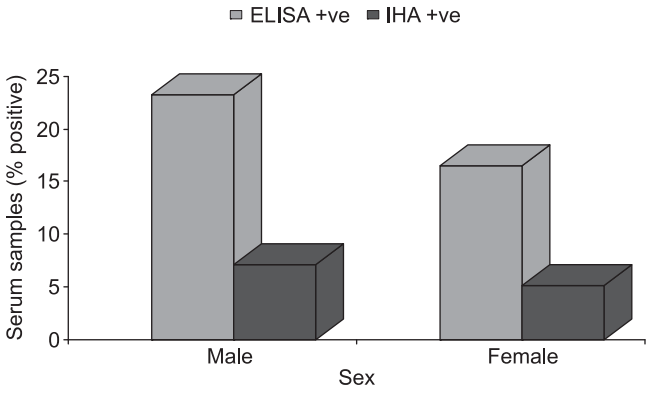

Fig. 2. Sex-wise seroprevalence of *P. multocida* in bovine.

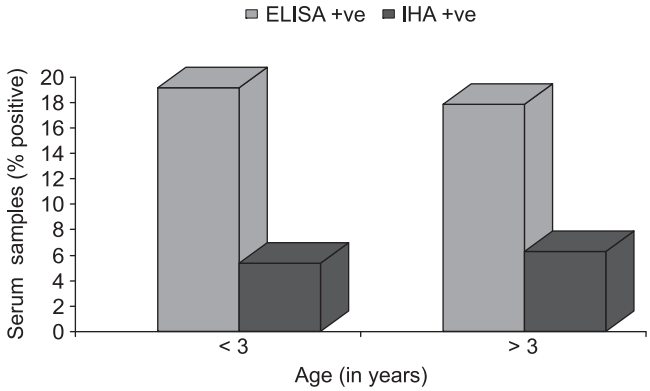

Fig. 3. Age-wise seroprevalence of *P. multocida* in bovine.

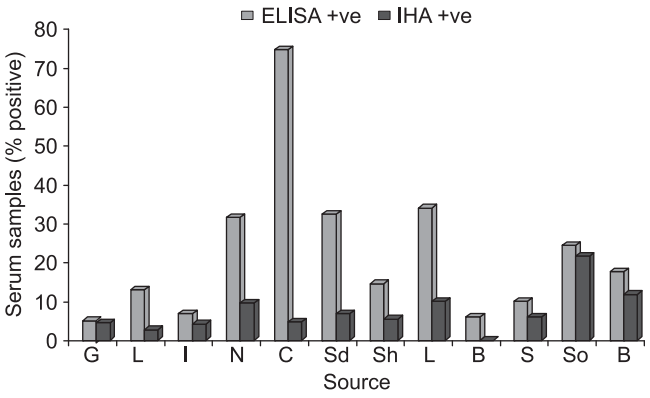

Fig. 4. Source-wise seroprevalence of P. multocida in bovine. *Source:* G, Guwahati: L, Ludhiana; I, Izatnagar; N, NJF, Ooty; C, CBF, Hosur: Sd, SSCC, Dharwad: Sh, SSCC, Hessarghatta; L, LBF, Hessaraghatta; B, Bargarh; S, Sambalpur; So, Sona farm, Uttarakhand; B, Bagalkot.

detect OMP or LPS antibodies which will be detectable when BEA was used in I-ELISA, although both SDS-PAGE and western blotting showed more number of bands in HEA than BEA. Actually IHA is not a standard test for serodiagnosis of pasteurellosis and is usually used for serotyping/capsular typing and enjoyed immense popularity worldwide and became the gold standard for capsular typing (Rhoades and Rimler 1987, and Rimler and Rhoades 1987).

Breed-wise, I-ELISA showed highest seroprevalence (50%) in Khillari breed and lowest of 2.27% in Tharparkar, while IHA showed highest seroprevalence 14.28% in Hallikar breed but none of the serum samples of Tharparkar, Amrithmahal and Surthi breed were found positive by IHA (Fig.3). The detection of high seroprevalence might be vaccination induced immunity as the serum samples of Khillari and Hallikar breeds were collected in August. Usually the vaccinal antibody titer goes down with increase in post vaccination duration. In fact, the sensitivity of IHA is low to reveal the vaccination induced immunity (De Alwis *et al.* 1982, 1990).

Sex-wise, there was no significant differences ($P \leq 0.01$) in seroprevalence rates between males and females by both I-ELISA and IHA, even though a greater positivity being recorded in males (Fig.4). It might be due to the fact that male serum samples were from well organized semen collection centres, where regular HS vaccination was in practice and also time of serum collection. But in contrast to present findings, Singh (2009) reported high seroprevalence in females than the males by both I-ELISA and IHA in caprine pasteurellosis.

HS tend to occur mostly in young animals between 6 months to 3 years of age. Therefore, serum samples were distributed in 2 age groups (Fig.5). I-ELISA showed the seroprevalence of 19.12 and 17.83% while IHA 5.39 and 6.28% in less than 3 years and more than 3 years age groups, respectively. However the difference is not statistically significant. Singh (2009) also reported similar findings in caprine pasteurellosis.

Source-wise, the I-ELISA detected the maximum seroprevalence (74.60%) in Cattle Breeding Farm, Hosur, Tamil Nadu while the IHA detected (21.62%) in Sona Farm, Udhamsingh Nagar, Uttarakhand. The lowest seroprevalence

by I-ELISA was found in Harnek Dairy Farm, Tharek, Ludhiana, Punjab (5.00%), while none of the serum samples of Bargarh District, Odisha were found positive by IHA (Fig.6). The vaccine status of bovine of Bargarh, Odisha was unknown. However, it was found that the sources from which blood were collected between December to February showed less seroprevalence in comparison to those collected between August to November. Probable reason for low level of antibody, not detected by serological tests, might be the post-vaccinal time elapsed when blood was collected, serum separated and tested.

Serological tests are not normally used for diagnosis of HS; however, high titre (1:160 or higher) by indirect haemagglutination in surviving in-contact animals are suggestive of the disease (OIE 2009), therefore, IHA was considered as standard test for evaluating the results of I-ELISA (Table 1). Only 58 serum samples were found positive by IHA while 188 samples were positive in I-ELISA. In I-ELISA very low antigen concentration (1.2 µg) was used to detect antibodies in highly diluted serum (1:200), which indicate high analytical as well as diagnostic sensitivity of I-ELISA than IHA. Several workers (Solano *et al.* 1983,Opuda-Asibo *et al.* 1985, Kawamoto *et al.* 1994, Seleim *et al.* 2003, Singh 2009) have reported the superiority of I-ELISA over other serological tests in terms of convenience and sensitivity. The relative sensitivity of I-ELISA in comparison to IHA was 94.83% suggesting that it was as effective as IHA in detecting true positive animals. But the relative specificity of I-ELISA was 86.03% signifying the inability of the ELISA to detect all the IHA negative animals as negative (Table 1). Similar to present findings, Dawkins *et al.* (1990) reported the specificity of 99% and sensitivity of 86% by using boiled extract antigen in ELISA. Singh (2009) reported a very high relative sensitivity (100%) but a low relative specificity (73.78%) of I-ELISA in comparison with IHA.

The predictive value of positive test result calculated was 29.25%. It signifies that there were 29.25% probability that bovines showing a positive reaction by I-ELISA were actually positive (true positive), while predictive value of negative test result was 99.63% that signifies that there were 99.63% probability that bovines showing a negative reaction by I-ELISA were actually negative (true negative). Singh (2009) report corroborated present findings. Accuracy of prediction of I-ELISA was 86.53% and error of prediction was 13.47%. Similar pattern was recorded by Singh (2009) also.

Concordance value between IHA and ELISA was 86.53%, which indicated an agreement in results of 86.53% between these 2 tests, which was further corroborated by the results of kappa statistics indicating a moderate agreement between these 2 tests (Table 1). However, Singh (2009) reported a lower concordance value (76.11%) and accordingly a fair agreement between IHA and ELISA.

Table 1. Comparison of results of I-ELISA with IHA

Serological tests		IHA		
		Positive	Negative	Total
I-ELISA	Positive	55	133	188
	Negative	03	819	822
	Total	58	952	1010
Relative sensitivity		94.83%		
Relative specificity		86.03%		
Accuracy		86.53%		
Positive predictive value		29.25%		
Negative predictive value		99.63%		
Concordance		86.53%		
Kappa statistic		0.41 (Moderate agreement)		

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