LIPOPOLYSACCHARIDE BASED INDIRECT ELISA FOR RAPID DETECTION OF *PASTEURELLA MULTOCIDA* ANTIBODIES IN BOVINES

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Haemorrhagic septicaemia is an important economic disease of bovines caused by *Pasteurella multocida* which is one of the major causes of livestock mortality in tropical regions of Asia. There is no reliable serological assay for distinct diagnosis of the disease. The aim of the present study is to develop an indirect ELISA (iELISA) assay for the detection of antibodies against *P. multocida* in cattle and buffaloes. To develop these assay lipopolysaccharide of *P. multocida* serotype B:2 used as antigen. The lipopolysaccharide (LPS) of *P. multocida* was characterized by silver stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A total of three hundred seventy three different field sera samples were collected. Using known negative and positive serum samples, it was found that LPS based indirect ELISA for the detection of *P. multocida* antibodies in sera was more sensitive when compared with other serological tests used for detection of antibodies against *P. multocida*. All the samples were screened by LPS based iELISA and have shown sensitivity of 91.04%, specificity of 96.41% when compared with Indirect haemagglutination test. It was concluded that LPS could be useful antigen for detection of *P. multocida* antibodies in bovines.

Key words: Antibody, Haemorrhagic septicaemia, iELISA, Indirect haemagglutination, SDS-PAGE

Hemorrhagic septicaemia (HS) is acute highly fatal bacterial disease caused by gram negative bacteria *Pasteurella multocida*. The causative organism *P. multocida* belonging to family Pasteurellaceae is grouped into five sero groups A, B, D, E and F based on their capsular typing and (1-16) serotypes based on somatic typing (Harper *et al.*, 2006). In India, HS is mostly caused by serotype B:2. It is one of the economically important livestock diseases with high mortality in many Asian countries. The disease has also been reported in 1974 in India.

According to the Indian epidemiological studies around 1974-1986, HS was placed first position in mortality rate compared with the other endemic diseases like FMD, anthrax and black quarter (Singh *et al.*, 2014). *P. multocida* serotypes mainly occurs in bovines (cattle and buffalo) while septicaemic pasteurellosis recorded rarely or only sporadically in swine, camel, water buffaloes, poultry, horses, elephants, rabbits and other animals (Sarangi *et al.*, 2014; Zhu *et al.*, 2020). The HS is transmitted by direct contact with infected animals,

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contaminated clothing, equipment, and through ingestion or inhalation of the organism. The clinical signs of the HS caused by serotype B:2 strain is characterized by fever, respiratory distress with nasal discharges and frothing from the mouth, leading eventually to leads to the death of the animal.

P. multocida serotype B:2 is a gram negative bacterium associated with HS has been found in Asia. The mechanism of the lipopolysaccharide, outer membrane proteins, etc. in virulence and protective immunity in effected animals has been described in various studies (Kharb and Charan, 2010; Wani *et al.*, 2011). The lipopolysaccharides are the endotoxins that play a vital role in the pathogenesis of the disease. It induces many critical hurtful biological effects in the host. Endotoxins are also activating polymorph nuclear cells, monocytes and macrophages. A proper diagnosis is needed for the identification of the disease. Although several serological tests such as tube agglutination test, indirect haemagglutination, dot immunoblotting and enzyme linked immunosorbent assay (ELISA) for detecting antibodies against *P. multocida* used previously, none is universally accepted. In view of these facts, the present study was undertaken to develop lipopolysaccharide (LPS) based indirect enzyme-linked immunosorbent assay (iELISA) for the detection of *P. multocida*-specific antibodies in bovines. *P. multocida* lipopolysaccharide (LPS) has been used as an antigen for the detection of *P. multocida* antibodies in cattle and buffalos.

MATERIALS AND METHODS

Culture of *P. multocida* and harvesting of culture: The lipopolysaccharide (LPS) antigen was extracted from *P. multocida* B:2 strain. For the extraction of LPS of *P. multocida* culture (MTCC-1148) was grown overnight on BHI agar (Himedia, India). The bacterial cells were harvested in normal saline and centrifuged at 8000 rpm for 10 min. After centrifugation, bacterial pellet was used for LPS extraction.

LPS extraction and purification: LPS was extracted by modified hot phenol-water extraction method as described earlier (Zankharia, 2015). Approximate 12 g (wet weight) of bacterial pellet was suspended in 105 mL pre-warmed distilled water to 65-70°C. After suspension of cells in water an equal volume of pre-warmed 65-70°C, 90% aqueous phenol (Fisher, India) was added to the cell suspension and the mixture was stirred vigorously at 65-70°C for 15 min followed by chilling on ice for 15 min. The mixture was then centrifuged at 8000 rpm at 4°C for 15 min for the separation of aqueous and phenol phases. After centrifugation upper aqueous phase (contains LPS) was collected and again the collected aqueous phase was centrifuged at 8000 rpm for 15 min to remove insoluble impurities. Then the LPS was precipitated by adding 0.15M sodium acetate (Fisher, India) and 95% ethanol (Merck) and sample was stored at -20°C overnight in order to precipitate the LPS. Precipitates of LPS were collected by centrifugation at 5000 rpm and the pellet was reconstituted in 2 mL distilled water. Sample was kept for dialysis against PBS at 4°C for 24hrs and collected LPS was stored at 4°C. The purified LPS were used further as an antigen in immunoassays.

The purity of LPS was checked by running SDS-PAGE and silver staining. SDS-PAGE was performed with discontinuous buffer system (SDS-PAGE running buffur: 0.25 M Tris, 1.92 M glycine, 1% SDS); the LPS sample was mixed with 2X sample buffer and boiled for 5 min. The electrophoresis was carried out with 12% resolving gel at 100mA on gel electrophoretic apparatus (Bio-Rad) with protein molecular weight marker (Spectra multicolour broad range protein ladder, Thermo Fisher Scientific, USA). Silver staining of the gel was performed according to the standardised protocol. The gel was fixed in fixation solution for 1 hour then renewed the fixation solution and kept for overnight incubation. The gel was sensitized in 10% tetrathionate solution for 45 minutes, then raised with 20% ethanol solution for thrice with 10 minutes of regular intervals along

with 2 times gentle wash with distilled water. Then the gel was treated with silver nitrate solution for 30 minutes and washed with distilled water twice. The gel was developed with developer solution (0.02% formaldehyde and 3% sodium carbonate) and reaction was stopped by adding stop solution (4% w/v Tris and 2% v/v acetic acid). Then the developing gel was stored in fixation solution (Chevallet *et al.*, 2006).

Sample collection: This study was conducted with approval from the Institutional Animal Ethics Committee of Genomix. A total 373 serum samples were randomly collected from dairy rearing households and local small dairy farms of Telangana and Rayalaseema region of Andhra Pradesh of India from July, 2018 to August, 2019 having no prior haemorrhagic septicaemia vaccination history. The blood samples were collected from jugular vein of each animal and serum was separated, stored at -20°C for further use. The reference sera in this study were provided by the Department of Veterinary Public Health and Epidemiology, PVNR Telangana Veterinary University, Hyderabad.

Development of Enzyme linked immunosorbent assay

Optimization of ELISA assay: The optimization of working concentration of different reagents like LPS antigen, serum dilutions and conjugate in the assay was done by using checker board titration. The optimal concentration of the reagents cut-off values were calculated through screening and validation of the test with positive and negative control sera samples. Cut-off values were calculated by Percent positivity value, $PPV = \frac{\text{Total Positive}}{(\text{Total Positive} + \text{False Negative})} \times 100$

LPS concentration was estimated by commercial endotoxin estimation kit (Pierce Chromogenic Endotoxin Quantification Kit, Thermo Fisher Scientific). Then concentration was adjusted to 2 mg/mL by freeze drying. LPS antigen (150 ng/per well) was coated in each well of micro titre plate (Nunc) with coating buffer (0.2 M

Na_2CO_3 and 0.2 M NaHCO_3) (Fisher, India). Plates were incubated over night at 4°C. After incubation the plates were washed three times with wash buffer (1X PBS+Tween20) (Fisher, India). The plates were blocked with 6% skimmed milk powder (Amul, India) and incubated for 1 hour at 37°C, after incubation plates were washed with wash buffer. The plates were dried under dehumidifier (Bryair, India). Once the plates were dried, they were packed with silica gels at 4°C till further use.

ELISA assay procedure: The test samples were diluted in a ratio of 1:100 in sample diluents buffer (1XPBS) along with controls i.e. known positive and negative serum samples in duplicates. Following 60 min incubation at 37°C, the plates were washed three times in wash buffer (1X PBS+Tween20). The 100 µL of Protein G HRP conjugate (1:10,000 diluted) (Sigma-Aldrich, USA) was added to each well and incubated for 60 min at 37°C. After incubation, plates were washed and 100 µL of TMB substrate (Abcam, USA) were added in each well and incubated in dark for 15 min or till the colour develops. Then the reaction was stopped with stop solution (0.5M H_2SO_4) (Fisher, India). The absorbance was measured at 450 nm using an ELISA reader (Robonik, India).

Validation of the assay: Validation of the indirect ELISA was carried out by using 373 bovine serum samples with no history of vaccination and the assay was compared with the mostly used assay i.e., Indirect haemagglutination (IHA) test (El-Jakee *et al.*, 2016). Briefly, overnight culture of *P. multocida* on BHI agar media was checked for its purity and the growth was harvested by washing the plates using 2-3 mL of 0.3% buffered formalin per plate. The capsular antigen of the bacterial suspension was separated by heat treatment at 60°C for 30 minutes followed by centrifugation at 2000 rpm for 30 minutes and supernatant was collected. 0.1 mL of 5% suspension of glutaraldehyde fixed sheep erythrocytes (Cho *et al.*, 1976) were added, and the mixture was further incubated at 37°C for

30 min. The sensitized erythrocytes were washed three times with phosphate-buffered saline by centrifugation and suspended in PBS containing 0.25% bovine serum albumin (BSA-PBS). The IHA test was performed in U bottom plates by adding two-fold serial dilutions of antiserum made in BSA-PBS and 0.025 mL of the sensitized erythrocytes. The plates were shaken and allowed to stand for 1 to 2 h at 25°C before erythrocytes settling patterns were read. The IHA titer was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment), as compared with the pattern of the negative control (smooth dot in the centre of the well) (Qureshi and Saxena, 2014)

RESULTS

SDS-PAGE and silver staining: The concentration of the extracted LPS from *P. multocida* by phenol-water extraction method was around 2.08 mg/mL. The SDS-PAGE of the water-phase LPS, reported pure LPS of 15KD molecular weight without any protein contamination (Fig. 1)

Validation of ELISA assay: The assay was optimized using purified LPS of *P. multocida* antigen. Out of total 373 test samples, 67 (17.96%) were found positive for *Pasteurella* antibodies with iELISA whereas with IHA, 72 (19.30%) samples were positive while 306 (82.04%) were negative with iELISA while by IHA, 301 (80.69%) were sero negative. The

sensitivity and specificity of the iELISA were 91.04% and 96.41%, respectively (Table 1).

DISCUSSION

Haemorrhagic septicaemia caused by *P. multocida* serotype B:2 is the second most reported infectious disease of cattle and buffaloes in India and is the cause of major economic losses in livestock world. It is characterized by an acute and highly fatal septicaemia with high mortality rate (60%) (Singh *et al.*, 2014). The diagnosis of *Pasteurella* infection in livestock was important to take the preventive measures to control the spread of the disease immediately without any delay. The diagnosis of HS was mainly done by isolation of *Pasteurella* species but culture isolation is time consuming technique, the molecular detection was rapid and sensitive method but requires specialized laboratory personnel (Sinha *et al.*, 2018). As a result, there is a need of rapid and reliable earlier identification of the bacterial infection in order to initiate appropriate antibiotic treatment at the earliest. There are few immunological assays available for the diagnosis of antibodies to the *P. multocida*. Rapid haemagglutination test is the most widely-used immunological assay for the diagnosis of HS disease. For accurate and appropriate disease diagnosis ELISA assay relatively robust and easier to perform, get the result quickly within 3 hours, affordable and, point of care testing format ELISA assay has been

Table 1. Sensitivity and specificity of iELISA for detection of *P. multocida* antibodies

		Indirect ELISA assay		
		Positive result	Negative result	Total
Indirect haemagglutination test	Positive result	61	11	72 (19.30%)
	Negative result	6	295	301 (80.69%)
	Total	67 (17.96%)	306 (82.04%)	
Total samples:				373
Sensitivity: $TP/(TP+FN) \times 100$				
Sensitivity: 91.04%				
Specificity: $TN/(FP+TN) \times 100$				
Specificity: 96.41%				

LPS based iELISA detection of *P. multocida* antibodies

Lane M Molecular weight marker, 1 LPS from *P. multocida*

Fig. 1. SDS-PAGE/silver staining of LPS from *P. multocida*

developing as important diagnostic technique for disease diagnosis and monitoring serological prevalence in animals against HS disease.

LPS is a major virulence factor of *P. multocida* (Harper and Boyce, 2017) and LPS is the most important bio-molecule present in the outer membrane of the *P. multocida* bacteria and having three distinct regions O antigen, core domain and lipid A. Lipid A part of the LPS is responsible for the toxicity also refer as endotoxin. This pathogenic nature of LPS causes systemic damage in the host. Besides LPS is also used for the detection of diseases as it induces innate immunity through toll like receptors (TLRs) (Farhana and Khan, 2020). According to the OIE Terrestrial Manual for Haemorrhagic Septicaemia (2018), the IHA assay as a tool to determine the antibody response in cattle. However, serological tests are not normally used for detecting antibodies or for disease diagnosis (OIE, 2018). Generally, haemorrhagic septicaemia is a disease that occurs mainly in farms that have poor biosafety or husbandry conditions. ELISA was recognized as a sensitive and accurate method and demonstrated to be able to detect the antibody against heat extracted antigen of *P. multocida* (El-Jakee *et al.*, 2016; Tankaew *et al.*, 2017) and ELISA can be used as best diagnostic assay than IHAT and Microtiter agglutination test (MAT) assays for the serological diagnosis of haemorrhagic septicaemia.

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The aim of the present study was to develop an in-house indirect ELISA based on LPS for the detection of antibodies against to *Pasteurella* in bovines to identify disease natural or vaccination challenges, to screen the carrier animal which cause the spreading of infections and for the serosurveillance. In summary, an in-house indirect ELISA was successfully developed where LPS antigen of *P. multocida* (serovar B:2) as a coating antigen and Protein G HRP conjugate were used. LPS antigen at a concentration of 150 ng/mL; sample serum at a dilution factor of 1:100 and Protein G-HRP in 1:10000 dilutions were optimal concentrations for the assay. In application, there were 17.96% (67/373) sera samples that were considered seropositive by the ELISA. This finding suggested that the iELISA can be used as a tool to detect the antibodies against haemorrhagic septicaemia in cattle in Indian context.

A sensitive and specific indirect ELISA based on LPS-antigen has been developed for the detection of *P. multocida* infection and the determination of the antibody titer against *P. multocida* in both cattle and buffaloes.

Conflict of Interest: None

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