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Development of in-house indirect enzyme linked immunosorbent assay (iELISA) for detection of *Salmonella enteritidis* dpecific antibodies in poultry

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Abstract

Salmonella enteritidis is a most important pathogenic bacterium of avian and mammals. *Salmonella* Enteritidis is the main cause of Salmonellosis in poultry flocks. *S. enteritidis* majorly infects the chicks, eggs and vertically transmitted to their off springs. The majority of the food infections to the humans are caused by *salmonella* by eating chicken meat and eggs. Monitoring of poultry farms with the bacteriological methods were time consuming and labour intensive process. The present study was development an in-house indirect enzyme linked immunesorbent assay (iELISA) for the detection of antibodies against *Salmonella enteritidis* in chicken serum samples. For detection of antibodies, *Salmonella enteritidis* LPS was used as antigen and rabbit anti chicken IgG HRP was used as the secondary antibody to detect antibodies against *Salmonella enteritidis*. The developed in-house ELISA was compared with the Rapid plate agglutination test. The purified LPS antigen 200ng/well, test sample serum at a dilution of 1:100 and rabbit anti chicken IgG HRP 1:10000 were used as optimal concentration of the assay and OD was measured at 450nm. A total of 1020 chicken serum samples were collected and performed the assay along with known Positive and negative controls. Out of these samples 592 and 566 samples were seropositive with iELISA and RPA respectively. Out of 1020 samples 58% samples shown positive immune response with iELISA and 55.6% samples were shown positive immune response to Rapid plate agglutination assay. The major prevalence of SE antibodies against SE antigen were shown in 20-25 weeks birds was 65.5%.The findings suggested that an in-house indirect ELISA based on *S.enteritidis* LPS can be a useful as a rapid and sensitive assay for the detection of antibodies to *S.enteritidis* and can be best assay for regular monitoring of *Salmonella Enteritidis* infection in flocks.

Key words: Antibody, antigen, LPS, RPA, HRP conjugate, ELISA

Abbreviations: SE-*Salmonella enteritidis*, LPS-Lipopolysaccharide, RPA-Rapid Plate agglutination ELISA – Enzyme linked immunosorbent assay; SDS-PAGE – Sodium dodecylesulphate – Polyacrylamide Gel Electrophoresis; HRP – Horse radish peroxidase; IgG – Immunoglobulin G; PBS – Phosphate buffered saline; TMB – 3,32 ,5,52 -Tetramethyl benzidine; nm – nanometer;

Introduction

Salmonella enteritidis is one of the important pathogen of many mammals and birds, it cause Salmonellosis.It is one of the dominant *salmonella*

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sero type and it is consided for its economical impact all over the world including India, it is also the major food borne pathogen associated with poultry meat and eggs. In recent years the majority of food borne pathogen outbreaks caused by the contamination of chicken meat and with contaminated eggs has increased.*Salmonella enteritidis* is a gram negative, flagellated, rod shaped, facultative aerobic bacterium.The contaminated environment with *Salmonella* serovars are main source of infection, by the reason of this *Salmonella* can remain in the environment for a long period of time, hereafterthe *Salmonella* have being transmitted to suitable hosts, where it can shed in their faeces for long time.(1) Poultry farms may carry few *Salmonella* serovars without showing any clinical symptoms of disease and without causing any harmful effects to chickens (2). To combat *salmonella* infections in flocks poultry sectorfacing most difficult problems and not only worried for poultry industry as well as concerned for public health hazard(3) because it causes problems in food safety.To overcome these problems in poultry there is a need for suitable assays to use as a screening test to detect *S.enteritidis* infection in poultry flocks based on clinical symptoms. Routine bacteriological procedures for the isolation and identification of *S.enteritidis* were laborious and time consuming method.Molecular methods such as PCR, ribotyping or restriction endonuclease analysis which have the epidemiological importance but these methods were more expensive to test each bird The diagnosis of infection traditionally has been done by serologically. Several serological assays have been used to detect SE antibodies they are the most reliable for flock screening rather than for testing individual birds.The most widely used serological assays are the serum agglutination test, ELISA, tube and micro agglutination tests.In the Serum plate agglutination test serum or whole blood samples from individual birds were tested. But the assay method is crude, inaccurate and insensitive (4).coming to the immunological assays Enzyme-linked immunosorbent assay (ELISA) methods were emerged as best tools for diagnosis the disease and monitoring the immune status of the poultry flocks and ELISA assays were developed for the detection of *salmonella* antibodies (5). Most ELISAs currently in use for the detection of *S.enteritidis* infected flocks identify antibodies to either crude surface-extracted antigens or flagellar antigens. Lipopolysaccharide (LPS) is the major antigenic and immunogenic structure on the surface of *S.enteritidis*, it consist three components: lipid A, inner core and O-side chain oligosaccharides. Lipid A is the endotoxic principle of LPS and activates macrophages. Several factors released from the macrophages exert biological effects associated with fever and shock (6).

The main objective of this study was to develop an indirect enzyme-linked immunosorbent assay (iELISA) for the detection *of S.enteritidis*-specific antibodies in chicken sera samples. *S.enteritidis* LPS has been used as an antigen for the detection of *S.enteritidis* in chicken flocks.

Materials and Methods

Bacterial strain and growth conditions : *Salmonella* enterica subsp enteric serovar Enteritidis (ATCC 13076TM) was obtained from American Type Culture Collection (ATCC). It was maintained as pure culture as recommended by ATCC and was revived periodically by successive transfers on same medium. The culture was grown on nutrient agar for 24 hrs at 37^{o}C.The culture was harvested in 5% phenol and it was centrifuged at 5000 rpm for 10 min. The weight of the harvested bacterial cell pellet was measured and further used for the extraction LPS. The entire procedure was carried out in a Class II bio-safety cabinet.

Extraction of LPS : Extraction of LPS was done by hot phenol water method without any modifications (7). 12 g (wet weight) of cells was suspended in 105 ml distilled water pre-warmed to 65-70°C. Equal volume of 90% aqueous phenol prewarmed to 65-70°C was added to the cell suspension. The mixture was stirred vigorously

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at 65-70°C for 15 min. The mixture was chilled on ice for 15 min. Mixture was centrifuged at 8000 rpm (Remi cooling centrifuge C-24 BL) at 4°C for 15 min for separation of aqueous and phenol phase. The LPS was collected from the aqueous phage in a fresh tube. Then carefully collected aqueous phase was dialyzed against distilled water in dialysis membrane of MWCO (Molecular weight cut off) 3.5 kd till absorbance at 260 nm of water outside dialysis membrane became zero. The aqueous phase was centrifuged at 8000 rpm for 15 min to remove insoluble impurities. The LPS precipitation was done by Ethanol precipitation in which sodium acetate was added to LPS at final concentration of 0.15 M. Tube was placed on ice and four volumes of chilled 96% (v/v) ethanol was added and kept at -20°C for 24 hours (8). Precipitates of LPS were collected by centrifugation at 5500 g. 10 mg LPS pellet was reconstituted in 2 ml phosphate buffered saline and stored at 4°C.

SDS-PAGE and silver staining : 10 microliter of LPS preparations were mixed with 10 ìlLaemmli sample buffer (62.5 mMTris-HCl with pH-6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% â mercaptoethanol) and heated at 90°C for 5 min. Samples were loaded in gel consisting of 12% resolving gel and 5% stacking gel. The SS-PAGE gel Electrophoresis was done by using (BIORAD's Mini Protean Tetra Cell apparatus). LPS was visualized by standard silver staining procedure (9).

Development of an immunoassay to measure immune response against *S.enteritidis*

Sample collection: This study was conducted with approval from the Institutional animal ethics committee of the Genomix Private Ltd. A total 1020 serum samples were randomly collected from 9 (MSB Farms, Gold chick hatcheries, Srinivasa hatcheries, GSV poultry Bindhu poultry farm VR poultry, Sneha farms, Gagana farms and Abhudaya hatcheries)different chicken flocks in and around Hyderabad, Telangana State of India in between June and July 2019 with no prior history of vaccination. . Three milliliter of blood was drawn from each bird randomly and all collected samples were subjected to centrifugation at 3000 rpm for 5 minutes. The clear sera were harvested using the pasteur pipette decantation method. The serum samples were then stored at 4°C and further used for the serological investigation in the present study. The reference sera used in this study for optimization of assay and field sample analysis were provided by Department of Veterinary Public Health and Epidemiology, P.V.N.R Telangana Veterinary University, Hyderabad.

Assay development and test procedure : The reagents used for ELISA were commercially procured to develop iELISA. The rabbit anti chicken HRP conjugate (Sigma Aldrich, USA), tetramethy lbenzidine/H_2O_2 (Abcam, USA) and 96 well ELISA plates (Nunc polysorpthermo fishers) were used. Positive serum sample from PCR confirmed cases of *S.enteritidis* were obtained by Department of Veterinary Public Health and Epidemiology, P.V.N.R Telangana Veterinary University, Hyderabad. The optimal working condition for dilutions of the coating Antigen LPS-SE, blocking solution, Serum samples, HRP conjugate were found out by checker board titration for their usage in developing ELISA. Briefly, the antigen concentrations optimized by assaying 0.5ìg/ml to 16 ìg/ml concentrations and antibody dilutions tested at 1:25, 1:100, 1:200, 1:400, 1:800, 1:1600 and secondary antibody that is rabbit anti chicken IgG HRP antibody tested at 1:5000, 1:10,000, 1:20,000.The known positive (PCR confirmed) and negative (SPF Sera) controls used for standardizing the assay and in order to get the high differential ratio between positive and negative sera. The validation of the In-house iELISA was carried out by 156 reference serum samples provided by P.V.N.R Telangana Veterinary University. After validation, in-house iELISA were tested with field sera samples along with other laboratory serological assay(SE RPA test).Preparation of ELISA micro titer plates are done as follows the nunc poly sorp micro titter

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plates are coated with the LPS-SE antigen at concentration of 2ug/ml with coating buffer (0.2M sodium carbonate and bicarbonate) pH 9.2 and they were incubated at 4°C overnight. The plates were then washed thrice with PBS-Tween 20. The remaining protein sites were blocked by adding 300ìl of 3% (Amul) skimmed milk powder prepared with 1X phosphate buffered saline, respectively to all wells of the plate and incubated at 37°C for 1 hr. The plates were then washed thrice with wash buffer. The test sera and control sera was diluted to 1:100 and they were added to the wells. The plates were then incubated at 37°C for 1 hr. The plates were washed thrice and then, the100 microliter of rabbit anti chicken immunoglobulin IgG in HRP (1:10000 diluted in 1XPBS) was added to each well and incubated for 1hr at 37°C. The plates were washed thrice and they were treated with 100 ìl of TMB/H_2O_2 for 15 min. Finally, the reaction was stopped by adding 100 ìl of stop solution (0.5M H_2SO_4). The OD readings were taken with an Elisa micro titter plate reader instrument (Robonik, India) at 450 nm. The values obtained by the assay were used to calculate by the percent positivity (PP) value.

Percent positivity value (PPV=True Positive / (True Positive+ False Negative) X 100).

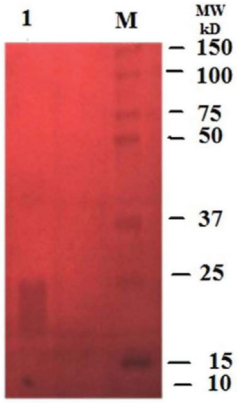

Fig. 1. SDS-Page/silver staining of LPS from *Slamonella enteritidis,* Lane M : Bio-Rad precision plus protein standard, 1-LPS from *Slamonella enteritidis.*

The SE RPA (*Salmonella enteritidis* Rapid Plate Agglutination) Test : The SE RPA test is carried out by using Biovac SE-RPA (Biovac SERPA TESTS AS2 Biovac animal health care) assay is a colored inactivated suspension of *Salmonella Enteritidis* antigen that are used for the detection of antibodies against *S.Enteritidis* in chickens by making a visible agglutination reaction.

Procedure : In the biovac rapid plate agglutination test, a drop of test sample was placed on a clean glass plate and a drop of biovac*S.enteritidis* antigen from was taken with dropper and deposited on plate, then the glass plate was gently rocked and rotated 30 to 40 seconds to ease the agglutination process. Read the results within 50 sec. Positive reaction was noted by the formation of clumps and negative reaction by without any clumps or clear solution.

Results

The LPS extracted from the *S.enteritidis* was run by SDS-PAGE, the gels were stained by Silver nitrate LPS was visualized and characterized upon staining the gel by silver staining method. SDS-PAGE followed by silver staining of LPS showed that molecular weight the LPS of *S.enteritidis* was ranging from ~14 kD to 25 kD. The Concentration of the Extracted S.E LPS was 2.07 mg/ml (Fig. 1).In house iELISA and RPA were compared with 156 reference serum samples. Cut off valuewas selected according to differentiate ratio between positive and negative values based on PP values.The sensitivity, specificity and accuracy of indirect ELISA using SE Antigen are sensitivity is 94.4%, specificity is 96.6% and accuracy is 93.5%.The Indirect ELISA assay for field samples were performed with a total number of 1020 serum samples from 9 individual commercial flocks and with a set of known reference samples, along with iELISA and another laboratory rapid plate agglutination assay also performed for all the collected serum samples. Among these, 592 (58.03%) serum samples are positive by iELISA and 566 (55.4%) serum samples were positive by Rapid plate

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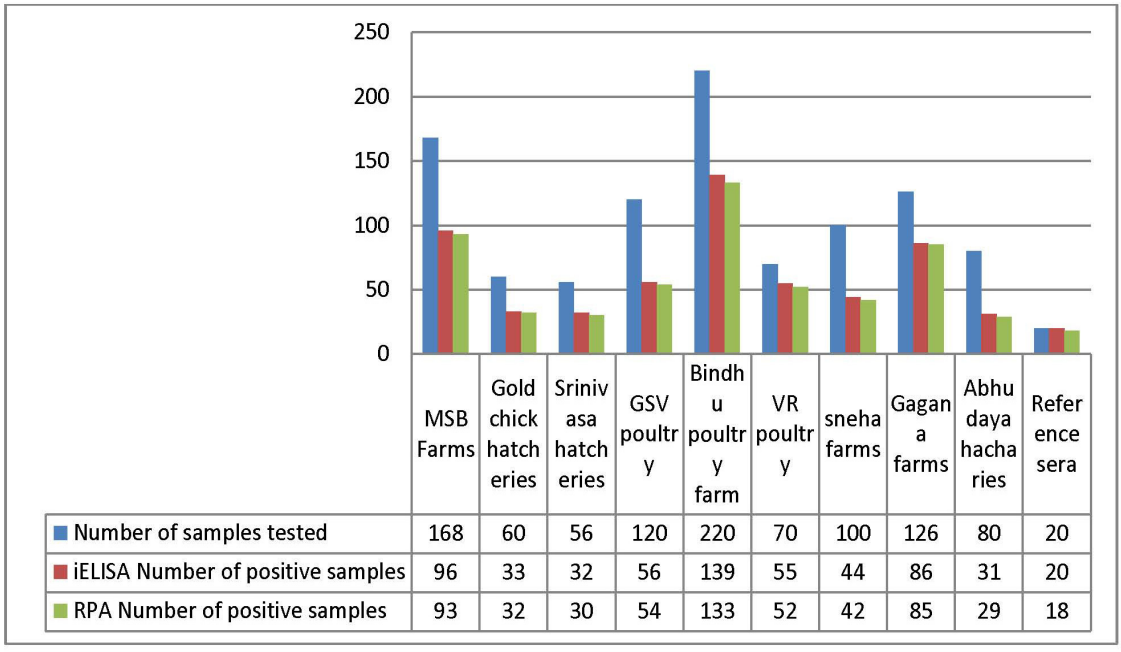
agglutination test respectively. The Percentage positivity of the SE antibodies in commercial flocks are MSB Farms (57.1%) and 55.3%, Gold chick hatcheries was 55% and 53.3%, Srinivasa Hatcheries was 57% and 53.5, GSV poultry was 47% and 45%, Bindhu poultry farm was 64% and 60.4%, VR poultry was 78% and 74%, Sneha farms pvt, ltd was 44% and 42%, Gagana farms was 68% and 67%, Abhyudaya hatcheries was 39% and 36.2 % and the positive % of reference sera was 100% and 98% (Fig.2). In this study the age of the chickens used was in between 20 to 35 weeks and according to the age of the birds the serological response of the SE antibodies against SE pathogen 20-25 weeks was given 65.5%, 26-30 weeks were given 47% and 31-35 weeks were given 47.2% immune response (Table 1). Among the birds of all age groups, young birds are given higher serological response to the *Salmonella enteritidis* antigen.

Discussion

The objective of the present study was to gauge the development of an indirect ELISA for the detection of IgG antibody against *Salmonella enteritidis* in poultry flocks. To develop this assay *Salmonella enteritidis* LPS (Lipopoly sacharide) used as antigen in ELISA were assessed together with serum plate agglutination test. For this study a total 1020 number of chicken serum samples were collected from birds of different age groups of 9 commercial poultry farms and performed iELISA and RPA test. In this study, we isolated LPS (Lipopolysacharide) from *Salmonella* Enteritidis purified cells by hot phenol-water extraction method. The LPS was obtained in upper aqueous phase and it was subjected precipitate the LPS by ethanol. Thereafter LPS was dialyzed and the isolated LPS concentration was determine it was 2.07 mg/ml. The LPS had given a band in between 14 to 25 kD on SDS PAGE. The SDS-



	MSB Farms	Gold chick hatcheries	Srinivasa hatcheries	GSV poultry	Bindhu poultry farm	VR poultry	sneha farms	Gagana farms	Abhudaya hacharies	Reference sera
Number of samples tested	168	60	56	120	220	70	100	126	80	20
iELISA Number of positive samples	96	33	32	56	139	55	44	86	31	20
RPA Number of positive samples	93	32	30	54	133	52	42	85	29	18

Fig. 2: Graph showing the sero positivity of chicken samples

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Age of birds(weeks)	Number of sera tested	Number of positive	Prevalence % samples
20-25	604	396	65.5
26-30	120	56	47
31-35	296	140	47.2

Table 1: Table showing the Age wise analysis of *Salmonella* Enteritidis infection

PAGE showed low molecular weight LPS was the characteristic for the repeated some sugar units as reported earlier by others (10,11).The iELISA With SE purified LPS was developed, to know the seroprevalance of *Salmonella enteritidis* in chickens and this showed that iELISA was robust tool for its mass screening and has many advantages than the other diagnostic methods. Similar reports have been showed by cooper (12) who detects the immune status of the SE antibodies in three flocks which gave high titer value than the other tests (12). In this study more than 1,000 sera were sampled among these chicken serum samples 592 (58.03%) and 566 (55.4%) were found to be seropositive for iELISA and RPA test respectively. Serological investigation of age-wise analysis shown that highest infection rate was 65.5% in 20-25 weeks age group followed by 47% in 26-30 weeks and 47.2% in 31 to 35 weeks. Among the birds of all age groups, younger birds are given higher serological response to the *Salmonella enteritidis* antigen.

Conclusion

This study stated that the iELISA immunoassay of SE was superior, rapid, easy to perform and most reliable test to screen the large number of birds for *S.enteritidis* infection and best use full tool for serosurvivalance to control the vertical and horizontal transmission of *S.enteritidis* in flocks.

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