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Evaluation of *Salmonella* Gallinarum Outer Membrane Protein Based Enzyme Linked Immunosorbent Assay for Detecting Antibodies in Vaccinated and Infected Chicken

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Abstract: Indirect enzyme linked immunosorbent assay was developed to measure specific antibody activity to outer membrane proteins in sera and egg yolk of chickens exposed to *Salmonella* Gallinarum infection and also following vaccination. The observed end point titers were used to formulate regression equation and the absorbance data obtained at a single serum dilution were converted directly to antibody titer by subtraction method. The assay was carried out at a single serum dilution of 1:320 standardized and the antibody titers of the *Salmonella* Gallinarum infected flocks were found to be of higher magnitude when compared to vaccinated flocks. The growth-decline trend of the egg yolk antibody levels was found to be similar to that of the serum antibody levels.

Key words: OMP, ELISA, *Salmonella* gallinarum, egg yolk, antibodies

Introduction

Fowl typhoid a septicemic disease caused by *Salmonella enterica subsp. enterica* serovars Gallinarum is widely distributed throughout the world (Shivaprasad, 2000). The upsurge of fowl typhoid in many countries in recent years has led to several studies on the use of both live and killed vaccines. Various authors have described the efficiency of vaccines in terms of clearance of the organisms from the organs and also protection against challenge. However, the immune response in terms of antibodies measurements are few.

Enzyme linked immunosorbent assays (ELISA) have been developed for detecting antibodies to various salmonellae. These ELISAs have been used with some success, particularly in Europe, but concerns about their specificity still persists. Screening assays for *Salmonella* in poultry are most useful if they could both consistently detect infection and predict the likelihood that infected hens will produce contaminated eggs. Egg yolks are an alternative source of antibodies that can be detected by methods similar to those applied to serum (Dadrast *et al*., 1990; Nicholas and Andrews, 1991). Antibodies to *Salmonella* have been found at relatively high titers in the sera of both experimentally (Gast and Beard, 1990; Timoney *et al*., 1990; Kim *et al*., 1991; Nicholas and Cullen, 1991) and naturally infected chicken (Chart *et al*., 1990; Poppe *et al*., 1992). Specific antibodies have been successfully detected using both the agglutination and ELISA test formats.

The major antigenic determinants of the salmonella like other gram-negative bacteria contain protein antigen besides lipopolysaccharides. Induction of protective immunity by such protein antigen has been demonstrated (Plant *et al.,* 1978). Since proteins are better antigens than carbohydrate substances, the use of outer membrane proteins (OMP) from *Salmonella* Gallinarum was examined for its protective ability in a vaccine preparation (Bouzoubaa *et al*. 1987; Bouzoubaa *et al*. 1989).

The objectives of the present study, was to standardize an OMP based immunoassay for detection of specific antibodies in serum and egg yolks of vaccinated chicken and infected flocks.

Materials and Methods

Culture: Standard *Salmonella* Gallinarum 9R (SG 9R) strain was obtained from the Indian Veterinary Research Institute, Izatnagar, U.P, India.

Antigen preparation: The extraction of OMP was performed as described previously (Prakash and Krishnappa 2002; Arun and Krishnappa, 2004). Briefly, SG 9R was grown in 3.0% tryptic soya broth for 16 hrs at 37°C. The growth was harvested by centrifugation at 4000 x g for 15 min and washed thrice in 0.001M PBS. The pellet was then suspended in 10 mM HEPES buffer and disrupted by sonication using 60 s pulses, 10 times in an ice bath at 30 sec interval. The enraptured cells and debris was removed by centrifugation at 4000 x g for 20 min at 5°C. The resultant supernatant containing cell membranes was subjected to ultra centrifugation at

1,05,000 x g for 45 min at 5°C. The pellet was resuspended in 20 mM Tris solution (pH 7.2) containing 1% Sodium N-lauryl sarcosine and incubated for 30 min at 22°C. The detergent insoluble OMP enriched fraction was collected by centrifugation at 1,05,000 x g for 45 minutes at 5°C. The pellet was suspended in 20 mM Tris solution and stored in small aliquots at – 70°C until use. The antigenic OMP content of the preparation was estimated using a protein-dye-binding method as described by Bradford (1976).

Reference positive serum: Antiserum to SG 9R was prepared using 13 weeks old roosters hatched from a flock with no previous history of *Salmonella* infection. The birds were raised under the cage system of rearing. Inactivated culture containing 10^9 cells/dose, emulsified with freund's incomplete adjuvant was administered through the subcutaneous route. Totally, three doses were administered at weekly intervals. The birds were test bled after the first booster to check for seroconversion by agar gel immunodiffusion. Ten days after the last booster, blood was collected by sacrificing the birds. The serum was separated and stored in small aliquots at –20°C, until further use.

Reference negative serum: Serum collected from the 13 week old roosters used for the production of SG 9R positive serum, one week before the inoculation was used as negative control.

Conjugate: Rabbit anti-chicken IgY conjugated to horseradish peroxidase (HRP) was obtained commercially from Bangalore Genei, Bangalore, India.

Substrate solution: Freshly prepared O-phenylene diamine-dihydrochloride (10mg) (Sigma Chemicals, USA) in 15 ml citric acid phosphate buffer containing 3% H_2O_2 was used as the substrate.

Diluents and Washing solution: Antigen was diluted in carbonate bicarbonate buffer (0.05 M, pH 9.6 ± 0.05) prior to coating. The serum and conjugate diluents and washing solution were phosphate buffer solution (PBS) containing 0.05% Tween-20 and 5% skim milk powder.

Protocol of immunoassay: The optimum working dilution of antigen and that of conjugate were determined by several checkerboard titrations (employing positive and negative reference sera). Microtiter plates (Nunc, Maxisorb(R), USA) were coated with antigen using coating buffer at a final concentration of 25 ng/well (1:40 dilution of OMPs) and incubated overnight at 4°C. Following incubation, excess antigen was removed and the plate was washed three times (three minutes each) with PBST. The unbound sites were blocked with 5% skim milk powder in PBST for one hour at 37°C. The coated plate was allowed to react with appropriately diluted test sera, followed by conjugate (1:10000), and finally the substrate solution. All the reagents including the substrate were added in 100 µl/well and incubated at 37°C for one hour. Between each step washing was repeated. Finally, the enzyme substrate reaction was stopped by adding 50 µl of 2.5 N HCl. The absorbance values were read using a software based labsystem, Megallan Plus microplate ELISA reader with filter of 492 nm. Reading was taken after the wells with only substrate/chromogen and HCl were blanked to 'zero' at 492 nm.

Prediction of antibody titers by end point titration: The ELISA antibody titers were determined by serial two fold dilution (beginning from 1:40 to 1:40960) and the end points were calculated by the subtraction method as described by Snyder *et al.* (1983). A total of five ELISAs were run with fifteen negative serum pools for the construction of a positive negative threshold (PNT) baseline to determine the observed ELISA antibody titers. First, the mean absorbance plus three standard deviation units above the mean were calculated from the replicates of all the trials at each negative serum dilution. The resultant mean ± three standard deviation unit values were plotted on log-log paper and employed as a baseline against the corresponding dilution for the calculation of the observed antibody titers. The absorbance of the test samples dilution was corrected by subtracting the appropriate absorbance of the internal negative control serum dilutions. The corrected average absorbance of the test samples were then graphed and the observed end point antibody titers were defined as the point, which intersected the PNT baseline.
A total of 19 positive antiserum were serially titrated and the observed end point titers were determined as described above.

Prediction of antibody titers in single serum dilution: Three dilutions (1:160, 1:320 and 1:640) were employed to derive the standard curves and the corresponding regression line equations for predicting antibody titers of the same nineteen samples containing a wide range of antibodies (employed earlier). The regression line equations for the corresponding single serum dilution was calculated using the equation Log_{10} titer = (Log_{10} absorbance - Y - intercept) / Slope. By solving the regression equation the titers were then predicted using the corrected absorbance from single dilution.

Antibody titers in salmonella infected flocks: Samples of serum and eggs were collected from breeder flocks, which were confirmed positive to *Salmonella* Gallinarum infection by way of isolation (Swayne *et al.*, 1998). A total of 20 samples per flock were collected and screened by ELISA standardized at 1:320 dilution. The yolks were

Table 1: Comparison of observed and predicted *Salmonella* Gallinarum antibody titers of test samples as determined by ELISA

Original end point titer	Predicted antibody titers at different dilutions		
	1:160	1:320	1:640
12589	8913	8278	7285
10000	7745	7834	6060
9762	7194	7144	5316
6309	6607	5140	4878
3162	3467	3123	3395
2089	3155	3040	3271
2018	2624	2851	3198
1949	2606	2691	2787
1905	2333	2606	2564
1819	1945	2333	2436
1698	1729	2023	1654
1584	1124	883	1160
1000	893	810	1130
618	868	629	1016
404	365	588	806
371	336	424	358
331	328	359	286
316	325	349	206
311	246	177	206

* and ** are significantly different (P<0.05), but doesn't vary more or less than two fold.

separated and the yolk antibodies were extracted with chloroform as described by Mingyong (1993).

Antibody response to vaccination: Two breeder flocks free of salmonellosis were vaccinated with a killed bentonite clay adjuvanted bacterin containing 10^9 cells / bird by subcutaneous route. One of the flock was vaccinated on 8th week and 15th week of age, while the other flock received a single dose of vaccine at 15th week of age only. Blood samples were collected from the immunized hens randomly at regular intervals of 5 weeks from 15th week onwards while the eggs laid were collected from the 20th week. All the serum and egg yolk antibodies were screened by ELISA at 1:320 dilution for prediction of antibody titers.

Results

Optimization of ELISA reagents: Checkerboard titration of antigen revealed a maximum absorbance at a dilution of 1:40 (containing 25ng/well) and was chosen as the working dilution for the OMP preparation. Likewise a 1:10000 dilution of rabbit anti-chicken HRP conjugate was found to be active and optimal for the assay.

Observed and predicted antibody titers: The PNT baseline absorbance was found to be 0.138 for the 1:320 dilution. The observed antibody titers were derived by plotting the average of the corrected absorbance in triplicate on log-log paper till the baseline was intersected and the antibody titers for the 19 samples analyzed is depicted in Table 1.

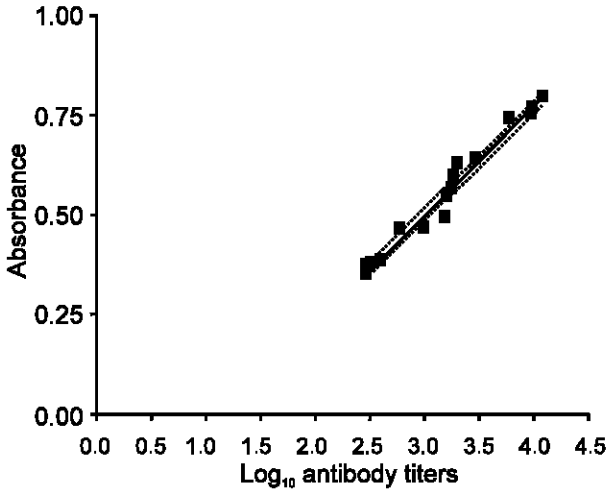

Fig. 1: The linear relationship between observed *Salmonella* Gallinarum OMP antibody activity titers of 19 test serum samples in ELISA and their corrected absorbance at 1:320 dilution.

Table 2: Constants obtained after linear regression analysis at different dilutions

Constants	1:160	1:320	1:640
r	0.982	0.965	0.943
Slope	0.2393±0.01	0.4596±0.03	0.6802±0.06
Y-intercept	-1.074±0.04	-2.001±0.09	-2.878±0.19

A linear relationship was found to exist between the $\log_{10}$ of the corrected absorbance at a single dilution and the corresponding observed titer, derived for the different dilution chosen (1:160, 1:320, and 1:640). Regression analysis revealed high correlation coefficients for all the three serum dilutions employed. The slope, Y-intercepts, 'r' values are shown in Table 2.

The predicted titers and the observed titers were found to be with differences of less than two fold. For optimal sensitivity and specificity of the assay 1:320 dilution was chosen for the assay so as to eliminate false positive/negatives, which are probable at very high or very low dilutions. The corrected absorbance versus the end point titers is presented graphically in Fig. 1.

Antibodies titers in infected flocks: The antibody titers in the flocks infected with *Salmonella* Gallinarum were found to be of a higher magnitude (Table 3) in the serum and the antibodies were also demonstrated in egg yolk.

Antibody response to vaccination: Antibody titers from the flocks vaccinated are shown in Table 3. The peak antibody titers were seen at 25 weeks following the booster dose at 15th week of age. A parallel response was observed in egg yolk titers derived from the vaccinated flocks. The flock, which received a single dose of vaccine revealed titers of a lower magnitude as compared to the flock, which received booster dose (Fig. 2).

Table 3: Mean (± SE) antibody titers evaluated using ELISA at single serum dilution (1:320) in infected and vaccinated flocks

Samples	Number of flocks	Serum Titers	Egg Yolk Titers
Salmonella infected flock	I	14589±1168	6913± 765
	II	11020±968	5745 ± 645
	III	10762±889	5194 ± 661
	IV	8509±666	4601 ± 541
	V	8264±770	5250 ± 495
Age of collection (in weeks)	15	2320±242	NA
Vaccinated flock I *	20	4254±239	2416 ± 186
(Primary vaccination on	25	6285±191	4254 ± 183
8th week and boosted	30	6012±185	3969 ± 175
on 15th week)	35	5824±200	3200 ± 150
	40	4856±196	2850 ± 156
Vaccinated flock II **	15	Negative	NA
(Primary vaccination on	20	2413±450	1656 ± 353
15th week and no	25	2159±475	1111 ± 345
booster)	30	1802±601	1063 ± 445
	35	1795±556	1004 ± 448
	40	1625±551	959 ± 496

NA - Not applicable. * batch antibody titers were significantly higher (P <± 0.05) than ** titers at different intervals of sample collection

Discussion

Various serological tests have been described for the assay of circulating antibodies to *Salmonella* Gallinarum, which included the whole blood plate test, rapid serum plate test, standard macroscopic tube agglutination, microagglutination, microantiglobulin tests, and ELISA (Swayne *et al.*, 1998). ELISA was preferred as it was found to be more sensitive, cheaper, less time consuming, and permitted handling a large number of samples simultaneously. In the present study the OMP fractions was utilized in the assay as it was reported to be of immunogenic value, in the studies conducted by Bouzoubaa *et al.* (1989). They reported that flocks vaccinated with OMP component of *Salmonella* Gallinarum, revealed better clearance and decreased transmission of pathogenic strain to zero by three weeks when compared to live SG 9R vaccine.

The OMP based indirect ELISA was found to be sensitive, rapid and adaptable in evaluating antibodies in the serum and egg yolk of seroconverted flocks. The OMP antigen coated, using the conventional carbonate-bicarbonate buffer was observed to be satisfactory as evidenced by low background in the assay. Further checkerboard titration of antigen and conjugate permitted optimal dilution of the reagents to be employed in the assays. The reproducibility of end point titrations appeared much higher in the present investigation, which could be due to samples diluted on a two-fold scale, rather than 10-fold scale (Malvano *et al.*, 1982).

Although, various methods were employed for expressing ELISA data, in the present study the subtraction method as described by Snyder *et al.* (1983) was employed. The subtraction method permitted correction of the absorbance of the test samples for each of the dilutions, resulting in minimal inter-assay variations. Further, it allowed the negative baseline to reflect the actual noise reduction following serial dilution of the negative sera resulting in better quality control. Also, high degree of correlation was observed for each of the dilutions tested (Table 1). The 1:320 dilution was chosen to predict titers, so as to reduce prozone effects, which probably occurred at very high or very low dilutions (Fig. 1 and Table 2). In addition, the occurrence of nonspecific reactions in avian ELISAs was often observed, particularly at low serum dilutions (York *et al.*, 1982). These reactions have been reported to be reduced by various modifications of the assay (Slaght *et al.*, 1978; Soula and Moreau, 1981) or by increasing the initial dilution of the serum being tested (Mockett and Darbyshire, 1981).

In addition, the use of single test dilution permitted a large number of samples to be screening, thereby reducing reagent costs. Further, the regression approach for predicting titers was employed, since it incorporated several absorbance values for the computation of titers; and was less sensitive to a single erroneous reading (Snyder *et al.*, 1983). High degree of correlation was observed between the observed and predicted titers (Table 1), which varied less than two fold for different dilutions employed.

The OMP based indirect ELISA was effective in demonstrating seroconversion against *Salmonella* Gallinarum in both vaccinated and infected flocks. The antibody titers in the infected flocks were of a very high magnitude (> two fold) when compared to the vaccinated flocks (Table 3). In addition, yolk sac immunoglobulins derived from vaccinated and infected flocks assayed

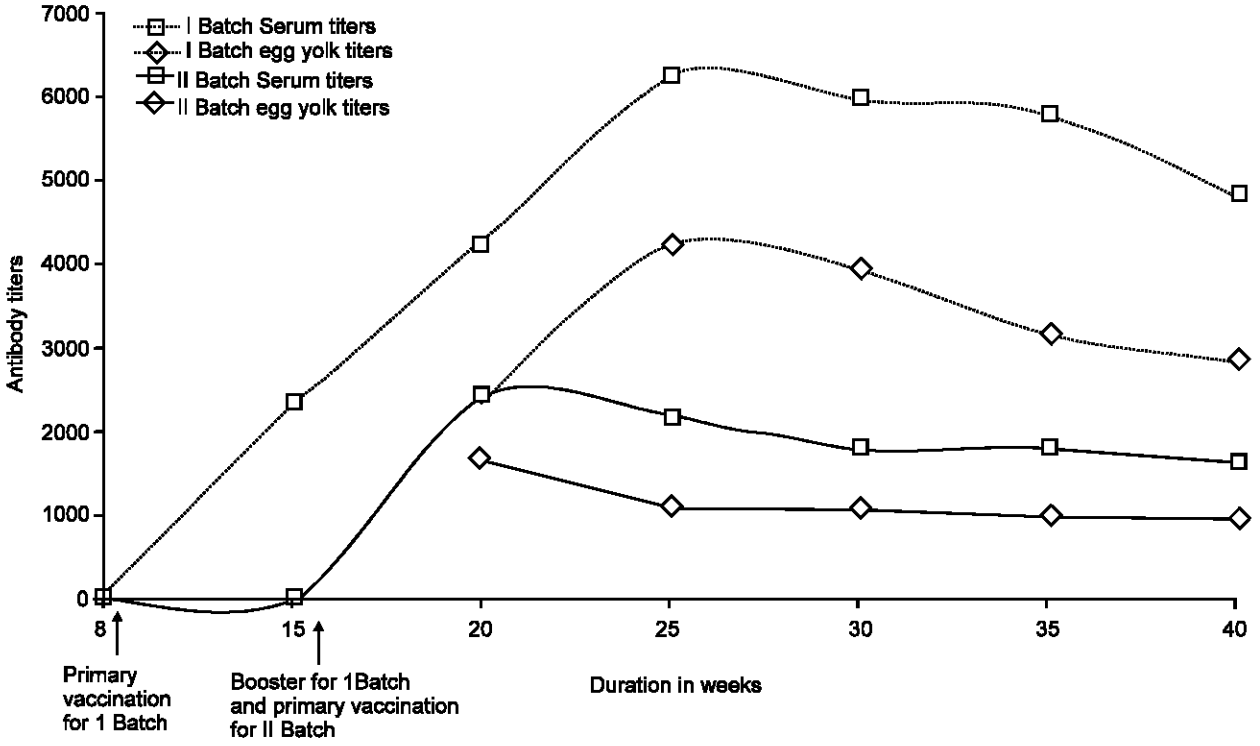

Fig. 2: Antibody titers in serum and egg yolk of breeder flocks following vaccination to Salmonella Gallinarum

revealed antibodies to *Salmonella* Gallinarum OMPs (Table 3), which was indicative of passive transfer of antibodies from the birds to the eggs, which is in concurrence with the findings reported by Larsson *et al.*, (1993) and Losch *et al.* (1986). The flock, which received a booster dose revealed a peak response around 25 weeks of age, which maintained the antibody level till 35 weeks, thereafter a perceptible drop was observed (Fig. 2). The antibodies in yolk also revealed a similar trend. The titers were invariably higher in the serum and egg yolk of the flocks vaccinated twice when compared to the flock vaccinated only once. The OMP based ELISA was found to be a sensitive tool in our study to screen chicken serum and egg yolk for antibodies against salmonella and was similar to the findings reported to lipopolysaccharides (Wysocki *et al.*, 2002; Tomaszewska *et al.*, 2003) and to whole cell antigen and OMPs (Lee and Lee., 2003).

To conclude, the OMP based indirect ELISA was found to be a useful serological test, for predicting the prevalence of salmonellosis and also for monitoring of immune response following vaccination in both serum and egg yolk. However, additional studies need to be conducted on the meaning of ELISA titers following vaccination with regard to protection in terms of survival and elimination of challenge infection. By translating the ELISA titers, the probability of survival and presenting the results in a profiling format, ELISA results could be easily understood by clinician and producer alike.

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