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## Validation and Refinement of *Salmonella pullorum* (SP) Colored Antigen for Diagnosis of *Salmonella* Infections in the Field

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**Abstract:** *Salmonella* infections are major problems for the poultry farming in Bangladesh. The cultural method to identify avian *Salmonella* infections is laborious and expensive, thus a rapid, sensitive and cost-effective method for the diagnosis of salmonellosis is anticipated. In the present investigation *Salmonella pullorum* organisms was obtained from the Department of Pathology and it was characterized by culture, biochemical tests and PCR. A neotetrazolium stained *Salmonella pullorum* antigen was prepared from local isolate of *Salmonella pullorum*. The protein concentration of stained antigen was measured by BSA standard curve. Prepared antigen was diluted in 2 fold dilution and minimum 72.5 µg/µl antigen concentration showed the positive reaction. Different preservatives (0.5% phenolized saline, 0.5% formalized saline and 0.09% sodium azide) were used to maintain the shelf life of prepared antigen. All the preservatives showed the similar results up to six months. Slide agglutination tests were carried out with un-diluted and diluted anti-sera having known ELISA titre and end point agglutination titre was determined. Serum titre 13942-21362 gave positive result to 2<sup>5</sup> fold dilution of serum and serum titre 412-771 showed negative result. Different groups of antigens were developed while antigen group-1 (48 h bacterial culture treated with 24 h in neotetrazolium and 2 h in thiomersal) gave the striking positive result. As Group-1 antigen exhibited highest protein concentration (1240 µg/µl) and gave the best result with positive sera, so it was selected for field trial. The seroprevalence of *Salmonella* infection was 44.39% in a particular poultry farm. The stained antigen was then stored at 4°C. As all used preservatives revealed similar trend of results, so it may be recommend that 0.5% phenolized saline as preservative because it is cost effective. In the present study, the slide agglutination test was found easy, sensitive, reliable, cost and time effective and needed very small amount of antigen, sera and as well as accessories. *Salmonella pullorum* antigen from a local isolate was successfully developed which could be used to screen the *Salmonella* infection in the poultry flocks at the farm premises. It may also be used to determine the antibody titer of the vaccinated flocks.

**Key words:** *Salmonella* infections, antibody titer, ELISA titre

### INTRODUCTION

Poultry rearing may play a vital role for the poverty alleviation. It fulfills one of the important sources of animal protein needed for the people. At present, there are more than 130 hatcheries producing 3.4 million day-old chick per week and about 30,000 commercial broiler and layers farms supplying 0.26 million metric tons of poultry meat and 2510 million table eggs per year (Rahman, 2003). Among the bacterial infectious diseases, *Salmonella* infections are major problems for the poultry farming in Bangladesh (Haider *et al.*, 2008). It causes a variety of acute and chronic diseases of poultry in Bangladesh (Bhattacharjee *et al.*, 1996).

For many years, the test of choice in diagnosis of *pullorum* disease and other *Salmonella* infections have been the slide agglutination test which was originally

developed by Runnels *et al.* (1927) for use with serum and adapted by Schaffer *et al.* (1931) for whole blood by using stained antigen (Tuchili *et al.*, 1995). The advantages of the microagglutination test are: (i) it requires fewer man powers to perform; (ii) it requires only one-twentieth of the amount of antigen; and (iii) it is easier to read (Brown *et al.*, 1980). Newly developed stained somatic antigen produced distinct tiny clumps with positive anti-sera of *Salmonella* spp. The present slide agglutination system was found to be easy, sensitive, reliable, cost and time effective and needed very small amount of antigen, sera and as well as accessories. This method may be used to screen the *Salmonella* infections in the poultry farms and to calculate the titre of the anti-*Salmonella* antibody in the infected and vaccinated chickens under farm conditions (Parvin, 2007).

## MATERIALS AND METHODS

The research work was conducted at the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh during the period of November 2007 to October 2008.

The research work was conducted through following methods: 1. Maintenance and characterization of *Salmonella pullorum* organism, 2. preparation of *Salmonella pullorum* colored antigen and 3. Validation with field sample.

**Sample collection:** *Salmonella pullorum* organisms were provided by Dr. Md. Golam Haider, PhD Fellow Department of pathology, Bangladesh Agricultural University, Mymensingh. The organism were then maintained and characterized culturally and biochemically and by PCR.

**Gram's staining procedure:** A small colony of representative *Salmonella* organisms was picked up by a bacteriological loop which was red hot and cooled down in agar media and a thin smear of bacteria made on glass slide. The smear was fixed by gentle heat. Then the smear was flooded by crystal violet solution for two minutes and then washed with running water. Gram's iodine was then added to act as mordant for one minute and then again washed with running water. Decolorizing agent (acetone alcohol) was then added for few seconds. It was washed with water and safranin (counter stain) was added and allowed to stain for 2 min. The slide was then washed with water, blotted and dried in air and then examined under microscope with high power objective (x 100) using immersion oil.

**Carbohydrate fermentation test:** The carbohydrate fermentation test was performed by inoculating 5 ml of nutrient broth culture of the organisms into the tubes containing different sugar media and incubated for 72 h at 37°C. Acid production was indicated by the color change from red to yellow of the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube.

**Biochemical test:** Several types of biochemical media and reagents like bacteriological peptone, methyl red, phenol red, liquid paraffin wax, MR-VP media, potassium hydroxide,  $\alpha$ -naphthol, alcohol and dulcitol were used in this study.

**Methyl red test procedure:** After incubation at 37°C, 2-4 drops of methyl red solution were added to the test tube which was incubated for 5 days for MR test. Positive test was indicated by the persistence of red color, indication of acidity and the negative one by the yellow color.

**Voges-Proskauer (V-P) test procedure:** The VP test was performed by adding 6 ml of VP reagent-1 and 0.2

ml of VP reagent-2 for each ml of culture. The ingredients were mixed thoroughly and allowed to stand for a while. The appearance of pink color indicated positive test.

**Indole test procedure:** 2 ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 h. 0.5 ml Kovac's reagent was added, shaken well and examined after 1 min. A red color in the reagent layer indicated indole. In negative case, there was no development of red colour.

**Dulcitol fermentation test:** This test was performed as like as carbohydrate fermentation test as described earlier and reaction profile was recorded.

**Motility test:** One drop of cultured broth was placed on the cover-slip and was placed inverted over the concave depression of the hanging drop slide to make hanging drop preparation. Around the concave depression of the hanging drop slide vaseline was used for better attachment of the cover-slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100-power objective of a compound microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with to and fro movement of bacteria.

**Total protein concentration (TPC) of the *Salmonella pullorum* stained antigen procedure:** Bovine Serum Albumin (BSA) standard was prepared by taking 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90  $\mu$ l with distilled water in separate eppendorf tube. 10  $\mu$ l protein samples (SP colored antigen) were also taken into separate eppendorf tubes and the volume was equaled to 100  $\mu$ l with distilled water. 1 ml solution-C was added in all tubes and incubated at room temperature for 10 min. 50  $\mu$ l Folin-ciocalteris reagent (Sigma) was added and made vortex immediately. Then it was incubated 30 min at room temperature at dark. Absorbance was taken at 660 nm at Spectronic® 20 Genesys™. Absorbances of standard proteins were plotted against concentration of BSA and the concentrations of unknown proteins were measured from standard curve (Fig. 1). Total protein concentration of stained antigen was calculated by multiplying dilution factor 20.

**Development of slide micro-agglutination test:** For this, 20  $\mu$ l of colored antigen and 20  $\mu$ l chicken sera were placed on a sterile glass slide by a micropipette and mixed thoroughly by stirring. The results were read within 02 min. In positive case, granules (agglutinates) were formed rapidly (within 2 min) due to combination of homologous antigen and antibody which was seen during rocking. In the absence of antibody, no such granules (agglutinates) were formed.

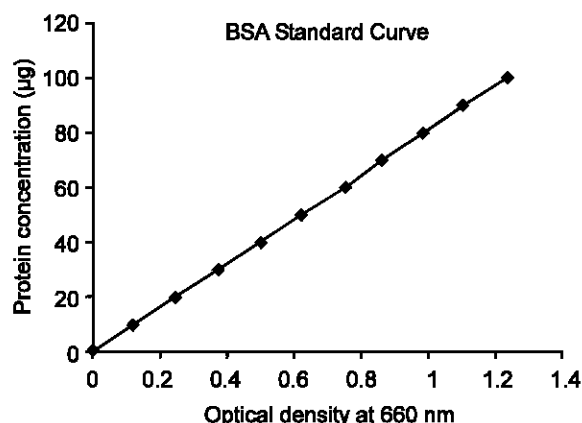


Fig. 1: Standard curve of BSA for total protein concentration in stained antigen

**Determination of the antibody titer:** 20 µl deionized water was placed on each eppendorf tube. 20 µl serum was added and mixed thoroughly. Again the procedure was repeated for several times up to obtaining positive reaction.

**Slide micro-agglutination test of diluted sera:** The serially diluted anti-sera were subjected to slide micro-agglutination test by the method that was previously described and the results were recorded.

**Comparative study with different group of prepared antigens:** Comparative study of prepared *S. pullorum* colored antigen was done by taking 20 µl of colored antigen and 20 µl chicken sera which were placed on a sterile glass slide by a micropipette and mixed thoroughly by stirring. The results were read within 02 minutes. In positive case, granules (agglutinates) were formed rapidly (within 2 min).

**Validation with field sample by whole blood agglutination test:** A clean slide was used. 1 drop (about 20 µl) of *S. pullorum* colored antigen was placed in the centre of each slide. A sample of fresh whole blood was obtained by making a stab of a wing vein using a needle with a triangular point. The drop of antigen and blood was mixed using a fine glass rod, which was wiped clean between samples. A gentle rocking motion was used to keep the drops agitated for up to 2 min. The drops should not be allowed to dry out during this time. A positive reaction was indicated by easily visible clumping of the antigen within 2 min. A negative reaction was indicated by absence of clumping of the antigen within 2 min.

**Micro-agglutination test:** The slide micro-agglutination test was performed with the newly developed *Salmonella pullorum* color antigen. Total sera of each

flock was tested and recorded individually. The agglutination results were recorded as Positive (+) and Negative (-).

**Storage and shelf life determination:** To determine the shelf life of stained antigen three preservatives 0.5% phenolized saline, 0.5% formalized saline and 0.09% sodium azide were chosen and observed for six months from the day of preparation.

## RESULTS

In the present research at first *Salmonella pullorum* organisms were obtained from Dr. M.G Haider, PhD Researcher in the Department of Pathology, Bangladesh Agricultural University, Mymensingh for the preparation of colored antigen. The antigen was tested with known positive and negative serum by serial folding. Validation with field sample was performed from 10 selected layer poultry flocks in the Trishal Upazilla of Mymensingh districts. About 446 birds were tested for Whole Blood Agglutination Test (WBA).

**Cultural character of *Salmonella* in different media:** *Salmonella* organisms showed different cultural characteristics in different media. These were turbidity in TTB, pink white color colonies in BGA, gray white colony in nutrient agar, slightly grayish color colonies in SS agar, black color colony in TSI agar, pale color colonies in Mc Conkey's agar, well defined glistening colonies in blood agar and pinkish colonies in EMB agar.

**Gram's staining:** From pure culture of different media, Gram's staining was performed to observe *Salmonella* organism. Under compound light microscope the organism was identified as Gram-negative, rod shaped and arranged in single. Chains of more than two bacilli were normally absent (Fig. 5).

**Carbohydrate fermentation tests:** In biochemical test the isolate was lactose and dulcitol non-fermenter, glucose and mannitol were fermented with production of acid or acid and gas. From red to yellow color change was the indication of acid production. Gas production was indicated by the deposition of gas bubbles in the Durham's tube. So the organism was confirmed as *Salmonella pullorum*.

**Different biochemical tests:** Different biochemical tests were used to identify the *Salmonella* organism. *Salmonella pullorum* was dulcitol negative. All the isolates of *Salmonella pullorum* were MR positive and VP negative. Indole test was also negative.

**Motility test:** *Salmonella pullorum* isolates were identified as non-motile.

Table 1: Protein concentration of prepared *Salmonella pullorum* colored antigen

Antigen group	Optical density	Protein concentration (µg/µl)
Group 1	0.619	1240
Group 2	0.595	1200
Group 3	0.585	1180
Group 4	0.575	1160
Group 5	0.560	1120
Group 6	0.545	1100
Group 7	0.545	1100
Group 8	0.519	1040

Group 1: 48 h culture 24 h Neotetrazolium and 2 h in Thiomerasol  
 Group 2: 48 h culture 24 h Neotetrazolium and 2 h in Phenol  
 Group 3: 48 h culture 2 h Neotetrazolium and 2 h in Thiomerasol  
 Group 4: 48 h culture 2 h Neotetrazolium and 2 h in Phenol  
 Group 5: 24 h culture 24 h Neotetrazolium and 2 h in Thiomerasol  
 Group 6: 24 h culture 24 h Neotetrazolium and 2 h in Phenol  
 Group 7: 24 h culture 2 h Neotetrazolium and 2 h in Thiomerasol  
 Group 8: 24 h culture 2 h Neotetrazolium and 2 h in Phenol

Table 2: Result of agglutination with diluted antigen

Antigen group	Protein concentration (µg/µl)	Positive up to fold
Group 1	1240	2 <sup>4</sup>
Group 2	1200	2 <sup>3</sup>
Group 3	1180	2 <sup>3</sup>
Group 4	1160	2 <sup>3</sup>
Group 5	1120	2 <sup>3</sup>
Group 6	1100	2 <sup>3</sup>
Group 7	1100	2 <sup>3</sup>
Group 8	1040	2 <sup>3</sup>

**Characterization by PCR:** Characterization of locally isolates of *Salmonella pullorum* organisms supplied by Dr. M.G. Haider, PhD researcher Department of Pathology, Bangladesh Agricultural University, Mymensingh. Locally isolates of *Salmonella pullorum* were tested using 139(F) and 141(R) primer with targeting gene *invA* and showed 284-bp products from each field samples after 1.5% agarose gel electrophoresis. But control sample did not show any amplicon (Fig. 6).

**Preparation of *Salmonella pullorum* colored antigen**  
**Bulk culture in Difco™ veal infusion agar:** Bulk culture is necessary for the preparation of *Salmonella* antigen. Thus a single colony of *Salmonella pullorum* from Difco™ veal infusion agar was inoculated in to Difco™ veal infusion broth. The inoculum formed confluent growth that showed more turbidity and sliminess.

***Salmonella pullorum* colored antigen:** *Salmonella pullorum* color antigen was prepared. The neotetrazolium stained *Salmonella* antigen showed bluish in color so that the agglutination result was easily seen. Eight different groups of colored antigen were prepared in different incubation period and by using different chemicals. All groups were used for comparative study.

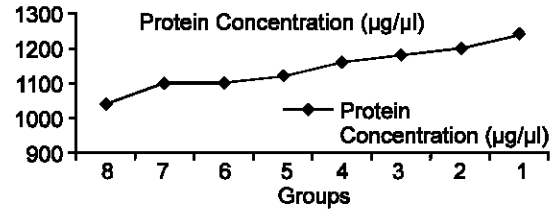


Fig. 2: Graphical presentation of protein concentration of different antigen groups

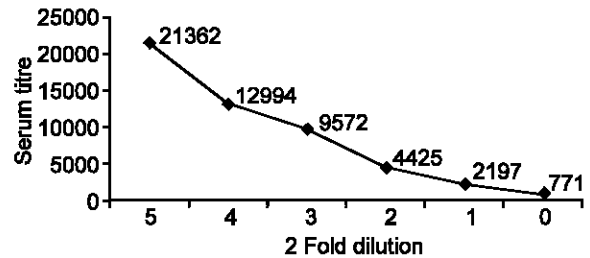


Fig. 3: Proportional curve between 2 fold dilution and ELISA titer

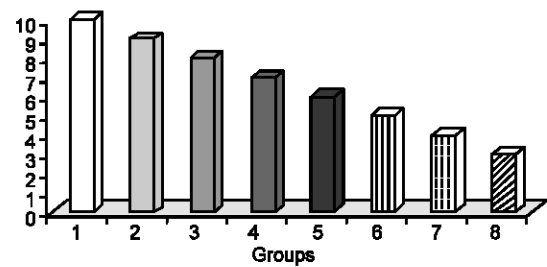


Fig. 4: Graphical presentation of comparative results of different antigen groups

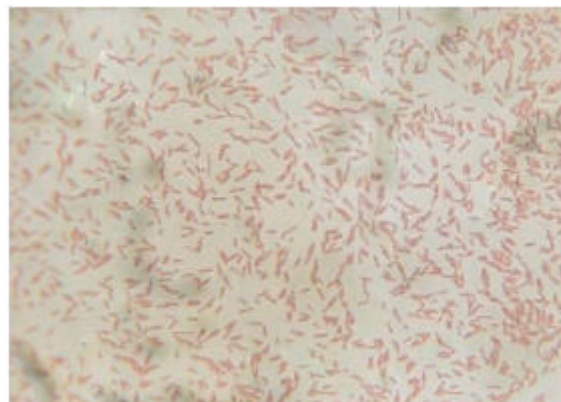


Fig. 5: *Salmonella pullorum* shows Gram negative, rod shaped, pink color bacilli in Gram's staining (83 x)

**Protein estimation of *Salmonella pullorum* colored antigen by BSA standard curve:** Total protein concentration of stained antigen was calculated by

multiplying 20 corresponding BSA standard curve as the dilution factor was 20.

Table 1 and Fig. 4 show that Group 1 antigen contained highest protein concentration.

**Reaction with diluted antigen:** The newly developed *Salmonella pullorum* colored antigen with the known positive sera were diluted as 2 fold pattern and agglutination test was performed. The agglutination results of the 2 fold diluted antigen were shown in the Table 2. The positive agglutination was recorded maximum up to  $2^4$  dilution with antibody having antigen concentration 1240  $\mu\text{g}/\mu\text{l}$ .

**Agglutination test with known antibody titre of serum:** The slide micro-agglutination test was performed as per procedure. The results were read within 2 min. In positive case, granules (agglutinates) were formed rapidly due to combination of homologous antigen and antibody which was seen during rocking. In the absence of antibody, no such granules (agglutinates) were formed. The reaction started to develop within 30 sec and completed within 2 min. The color of the agglutinins was bluish and agglutinins were fine granular (Fig. 7). The specificity of the stained antigen was high as it did not react with the negative control serum and water (Fig. 8).

**Reaction with diluted serum:** The collected known positive sera were diluted as 2 fold pattern and agglutination test was performed with the newly developed *Salmonella pullorum* colored antigen. The positive agglutination was recorded maximum up to  $2^5$  dilution with antibody having titer 21362 (Fig. 3). The serum titre 13942-21362 gave to  $2^5$  fold positive result, titre 10006-12825 gave to  $2^4$  fold positive result, titre 4864-9572 gave to  $2^3$  fold positive result, titre 2517-4425 gave to  $2^2$  fold positive result, titre 2197-1190 gave to  $2^1$  fold positive result and titre 412-771 showed negative result (Fig. 3).

**Comparative study with different group of antigen:** Group 1 antigen showed the best result among the 8 groups of antigens (Fig. 4). The highest protein concentration was 1240  $\mu\text{g}/\mu\text{l}$  in group-1 and the lowest protein concentration was 1040  $\mu\text{g}/\mu\text{l}$  in group-8.

**Validation with field sample:** According to above result Group-1 antigen was selected for validation in the field. In positive cases *Salmonella pullorum* color antigen reacted with blood and produced fine granules (Fig. 9). In negative cases no such granules were found (Fig. 10).

Table 3 shows that prevalence of *Salmonella* 44.39%. The highest prevalence is 65% found in flock-1 and lowest prevalence is 30% found in flock-10.

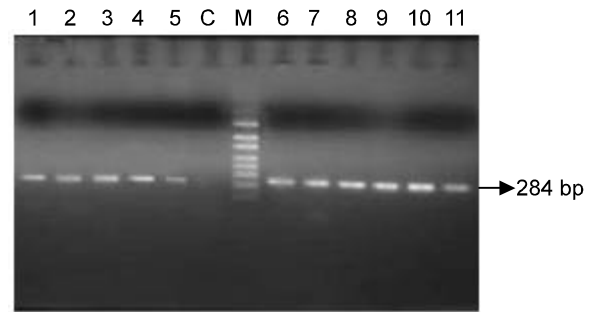


Fig. 6: Electrophoresis on agarose gel showing the 284 bp PCR product (from lane 1 to lane 11) after amplified with *invA* genes in field samples of locally isolated *Salmonella pullorum* and lane C and lane M showing the negative control and marker, respectively

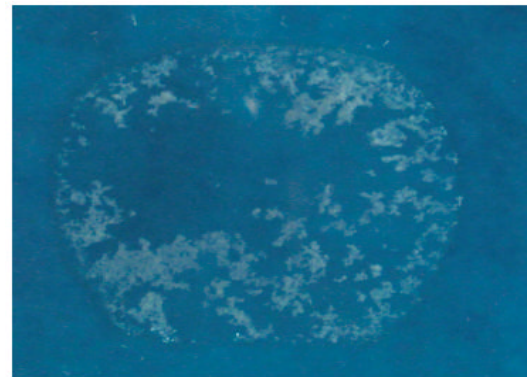


Fig. 7: Agglutination test with known positive serum

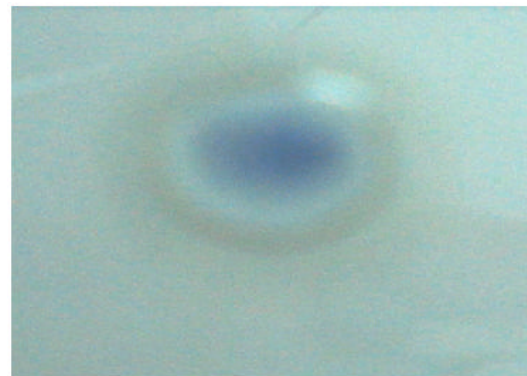


Fig. 8: Agglutination test with known negative serum

**Preservative:** It was observed that all three preservative 0.5% phenolized saline, 0.5% formalinized saline and 0.09% sodium azide give similar result reaction with known positive serum.

**Storage and shelf life determination:** Shelf life of stained antigen with three preservatives 0.05%

Table 3: Reaction with field samples

Flock No.	Age day	Total birds	No. of blood test	Seropositive	Prevalence (%)
1	27	5027	40	26	65
2	189	5185	42	22	52.3
3	301	4216	34	14	41.17
4	52	6050	48	25	52
5	112	5985	48	27	54
6	157	5122	40	16	38
7	196	6025	48	19	39
8	201	6322	52	20	38
9	247	6411	54	17	31
10	266	4911	40	12	30
Total		55254	446	198	44.39

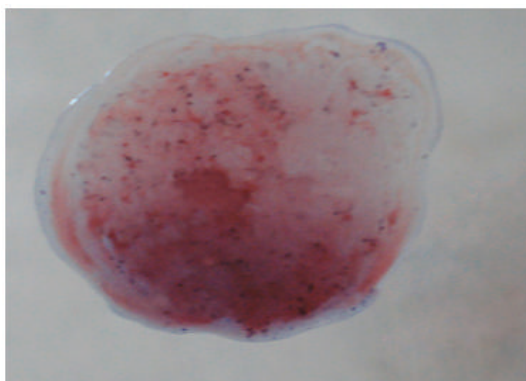


Fig. 9: Whole blood agglutination test with field sample positive result



Fig. 10: Whole blood agglutination test with field sample negative result

phenolized saline, 0.05% formalized saline and 0.09% sodium azide were chosen and was tested three times at day of preparation, after three months of preparation and after six months of preparation during the study period. All preservative showed similar result during test period. Antigen showed good result within study period. The stained antigen was then stored at 4°C.

## DISCUSSION

The present study was conducted to prepare *Salmonella pullorum* colored antigen from a local isolates. Efficacy test was done by reaction with known positive and negative serum and by whole blood agglutination test at Krishibid Farm Limited, Trishal upazilla. Mymwnsingh. *Salmonella pullorum* organism was characterized by using different types of cultural media, Gram's staining, biochemical test, sugar fermentation test and PCR.

Different media were used for the isolation of *Salmonella* organisms. The media includes TTB, BGA, BA, Mc Conkey's agar, LIA, SS agar, TSI, EMB agar and nutrient agar (Rybolt *et al.*, 2005). In Gram's staining, the organisms were Gram-negative, rod shaped, pink color and short to long chains and single or paired in arrangement. This study was supported by many investigators (Goswami *et al.*, 2003; Haider *et al.*, 2003). Different biochemical tests were used for identification

of *Salmonella pullorum* organism in this study. *Salmonella pullorum* were MR positive and VP and indole negative. In the dulcitol fermentation test, *Salmonella pullorum* was dulcitol non-fermenter (Sujatha *et al.*, 2003; Batabyal *et al.*, 2003; Haider *et al.*, 2003). Different carbohydrate fermentation tests were used to *Salmonella pullorum*. *Salmonella pullorum* were lactose and sucrose negative but fermented glucose and mannitol with acid and gas production. *Salmonella pullorum* did not ferment maltose (Sujatha *et al.*, 2003; Haider *et al.*, 2003). Motility tests were performed to characterize *Salmonella pullorum* which was non-motile (Haider *et al.*, 2003; Islam *et al.*, 2006). *Salmonella pullorum* were tested using 139(F) and 141(R) primer with targeting gene *invA* and showed 284-bp products from each field samples after 1.5% agarose gel electrophoresis.

In the present study, neotetrazolium stained *Salmonella pullorum* antigen was prepared from a local isolates and a micro-agglutination procedure was developed for the detection of *Salmonella* infection in farm level. The neotetrazolium stained *Salmonella* antigen showed enhanced sensitivity than the conventional agglutination test as previously described by Parvin (2007) and Williams and Whittemore (1972).

The protein concentration of prepared *Salmonella pullorum* colored antigen was determined by BSA



standard curve the highest protein concentration was observed in Group-1 and a consecutive decrease in Group-8. Highest protein concentration in Group-1 was due to culturing at higher duration which caused increased organismal propagation and ultimately made higher protein concentration per unit of solution. Group-7 and Group-8 took lowest culturing time and resulted lowest protein concentration. BSA standard curve showed higher protein concentration with increasing Optical Density (OD) value. Same was observed in the present finding that when OD value increased, simultaneously protein concentration increased at a same rate (Fig. 3). Antigen was diluted as 2 fold pattern and reaction was performed with known positive serum. Group-1 antigen gave upto 2<sup>4</sup> fold reaction due to high protein concentration and higher incubation period. From findings, it was observed that at least 72.5 µg/µl antigen (protein concentration) gave positive reaction with known sera.

Micro-agglutination procedure was developed by reaction with known positive and negative sera. The reaction started to develop within 30 sec in positive case and in susceptible case it may need some more time but not more than 2 min. It required only 20 µl sera and 20 µl antigen. The color of the agglutinins was bluish and it was fine granular which were easily visible by the naked eye without the problems of being doubtful. The specificity of the stained antigen was high as it did not react with the negative serum and water control. The results are in agreement with a previous study, Parvin (2007) and Williams and Whittemore (1972).

Reaction with known positive sera showed that serum titre 13942-21362 gave to 2<sup>5</sup> fold positive result, titre 10006-12994 gave to 2<sup>4</sup> fold positive result, titre 4864-9572 gave to 2<sup>3</sup> fold positive result, titre 2517-4425 gave to 2<sup>2</sup> fold positive result, titre 2197-771 gave to 2<sup>1</sup> fold positive result and titre 412-694 showed negative result. Comparative study with different antigen groups was performed by reacting with known positive sera. From above findings Group-1 antigen gave positive result with all the sera due to higher protein concentration and higher incubation period. Group-8 gave lowest positive reaction due to lower protein concentration. So highest protein concentration and higher incubation period gave best result.

As Group-1 antigen exhibited highest protein concentration (1240 µg/µl) and gave best result with positive sera, it was selected for field trial. The seroprevalence of *Salmonella* infection was 44.39% which is supported by Ahmed *et al.* (2008), Arsenault *et al.* (2007) and Islam *et al.* (2006). However, Jai-Sundar *et al.* (2007) and Hoop and Pospischil (1993) reported 61.68, 63.5, 64.2 and 64.9% seroprevalence, respectively which were higher than the present findings. The highest prevalence was recorded in the Nicobar Islands, with all the sera samples (100%) being positive to Salmonellosis (Jai-Sundar *et al.*, 2007). The variation

was due to the environmental, managerial and differences in the geographical distribution. Mdegela *et al.* (2000) reported 2.6% seroprevalence, Robinson *et al.* (2000) 18.4% Jha *et al.* (1995) 21.3% and 12% in two village flock, Lu *et al.* (1992) 2.0%, 2.9% and 4.2% and Bell *et al.* (1990) 6.2% seroprevalence which were much more lower than the present study. This was because of all the prevalence study were done on village chickens where the prevalence of *Salmonella* infection were generally lower than the commercial chickens (Mdegela *et al.*, 2000; Robinson *et al.*, 2000). In the present study, the highest seroprevalence was observed in the starter group (65%) and lowest in the layer group (30%). Within the different aged flocks, one of the flocks showed the maximum 65% at the age of 27 days. The seroprevalence percent declined with the advancement of age in the present study. This study was strongly supported by Ahmed *et al.* (2008).

Shelf life of stained antigen with three preservatives 0.5% phenolized saline, 0.5% formalized saline and 0.09% sodium azide were chosen and was tested three times at day of preparation, after three months of preparation and after six months of preparation during the study period. All preservatives showed similar result during test period. Antigen showed good result within study period. The stained antigen was then stored at 4°C (Parvin, 2007; Williams and Whittemore, 1972; Williams and Whittemore, 1979). We can recommend 0.5% phenolized saline as preservative because it is cost effective.

From the above discussion, it may be concluded that a neotetrazolium stained *Salmonella pullorum* antigen from a local isolate was successfully developed which could be used to screen the *Salmonella* infection in the poultry flocks at the farm premises. It also may be used to determine the antibody titer of the vaccinated flocks.

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