

Investigation on the Efficacy of Salmonella Bivalent Vaccine

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Abstract: The work was performed to investigate the efficacy of Salmonella bivalent vaccine containing *Salmonella gallinarum* and *Salmonella pullorum* prepared at the Livestock and Poultry Vaccine Research and Production Centre (LPVRPC) of the Bangladesh Agricultural University (BAU). Purity and safety test of the vaccine was carried out as per OIE (2008). For efficacy test, vaccination was performed in Shaver brown chicken of group A containing 10 birds while group B comprising of 10 birds was maintained as unvaccinated control. Birds were inoculated primarily via intramuscular route at 7 weeks of age with 0.5ml (4.7×10^7 CFU/ml) of vaccine followed by a booster dose at 35 days of primary vaccination (DPV). At 21 DPV (3 weeks), mean PHA antibody titre of sera samples were recorded as 104.00 ± 11.71 with *S. gallinarum* and 112.00 ± 10.47 with *S. pullorum* antigen whereas sera samples obtained at 35 DPV (5 weeks) showed mean PHA antibody titre of 96.00 ± 12.09 and 80.00 ± 10.47 respectively. At 2 weeks of booster vaccination such mean PHA antibody titres were 144.00 ± 16.00 and 136.00 ± 24.00 . LD_{50} were determined to calculate the challenge dose. Prior to challenge given at 4 weeks of booster vaccination the mean PHA antibody titres were found to be 104.00 ± 11.71 with both the experimental antigens while unvaccinated control group B had $\leq 4.0 \pm 0.00$. It was observed that the birds vaccinated with the schedule of bivalent vaccination and exhibiting mean titres of 104.00 ± 11.71 with either *S. gallinarum* or *S. pullorum* antigens withstood the challenge infection given IM with 1ml containing 8.6×10^{13} CFU/ml and 8.9×10^{13} CFU/ml of virulent experimental bacterial cultures respectively. The PHA titre of group A birds analyzed by student t-test to determine the protective capacity of vaccinated chickens against challenge exposure. It was demonstrated that experimental Salmonella bivalent vaccine conferred protection against challenge infection and was found to be safe.

Keywords: *Salmonella gallinarum*, *Salmonella pullorum*, vaccine, immunogenicity, PHA titres.

I. Introduction

Salmonellosis is one of the most important bacterial diseases in poultry industry causing heavy economic loss through mortality and reduced productivity (Begum *et al.* 1992, Haque *et al.* 1997). The disease is most significant because the causal agents of the disease are transmitted vertically from parents to offsprings. The importance of Salmonellosis in poultry sector has drawn increased day by day attend on throughout the world. It is potentially responsible for various pathogenic processes in man and animals including poultry (Freeman, 1985).

There are mainly two types of *Salmonella* spp. namely *S. gallinarum* and *S. pullorum* that cause fowl typhoid and pullorum disease respectively. These two species of *Salmonella* are very important in poultry health because they are responsible for massive destruction. Salmonellae may cause varieties of clinical signs from acute systemic disease and gastrointestinal symptoms in the poultry flocks to embryonic problem in hatchery (Gast, 1997). Pullorum disease also known as bacillary white diarrhoea (BWD) caused by *S. pullorum* is usually confined to the first 2-3 weeks of age and occasionally occurs in adults (Shivaprashad, 1997). Fowl typhoid (FT) caused by *S. gallinarum* is frequently referred to as a disease of adult birds but there are also reports of high mortality in young indistinguishable from those associated with pullorum diseases (Threlfall and Frost, 1990). With great expansion of the poultry rearing and farming, pullorum disease and fowl typhoid have become widespread problem in Bangladesh like other countries of the world (Rahman *et al.*, 1979) causing heavy economic losses in broiler, layer and breeding flocks. Such a loss occurs in both broiler and layer flocks due to morbidity, mortality, reduced production and chick quality. Mortality may vary from negligible to 10% and upto 80% or higher in severe outbreaks (Kumar and Kaushi, 1988, Kaura *et al.* 1990, Kleven and Yoder, 1998). The major emphasis for preventing infections is to avoid the introduction of pathogens into the farms by increased bio-security (Gifford *et al.*, 1987) along with vaccination. Vaccine available is both live (usually based on the Houghton 9R strain) and bacterins (killed / inactivated vaccine).

Salmonella vaccines of both live and killed type are imported and marketed in Bangladesh by different commercial companies beside those of local manufacture. It is necessary to monitor sterility, purity, safety and protective efficacy of any biologics or vaccines by respective controlling agency or an accredited agency prior to introduce it within the country for field use. Poultry Biologics Unit (PBU) recently renamed as "Livestock and Poultry Vaccine Research and Production Centre (LPVRPC) incorporated with its parent organization the

Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU) Mymensingh produces a bivalent vaccine employing *S. pullorum* and *S. gallinarum* which are distributed for field use. The present work was undertaken with the objectives of determine the efficacy along with determination of PHA titre of sera obtained from the vaccinated birds. Hence, a thorough investigation on protective efficacy of experimentally prepared salmonella bivalent vaccine was performed in Shaver brown chicken

II. Materials And Methods

2.1. Experimental layout

The study included vaccination of *Salmonella* bivalent vaccine followed by measurement of antibody titre by the passive haemagglutination (PHA) test, determination of lethal dose fifty (LD₅₀) of the bacteria and performance of challenge test in the vaccinated and control birds (figure 1).

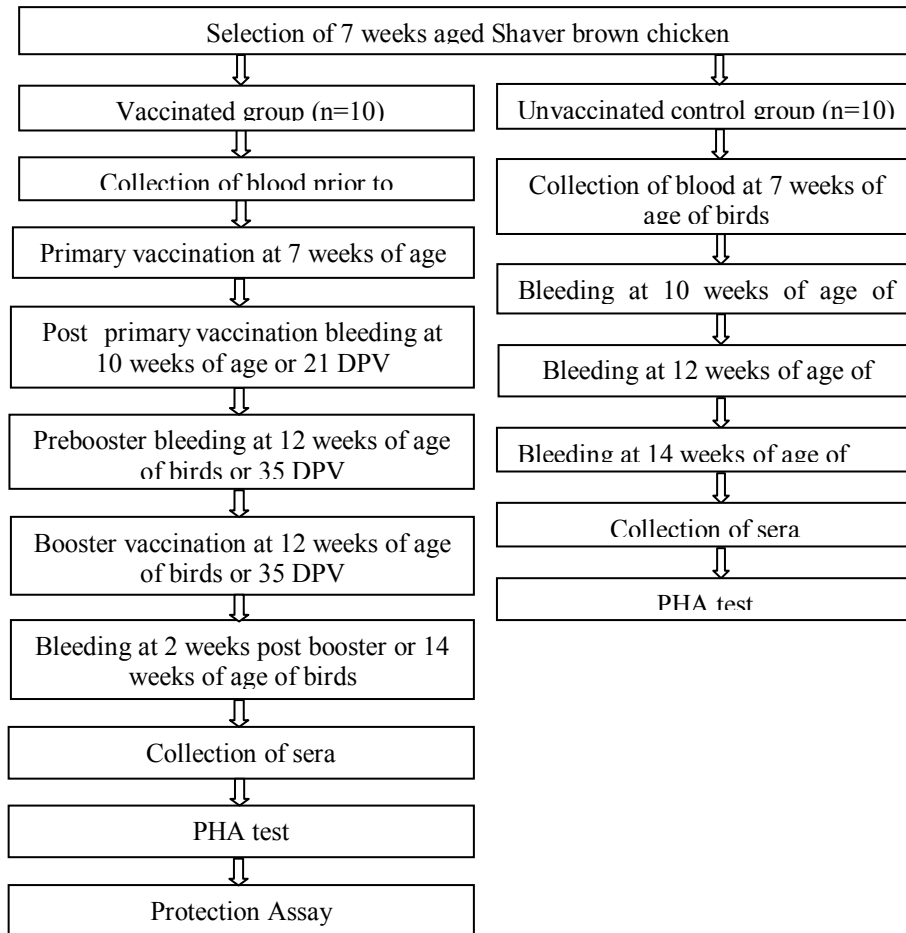


Fig- 1: Experimental layout

2.2. Passive haemagglutination (PHA) test

The test was used to determine the antibody titres in birds against *Salmonella spp.* following of vaccination as described by Carter (1979), Tripathy *et al.*, (1970), Chowdhury *et al.*, (1985), Sarker *et al.* (1976) and Siddque (1997) with slight modification.

2.3. Principle of the test

The sensitivity and specificity of PHA test depends on the use of soluble antigens. In this case, somatic antigens of *Salmonella spp.* were coupled with chemically modified horse erythrocytes so that antigen-coated erythrocytes readily react with specific antibodies and results in haemagglutination.

2.4. Collection and preparation of 2.5% horse red blood cells (HRBC)

Blood was collected from the right jugular vein of a normal adult horse with sterile syringe and needle containing 5 ml of Alsever's solution per 10 ml of blood. The blood was centrifuged in graduated centrifuge tube at 1500 rpm for 10 minutes. The supernatant was then pipetted off and the blood cells were resuspended with PBS and then centrifuged. The process was repeated for at least three times for washing the blood cells. During

last washing the cells were maintained in PBS for 15 minutes and then centrifuged at 2000 rpm for 10 minutes to obtain the packed cells. The sediment blood cells were diluted with PBS to make 2.5% suspension of the blood cells and preserved at 4 to 8°C.

2.5. Tannic acid solution (1:200)

Tannic acid solution (1:200) was prepared by dissolving 1 gm of tannic acid powder in 200 ml of distilled water in a conical flask and the solution was sterilized by autoclaving at 121°C maintaining a pressure of 15 lb pressure per sq. inch for 15 minutes (1 kg/Cm²) and kept at 4°C to 8°C until used. Finally, tannic solution was prepared by mixing one ml (1:20,000) of the stock solution (1:200) with 99 ml of PBS taken in a conical flask and mixed thoroughly (Siddique, 1997)

2.6. Inactivation and preparation of 1% normal rabbit serum diluents (NRSD)

Collected rabbit serum was inactivated at 56°C in hot water bath for 30 minutes and then one ml of the inactivated serum was added with 99 ml of PBS (pH 7.2) in a conical flask to obtain 1% solution. The serum solution then kept at 4 to 8°C.

2.7. Tannic acid treatment of horse red blood cells

Five milliliters of 2.5% HRBC and 5 ml of 1: 20,000 dilution of tannic acid was taken in a test tube and mixed thoroughly. The mixture was then incubated at 37°C for 10 to 15 minutes in water bath according to the methods of Tripathy *et al.*, (1970). The cells were centrifuged at 2000 rpm for 10 minutes; the sediment was then washed with PBS. Washed tanned HRBC was again diluted to make 2.5% suspension with PBS and used for the test.

2.8. Preparation of somatic antigen

The isolates of *S. gallinarum* and *S. pullorum* organism were cultured on SS agar media. Incubated overnight at 37°C and selected a smooth colony and carried out slide agglutination test to ensure that the required somatic antigen is present. A pure culture on nutrient agar slope after incubation for 8-12 hours at 37°C was washed off the plate by using Pasteur pipette with 2 ml of absolute alcohol. It was then transferred into a sterile container. The antigen was left for 4-6 hours at room temperature to enable the alcohol to kill the bacteria. The container was spined for 5 minutes at 1000 rpm. The liquid was poured off and added enough phenol saline to make the antigen up to opacity. Standard titration was carried out with known serum to ensure that the antigen is positive for the required factor and stored at 4°C.

2.9. Sensitization of somatic antigen with tannic acid treated horse red blood cell

Three ml of sensitized HRBC (2.5%), 1 ml of somatic antigen (1:10 dilution) and 8 ml PBS were mixed together. This mixture was incubated at 37°C for 20 to 30 minutes. After sensitization, the cells were centrifuged at 1500 rpm for 10 minutes, then the supernatant fluid was discarded and the sedimented HRBC was collected and diluted with 1% normal rabbit serum diluents (NRSD) at the ratio of 1: 4. This was then mixed thoroughly and kept at room temperature for an hour and centrifuged for 10 minutes. The cells were resuspended in 1% NRSD to make 0.5% sensitized cells for use in microtitre plate method and stored at 4°C until used (Tripathy *et al.*, 1970).

2.10. Microtitre plate method

The procedure of the PHA test was followed according to the method described by Tripathy *et al.*, (1970).

- i. An amount of 50 µl of PBS was first poured in each well up to 8th well of horizontal row of microtitre plate. 50 µl of test serum was added in the 1st well.
- ii. Two fold dilutions of serum ranging from 1: 2 to 1: 256 were made by transferring 50 µl of the mixture from the 1st well to 2nd well and thus continuing successively up to the 8th well from where an excess amount of 50 µl of the mixture was poured off.
- iii. A volume of 50 µl 0.5% somatic antigen sensitized hRBC was taken in each of the eight wells.
 - a. Control system, horizontal row of microtitre plate
 - 9th well: equal volume of 50 µl of normal serum and PBS.
 - 10th well: equal volume of 50 µl of sensitized tanned RBC and PBS.
- iv. The content of the wells of the test system and control were mixed by gentle agitation of the microtitre plate and kept at room temperature for 4 to 5 hours.
- v. Agglutination resulting from mixing of test serum and somatic antigen sensitized HRBC in the test system was observed very carefully.
- vi. Deposition of diffuse thin layer of clumping of HRBC on the bottom of the well considered as positive and the result were recorded.

vii. The PHA titre was the highest dilution of test sera were complete haemagglutination occur due to the reaction of specific antibody and antigen sensitized tanned HRBC.

The reciprocal of the end point of highest dilution of test sera and sensitized tanned HRBC was considered as titre of the serum. Agglutination was indicated by a flat deposition of a diffuse thin layer of clumping of RBC on the bottom of the wells. The results were recorded after deposition of a diffuse thin layer of clumping of RBC on the bottom of the well, which indicated HA positive, and a compact buttoning with clear zone indicated HA negative. The reciprocal of the highest dilution of sensitized tanned HRBC was considered as titre of the serum.

III. Results

3.1. PHA antibody titre

The prevaccination mean PHA antibody titre with standard error (\pm) was $\leq 4.0 \pm 0.00$ in chickens of all groups. After three (3) weeks or 21 DPV the mean PHA antibody titres were 104.00 ± 11.71 and 112.00 ± 10.47 in groups of birds inoculated with *S. gallinarum* and *S. pullorum* respectively. At prebooster bleeding on 35 DPV the mean PHA antibody titres were 96.00 ± 12.09 in *S. gallinarum* and 80.00 ± 10.47 in *S. pullorum*. After two weeks of booster vaccination, the mean PHA antibody titres were 144.00 ± 16.00 and 136.00 ± 24.00 in birds vaccinated with *S. gallinarum* and *S. pullorum* respectively. Before challenge, provided at 4 weeks of booster vaccination, the mean PHA antibody titres were 104.00 ± 11.71 in both the groups of birds administered with *S. gallinarum* and *S. pullorum*. The mean \pm SE PHA titre of vaccinated birds and control birds are presented in Table 1.

Table 1: Mean PHA titre with standard error of sera of chickens vaccinated with *Salmonella bivalent* vaccine prepared at BAU.

| Weeks of vaccination | Used antigen | Pre- vaccinated PHA titre of all vaccinated and control birds (Mean \pm SE) | Post Vaccination PHA titre of vaccinated group (Mean \pm SE) | P value |
|----------------------|----------------------|---|--|---------|
| 10 | <i>S. gallinarum</i> | <4 \pm 0.00 | 104.00 \pm 11.71 | 0.0001 |
| 10 | <i>S. pullorum</i> | <4 \pm 0.00 | 112.00 \pm 10.47 | |
| 12 | <i>S. gallinarum</i> | <4 \pm 0.00 | 96.00 \pm 12.09 | |
| 12 | <i>S. pullorum</i> | <4 \pm 0.00 | 80.00 \pm 10.47 | |
| 14 | <i>S. gallinarum</i> | <4 \pm 0.00 | 144.00 \pm 16.00 | |
| 14 | <i>S. pullorum</i> | <4 \pm 0.00 | 136.00 \pm 18.88 | |
| 16 | <i>S. gallinarum</i> | <4 \pm 0.00 | 104.00 \pm 11.71 | |
| 16 | <i>S. pullorum</i> | <4 \pm 0.00 | 104.00 \pm 11.71 | |

** means (P<0.01)

Legends: PHA=Passive haemagglutination

Mean=Geometric mean of 10 birds, SE=Standard error

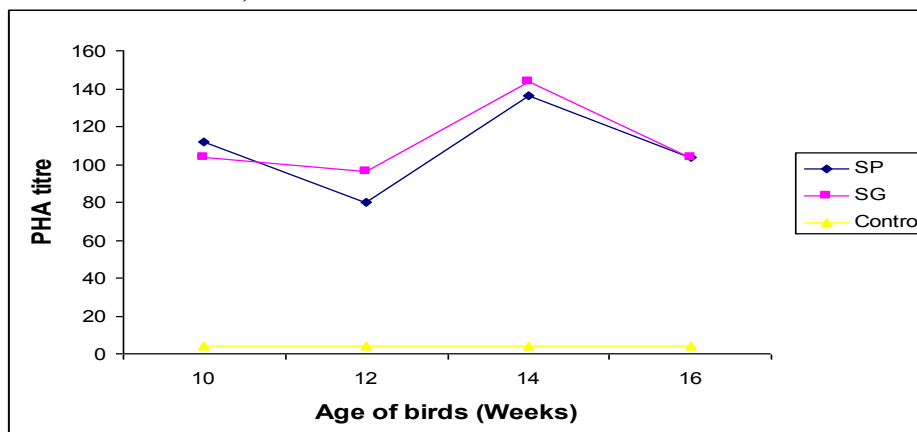


Fig-2: Graphical representation of mean PHA titres with standard error of sera of chickens vaccinated with *Salmonella bivalent* vaccine prepared at BAU.

3.2. Results of challenge infection

Challenge infection at the dose rate of 1 ml (*S. pullorum*: 8.9×10^{13} CFU/ml and *S. gallinarum*: 8.6×10^{13} CFU/ml) was given separately to the chickens of group both A, B. All birds of the group A were resistant to virulent challenge exposure. All birds of the group B showed sign and symptoms of infection within one day of challenge. It was demonstrated that experimentally prepared *Salmonella bivalent* vaccine conferred 100%

protection against challenge infection given after 4 weeks of secondary (booster) vaccination when unvaccinated control birds were found to be affected with challenge organisms.

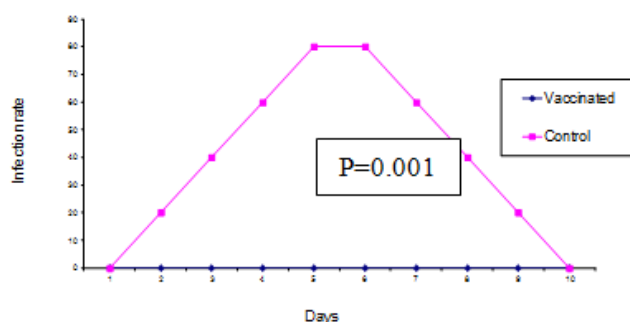


Fig: 3 Graphical representation of post challenge infection

IV. Discussion

In Bangladesh a good number of commercial companies import *Salmonella* vaccine for marketing. Such imported vaccines are used without any field trial which should have been mandatory in terms of testing of efficacy. In consideration of this, the efficacy of *Salmonella* bivalent vaccine produced at LPVRPC, BAU, Mymensingh, was selected for the determination of the serum antibody titre by PHA test. As an essential part of efficacy test, the LD₅₀ of virulent *Salmonella gallinarum* and *Salmonella pullorum* was also determined individually so that protection test might be carried out without any question whatsoever. The purity (sterility) and safety test of the concerned vaccine was carried out as per instruction of OIE (2008).

PHA test of sera from vaccinated and control birds were conducted for determination of antibody titre as per the method described by authors mentioned earlier. The pre-vaccinated PHA titre of sera samples of all vaccinated chickens were recorded as $\leq 4 \pm 00$ which was closely related to the findings of Ferdous (2008), Yeasmin (2010) and Jannatun (2010).

Sera collected on 21 and 35 DPV and those collected at two weeks following secondary (booster) vaccination were subjected to PHA test. The test conducted with both *Salmonella gallinarum* and *Salmonella pullorum* antigen revealed titre of 104.00 ± 11.71 and 112.00 ± 10.47 on 21 DPV and 96.00 ± 12.09 and 80.00 ± 10.47 on 35 DPV and 144.00 ± 16.00 and 136.00 ± 24.00 at two weeks and 104.00 ± 11.71 at four weeks of booster vaccination.

LD₅₀ of *S. gallinarum* and *S. pullorum* was found to be containing 8.6×10^{13} CFU/ ml and 8.9×10^{13} CFU/ ml. The experimental birds, both vaccinated and control, were exposed to protection test (challenge test) after four weeks of booster vaccination. These results were in agreement with the statement of Rahman *et al* (2005), who found that the antibody titre reached peak at 4 weeks vaccination in chickens.

Based on the results of the study; it may be calculated that

- i. The experimental birds having vaccinated with the schedule of primary and secondary (booster) vaccine and exhibiting mean PHA titre of 104.00 ± 11.71 and 104.00 ± 11.71 at four weeks of booster vaccination were protected following challenge infections with virulent cultures of *S. gallinarum* and *S. pullorum* with a dose of 1ml (8.6×10^{13} CFU/ml and 8.9×10^{13} CFU/ml) administered via IM.
- ii. *Salmonella* bivalent vaccine containing *S. gallinarum* and *S. pullorum* prepared at LPVRPC fulfilled the criteria of safety, purity, and efficacy and appeared to be dependable for inducing satisfactory level of immunity.

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