

## INVESTIGATION ON THE EFFICACY OF *SALMONELLA GALLINARUM* KILLED VACCINE

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### ABSTRACT

The study was undertaken to investigate the efficacy of *Salmonella gallinarum* vaccine prepared at the Livestock and Poultry Vaccine Research and Production Centre (LPVRPC), Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. The vaccine induced immune response both in chicken and mice are determined by PHA antibody titre and protection test. The highest PHA antibody titer was at 15 days after booster vaccination in both chickens and mice. The chickens and mice of vaccinated groups conferred 100% protection following challenge infection with the virulent isolate of *Salmonella gallinarum* given at 2 weeks after final immunization through i/m route ( $p < 0.01$ ). Differential leukocyte count (DLC) was performed in vaccinated mice and it was revealed that 11% increase in lymphocyte count in vaccinated group compared to control group ( $p < 0.01$ ). Finally, passive protection test in chickens that the protective value in terms of overall survival rate was 100% ( $p < 0.001$ ). These results clearly demonstrated that the *Salmonella gallinarum* vaccine of LPVRPC induced satisfactory level of antibody titre.

**Key words:** *Salmonella gallinarum*, efficacy, vaccination, PHA test and protection

### INTRODUCTION

*Salmonella gallinarum* (SG) is a biovar of avian host specific serotype *Salmonella enterica* (Uzzau *et al.*, 2000). It is a non-motile, Gram negative rod and along with the closely related *Salmonella enterica* serovar *pullorum*, but rarely, if ever, presents a risk of zoonotic transmission to man (Shivaprasad *et al.*, 2000). *S. gallinarum* causes a severe systemic disease of chickens and other galliformes birds which is called fowl typhoid (FT).

FT is frequently referred as a disease of adult birds, although, there are also many reports of high mortality in young chicks (Samad, 2005). Most FT cases appear in brown layers, which occupy most of the commercial layer farms and sometimes in meat-type breeders (Lee *et al.*, 2003). Clinical signs of FT are typical of a septicemic condition in poultry and include increased mortality and poor quality in chicks hatched from infected eggs. Older birds show signs of anaemia, depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. The highest mortality occurs in birds of 2-3 weeks of age. In breeding flocks reduced egg production and hatchability may be the only signs and trans-ovarian infection resulting in infection of the egg and hatched chicks or poults is one of the most important vertical transmission routes for the disease (OIE, 2008). Infection in chickens may occur at all ages and is typified by severe hepatosplenomegaly accompanied by characteristic liver 'bronzing', anaemia and septicaemia (Shivaprasad *et al.*, 2000). *S. gallinarum* is primarily associated with the mononuclear phagocyte system and resides primarily within macrophages in the liver and spleen (Barrow *et al.*, 1994 and Wigley *et al.*, 2002). It is only found in the gastrointestinal tract early in the infection and at the end stage of fowl typhoid where bacteria shed into the intestines causing substantial haemorrhage of the intestinal wall (Wigley *et al.*, 2002).

The major emphasis for preventing infections is to avoid the introduction of pathogens into the farms by increased biosecurity (Gifford *et al.*, 1987) along with vaccination (Seo *et al.*, 2000). A number of investigations were performed with *Salmonella gallinarum*, *Salmonella pullorum* and bivalent vaccine concerned with investigation of humoral immunity. No study has yet been accomplished to evaluate both humoral and cellular immunity together essential to protect birds from FT.

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*Salmonella* vaccines of both live and killed type are imported and marketed in Bangladesh. Prior to introducing a vaccine within the country by different commercial companies for field use, it is mandatory to monitor sterility, purity, safety and protective efficacy of any biologics or vaccines by respective controlling agency or an accredited agency. Such legal provisions are not at all followed. Livestock and Poultry Vaccine Research and Production Centre (LPVRPC), Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh produces and distributes vaccine to protect birds against both (*S. pullorum* and *S. gallinarum*). Rahman (2011) and Basak (2011) reported that SG vaccine produced in Department of Microbiology and Hygiene effectively induced antibody production and protected the vaccinated chicken against challenge infection, but the mechanism of protection still remains unknown. Therefore, the present research was undertaken in Shaver brown chicken to investigate the immunogenicity and protective activity of *Salmonella gallinarum* vaccine with mechanism of protection produced by LPVRPC, Department of Microbiology and Hygiene, Bangladesh Agricultural University.

## MATERIALS AND METHODS

### Study area and duration

The research was conducted during the period of July 2011 to December 2011 in the Bacteriology laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh.

### Experimental mice and chickens

A total of 70 mice (Balb/c) were obtained from the experimental animal shed of Department of Microbiology and Hygiene, BAU, Mymensingh and used for safety, efficacy test and determination of LD<sub>50</sub> dose. Twenty (20) eight-week-old layer birds (Shaver brown strain) were obtained with the courtesy of Phenix Poultry Hatchery Limited. Another twenty (20) Hisex white chickens of eight-week-old were taken and used for passive protection test. The mice and birds were provided with balanced diet, pure water, and other managerial requirements for proper bio-security.

### Purity and safety test of *Salmonella gallinarum* vaccine

Five blood agar (BA) plates were inoculated with five *Salmonella gallinarum* vaccine and incubated at 37 °C for 24- 48 hours in the incubator for the growth of aerobic and anaerobic organism. The *Salmonella gallinarum* vaccine which did not exhibit the growth of aerobic and anaerobic organism was used in the experiment (Heddleston and Reisinger, 1960). The safety test was carried out following the method of Matsumoto and Helfer (1977) and Dorsey (1963). Five mice were inoculated subcutaneously (S/C) with 0.5 ml of each vaccine and the vaccine considered safe if the inoculated mice remained alive and healthy during the observation period of 5 days.

### Determination of LD<sub>50</sub> dose in mice

*Salmonella gallinarum* was cultured in nutrient broth overnight at 37 °C in a shaker incubator. After 12 hours growth, centrifuged at 1500 rpm for 5 minutes. Then the supernatant was decanted off and bacterial mass was re-suspended in PBS by shaking. This process was repeated three times. The pellet was then dissolved in sufficient amount of PBS and OD value was measured at 550 nm and OD value was adjusted at 2.8. A 250 µl, 100µl, 50µl and 25 µl bacterial suspension or PBS were injected intra-peritoneally in mice (n=10) of each group. Death pattern was observed for subsequent 10 days. A 10-fold dilution of the bacterial suspension was made and spread on nutrient agar (NA) media for counting of CFU (8.6×10<sup>13</sup> CFU/ ml). A 25 µl of 8.6×10<sup>13</sup> CFU/ml contain LD<sub>50</sub> dose in mice.

### Immunization of chicken and mice

Experimental chickens (Shaver brown strain) were divided into two groups namely A and B. The chickens of group A (n=10) were vaccinated with *Salmonella gallinarum* vaccine @ 0.5 ml (3.75 × 10<sup>9</sup> CFU/ml) through subcutaneous (s/c) route at 8-week of age for primary vaccination. These birds were revaccinated with booster dose of vaccine through same route after 4 weeks (at 12-week of age) of primary vaccination. The chickens of group B (n=10) were kept as non-vaccinated control. Among 70 mice (Balb/c), 20 was divided into 2 groups namely group A (n=10) and group B (n=10). The mice of group A was used for vaccination and group B was maintained as unvaccinated control. Mice of group A was vaccinated with *Salmonella gallinarum* killed vaccine @100 µl (3.75 x 10<sup>9</sup> CFU/ml) through intramuscular (i/m) route at 10

week of age for primary vaccination. After 2 weeks of primary vaccination (at 12 weeks of age), the vaccinated mice were boosterd with same dose through same route.

#### **Collection and preservation of sera from chicken and mice**

The sera from vaccinated and non-vaccinated chicken and mice were collected and preserved following the method described by Heddleston and Reisinger (1960). The sera were collected from vaccinated chickens at 8 (Pre-vaccinated), 10 (Post vaccinated), 12 (Pre-boosterd), 14 (Post boosterd) and 16 (Post challenged) weeks of age and from mice at 10 (Pre-vaccinated) and 12 (Post boosterd) weeks of age. Sera were also collected from non-vaccinated control chickens and mice at the same date.

#### **Preparation of challenge dose**

*Salmonella gallinarum* was cultured in 5 ml nutrient broth at 37 °C for overnight. Then an aliquot of the culture was inoculated into 50 ml of fresh medium and further incubated at 37 °C for another 12 hours in a shaker incubator. The cultures were then centrifuged, washed twice with PBS and adjusted to an OD of 2.80 at 550 nm wave length.

#### **Passive haemagglutination (PHA) test**

The PHA test was performed to determine the antibody titre of collected serum from both vaccinated and non-vaccinated chickens and mice according to the method described by Tripathy *et al.* (1970).

#### **Differential Leukocyte Count (DLC) in mice**

Ten 10-week-old mice were selected to calculate the DLC. Mice were divided into two groups (A and B). Mice of group A were immunized at 10 weeks age and boosted at 12 weeks age through i/m route at the dose rate of 100 µl/ mice ( $3.75 \times 10^9$  CFU/ml of SG). Mice of group B was kept as non-vaccinated control. For blood profiling, a drop of blood was taken from tip of the tail of mice of both groups at 10, 12, and 14 weeks of age. DLC was performed after staining with Wright's stain.

#### **Protection test in chickens**

Two weeks (14 weeks of age) after final immunization of both vaccinated (Group A) and control (Group B) chickens were challenged with 10LD<sub>50</sub> ( $8.6 \times 10^{13}$  CFU/ml of *S. gallinarum*) of mice through i/m route which corresponds about 90% infection in chicken and the mortality of challenged chickens were monitored for the subsequent 10 days.

#### **Protection test in mice**

Two weeks after final immunization, mice were challenged intramuscularly with 1.5 mice LD<sub>50</sub> of *S. gallinarum* and the mortality of challenged mice were monitored for the subsequent 10 days.

#### **Passive protection test in chickens**

Sera were obtained from vaccinated birds (Group A) after 2 week of booster vaccination and twenty (20) Hisex white chickens of eight-week-old were taken to perform the passive protection test (PPT). The chickens were divided in to two groups (A and B). A 250µl of pooled serum was mixed with 10LD<sub>50</sub> dose ( $8.6 \times 10^{13}$  CFU/ ml) of SG antigen and incubated at 37 °C for 45 minutes. These suspensions were then administered intramuscularly to chickens of group C @ 500µl /chicken. On the other hand 250 µl of PBS was mixed with 10 LD<sub>50</sub> dose ( $8.6 \times 10^{13}$  CFU/ ml) of SG antigen and incubated at 37 °C for 45 minutes and then administered through i/m route to the chickens of group D @ 500µl/chicken. The mortality of the chickens of both groups was monitored for subsequent 10 days as per the method described by Saha *et al.* (2006).

#### **Statistical analysis**

Statistical analysis of PHA antibody titer was performed using Student's paired t-test ( $p \leq 0.05$  was considered statistically significant). Differences in leukocyte count were analyzed by Mann-Whitney test ( $p \leq 0.01$  was considered statistically significant). Challenge infection and passive protection test were analyzed by Mantle-Cox Log rank test ( $p \leq 0.01$  was considered statistically significant).

## RESULTS

### Purity and safety test of *Salmonella gallinarum* vaccine

The purity of the SG vaccine was evaluated by inoculating 0.1 ml of vaccine onto blood agar media. The vaccine was biologically pure as no growth of organisms was detected after inoculated media at 37 °C for 24-48 hours. After inoculation of 0.5 ml of *Salmonella gallinarum* vaccine into the mice subcutaneously, the mice were kept under observation for five days. No clinical signs or mortality was detected within the observation period. The results revealed that the vaccine was safe for vaccination.

### PHA test in chickens

Serum was collected from the chickens of both vaccinated and non-vaccinated group at different interval and subjected to PHA test. The pre-vaccinated PHA antibody titres of chickens of both groups (A and B) were  $\leq 4 \pm 0.0$ . The PHA antibody titres of vaccinated chickens (Group A) at 10 (Post vaccinated), 12 (Pre-booster), 14 (Post booster) and 16 (Post challenge) weeks of were  $102.4 \pm 15.676$ ,  $70.4 \pm 15.676$ ,  $153.6 \pm 43.40$  and  $96.00 \pm 20.23$  respectively. The PHA antibody titres of non-vaccinated control group were  $\leq 4 \pm 0.0$  at both 10, 12, 14 and 16 weeks of age of chickens (Figure 1).



Figure 1. Serum antibody titres against SG vaccination in chicken. Chickens were immunized with SG vaccine at 8 week age of birds and boosted at 12 weeks through S/C route at the dose rate of 0.5 ml / bird ( $3.75 \times 10^9$  CFU/ml). Serum was collected at 8, 10, 12, 14 and 16 weeks age of chicken and subjected to PHA test. The graph shows the mean  $\pm$  SE values of serum PHA antibody titre (n=5 chickens/group). \* $p < 0.05$  by Student's t-test.

### Protection test in chicken

Chickens of vaccinated and non-vaccinated groups were challenged with SG antigen and mortality rate was observed for subsequent 10 days. All birds of the group A were resistant to virulent challenge exposure whereas all birds of group B showed signs and symptoms of infection within two days of post challenge and all control birds died within 7 days of post challenge (Figure 2).

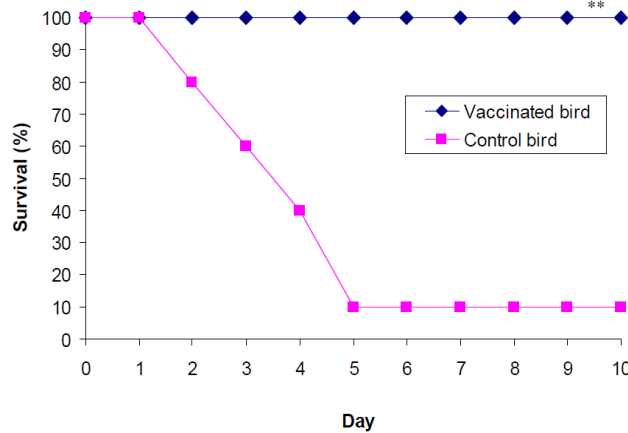


Figure 2. Survival rate of chickens following challenge infection. Chickens were immunized twice with SG antigen at 8 week age of birds and boosted at 12 weeks age through s/c route at the dose rate of 0.5 ml / bird ( $3.75 \times 10^9$  CFU/ml). Survival rate of chicken was monitored following i/m challenge infection with  $10LD_{50}$  dose of mice ( $8.6 \times 10^{13}$  CFU/ml) for subsequent 10 days. (n=10 chickens/group). \*\* $p < 0.01$  by Mantel-Cox Log rank test.

**PHA test in mice**

The PHA antibody titres (Mean± SE) of mice belonged to group A at 10 and 14 weeks of age were recorded as  $5.2 \pm 1.2$  and  $64.17 \pm 5.27$  respectively. On the contrary, PHA antibody titers of mice belonged to group B were  $5.2 \pm 1.2$  at both 10 and 14 weeks of age (Figure 3).

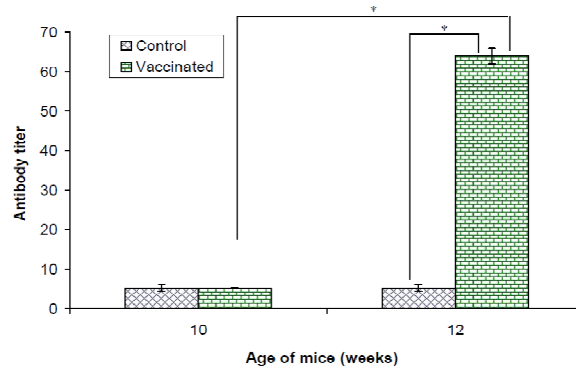


Figure 3. Serum PHA antibody titres of mice immunized with *Salmonella gallinarum* vaccine. Mice were immunized at 10 weeks age of mice and boosted at 12 weeks age through i/m route at the dose rate of 100 µl/mice ( $3.75 \times 10^9$  CFU/ml). Serum was collected from both vaccinated and non-vaccinated mice at 10 and 14 week age of mice and subjected to PHA test. The PHA antibody titers were expressed as mean ± SE. (n=5 mice/ group). \* $p < 0.05$  by Student's t-test.

**Protection test in mice**

The mice of vaccinated and non-vaccinated groups were challenged with SG antigen and mortality rate was monitored for subsequent 10 days. All vaccinated mice (Group A) were resistant to virulent challenge exposure. Whereas all non-vaccinated mice (Group B) were showed signs of diseases within one day of post infection and died within 5 days of challenge infection (Figure 4).

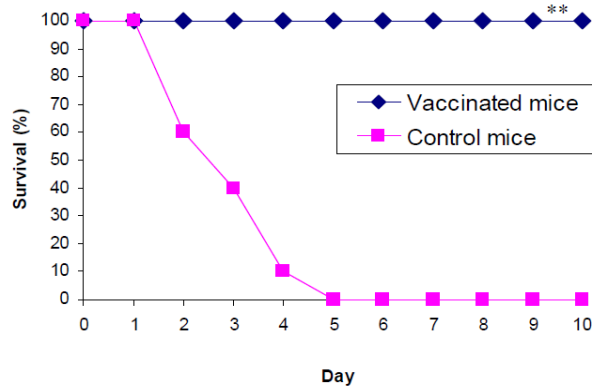


Figure 4. Survival rate of mice challenged intramuscularly with virulent isolate of SG antigen. Mice were immunized with SG antigen at 10 week age and boosted at 12 weeks age through i/m route at the dose rate of 0.5 ml/mice ( $3.75 \times 10^9$  CFU/ml). Survival rate of mice was monitored for subsequent 10 days following i/m challenge with 1.5 LD<sub>50</sub> doses of mice ( $8.6 \times 10^{13}$  CFU/ml). (n=10 mice/group). \*\**p* < 0.01 by Mantel-Cox Log rank test.

**Differential leukocytes count (DLC) in mice**

The mean±SE of leukocyte count of vaccinated mice (Group A) at 10 (Pre-vaccinated), 12 (Pre-bolstered) and 14 (Post bolstered) weeks of age were 61.2±0.66, 69.2±1.96 and 72± 1.41 respectively. Whereas the mean±SE of leukocyte count of non-vaccinated mice (Group B) were 61.2±0.66 at 10, 12 and 14 weeks of age of mice (Figure 5).

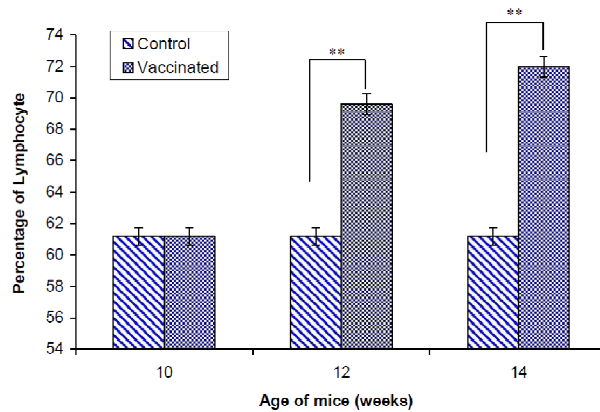


Figure 5. Percentage of leukocyte against SG antigen vaccination in mice. Mice were immunized at 10 week age of mice and boosted at 12 week age of mice through i/m route at the dose rate of 100 µl/mice ( $3.75 \times 10^9$  CFU/ml) and DLC was performed at 10, 12 and 14 week of age. The figure shows the mean±SE of leukocytes. (n=5 mice/group). \*\**p* < 0.01 by Man-Whitney test.

**Passive Protection test in chicken**

Bacteria and PBS administered birds showed typical clinical signs of FT such as diarrhoea, lethargy, off feeding etc. and finally die within 10 days. On the other hand mixture (bacteria + serum) administered birds did not expressed any signs of illness. That means no clinical signs of FT were showed within 10 days of observation period. This indicated that 250 µl of hyper immune serum was able to neutralized 250 µl of infective dose of bacteria (Figure 6).

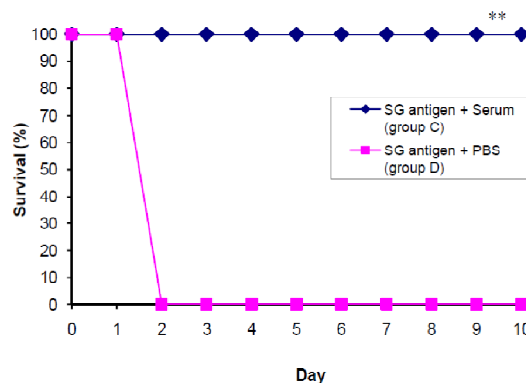


Figure 6. Survival rate of chicken after passive protection test. A 250 $\mu$ l of pooled serum was mixed with 10 LD50 dose ( $8.6 \times 10^{13}$  CFU/ ml) of SG antigen and incubated at 37  $^{\circ}$ C for 45 minutes. These suspensions were then administered intramuscularly to chickens of group C @ 500 $\mu$ l /chicken. On the other hand 250  $\mu$ l of PBS was mixed with 10 LD50 dose ( $8.6 \times 10^{13}$  CFU/ ml) of SG antigen and incubated at 37  $^{\circ}$ C for 45 minutes and then administered to the chickens of group D @ 500 $\mu$ l/chicken. The mortality was monitored for subsequent 10 days. \*\* $p < 0.01$  by Mantel-Cox Log rank test.

## DISCUSSION

Vaccination against *S. gallinarum* is commonly used as preventive measure and is regarded as an additional measure to increase the resistance of birds against *Salmonella* exposure and decrease shedding of *Salmonella*. A number of authors (Collins, 1973; Brito *et al.*, 1993; Babu *et al.*, 2004) have demonstrated that the expression of acquired resistance to salmonellosis depends upon the combined humoral and cellular responses of the infected host. Specific serum immunoglobulin play a primary role during the early clearance phase of the infection (Collins, 1969), but once the organisms become established in an intracellular environment within the liver and spleen, their subsequent elimination depends upon a cell-mediated type of immune response (Mackaness, 1971). Cerquetti and Gherardi (2000) also reported that *Salmonella enteritidis* live vaccine conferred excellent protection with the additional advantage of producing limited invasion of the deep tissues.

The purity and safety test of experimentally prepared FT vaccine was performed prior to vaccination. The result of the study revealed that this vaccine was biologically pure. The purity (sterility) and safety test of the concerned vaccine was carried out as per instruction of OIE (2008).

The pre-vaccination PHA antibody titre of all vaccinated and unvaccinated control birds were less than  $4 \pm 0.0$  (Figure 1) and similar reports were recorded by other investigators (Ferdous, 2008; Yeasmin, 2010; Jannatun, 2010; Basak, 2011). In control birds (Group B), the PHA antibody titres were found  $\leq 4.00$  throughout the study. Vaccinated group exhibited significantly higher antibody titers as compared to the unvaccinated group ( $p < 0.05$ ). The PHA antibody titres following primary vaccination were increased that varied from 32-128 but in some cases declines just before administration of secondary vaccination after 28 days of primary vaccination. Similar findings were observed by Bhattacharya *et al.* (2004).

The lowest PHA antibody titres induced by secondary vaccination ranged from 32 to 64 that obtained at 45 and 58 days post vaccination (After 15 and 28 days of secondary vaccination) and highest titres of post secondary vaccination ranged from 128 to 256 that obtained after 15 days of second vaccination and maintained up to 28 days of second vaccination. The chickens received booster dose of vaccine had significantly higher antibody titres than the chickens of primary vaccinated groups ( $p < 0.05$ ). But there was no significant difference in antibody titres after 15 days and 28 days of booster vaccination. These results are similar to the findings of Basak (2011), Jannatun (2010) and Rahman *et al.* (2005). Immune response at 15 days after booster vaccination was better than the 28 days after primary vaccination. Vodas (1978) recorded that agglutinin formation against *S. gallinarum* in chickens directly correlated with age. Agglutination formation began more quickly and the immune response was greater with increasing age and persisted longer in the blood.

Protection against challenge with *S. gallinarum* was satisfactory where vaccinated chickens possessed a mean PHA titre of  $96.00 \pm 20.23$ . The survivability rate and mortality rates of the control group was 10% and 90% respectively and in vaccinated group the survivability rate and mortality rates were 100% and 0% respectively. These results indicate that *Salmonella gallinarum* vaccine induce antibodies that provided protective immunity in the vaccinated birds ( $p < 0.001$ ).

In this study 250  $\mu$ l of hyper immune serum was able to neutralized 250  $\mu$ l of infective dose of bacteria. These results clearly demonstrated that *Salmonella gallinarum* participated in the induction of the passive protective immunity and the humoral immune response might be one of the mechanisms involved in the establishment of this protection. After passive protection test the survival rate of the experimental group and control group were 100% and 0% respectively and found that the passive serum treatment had a definite protective value in terms of overall survival ( $p < 0.01$ ). This study also recorded a better immune in mice belonged to vaccinated group A response at 15 days after booster vaccination was better than the control group B ( $p < 0.05$ ) the vaccinated mice resisted to virulent challenge exposure and nonvaccinated mice found mortality and survivability rates were 100% and 0%, respectively. It could be concluded that vaccinated mice show significantly higher survivability rate after challenge infection than the control mice ( $p < 0.01$ ). Lymphocyte percentage in blood of normal mice was 62.44% (Simonds, 2005). This study revealed that 11% increase in the lymphocyte count in vaccinated mice than in the control group ( $p < 0.01$ ) at both 15 days after primary vaccination and the 15 days after booster vaccination and high leukocyte count persisted for longer periods ( $p < 0.05$ ).

## CONCLUSION

*Salmonella gallinarum* vaccine produces higher level of antibodies both in chicken and mice as revealed by PHA test ( $p < 0.05$ ). This study revealed that an 11% increase in the lymphocyte count in vaccinated mice than in the control group ( $p < 0.01$ ). Vaccinated chickens protected from lethal challenge ( $p < 0.01$ ) and the passive serum treatment had a definite protective value in terms of overall survival indicating *Salmonella Gallinarum* vaccine induce passive protection immunity through humoral immune response ( $p < 0.01$ ).

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