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RESEARCH ARTICLE

Seroprevalence and Immunopathological Studies of *Salmonella pullorum* in Broiler Birds in District Faisalabad Pakistan

Nasir Mahmood[1], Farzana Rizvi[1*], Muhammad Kashif Saleemi[1] and Muhammad Aamir Aslam[2]

[1]Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan
[2]Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: farzana.rizvi@uaf.edu.pk

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A B S T R A C T

Salmonella pullorum (*S. pullorum*) seroprevalence was determined, followed by isolation and molecular characterization in commercial poultry in district Faisalabad, Pakistan. The bacterin vaccine was prepared from the local isolate of *S. pullorum* and its efficacy was evaluated in broiler chicks. For seroprevalence, a total of 384 boiler farms were screened. Seroprevalence of *S. pullorum* in broiler was 11.72%. The collected tissue samples (liver, spleen, and intestine) from seropositive birds were processed on different media (Xylose lysine deoxycholate agar, Salmonella shigella agar and MacConkey agar) for bacterial isolation of *S. pullorum*. Molecular detection was done with the help of polymerase chain reaction followed by bacterin preparation. An experimental trial was done to compare the efficacy of bacterin with the commercial vaccine (Poulvac ®, USA). A total of 120 broiler birds were divided into four groups (G1-G4) designed as G1 (negative control), G2 (infected with *S. pullorum*, G3 (bacterin + *S. pullorum* infection), and G4 (commercial vaccine (Poulvac®) + *S. pullorum* infection). Higher morbidity (80%) and mortality rates (60) were in group G2 as compared to the group (G3 and G4). Gross and histopathological lesions were highest in group G2 as compared to other groups. In conclusion, the locally prepared bacterin provides better protection in terms of mortality and morbidity and enhances humoral immune response in broiler birds.

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INTRODUCTION

Poultry is the largest sector of livestock and one of the most valuable sources of food and income worldwide. It is particularly important for country's economy being inexpensive, manageable, protein rich, and has a rapid growth rate when compared to other livestock animals (Abdela and Markos, 2016; Sulieman et al., 2020). However, multiple constraints such as poor management, disease occurrence, vaccine failure, and financial limitations hinder its development across the globe. About 2600 serotypes of *Salmonella* have been recognized and the majority can adopt various hosts including humans. *S. pullorum* and *Sallmonella gallinarum*, which is the causative pathogen of fowl typhoid (FT) whereas, *S. pullorum* is that of pullorum disease (PD), infecting young and adult chicks respectively and are rarely linked to human (Sarba *et al.*, 2020).

The mortality rate in PD is up to 90% in second-week-old chicks (Kebede *et al.*, 2016). Transmission of PD occurs via direct or indirect contact with infected chicken (respiratory or fecal). Vertical transmission complicates the control of the disease. Hens become sub-clinically infected, carriers and pass the infection to their embryo in the egg (Taddese *et al.*, 2019). *Salmonella* evades host defense in the stomach and enters the intestine and where it interacts with the non-phagocytic cells such as epithelial cells of the intestinal mucosa.

The *S. pullorum* has a many virulence structure such as a toxin, virulence plasmids, flagella, and fimbriae that assist in establishing disease (Lou *et al.*, 2019). *S. pullorum* infects and reproduces inside the cells of the mononuclear phagocytic system of the chickens and the prime site of reproduction is the digestive system, which may consequence of extensive contamination of the atmosphere due to bacterial excretion through feces. *S. pullorum* specifically targets the bursa of Fabricius before provoking inflammation in the whole GIT of chicks, where it is taken up by macrophages and ultimately enters

into blood circulation causing bacteremia (Markos, 2016). Sick chicks huddle close to the heat source and appear weak with decreased appetite and stunted growth rate. Affected chicks show a characteristic "white pasting of vent". Maximum mortality occurs during the 2nd to 3rd week of infection (Martelli *et al.*, 2017).

Many methods are adopted to reduce and eliminate the infection from poultry birds. Both inactivated and live vaccines are available which mostly contain the *S. gallinarum* 9 R strain and *S. enteritides* which are used to immunize the birds against *S. pullorum* (Corrêa *et al.*, 2018). This vaccine gives the cross-protection against the PD, but some time antibody titer is not reached to a protective level against the infection (Revolledo *et al*., 2009). PD and FT are economically important infectious diseases, without their actual control through proper diagnostic tools and expensive treatment, it would not be possible to raise poultry profitably (Aragaw *et al*., 2011). So, keeping in view the severity of the disease and the suboptimal response of the available remedies, the project was planned to study the prevalence of *S. pullorum* in the Faisalabad district, its pathogenesis, and immuno-prophylaxis using the locally prepared vaccine.

MATERIALS AND METHODS

Collection of samples and study area: Screening of 384 broiler farms was done through rapid serum plate agglutination test. Samples size was calculated with formula of (Thrusfield, 2007) and samples were collected based on different seasons (Spring, autumn, winter, and summer) and age (1, 3, and >4 week) groups, following the sample collection the collected tissue samples (liver, spleen, and intestine) of seropositive infected flocks were brought to the Department of Pathology, University of Agriculture, Faisalabad for further processing.

Isolation of *S. pullorum:* All the collected tissue samples were washed with normal saline enrichment of bacteria was done with tetrathionate broth. After 24h incubation at 37ºC, the sterile loop was used to transfer the enriched bacterial culture from broth to MacConkey agar**,** Xylose lysine deoxycholate agar (XLD) and Salmonella Shigella agar (SSA) for observing the colony morphology followed by Gram staining and motility test.

Biochemical characterization: To differentiate between *S. pullorum* and *S. gallinarum* isolates, different biochemical tests i.e., sugar fermentation test, methyl red (MR) test, indole test, triple sugar iron (TSI) test, dulcitol fermentation test, and citrate utilization test was used according to the standard procedures as described by OIE manual.

Molecular detection of *S. pullorum* through PCR: For molecular identification, DNA was extracted. Briefly, few colonies of *S. pullorum* were picked and poured into 1.5 ml Eppendorf containing the 0.5ml phosphate buffer saline (PBS) and mixed it followed by DNA extraction by kit method (Thermo Scientific, USA). Quantification of the extracted DNA from *S. pullorum* isolates was done through nanodrop (NAS-99, USA).

Preparation of PCR reaction mixture: The extracted DNA was amplified by using the specific set of primers (F 5′-TACGGGACGAGTGGGTACTT-3′: R 5′-AGATGCC CCACCACTCAAAG-3′) in a thermal cycler (Bio-Rad, USA). A single PCR reaction mixture was comprised of 25 µl (Felipe *et al.*, 2014). Thermal cycler conditions for amplification of rat*A* gene of *S. pullorum* are shown in Table 1.

Table 1: Thermal cycler conditions for amplification of rat*A* gene

Reactions	Temperature/time	Cycles
Initial denaturation	5 min at 95°C	1 cycle
Denaturation	30 second at 95°C	
Annealing and	30 second at 57°C	27 cycles
Elongation	42second at 72°C	
Final extension	5 min at 72°C	1 cycle
Storage	4°C	∞

Preparation of Bacterin from local *S. pullorum* isolates
Formalin-killed bacterin was prepared from the PCR confirmed isolates of *S. pullorum*. *S. pullorum* was grown on XLD then kept in a bacteriological shaker incubator at 37^0C for 24 hours. Isolated colonies of *S. pullorum* were transferred to nutrient broth (NB). The inactivation was done with 37% formaldehyde solution which was added to the bacterial suspension and adjusted 0.2% final concentration. *Salmonella* bacterin emulsified with mineral oil in the ratio of 1:4. Inactivated bacterial suspension mixed with 4% of tween 80. Bacterial contents in bacterin were adjusted to 10 CFU/0.5 ml bacterin. Thiomersal was added in a concentration of 0.05-0.1g/liter of the vaccine as a preservative (Soliman, 2015).

Sterility test: For sterility testing, 0.1ml of *S. pullorum* bacterin was inoculated onto the brilliant green agar (BGA) medium and incubated at 37°C. The culture plates were then examined after 24h and 48h of incubation for the appearance of any *S. pullorum* growth on the media.

Safety test: Safety studies of locally prepared bacterin vaccine were performed in mice. A total of ten mouse were used in the safety studies. Each mouse was inoculated with 0.5ml of formalin killed *S. pullorum* bacterin, subcutaneously (S/c). After administration of bacterin, all the mice were daily examined for the appearance of clinical signs and mortality for 10 consecutive days.

Comparative study: To determine the comparative *in vivo* efficacy of locally prepared bacterin and commercial *S. pullorum* vaccine (i.e., Poulvac ®), the experimental trial was conducted in the young broiler chicks (Table 2). A **t**otal of 120 (1-day-old) broiler chicks were procured from a local hatchery and kept in an experimental room. These broiler chicks were divided into 4 groups (G1-G4) with each group comprising of 30 birds.

Table 2: Experimental design for comparative efficacy

Groups	No. of Birds	Treatment
G1	30	Control Negative (PBS)
G2	30	Control Positive (*S. pullorum* Infection ($8x10^8$CFU/ml/bird)
G3	30	Bacterin + Infected with *S. pullorum* ($8x10^8$CFU/ml/bird)
G4	30	Commercial vaccine (Poulvac®) + Infected with *S. pullorum* ($8x10^8$CFU/ml/bird)

Birds in G1 were kept as negative control (treated with PBS only) whereas G2 birds were kept as the positive control (infected with *S. pullorum*). On the 7th day, each bird in group G3 and G4 was injected intramuscularly (I/M) with 0.5 ml of the primary dose of bacterin and commercial vaccine, respectively. On the 14th day, all the birds in G3 and G4 were again administered with a booster dose (0.5ml/bird) of bacterin and commercial vaccine through the I/M route, respectively (Abed and Ali, 2018). On day 28, each bird in G2, G3, and G4 was challenged with $8x10^9$ CFU/ml of *S. pullorum* through the oral route. The comparative efficacy of both vaccines was determined. Indirect ELISA for serogroup D (ID Screen®, France) test was carried out with the sera to determine the potency of the vaccine. Calculate the S/P ratio and antibody titer as follows:

$$S/P = \frac{OD(Sample) - OD(NC)}{OD\ (PC) - OD\ (NC)}$$

Log_{10} (titer) = 1× Log_{10} (S/P) +3.4; Titer = $10^{log_{10}(titer)}$

Statistical analysis: The experimental data were subjected to one-way ANOVA by using R-studio and means were compared with tukey's test. The level of significance was 0.05 or lower.

RESULTS

Seroprevalence of *S. pullorum*: The overall prevalence of *S. pullorum* was 11.72% through rapid plate serum agglutination test (RSA). The seasonal seroprevalence of *S. pullorum* in broiler was found highest 18.75% during the winter (Dec-Feb) followed by 13.54% autumn (Sep-Nov), 9.37%, spring (March-May), and summer (June-August) 5.20%. The seroprevalence of *S. pullorum* in different age group was highest in broiler birds of the 2nd week (17.07%) followed by 1 week 13.54%, and 3rd week 9.37%, and >4 weeks older 6.25%.

Cultural characteristics of *S. pullorum*: *S. pullorum* showed opaque, translucent, smooth, and round colonies on SS agar, whereas pink colonies with a black centre were observed on XLD agar. On MacConkey agar, pink circular and smooth colonies of avian Salmonella were observed. Microscopically, *S. pullorum* appeared as small rod-shaped bacilli with single or paired arrangements. All *S. pullorum* isolates were non-motile.

Biochemical characterization of *S. pullorum* Tests	*Salmonella gallinarum*	*Salmonella pullorum*
Lactose fermentation	-	-
Glucose	(Acid) +	(Acid and Gas) +
Dulcitol	+	-
Maltose	(Acid) +	(Acid and Gas) +
Indole Production	-	-
Methyl red test	+	+
Motility	-	-

Molecular confirmation of *S. pullorum*: Molecular confirmation was done with the help of PCR. The appearance of 243bp band for *rat*A gene on gel electrophoresis apparatus confirmed the *S. pullorum* isolates as shown in Fig. 1.

Sterility and safety of bacterin vaccine: There was no growth of any type of Gram-negative and Gram-positive bacteria after incubation in on XLD agar. Furthermore, no clinical signs and lesions were observed in mice up to 10 days of a safety testing trial showing bacterin was sterile and safe for birds. While control negative group also showed no clinical signs.

Efficacy of bacterin and commercial vaccine (Poulvac ®)
Morbidity and mortality rate (%): The positive control group (G2) exhibited the highest morbidity (80%) and mortality rate (60%) compared with bacterin vaccinated group (G3) in which 30% (morbidity) and 10% (mortality) was observed. The morbidity and mortality rate in group commercially available bacterin vaccine group (G4) was 40% and 20%, respectively.

Gross lesions of experimental trial: No gross lesions were observed in group G1 throughout the experiment (Fig. 2). In this study, the maximum gross lesions were observed in broiler birds of group G2. While G3 and G4 exhibited mild gross lesions as compared to the control group G2 as shown in Fig. 2.

Histopathological studies
Liver: The hepatic tissue of G2 showed the petechial hemorrhages (Ph), along with a disrupted hepatic cord pattern. Mononuclear cells infiltration were also observed at some area of hepatic tissues. Locally prepared bacterin vaccine group (G3) and commercially available vaccine group (G4) showed mild haptic tissue damage, mild congestion, petechial hemorrhages (ph), and small foci of degenerated hepatocytes observed in the tissue section as shown in Fig. 3.

Spleen: The splenic micrograph of group G1 showed the normal splenic structure with trabular artery (Ta) and diffused white pulp (Wp) and red pulp (Rp). The splenic tissue of group G2 (positive control) showed congestion (small arrow) of the central artery and diffused proliferation of mononuclear cells disturbing the nodular pattern of the white and red pulp (Wp & Rp) (Fig. 4).

Heart: Myocardium histograph of G1 showed normal cardiac muscles (M) with the syncytial pattern while in G2 (positive control), congestion of blood vessels in cardiac tissue with necrosis of myocytes, infiltration of heterophils and mononuclear cells at some areas. Group G3 and group G4 showed minimal vascular congestion and loss of striation of myocytes as shown in Fig. 5.

Intestine: The intestine of group G1 showed normal cellular structure. In group G2 villi (v) was seen with disrupted epithelium, atrophy, and damage of the submucosal gland (dg). There was an infiltration of inflammatory cells in lamina propria and submucosa. Broiler birds of groups bacterin vaccinated group (G3) and commercially vaccinated group (G4) showed less severe pathological changes (Fig. 6).

Immunological study: During the experimental period, the positive and negative control (G1 and G2) showed non-significant differences up to 28th days of age. After infection, the group (G2) showed a significant increase ($P \leq 0.05$) in values at 35 days of age to reach 1145 (optical

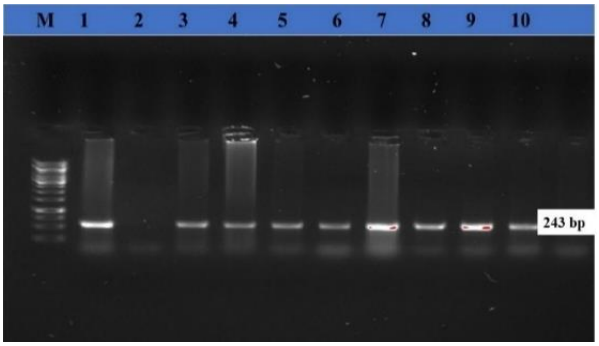

Fig. 1: Gel electrophoresis of PCR product showing the band at 243bp for *rat*A gene which is positive for *S. pullorum*. Lane 1, 3, 4, 5, 6, 7, 8, 9, and 10 the 243bp band for *S. pullorum*.

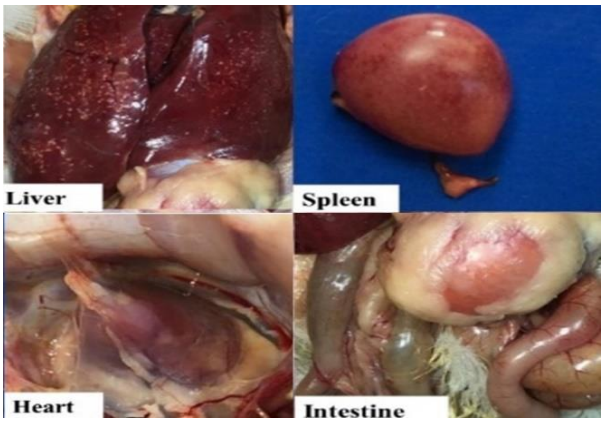

Fig. 2: Gross pathological lesions in broiler birds vaccinated with bacterin and commercial vaccine (Poulvac ®) infected with *S. pullorum*. Total no of birds was in each group= 30. Liver: Hemorrhagic, white foci, Spleen: Splenomegaly, Heart: Pericarditis with whitish nodules, Intestine: cheesy material and enteritis.

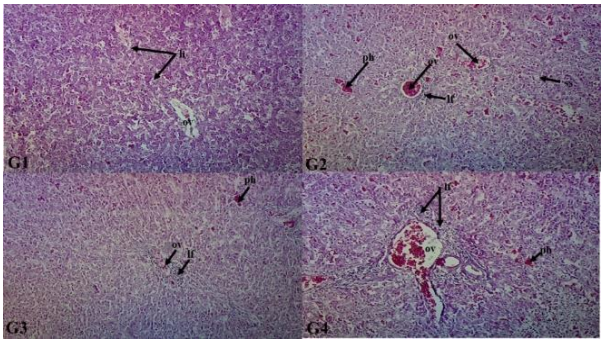

Fig. 3: Histopathological changes in the liver of different groups. **h**: hemorrhages, **cv**: central vein, **lf**: infiltration, **ph**: petechial hemorrhages, **ss:** Sinusoidal space.

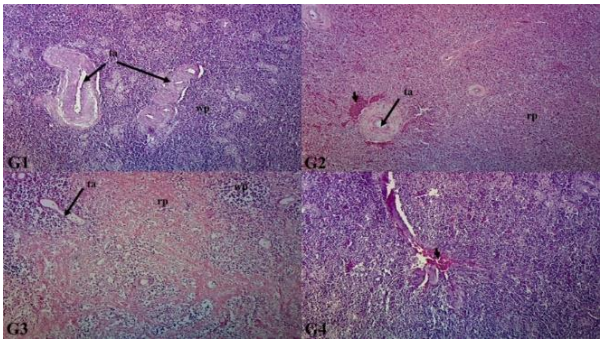

Fig. 4: Histopathological changes in spleen of different groups. **Small arrow**: Hemorrhages, **rp**: Red pulp, **wp**: White pulp, **ta**: Central artery.

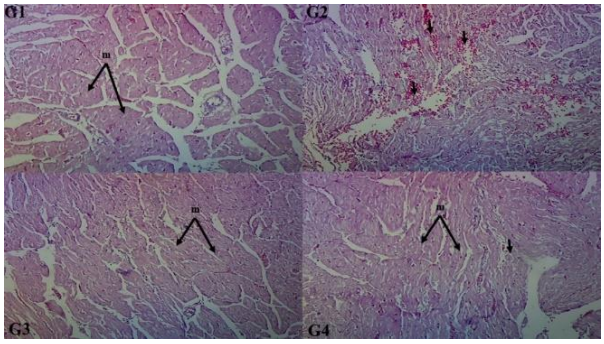

Fig. 5: Histopathological changes in heart of different groups. **Small arrow** : congestion of cardiac myocytes, **m**: cardiac muscles.

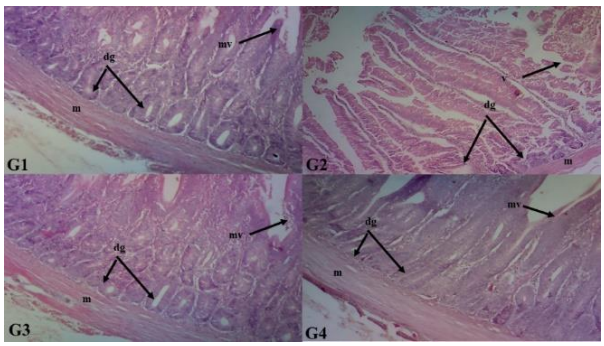

Fig. 6: Histopathological changes in intestine different groups. **g**: Submucosal gland, **mv:** microvillai, **m:** muscularis.

density 450 nm) and decrease to 954 at 42nd days of age. The groups G3 and G4 showed gradual and significant (P≤0.05) increase in optical densities values from 345 prior vaccinations to 1856 and 1650 respectively at 35th days of then significant (P≤0.05) increase in these values at 42 days old (2354 and 2060 respectively) were observed.

DISCUSSION

Among bacterial diseases such as, fowl cholera, mycoplasmosis, and necrotic enteritis, salmonellosis is one of the major problems and its spread is increased due to various environmental factors including temperature, humidity, and stress in poultry farms. Salmonella causes substantial economic losses resulting from morbidity, mortality, and poor growth rate. Khan *et al.* (2014) reported a prevalence of 56.3% in selected six broiler farms of district Faisalabad and Sarker, and Rahman, (2021) reported 45.5% in Bangladesh. In the current study, seroprevalence was higher than the previous studies (Ahmed *et al.*, 2008) which reported 1.01% in Bangladesh. These reports show different incidences than the current study and it may be due to differences in geographical variation and management conditions. The occurrence of disease was more in the winter season and the similar results were reported previously (Grimont and Weill, 2007). It was observed that during the winter season there were more incidences of diseases which might be due to more predisposition of birds to these pathogens when the temperature is low. RSA test occasionally gives a very high prevalence which may be due to false-positive results because of cross-reactivity among serotypes, administration of killed vaccines, contaminated serum samples, and age of flock (Corrêa *et*

al., 2018). For proper diagnosis and control, programs based on seroconversion may be inadequate, so sero-monitoring should be combined with culture and molecular techniques (Shoaib *et al.*, 2020).

The colony characters of *S. pullorum* on SSA were black, while single or in pairs, rounded, small, and centrally black on XLD. Colonies on MacConkey agar were transparent, pink, and smooth. These all findings corresponded with the previous studies (Ahmed *et al.*, 2008; Perez *et al.*, 2004). In Gram staining, the morphology of the isolated bacteria appeared as Gram-negative small rods with the single or paired arrangement that was in line with the previous findings (Hossain *et al.*, 2006). Similar findings were previously reported by several previous studies (Xiong *et al.*, 2017). Many findings have shown that standard PCR-based detection of *Salmonella* serovars is more susceptible, easier, and quicker than conventional microbiologic methods (Soria *et al.*, 2013). Therefore, a PCR assay based on the specific gene (*rat*A) was used for the detection and separation of *Salmonella serovar Gallinarum biovars gallinarum* & *pullorum*. In the current study, findings based on the mentioned genes were like the previous results (Batista *et al.*, 2013).

Comparative efficacy of bacterin in broiler chicks: Formalin killed vaccine was prepared from the local isolates of *S. pullorum* following the method described by (Samina *et al*., 2013) based on principle for the development of an inactivated vaccine (Nunnally *et al*., 2015). In the sterility test, bacterin gave no growth on brilliant green agar, and in the safety test, there was no lesion produced in mice after which showed that bacterin was safe for birds results were in line with guidance for the preparation of bacterin (World Organisation for Animal Health, 2006). The clinical trial was constructed to check the efficacy of bacterin, and results were compared with different studies.

The morbidity and mortality rate were increased in the control positive group (G2) as compared to groups (G3 and G4) that were immunized before infection with *S. pullorum.* These findings are supported by the previous study (Abed and Ali, 2018). In necropsy findings of the infected birds, the liver was found congested, bronze discolored with white necrotic foci. The spleen was congested and showed splenomegaly. The heart showed pericarditis with white nodules. Ceca were filled with semisolid, cheesy material and showed catarrhal enteritis. Microscopically, the liver showed petechial hemorrhages along with disrupted hepatic cord pattern and necrosed hepatocytes. The liver, spleen, heart, and intestine showed infiltration of mononuclear cells. These gross and histopathological findings were more in the positive control as compared to groups G3 and G4. These findings are in line with the previously published studies (Haider *et al.*, 2012; Yin *et al.*, 2015b). In groups G3 and G4 that were immunized before infection with *S. pullorum* showed the normal histogram of broiler birds which showed that bacterin and commercial vaccine (Poulvac®) minimize the risk of disease by boosting immunity and combat the infection before producing clinical lesions.

In the present study, the systemic response of IgG was significantly increased against *S. pullorum* antigens in groups G3 and G4 which were immunized before infection with *S. pullorum*. Furthermore, immunization of chickens with inactivated *S.* pullorum vaccine in two shots induced increasing in ELISA seroconversion (serum IgG response) which persisted up to from 3 to 32 weeks post-vaccination (Freytag and Clements, 2005; Rahaman *et al.*, 2014). The success of immunization against intracellular pathogens such as *S. pullorum* is largely accredited to the improvement of systemic and mucosal immune responses (Titball, 2008). The bacterin would be more potent than the commercially available vaccine because it was produced by the locally isolated *S. pullorum*.

Conclusions: In Asian countries, in which avian salmonellosis is frequent due to difficulty in restriction of the live poultry movement, infection.

Authors contribution: NM performed all experiments, analyzed the sera and tissue samples. FR designed the research plan, interpreted data, and drafted the manuscript. MKS and MAA provided the technical support about experiments and data analysis.

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