Research Article
Volume 6:1, 2022

Journal of Microbial Pathogenesis

Open Access

Molecular Detection of *Salmonella gallinarum* and its Prevention using Autogenous Bacterin in White Crystal Neck Commercial Layer Birds

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Abstract

Fowl typhoid is one of the most significant bacterial diseases of layer birds. Its significance is drawn from the cost of productivity in commercial layer poultry. Vaccination is a superlative way of preventing disease in commercial poultry. In the current study, immunization, and protective efficacy of local strain in a prepared autogenous bacterin had been studied. A total of 120-layer birds of crystal neck breed were equally divided into three groups (A, B and C) having 40 birds in each. The birds of group A were kept as negative control (non-infected and non-treated), the group B was kept as a positive control (challenged with *Salmonella gallinarum* and non-treated) and group C was kept as a vaccinated group (challenged and vaccinated). All the experimental groups were retained under surveillance for the 3rd-week post-challenge. The protection assay was evaluated according to the clinical signs, mortality, fecal shedding of challenged birds. On weekly basis, the blood was collected and humoral immune response against *Salmonella gallinarum* was assessed by using Micro-agglutination Test (MAT) and ELISA. The results showed that autogenous bacterin increases the performance of chickens by reducing clinical signs, mortalities, gross lesions, and fecal shedding. The local bacterin increased the humoral immune response in the vaccinated group (C) as compared to the positive control group (B). In conclusion, the locally prepared autogenous bacterin protects against challenged infection of *Salmonella gallinarum*.

Keywords: *Salmonella gallinarum* • Autogenous bacterin • Humoral immune response

Introduction

The success of the flock, growth, and welfare in commercial poultry depends upon the use of a good vaccination program. A vaccine can help to prevent or reduce the effect of harmful diseases when used in conjugation with good biosecurity and managemental conditions. Many vaccines are used to control *Salmonella* infection such as live vaccine may become ineffective in immunocompromised individuals and the killed vaccine are serovar specific, produces only short immunity [1]. Live and inactivated vaccines are made from rough 9R strain and have been widely used to control fowl typhoid. The number of viable organisms/doses is important to control its transmission (through eggs and from bird to bird). Vaccination with 9R produces transient antibodies and may precipitate high mortality in the infected flocks. The primary dose of the 9R vaccine is usually done at the 8th week of age & booster dose at the 16th week of age 5. Despite these limitations, a broad-spectrum killed bacterin will be good to control *Salmonella*. Bacterin is a major component of the vaccination program and is used to stimulate high-level immunity. These contain inactivated organisms suspended in aluminum hydroxide or water in oil adjuvants. They induce an immune response by producing protective antibodies, protecting the birds from harmful bacterial pathogens such as *Salmonella* spp., during the laying period [3]. To our knowledge, very little work has been done yet in Pakistan to characterize the non-motile poultry *Salmonella* at the molecular level and to prepare bacterin from a local isolate. Keeping in view the economic importance of this disease the comprehensive research will help in the formation of preventive strategies, in the long run to curtailing economic losses to the poultry industry. Therefore, the current study was designed to prepare of bacterin vaccine from local isolated *S. gallinarum* and its comparative efficacy study of commercially available and locally prepared bacterin against *S. gallinarum*

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Received 02-Feb-2022, Manuscript No: jmp-22-55822; **Editor assigned:** 05-Feb-2022, PreQC No. P-55822; **Reviewed:** 16-Feb-2022, QC No. Q-55822; **Revised:** 21-Feb-2022, Manuscript No. R-55822; **Published:** 28-Feb-2022; DOI:10.37421/jmp.22.06.108

Materials and Methods

Isolation of *S. gallinarum* was done from cloacae swabs of birds, briefly; a sterile cotton swab was incorporated into the cloaca of clinically sick birds to collect cloacal contents. After collection, every swab was shifted to a 10 ml tube containing tetrathionate broth and incubated at 37°C overnight. A loopful of broth was streaked on SS agar for examination of *S. gallinarum* colonies. Suspected colonies were examined biochemically and morphologically.

Salmonella specific gene amplification by PCR

Isolated bacterial colonies were picked up and mixed with distilled water for isolation of DNA template for PCR. The *Salmonella*-specific set of forward and reverse SPG primers to target *SG* gene were used [4]. A PCR reaction mixture of 25 µl was prepared by taking 12.5 µl of Master mix, 1 µl of each primer, 2 µl of DNA template, and 8.5 µl of water in the tube. The reaction was carried out in thermocycler with 35 cycles to amplify *SG* gene with the following conditions: initial denaturation for 3 minutes at 95°C, denaturation at 94°C for 20 seconds, annealing at 59°C for 30 seconds, extension at 82°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR product was analyzed through gel electrophoresis at 60V/cm for 50 minutes in a 1.5% agarose gel stained with 2.5 µl of ethidium bromide.

Challenge inoculum

Broth culture of S. *gallinarum* was taken and centrifuged at 3000 r.p.m for 10 minutes. The supernatant was collected and discarded and the remaining was mixed and diluted with buffer saline using MacFarland alike tube to have 10^9 CFU/ml, infected dose was prepared according to Timms LM, et al. [5].

Bacterin preparation

The autogenous bacterin was made from the locally isolated field strain of *S. gallinarum* following the technique of Timms LM, et al. [5]. The isolated field strain was cultured in nutrient broth followed by nutrient agar and incubated at 37°C for 24 hours. Thereafter brain heart infusion agar was used for culturing and incubated at 37°C for 48 hours. The growth of *S. gallinarum* was shifted in normal saline at room temperature for 24 hours and 1% formalin was used for inactivation. The washed concentration of inactivated bacterin was adjusted to contain 10^{11} CFU/ml by using MacFarland matching tubes. Sterile concentration was obtained by adding equal volumes of washed inactivated bacterin and Montanite oil and stored in the refrigerator for further use. The prepared local whole-cell deactivated bacterin was provided to layer birds at the 6th week of age and a booster dose (0.2 ml/bird) was given at the 9th week of the period. The whole-cell deactivated bacterin was offered in two shots intramuscularly (I/M) in the thigh muscle.

Quality control test of autogenous bacterin

The formulated whole-cell deactivated bacterin was verified for purity, safety, and efficacy test according to the basic procedures described by British veterinary codes (1970).

Purity test of inactivated bacterin

A purity test was performed before the formalin deactivation of the *S. gallinarum* strain. This test confirms the purity of broth culture and did not contain any organism before inactivation. This purity test was done by inoculating the broth culture on SS agar and incubating it at 37°C for 24 hours. The presence of pure colonies of *S. gallinarum* on SS agar confirms the purity of broth.

Safety test of prepared bacterin

Safety was done following the method of Dorsey (1963). Mice (n=10) were injected (0.2 ml of vaccine) subcutaneously (S/C). The vaccinated mice were kept under observation for 14 days to observe any clinical signs and symptoms.

Efficacy test of prepared autogenous bacterin

Layer birds (n=120) of crystal neck breed were used in this study. Day-old layer birds were purchased from a local hatchery and kept under good managemental conditions. Fresh and clean water was ensured, and commercial Mukhtar feed of layer was given throughout the experimental trial. The layer birds were kept under understudy for 15th weeks of age.

Experimental design to check the efficacy of bacterin vaccine

One hundred- and thirty-layer birds, day-old were purchased and divided into three groups having 40 birds in each. Upon arrival, ten birds were slaughtered and observed bacteriologically to ensure *Salmonella*'s free flock. The layer chicks were divided as follows.

Group A: Control negative (Non infected and non-treated)

Group B: Control positive (*S. gallinarum* infected birds)

Group C: Vaccinated and *S. gallinarum* infected birds

Parameters measured during experimental trial for the efficacy of bacterin

Birds were monitored for clinical signs, mortality, and morbidity rate for three weeks post-infection. Deceased birds were exposed to postmortem assessment for reflection of gross and histopathological study.

Detection of shedding of bacteria

To ensure birds were free from *S. gallinarum* infection before the experimental trial, the cloacal swabs were taken from each group.

Subsequently the challenge, swabs were taken from infected as well as a control group on weekly basis and assessed bacteriologically to check shedding of *S. gallinarum* infection. The cloacal contents were collected using the cloacal swab, swab was inserted and rotated clockwise to collect contents. After which the collected swabs were transferred to tetrathionate broth for the enrichment and incubated at 37°C for 24 hours. Colonies were studied morphologically and bacteriologically.

Upon arrival, the birds were weighed and weekly production parameters such as body weight and Feed Conversion Ratio (FCR) were measured.

Humoral immune response

Just before 1st vaccinal dose, the 10 birds were selected randomly and from the wing vein blood samples were taken. Also, before the booster dose, the 10 birds were chosen haphazardly from each group and blood samples were taken from the same marked birds. On weekly basis, the blood was collected from each group post-infection and sera were collected. The collected sera were stored at -20°C for further use.

The antibody titer against *S. gallinarum* was determined through ELISA according Muktaruzzaman M, et al. [6], and the Microagglutination test according to Brown, etc., 1981. In MAT the antibody was expressed as Geometric Mean Titer (GMT), while in ELISA the antibody titer was assessed through S/P ratio by using the following formula:

S/P ratio: Sample mean- Negative control/ positive control- negative control

Calculation of Antibody Titer: Log10 Titer=1.13Log $^{(SP)}$ +3.156.

Anti-Log= Antibody titer

Histopathological examination

The formalin-fixed tissue samples of the liver, kidney, spleen, intestine and heart were collected aseptically. The collected tissue samples were processed using the paraffine embedding technique. Tissue sections of 5 µ thickness were cut using the microtome followed by hematoxylin and eosin staining and analyzed under a microscope to check changes.

Results

Results of quality control of autogenous bacterin

The locally prepared bacterin of *S. gallinarum* proved to be pure, safe, and free from any adverse side effects on crystal neck white commercial layer birds and weekly body weight gain. The affected mice did not show any clinical signs and symptoms.

Results of protective efficacy of autogenous bacterin

The locally prepared autogenous bacterin of *S. gallinarum* showed an 82% protection rate against wild type *S. gallinarum* strain in group C while in the positive control group (B) the survival rate was only 22% as shown in Table 1.

Results of shedding of bacteria (***S. gallinarum***) from vaccinated and challenged layer birds

The rei-solation of *S. gallinarum* from positive control (Group B) was 55.88%, 41.17%, 18.18%, and 9.09% at 1st, 2nd, 3rd, and 4th-week post-infection respectively compared to 15.21%, 12.19%, 4.87%, and 0% in those vaccinated with the locally prepared autogenous vaccine (Group C) as shown in Table 2.

Results of clinical signs and gross pathology

The layer birds vaccinated with autogenous bacterin showed mild clinical signs with a slight gross lesion in the intestine (enteritis). The layer birds of a positive control group (B) showed perfused white watery diarrhea, depression and birds were hesitant to move. On postmortem examination, the positive control group showed bronze discoloration of the liver having white necrotic foci, swelling of the spleen and liver. In a few birds, the pericardium was wrapped with yellowish necrotic material.

Results of Microagglutination test for measurement of antibody

The GMT of layer birds vaccinated with autogenous bacterin of *S.*

Table 1. Protective efficacy of autogenous bacterin in layer birds challenged with *S. gallinarum* strain.

Groups	Total No. of Birds	No of Dead Birds/Weeks Post-challenge				Dead/Total	Survival/Total	Mortality Rate %	Survival Rate
		1st	2nd	3rd	4th				
A	50	0	0	0	0	0/50	50/50	0%	100%
B	50	16	17	6	0	39/50	11/50	78	22
C	50	4	5	0	0	9/50	41/50	18	82

A= Negative control; B= Positive control; C= vaccinated and infected.

Table 2. Results of *Salmonella* shedding from vaccinated and control groups after challenge with virulent *S. gallinarum* strain.

Groups	No. of Positive Birds for Isolation/Total No. of Live Birds			
	1st Week	2nd Week	3rd Week	4th Week
A	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)
B	19/34 (55.88%)	7/17 (41.17%)	2/11 (18.18%)	1/11 (9.09%)
C	7/46 (15.21%)	5/41 (12.19%)	2/41 (4.87%)	0/41 (0%)

A= Negative control; B= Positive control; C= vaccinated and infected

gallinarum increased from (0) pre-vaccination to 64 at the 3rd-week post-primary vaccination and 173 after the 3rd week of a booster vaccination. After the challenge, the geometric mean titer of antibody had increased in group C (vaccinated and infected) from 173 to 275 against *S. gallinarum* infection while it was 65 in the positive control group (B) at 3rd-week post-challenge (Table 3).

Results of ELISA of autogenous bacterin

At 3rd-week post-primary vaccination the ELISA antibody titer in group C (vaccinated and infected) was 850.3 against *S. gallinarum* infection. Moreover, a gradual increase in antibody titer was shown in group C at 3rd-week post booster vaccination, it was 2258.7. After 3rd week of the challenge, the antibody titer in the vaccinated group (C) had increased up to 2272.4 against *S. gallinarum* infection (Table 4).

On the other hand, a sudden increase of antibody titer was noted in the control unvaccinated group (Group B), where the antibody titer was 159.2 at the 3rd-week post of primary vaccination, 209.6 at 3rd-week post booster vaccination, and 898.4 at 3rd-week post-challenge (Table 4).

Molecular confirmation of *Salmonella gallinarum*

Conventionally confirmed isolates of *S. gallinarum* were confirmed by PCR targeting *SG* gene. All the tested isolates were successfully amplified targeting *SG* gene (Figures 1 and 2).

Discussion

Salmonellae gallinarum is responsible for major economic losses in poultry in terms of mortality and decrease in egg production. In our study the tested organism (*S. gallinarum*) was gram-negative, rod-shaped occurred singly or in paired. Small-round and black-centered colonies on XLD; pink, transparent, smooth raised colonies on MacConkey agar; black-colored colonies due to production of H_2S on SS agar were observed. The isolate fermented maltose, dextrose, and mannitol produced both acid and gas, which were in line with the previous findings [7].

In the next step, the PCR-confirmed *S. gallinarum* isolates were used for the preparation of bacterin followed by determination of immunogenicity potential of the bacterin in layer birds challenged with *S. gallinarum*. Our results of the challenge protection study showed that the birds immunized with autogenous bacterin (C) showed significant protection to *S. gallinarum* infection as compared to the birds of the positive control (B). Only mild or no clinical signs of fowl typhoid were observed in autogenous bacterin (C). Similar findings regarding the protective potential of S. gallinarum-bacterin had also been reported by the previous reports of several studies [8]. It is summarized that locally autogenous bacterin of fowl typhoid and probiotics are effective in reducing clinical signs, mortality rate, and gross lesions in *S. gallinarum* infection, similarly, the protective effect of formalin killed bacterin previously reported by [9,10]. Typical clinical signs of fowl typhoid such as

Table 3. Microagglutination test for measurement of antibody against *Salmonella* in sera of layer birds vaccinated with autogenous bacterin.

Groups	Pre-vaccination	Geometric Mean Titer		
		3rd-week post-primary vaccination	3rd-week post booster vaccination	3rd-week post-challenge
A	0	0	0	0
B	0	0	0	65
C	0	64	173	275

A= Negative control; B= Positive control; C= vaccinated and infected

Table 4. Result of ELISA for antibody assessment against *Salmonella* in sera of layer birds vaccinated with autogenous vaccine.

Groups	Geometric Mean Titer		
	3rd-week post-primary vaccination	3rd-week post booster vaccination	3rd-week post-challenge
A	0	0	0
B	159.2	209.6	898.4
C	850.3	2258.7	2272.4

A= Negative control; B= Positive control; C= vaccinated and infected.

Figure 1. Polymerase chain reaction products from *Salmonella enterica* subsp. *enterica* serovar *Gallinarum* biovars *Gallinarum* and *Pullorum* strains using the *SG* gene differential primers. Lane 1 to 5 represents *S. gallinarum* field isolates from clinical avian samples.

fever, anorexia, ruffled feathers, diarrhea, and anemia were observed in the positive control group (B). Similar clinical signs were reported previously by Lopes PD, et al. [11], Freitas Neto OC, et al. [12], Ezema WS, et al. [13], Garcia KO, et al. [14] and Shivaprasad HL [15]. An incubation of seven days was observed in our study which was in contrast to the previous findings of [14] in which three days of incubation had been reported. This variation in the disease incubation period could be associated with several factors including the infective dose, virulence, host pathogenicity, and immune response to fight against pathogenic organisms like *S. gallinarum* by Lahiri A, et al. [16]. A mortality and survival rate of 18% and 82% respectively was observed in birds administered with autogenous bacterin (C) in comparison to the positive

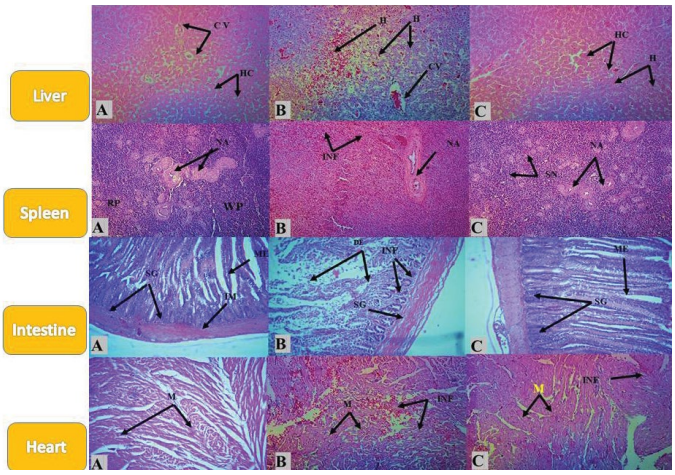

Figure 2. Liver: Liver of control negative (A) exhibited the normal hepatocyte (HC) and central vein (CV), while the positive control (B) showed the severe disruption of hepatocyte (HC), extensive hemorrhages (H) dilated central vein (CV). While the vaccinated group (C) exhibited the recovery of hepatocyte (HC) towards normal and less severity of hemorrhages (H); Spleen: Histopathology of spleen showed the normal nodular arty (NA), the red pulp (RP), and white pulp (WP) in the control negative group (A), while the positive control group (B) exhibited severe inflammation (INF) and disrupted nodular artery (NA) and vaccinated group (C) showed the recovery of inflammatory cells in red and white pulp; Intestine: The histomicrograph of the intestine showed the intact epithelial cell, mucosa (M), submucosal gland (SG) in the control negative group (A), while disrupted submucosal gland (SG) and inflammatory cells were found in the positive control group (B) and recovery of the submucosal gland (SG) towards normal in the vaccinated group (C); Heart: Histomicrograph of the heart showed normal cardiac muscle (M) in control negative (A), while severe inflammatory cells are seen in the positive control group (B) and the vaccinated group (C) showed less inflammation (INF) and recovery of lost myocytes (M).

control (B) in which 78% mortality and 22% survival rate was observed. These results suggest that autogenous bacterin could be a potential candidate as a prophylactic vaccine. Our results of immunogenicity potential of autogenous bacterin were following the previous studies [17-19].

In the present study, the protective efficacy of locally prepared autogenous bacterin was more as compared to the positive control group (B) and these results are in agreement with Penha RAC, et al. [20]. Fecal shedding of *Salmonella gallinarum* organisms in the group of chickens (vaccinated with locally prepared bacterin) reached 4.87% which was lowered than the positive control group (*Salmonella* infected group) similar results of fecal shedding was reported by El-Enbaawy MI, et al. [21].

Conclusion

The protection with local prepared autogenous bacterin to be safe and help the birds with fast recovery from *Salmonella gallinarum* infection. It helps to prevent the birds from infection with increased FCR, decreased clinical signs, and mortality. It enhances the humoral immune response in vaccinated birds as compared to non-vaccinated birds.

Conflicts of Interest

The author declares no conflict of interest as there are no direct financial or other connections with other people or organizations or that can inappropriately influence our work.

Acknowledgments

We would like to thank all participants. The authors would like to thank Dr. Farzana Rizvi for assisting with project layout and Statistical analysis.

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How to cite this article: Shakir, Muhammad Zulqarnain, Farzana Rizvi, Muhammad Kashif Saleemi and Muhammad Wasim Usmani. "Molecular Detection of *Salmonella gallinarum* and its Prevention using Autogenous Bacterin in White Crystal Neck Commercial Layer Birds." J Microb Path 6 (2022): 108.