Using T cell lymphokines of hyperimmunized chickens with *Salmonella pullorum* to protect layer hens against *Salmonella pullorum* infection

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**Abstract**

*Salmonella pullorum* (SP) is well adapted to cause an acute systemic infection in hens with a high mortality rate. This study aimed to assess the efficacy and safety of a salmonella-immune lymphokine (SILK) produced by hyperimmunized pullets with *Salmonella pullorum* during the layer hens' production stage to boost their immune response against *Salmonella pullorum* infection. In this study, two groups of 25 pullets were used to produce lymphokines; the first group received three doses of the *Salmonella pullorum* vaccine at 12, 14, and 16 weeks, while the second group only received phosphate buffer saline (PBS), which served as the control. At 18 weeks, non-immune lymphokines (NILK) were isolated from the T cells of the second group, and salmonella-immune lymphokines (SILK) were isolated from the T cells of the first group. Then, 100-layer hens (ISSA Brown), at 30 weeks old, which entered the production stage, were separated into four groups, each with 25 chickens. G1: infected with SP and treated with SILK. G2: infected with SP and treated with NILK. G3: untreated and SP-challenged. G4: no treatment or challenge. The current study aimed to describe the host humoral immune responses to infection in serum samples and bacterial shedding in hens challenged with SP during 31-33 weeks by qPCR techniques. Internal organ bacterial loads were estimated to evaluate the persistence of bacteria. The results show that the spleen, liver, and caecal tonsils tested were positive for bacteria in both groups (G2, G3), proving that Salmonella was not eliminated from the birds and suggesting that internal organ colonization bacteria may act as a reservoir for ongoing bacterial shedding with low IgG titer in compared to G1 with extremely low persistence and high IgG titer in weeks 31, 32, and 33. The current investigation shows that using SILK provides layer hens with better homogenous protection against SP and less internal organ persistence.

**Keywords**

SILK, ISSA brown, Bacterial load, IgG, Bacterial shedding

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**Introduction**

*Pullorum disease* (PD), often known as white diarrhea, is a pervasive and extremely harmful illness caused by the highly adapted *Salmonella pullorum* in poultry (1). Infected hens exhibit a variety of symptoms, such as anorexia, depression, diarrhea, and recurrent cloacal infection; PD is a severe, systemic illness with a high death rate (2). Furthermore, *S. pullorum* infection in symptom-free adult hens can persist in the spleen and reproductive system for months, causing the vertical transmission to eggs and offspring (3). *S. pullorum* may infect chickens of all breeds and ages, although as they become older, infection mortality declines and more infected birds acquire latent and chronic disease (4). It was discovered that 20% of *S. pullorum*-infected birds had steadily declining bacterial counts in their spleen and liver, which cleared between 20 and 25 weeks of age. However, in females, the decline is
interrupted by the onset of sexual maturity, which results in decreased T cell responsiveness (5). Most laboratories have shown that T cells are derived from S. Enteritidis. In vitro production of lymphokines by SE immunological hens, these lymphokines cause an inflammatory response that is specific to heterophils, which dramatically boosts hens' natural defences against S. Enteritidis ability to infect their organs, and lead to protects neonatal chicks within 24 hours of challenge (6). In addition, the Salmonella protection offered by the Salmonella Enteritidis-immune lymphokines (SE-ILK) can last for at least 6 days when administered in eggs (7). The antibody response lasted for more than 40 weeks, indicating that S. pullorum may modify host immunity after infection (8,9). Therefore, extensive preventative and control methods are strongly recommended in addition to the creation of breeding flocks free of Salmonella in order to totally eradicate S. pullorum.

The goal of the current study is to analyze the potential of SILK in layer hens to minimize S. pullorum shedding in the faeces and the danger of horizontal transmission in the environment, as well as to investigating antibody formation in chickens with S. pullorum infection.

Materials and methods

Ethical approval

This work approved by College of Veterinary Medicine, University of Baghdad under the code 234 dated 1/2/2022.

Preparation lymphokines

The Iraqi Salmonella pullorum isolate of (OM988162.1) was provided from Department of Pathology and Poultry Diseases-College of Veterinary Medicine-University of Baghdad. The isolate's lethal dose (LD50), which results in 50% death, was calculated to be precisely 5×10^5 bacteria per bird at a dilution of 10^-7. Then, at Al-farms, Rashidiya's two groups, each with 25 pullets, were raised. The first group orally received three doses 1×10^8 of the SP vaccine at 12, 14, and 16 weeks the method of vaccine was prepared according to Revolledo (4); the second group was the control group and did not get any vaccinations. At 18 weeks, the T cells in the first group secreted immune lymphokines (SILK), whereas the T cells in the second group generated non-immune lymphokines (NILK). The T-cells were recovered from the crushed vaccinated avian spleens by centrifugation at 1500 rpm. The subsequent step involved adding Concanavalin A (Con-A) to T-cell cultures in RBM media to stimulate the release of lymphokines. The lymphokines were separated by centrifugation of the filtrate at 3000 rpm (10).

Experimental strategy

One-hundred-layer hens (ISSA brown) at 30 weeks of age, separated into 4 groups (25 in each group). First G1 was treated with 0.5 ml of S-ILK supernatants, after 5 hr. gavage-inoculated into the crop with 0.5 ml of Salmonella pullorum (5×10^6 CFU/ml). G2: received SP-infected and NILK therapy. G3: was not treated with either SILK or NILK and was received SP-challenged. G4: serve as control and not giving therapy or infection with Salmonella pullorum.

Counting the number of Salmonella in fecal samples

On week 31, 32, and 33 post infection, feces were aseptically sampled by using sterile plastic bags from all individual chickens in four groups. All fecal samples were counted for Salmonella using the previously reported three-tube most probable number (MPN) technique according to (11). Salmonella suspected samples were streaked over xylose lysine deoxycholate (XLD) and Salmonella Brilliance agar plates (Oxoid, Australia) to determine whether they contained Salmonella spp (12).

Extraction of bacterial DNA from internal organs

The Wizard genomic DNA purification kit (Promega, Australia), was used to extract the DNA from all the tissue samples in accordance manufacture guideline (12).

Internal organ standard curve and qPCR for Salmonella pullorum

A total reaction volume of 10 µL, the Quantifast SYBER Green qPCR kit (Qiagen, Australia) was used to carry out the PCR detection of Salmonella. This kit PCR reaction contains 2 µL of sample (5 ng/µL), 5 µL of Quantifast SYBER Green Master Mix, and 1 µM from each reverse and forward primers (Salmonella pullorum serovar-specific primers TSR3) were used (13). To find Salmonella pullorum (OM988162.1), the reaction condition included Denaturation of DNA at 95°C for 5 minutes followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. (Qiagen, Australia).

Microbial load of the internal organs

For bacteria culturing, the spleen, liver, and caecal tonsils were removed at weeks 1, 2, and 3 pi. In a nutshell, 0.1-0.2 grams of tissue samples were homogenized, and phosphate buffer saline was added in repeated tenfold dilutions (PBS) (14). Each dilution was applied in an amount of 100 microliters on XLD agar plates, and they were then incubated at 37°C overnight. Salmonella in tissues was reported as a mean log10 CFU/g of tissue after the bacterial colonies had been counted for 24 hours.

Serum sample collection and ELISA-based serological evaluation

On weeks 31, 32, and 33 pi, 10 hens were randomly selected to measure the humoral immunity (IgG) against SP in serum, 2 mL of blood was taken from each bird in the four groups and put into serum clot activator tubes. The Chicken Salmonella pullorum Antibody Kit LPS (BioChek,
Holland) was used to test for the presence of antibodies in serum and egg yolk samples, and antibody titers were determined according to the manufacturer's instructions.

**Statistical Analysis**

Using a two-way analysis of variance, the average log$_{10}$ CFU/qPCR and serum data were examined (ANOVA). GraphPad Prism version 6 and IBM SPSS Statistics$^b$ version 21 were used to analyze all the data, with p values less than 0.05 considered statistically significant.

**Results**

**Salmonella shedding and viable bacterial numbers in fecal samples**

In experimental treatment groups, bacterial shedding changed considerably over time between groups (Table 1). During weeks 31, 32, and 33 pi, with a mean MPN/g, the most viable bacteria were found in chickens infected with *Salmonella pullorum* in G2, and G3. The lowest mean MPN/g of *Salmonella pullorum* was seen in samples taken from the G1. In week 33, the three viable *Salmonella pullorum* strains were found.

**Table 1: Salmonella in feces counted using the most probable number (MPN) approach**

<table>
<thead>
<tr>
<th>Groups</th>
<th>31 weeks</th>
<th>32 weeks</th>
<th>33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3.1±0.62$^b$</td>
<td>4.7±0.4$^b$</td>
<td>5.2±0.53$^b$</td>
</tr>
<tr>
<td>G2</td>
<td>36.2±3.6$^a$</td>
<td>49.3±4.7$^a$</td>
<td>62.6±12.7$^a$</td>
</tr>
<tr>
<td>G3</td>
<td>38.6±4$^a$</td>
<td>52.3±8.2$^a$</td>
<td>63±14.6$^a$</td>
</tr>
<tr>
<td>G4</td>
<td>0±0$^b$</td>
<td>0±0$^c$</td>
<td>0±0$^c$</td>
</tr>
</tbody>
</table>

Five samples taken. A difference that is significant at the level of P≤0.05 is indicated by small letters.

**Immunity against Salmonella pullorum (IgG)**

After being divided into groups at 30 weeks (Table 2). The treatment group G1 provided the greatest mean antibody level of titer, whilst the treatment groups G2 and G3 provided the lowest mean antibody level of titer, but the control negative G4 group did not record any titer.

**Table 2: Specific IgG antibodies in serum, statistically described**

<table>
<thead>
<tr>
<th>Groups</th>
<th>31 weeks</th>
<th>32 weeks</th>
<th>33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>2131.4±156.2$^a$</td>
<td>2955.8±287.4$^a$</td>
<td>4217.8±798.2$^a$</td>
</tr>
<tr>
<td>G2</td>
<td>876.4±31$^b$</td>
<td>1320.4±86.1$^b$</td>
<td>1756.9±166$^b$</td>
</tr>
<tr>
<td>G3</td>
<td>922.7±12.3$^b$</td>
<td>1432±79.6$^b$</td>
<td>1803±134$^b$</td>
</tr>
<tr>
<td>G4</td>
<td>278.2±18.3$^c$</td>
<td>200±16.5$^c$</td>
<td>198.7±21$^c$</td>
</tr>
</tbody>
</table>

Five samples taken. A difference that is significant at the level of P≤0.05 is indicated by small letters.

**Salmonella persistence in internal organs**

The results show that SP was not present in any of the samples taken from control chickens. While in G1, G2 and G3 all tissues tested were positive for bacteria; however, the number of positive samples was highest in the spleen, followed by the caecal tonsils and liver (Table 3). In comparison to the mean bacterial load in G1, the mean bacterial load in G2 and G3 was substantially higher.

**Table 3: Statistical information (CFU/g tissue) reflecting the number of bacteria in internal organs**

<table>
<thead>
<tr>
<th>Groups</th>
<th>liver</th>
<th>spleen</th>
<th>Caecal tonsils</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>164±15$^b$</td>
<td>935.2±94$^b$</td>
<td>1280±87$^b$</td>
</tr>
<tr>
<td>G2</td>
<td>973.2±27$^a$</td>
<td>2467.8±351$^a$</td>
<td>4367.4±897$^a$</td>
</tr>
<tr>
<td>G3</td>
<td>867.1±19.3$^a$</td>
<td>2879±245$^a$</td>
<td>4876.3±567$^a$</td>
</tr>
<tr>
<td>G4</td>
<td>0±0$^c$</td>
<td>0±0$^c$</td>
<td>0±0$^c$</td>
</tr>
</tbody>
</table>

Five samples taken. A difference that is significant at the level of P≤0.05 is indicated by small letters.

**Discussion**

Once it has established an infection, *S. pullorum* may avoid the immune system of the host and parasitism the spleen and reproductive tract for more than 40 weeks before spreading through the digestive or reproductive tract. The infection cycle consists of three stages: (I) invading the intestine and parasitising the gastrointestinal tract epithelium (15); (II) invading macrophages and dendritic cells and establishing systemic infection in various tissues via the lymphatic system (16), which is essential for long-term persistence; and (III) developing the infection outcome, which can either be clearance, death, or carrier state (17). According to this study, layers treated with *Salmonella pullorum* had a chronic infection that resulted in irregular bacterial shedding in feces. The MPN count in the SP-infected (G2, and G3) groups at 3 weeks pi was substantially greater than in the group that had received SILK treatment (G1). In experimental birds, week 30 corresponded to the start of the production stage, and it was hypothesized that this rise may be attributed to physiological stress brought on by lay (18). High bacterial loads in feces can occur from stress stimulating the regrowth of germs from inside tissues (19). microorganisms in feces and measuring serum antibody levels (20). Detecting the regularity of SP shedding and antibody formation in chickens of various ages is crucial since both processes appear to depend on the immune system's maturation and capacity to eradicate germs. We noticed that *S. pullorum* shedding was greater in hens 33 weeks old than in chickens 10 days old. This may be due to the reproductive tracts of 33-week-old hens having seen a fast rise in S. pullorum population at this point due to their heightened vulnerability to *Salmonella* (21). According to our findings, SP remained in internal organs despite significant amounts of circulating IgG antibodies. Previous research suggested that Th-1-dominant responses and high levels of interferon-expression at roughly 14-28 days after
infection clear *Salmonella typhimurium* from the liver and other internal organs (22,23). According to certain research (24), *Salmonella enteritidis* was found infrequently in the liver and other internal organs for up to 22 weeks. Furthermore, it has been claimed that the age of exposure had no effect on *Salmonella typhimurium* liver recovery (25). The results were consistent with those reported by Ke Ding et al. (26), who demonstrated that lymphocytes play a role in regulating the host immune response and the cellular immune response is particularly important for the clearance of *S. pullorum* because it elicits a higher Th2-like response than the Th1-type response, which is more typically associated with *S. typhimurium* or *S. enteritidis* (27). After week 1 pi, the IgG antibody titers to *S. pullorum* grew and peaked at week 3 pi. Despite the immune response failing to completely remove *Salmonella* spp., birds remained seropositive until the trial’s conclusion at week 33 pi. Additionally, it’s crucial to remember that while the antibody response aids in the removal of external bacteria, intracellular bacteria can still survive in the host, necessitating the activation of the cell-mediated immune system in order to eliminate *Salmonella pullorum* (28).

However, the G1 that received SILK treatment increased the production of IgG against SP in the first week, all titer of IgG in all animals’ groups did not contribute to the clearance of SP. These findings concur with those of He et al. (29,30) SILK is essential for the development, recruitment, and quick aggregation of lymphocytes as well as for the defense against different infections. The *Salmonella pullorum* were elevated inflammatory cytokine IL-18, which can trigger IFN-γ, production by activated macrophages (Th1 immunological mechanism) (31,32), which boosts the Th1 immune system 7 to 35 days after ingestion. Our results are consistent with those reported by He et al. (33), who claim that activated cells unusually stimulate the production of IL-18. This verified SILK’s important role in reducing overall bacterial burden in tissues such as the liver, caecal tonsils, and spleen that were infected.

**Conclusion**

From our results we conclude that, SILK, when given to layer hens throughout the production stage, assists in boosting and activating the immune system and also gets the chickens ready to fight off infection at any moment. This helps prevent the chickens from contracting SP.

**Acknowledgements**

Greetings and blessings are sent to the Al-Rashidiya farm and Al-Nahda Veterinary Laboratories for meeting all of research demands. Also appreciate everyone who assisted me in any manner throughout the project’s completion.

**Conflict of interest**

There is no dispute or conflict between scientific fact and practical goals; rather, scientific activity is linked to new scientific activities and notions.

**Reference**

7. Setta AM, Barrow PA, Kaiser DE, Deboer JR, Hargis BM. Comparison of prophylactic and therapeutic efficacy of *Salmonella enteritidis* immune lymphocytes against salmonella in artificially contaminated chicken meat by cultured salmonells. Avian Pathol. 2002;31(5):569-76. DOI: 10.1080/0307945021000005879
استعملت المفونكتات الخلايا الناجحة لعلاج المعوز:

مناعاً بالسالونيلا بللورم لحماية الدجاج البياض ضد الإصابة بالسالونيلا بللورم.

مشتاق طالب الزهري

فرع الأراضي وأمراض الدواجن، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة

السالونيلا بللورم تتكيف بشكل جيد تسبب جهازية حادة في الدجاج مع معدل ثلاث مئات تم تقدير هذه الدراسة بتقييم عدوى السالونيلا بللورم السالونيلا النارية في إنتاج الفئران، بينما لقحت المجموعة تكيفت بشكل جيد لتسبب عدوى جهازية حادة البكتيريا في الدجاج المصاب بالسالونيلا بللورم. في هذه الدراسة، استخدمت 25 مجموعة من جرعة من الفئران في الأسّبوع الأول، 100 مجموعة من الفئران في الأسّبوع الثاني، 30 مجموعة من الفئران في الأسّبوع الثالث، 25 مجموعة من الفئران في الأسّبوع الرابع. تم إعطاء المجموعة الأولى مادة مضادة للفوسفات، بينما لم تتم علاج المجموعة الثانية. تم تقييم فعالية الأبحاث في الحالة والفصل.}


