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## Research Article

# Molecular Detection of *Salmonella pullorum* from Poultry Ceca and Vaccine Preparation

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## Abstract

**Objective:** Isolation of *Salmonella pullorum* (*S. pullorum*) from chicken ceca and confirmation by PCR after a trial to prepare a vaccine from this isolate by using the freezing and thawing method. **Materials and Methods:** A total of 69 samples were isolated from chicken ceca. Biochemical tests were performed and diagnoses were made. Twenty-one *Salmonella* positive samples were found, 12 of which were *S. pullorum*, which were confirmed by differential biochemical tests (glucose, sucrose, lactose, mannitol and maltose) and additionally confirmed by PCR. The second part of the study was to determine the LD<sub>50</sub> of *S. pullorum* at doses up to  $2 \times 10^{-7}$  after isolate exposure to slow freezing and after thawing, which effected isolation effectiveness. The third part of the study included the 28 day a challenge-dose test. **Results:** Mortality in the control group (n = 15) was 20%, with clinical signs. On the third day of challenge via oral inoculation with *S. pullorum* (0.5 mL of  $2 \times 10^{-7}$  CFU mL<sup>-1</sup>), there were two cases of lameness that improved in the vaccinated group. Over 2-3 days, signs of respiratory symptoms were minimal and nonspecific, with animals in the control group showing a dull appearance, depressed activity and a loss of appetite. Non-significant signs were noted 5 days after experimental infection in the vaccinated group. **Conclusion:** The isolation and characterization of *S. pullorum* from infected blisters and attempts to prepare a vaccine using a freezing and thawing method showed protective results after the challenge test with the same isolated strain compared to results in non-vaccinated chickens.

**Key words:** Genus *Salmonella*, *Salmonella pullorum*, PCR, vaccine, poultry cecum, broiler and layer production

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Salmonella enterica* includes more than 2500 serovars. This is the most common *Salmonella* species and is responsible for various diseases. Many *Salmonella enterica* (*S. enterica*) serovars play significant roles in medical and veterinary fields and approximately 99.5% of the isolated *Salmonella* serovars species belong to *S. enterica*, which causes acute intestinal inflammation and other systemic diseases<sup>1</sup>. Approximately 10% of these serovars have been isolated from poultry<sup>2</sup>. *Salmonella enterica* subsp. *enterica* serovar *Gallinarum* biovars *Gallinarum* and *Pullorum* cause fowl typhoid and pullorum, respectively, two septicemic diseases specific to poultry<sup>3</sup>. Pullorum in poultry is caused by *S. pullorum* and is an acute systemic disease that is considered more common in young birds (it rarely appears in adults) and infections results in bird's chronic carrier<sup>4</sup>. The *S. gallinarum* and *S. pullorum* cause specific avian diseases and differentiating between these diseases is necessary. Epidemiological differentiation and preventive measures depend on the phenotypic and the genetic methods used. The cost of equipment and reagents and the high level of experience and the analytical software required are important disadvantages of genotyping methods. Phenotyping methods, including biotyping, antimicrobial susceptibility tests and serotyping, can be used in epidemiological analyses and to differentiate between different *Salmonella* isolates<sup>5</sup>.

*Salmonella* spp. especially *S. pullorum*, are considered to spread disease in poultry. Control and preventative vaccinations are highly effective. All types of live, attenuated and killed vaccines show good results in the protection of layer and breeder flocks. The most important types of vaccines are *S. gallinarum* vaccine 9 (SG9R), which is a live vaccine<sup>6</sup>.

In the near future, vaccination is likely to play key role in protecting breeder and layer flocks from *Salmonella* pathogens due to research showing that *Salmonella* vaccination has reduced contamination in poultry farms<sup>7</sup>. Culturing bacteria through traditional methods requires 5-7 days to obtain accurate results, so test methods have been developed to reduce the time required to obtain results. One of these methods is real-time polymerase chain reaction (RT-PCR) analysis<sup>8</sup>. Most studies support the detection of *Salmonella* using PCR analysis, as its specificity and sensitivity are superior to that of culturing<sup>9</sup>. This study was designed for isolation of *Salmonella pullorum* from chicken ceca and confirmation by PCR after a trial to prepare a vaccine from this isolate by using the freezing and thawing method.

## MATERIALS AND METHODS

**Sample collection and experimental area:** A total of 69 chickens were collected between August and December, 2016 in the Governate of Salah al-Din Tikrit, Al-Alam and Samarra from broiler and layer populations with no history of *Salmonella* vaccination. Chickens showed symptoms of diarrhea and signs of respiratory infection at ages 1-3 weeks for broilers and 120-180 days for layers. The samples were immediately put in a small, sterile, plastic cooled box and were transferred to the Laboratory of Microbiology at Tikrit University, College of Veterinary Medicine and were investigated for the presence of *Salmonella*.

**Identification of *Salmonella*:** All 69 isolated samples were treated with care using sterile methods, where the surface of the cecum was sterilized with a hot spatula and then, a sterile cotton swab was inserted to take the sample. Then, cecal swabs were cultured on Selenite-F broth and incubated for 24 h at 37°C on *Salmonella-Shigella* agar (SS) and incubated at 37°C for 24 h on xylose lysine deoxycholate agar (XLD).

**Biochemical tests:** The specific biochemical tests for different species and types were carried out on isolated colonies believed to be *Salmonella*. Tests involved microscopy, Gram staining and motility testing, as well as a range of other tests, such as the triple sugar iron (TSI), carbohydrate fermentation (glucose, sucrose, lactose, mannitol and maltose), indole, urease production and Simmons citrate agar tests.

**Serological testing:** Results obtained from the samples sent to the Central Health Laboratory showed positive results that were obtained through *Salmonella* antisera serotyping using O and H polyvalent anti sera (Table 3).

**DNA extraction:** One colony of isolated *Salmonella* was taken for DNA extraction using the genomic DNA extraction kit from the Gene Net MacroGen sequencing service (Korea) following the manufacturer instructions. The extracted DNA was stored at -20°C for subsequent PCR assay.

**Polymerase chain reaction analysis:** PCR analysis was performed following the manufacturer's instructions using Gene Net PCR Premix. Specific gene PCR primers (forward primer, GTGAAATTATCGCCACGTTCCGGCAA; reverse primer TCATCGCACCGTCAAAGGAACC) were arranged from a specific *Salmonella* genus for the *invA* gene to amplify a 285 bp sequence<sup>10</sup>. Primers specific for *S. pullorum* were as follows: forward primer, GATCGAAAAAATAGTAGAATT; reverse primer GCATCAAGTGATGAGATAATC. MacroGen (Korea) made the

primers. PCR reactions were carried out with 5 µL of DNA template and 1 µL of 10 pmol forward and reverse primers and the reaction was then completed in a volume of 20 µL DEPC-H<sub>2</sub>O. A BIO-RAD thermal cycler (USA) was used with the following parameters, initial denaturation at 94°C for 5 min 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec and a final extension step at 72°C for 5 min.

**Electrophoresis and gel analysis:** Electrophoresis of the PCR products was done in a 1.2% agarose gel. First, 7 µL of PCR contents were colored with 3 µL of safe dye, loaded on the gel, visualized by a UV transilluminator and photographed using an UVETIC, U.K. The amplicon sizes were estimated according to the migration pattern of a 100 bp DNA ladder.

**Determination of median lethal dose (LD<sub>50</sub>) in chicks:** The median lethal dose (LD<sub>50</sub>) was determined according to Reed and Muench<sup>11</sup>. The LD<sub>50</sub> was determined for 2 days-old chickens. First, 32 chickens were randomly divided into 8 groups (n = 4). Experimental groups were inoculated with one of 7 different 10-fold-diluted doses in PBS: 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup> and 10<sup>-7</sup>. Each experimental group was administered 1 mL of the diluted dose and the control group was administered 1 mL of PBS.

**Freezing and thawing:** A *Salmonella* sample was placed in nutrient broth at the specified level and bacterial suspensions were frozen slowly at a rate of 1-2°C min<sup>-1</sup> to at least -70°C before being thawed at a rate of 1-2°C min<sup>-1</sup> as described by Calcott and MacLeod<sup>12</sup>. The bacteria were counted in 1 mL before and after the procedure. The samples were frozen and thawed up to 3 times.

**Experimental infection:** Birds were equally divided into a group to be vaccinated and a control group (n = 30 per group). In the vaccinated group, the birds were orally

inoculated with 0.1 mL of a bacterial suspension containing 2×10<sup>-7</sup> colony-forming units (CFU) of the live, attenuated *S. pullorum* (SP) at 7 days of age. For the control group, 0.1 mL of PBS was administered. At 14 days of age, birds received 0.1 mL of a 2×10<sup>-7</sup> CFU mL<sup>-1</sup> solution of the live attenuated SP.

**Challenge dose:** At 28 days of age, all birds in the 2 groups (n = 15) were challenged orally with 0.5 mL of a 2×10<sup>-7</sup> CFU mL<sup>-1</sup> suspension containing the virulent SP strain.

## RESULTS

From the microbiological analysis of 69 samples, about 21 samples were positive for presumptive *Salmonella* spp. (Table 1). The bacterial colonies appeared at the center of the SS as small, smooth and transparent round shapes with black color at their center due to hydrogen sulfide production. The colonies appeared smooth with a black center after culture on XLD. The *Salmonella* strain in the Gram stain appeared rod shaped and was pink in color. When grown on TSI, the colonies were unable to ferment lactose and sucrose and appeared yellow and the slant was red, with H<sub>2</sub>S gas production. When fermenting glucose, the slant appeared red at an alkaline or neutral pH and the bottom was yellow, indicating an acidic reaction (Fig. 1). The isolated bacteria were negative for the indole and urease tests, positive for citrate utilization, non-motile when cultured on sulfide-indole-motility (SIM) agar, negative for the fermentation of sugars such as lactose, sucrose and dulcitol and positive for maltose, mannitol and glucose fermentation. Figure 2 and Table 2 show the results of these biochemical tests.

The study showed that a 10<sup>-5</sup> dilution caused the death of 50% of the experimental chicks infected with *S. pullorum* bacteria, which contains 200 cells mL<sup>-1</sup>. Thus, the LD<sub>50</sub> is 2×10<sup>-7</sup> cells mL<sup>-1</sup>.

Table 1: Number of samples of isolates and corresponding percentages

No.	Total samples sent to laboratory	Broilers	Layers	Positive	Negative	Positive (%)
1	13	13	0	5	8	23.80
		18		2	16	9.60
2	22		4	0	4	-
	14	11		6	5	28.60
3			3	2	1	9.50
4	4	4	0	0	4	-
5	6	6	0	4	2	19.00
	10	8		2	6	9.50
6			2	0	2	-
Total	69	60	9	21	48	100.00

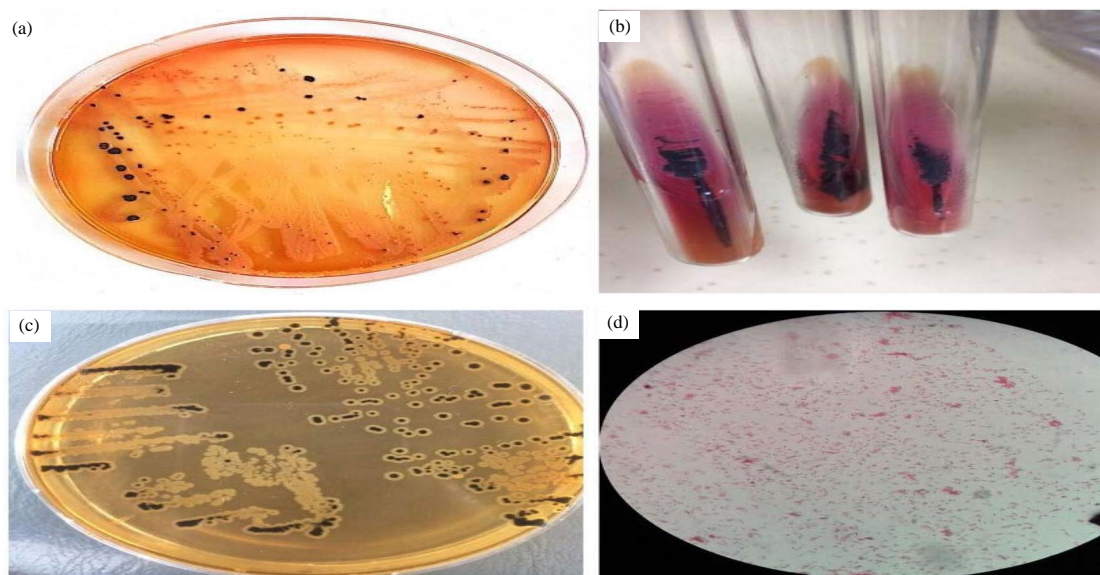


Fig. 1(a-d): (a) SP on XLD, (b) SP on TSI, (c) SP on SS agar and (d) Gram-stained SP appears rod shaped and pink in color

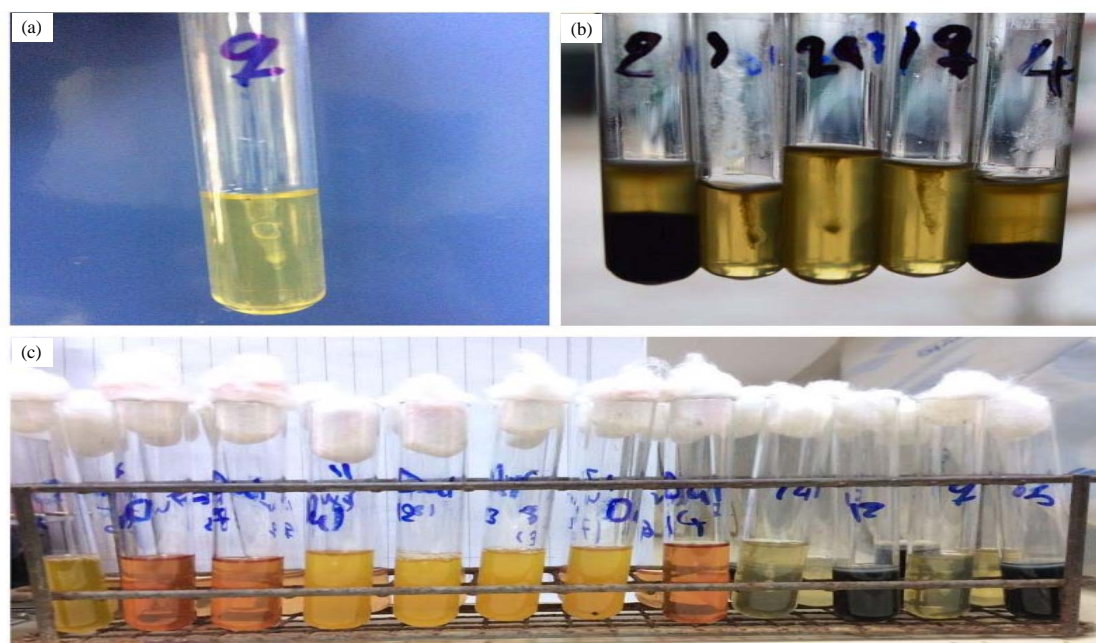


Fig. 2(a-c): SIM results show SP as (a) Motile, (b) Non motile and (c) Biochemical test of sucrose fermentation

**PCR results:** Isolates that tested biochemically positive for *Salmonella* species were confirmed by PCR analysis targeting the genus-specific *invA* gene, with an amplicon size of 285 bp, using self-designed primers for *Salmonella* spp. and 150 bp for *S. pullorum* (Fig. 3).

**Clinical signs:** After the 1st and 2nd dose was administered, on the 2nd day, loss of appetite and

inactivity in the birds, with mild diarrhea in some of them, were observed and after 3 days of oral inoculation with attenuated *S. pullorum* at  $1.0 \text{ mL } 2 \times 10^{-7} \text{ CFU mL}^{-1}$ , all the birds appeared normal in the vaccinated group. No mortality occurred in the two groups at this point in time. On the 3rd day of challenge ( $n = 15$  per group) via oral inoculation at  $0.5 \text{ mL } 2 \times 10^{-7} \text{ CFU mL}^{-1}$  with *S. pullorum*, there were two cases of lameness in the vaccinated group, which improved.



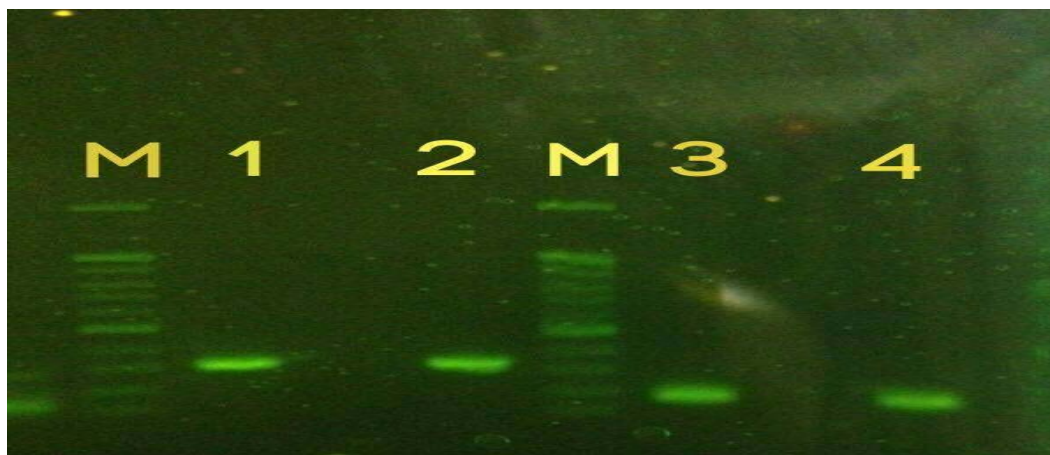


Fig. 3: Representative agarose gel image from PCR assay targeting the *invA* gene in *Salmonella* isolates, M: 100 bp DNA ladder, 1: 285 bp *Salmonella* amplicon, 2 and 4: Positive controls, 3: 150 bp SP amplicon

Table 2: Biochemical and sugar fermentation results for *Salmonella pullorum*

Test	Result
Gram reaction	- Ve
Indole production	-
Triple sugar iron (TSI) red/yellow/+H <sub>2</sub> S/+gas	-
Urease	-
Glucose fermentation	+
Dulcitol fermentation	+
Maltose fermentation	-
Mannitol fermentation	+
Sucrose fermentation	-
Simmons citrate agar	+
Sulfate-motility-indole (SMI)	-

Table 3: Identification of isolates by antisera according to the Central Health Laboratory

Sample	Result
Gut of poultry	<i>Salmonella hadar</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella hadar</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella hadar</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella hadar</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>
Gut of poultry	<i>Salmonella gallinarum-pullorum</i>

Over 2-3 days, the signs of respiratory symptoms were few and nonspecific in the control group, with birds showing a dull appearance, depressed activity and a loss of appetite. Five days post-challenge, no significant signs were observed in the vaccinated group. In the control group, the clinical signs were continuous at 8 days. In the present experiment, no mortality occurred in the vaccinated group (0%), while the mortality rate in the control group challenged via oral inoculation was 20% (3 chicks).

## DISCUSSION

***Salmonella* results in intracellular infections:** A variety of acute and chronic diseases in poultry are caused by *Salmonella*, meaning that poultry acts as a source and reservoir of *Salmonella* and can result in its spread. This can transmit the bacteria to humans via food. *Salmonella* has been isolated from poultry more than from any other animal species<sup>13</sup>. Current study of 69 broiler and layer chickens suspected of *Salmonella* infection showed 39 *Salmonella* infections, for an infection rate of 30.43%. This ratio is close to the 31.94% recorded in Syria in March<sup>14</sup>. This percentage is also higher than the percentage found in Jordan, which was 11.67%<sup>15</sup>. In Iran, 27 *Salmonella*-positive isolates out of a total of 1125 samples were identified, which is less than what was found in this study.

PCR analysis has emerged as a useful tool for the rapid and accurate detection of small pieces of DNA for molecular characterization and comparisons of serotypes. This is the method used by most researchers for targeting the *invA* gene in *Salmonella*<sup>16,17</sup> and the PCR results from this study for *S. pullorum* are shown in Fig. 1. For serotype tests, *Salmonella* has been profiled at the Central Health Laboratory/Ministry of Health, which is a certified national laboratory. This laboratory has the capacity to test for all of the serotype agents of *Salmonella* that infect humans and animals and that are common to each group. According to the findings shown in Table 3, all 11 isolates sent for analysis contained *Salmonella* species. This percentage differed from previously recorded percentages (9.18% of 370 samples)<sup>16</sup>. The chicks used in the study were found to have an LD<sub>50</sub> of  $2 \times 10^{-7}$  CFU mL<sup>-1</sup> and this dose is similar to that referred to by Yin *et al.*<sup>18</sup>.

The *Salmonella* isolates were affected by freezing and thawing, which decreased the number of live bacteria, similar to the results of Obafemi and Davies<sup>19</sup>, who found that *Salmonella typhimurium* was affected by double freeze-thawing, with viability reduced by 99.0 and 95.6% over subsequent cycles. Clinical signs of disease, including slight and temporary lethargy, anorexia, diarrhea and lameness, were observed in chickens from the vaccinated group and all these symptoms were absent 3 days after inoculation, which corresponds to the results found by Yin *et al.*<sup>18</sup> After experimental challenge, birds showed depressed activity, had a dull appearance and had mild diarrhea with vent pasting<sup>20</sup>. Barman *et al.*<sup>21</sup> found that out of 6 chicks derived from vaccinated birds, only 1 chick died after challenge with  $1 \times 10^{-9}$  CFU mL<sup>-1</sup> of *S. gallinarum*.

## CONCLUSION

It is concluded that in the challenge test with the same strain of vaccine, it is possible to vaccinate chicks against *S. pullorum* without relying on maternal immunity against infection, preventing the loss of chicks after infection, which was not the case for non-vaccinated chicks. Many vaccinations have been prepared and administered to laying hens to ensure that chicks are immunized against *Salmonella*, especially vaccines against *S. gallinarum*, to develop immunity and protect against early infection by these bacteria. There are several methods for the preparation of bacterial vaccines, including freezing and dissolving isolates to obtain vaccine antigens. Numerous studies have shown that vaccinating young chicks generates strong immunity against early *Salmonella* infections during the 1st weeks of life.

## SIGNIFICANCE STATEMENT

This study discovered an appropriate vaccine dose for the purpose of preparing a vaccine from isolates collected locally from infected chicks and it assessed how the isolated bacteria differed from other types by conducting biochemical tests. Then, *S. pullorum* was identified by PCR. Young chicks were inoculated with the *S. pullorum* vaccine to provide immunity against infection and perform a challenge test.

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