Research Article

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Investigating of Some Virulence Genes of *Salmonella* Isolated From Chickens and Their Eggs Using Biochemical and Molecular Methods

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Abstract

This investigation aimed for isolation and molecularly identify of *Salmonella* species from local chickens and their eggs. A total of four chicken flocks located inside Baghdad's urban limits provided the 100 cloacal swabs and 100 egg shells used to isolate Salmonella spp. These were cultured on basic medium, then subcultured on XLD then biochemical tests were done for identification of bacteria. PCR was done for confirmation of the results by using the three genes *flic, rfbsp*,and *rfbsg.* After t confirming he presence of Salmonella spp., the Kirby-Bauer disc diffusion assay was used to determine susceptibility of antibiotics in all of the isolates (16). The antibiotics that were included in the test were from Bioanalyse in Turkey and included the following: Trimethoprime & Sulphamethoxazole (25 µg), Azithromycin (15 µg), Florfenicol (30 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), and Tetracycline (30 g). The current results showed that Salmonella spp. was isolated from cloacal swabs at 15% while from egg shell the percentage was 2% and the total percentages of isolation was 8.5%. The genes *flic* and *rfbsg* had been were also found in all isolates. The investigation found the following antibiotic resistance rates among the isolates: Azithromycin (82.3%), Florfenicol (70.6%), Trimethoprime- Sulphamethaxezole (70.6%), Tetracycline (64.7%), Ciprofloxacin (58.8%), Ampicillin (52.3%), and Gentamycin (35.3%) *Salmonella spp.* was isolated significantly from cloacal swabs compared to egg shell which were characterized by multidrug resistance.

Keywards: ***Salmonella,*** **chicken, gen,** ***flic*** **,** ***rfbsg*****,** ***rfbsp***

دراسة بعض جينات الضراوة لبكتريا السالمونيلا المعزولة من الدجاج وبيضه باستخدام الطرق الكيموحيوية والجزيئية

يهدف هذا البحث إلى عزل وتشخيص أنواع السالمونيلا من الدجاج المحلي وبيضه. تمت دراسة اربعة انواع من الدجاج المحلي في بغداد وقد اخذت 100 مسحة مذرقية من الدجاج و 100من قشرة البيض لعزل السالمونيلا spp. ومن ثم زرعها على وسط اساسي ، ثم زرعت على وسط XLD وبعدها إجريت الاختبارات البيوكيميائية للتعرف على البكتيريا. تم فحص PCR لتأكيد النتائج باستخدام 3 جينات flic، rfbsp، rfbsg. بعد التأكد من وجود السالمونيلا، تم استخدام اختبار كيربي باور لتحديد مدى حساسية المضادات الحيوية في جميع العزلات (16). المضادات الحيوية التي تم تحديدها في الاختبار كانت من شركة Bioanalyse في تركيا وتضمنت المضادات ما يلي: تريميثوبريم وسلفاميثوكسازول (25 ميكروغرام)، أزيثروميسين (15 ميكروغرام)، فلورفينيكول (30 ميكروغرام)، جنتاميسين (10 ميكروغرام)، سيبروفلوكساسين (5 ميكروغرام)، الأمبيسلين (10 ميكروجرام)، والتتراسيكلين (30 جم). أظهرت النتائج الحالية أن السالمونيلا spp. تم عزله من مسحات المذرق بنسبة 15% بينما من قشرة البيضة كانت النسبة 2% وكانت نسبة العزل الكلية 8.5%. كما تم العثور على الجينات flic وrfbsg في جميع العزلات. وجدت الدراسة أن معدلات مقاومة المضادات الحيوية التالية بين العزلات: أزيثروميسين (82.3%)، فلورفينيكول (70.6%)، تريميثوبريم- سلفاميثاكسيزول (70.6%)، تتراسيكلين (64.7%)، سيبروفلوكساسين (58.8%)، أمبيسيلين (52.3%). والجنتاميسين (35.3%) السالمونيلا. تم عزله بشكل ملحوظ من مسحات المذرق للدجاج مقارنة بمسحات قشر البيض الذي تميز بمقاومته للأدوية المتعددة.

Introduction:

The eggs and the meat may be found in chicken. Economically significant agro-industrial components include the poultry production-related sector [1].*Salmonella* spp. is a leading cause of illness in birds and poultry [2]. There are substantial monetary losses due to the increased mortality rate and lower production rate of chicken caused by it [3]. There will be more than €3 billion in lost revenue in the European Union due to the Salmonella spp. pandemic, compared to an estimated $11.6 billion in the US [4].

One of the best ways for *Salmonella* spp. to infect people is via the consumption of contaminated poultry [6]. About 40% of *Salmonella* infections in people are associated with poultry [7]. This suggests that poultry is the most prevalent food source for *Salmonella* infections in humans. The Gram-negative bacteria known as *Salmonella* spp. are members of the Enterobacteriaceae family [8]. The opportunistic zoonotic bacteria *Salmonella* spp. may infect cells in humans and other animals via tainted food and water [9]. As an example, it infects epithelial cells, M-cells,and dendritic cells, as well as macrophages, among many others [10]. In other words, it doesn't need oxygen to live [11].

Salmonella spp. are often grouped into three categories according to the illnesses they cause in hens [12]. In the first group are *Salmonella* species that have evolved to infect hens; these include the avian typhoid-causing S. gallinarum and the chicken-sucking S. pullorum [13]. Septicemic infections such as fowl typhoid (FT) and pullorum disease (PD) which often manifest most severely in adult and increasing chicken populations. Egg production reduction, fertility reduction, decrease hatchability, decreased appetite, and an increased mortality rate are signs of FT and PD in adult poultry [14].

A second kind of *Salmonella* may infect birds; this type, known as paratyphoid *Salmonella*, is invasive and nonhost-specific, meaning it can infect both animals and people. One zoonotic worry is this particular strain of bacterium, which causes paratyphoid in birds. There are ten to twenty different serovars of paratyphoid *Salmonella*. The most significant serovars are S. *enteritidis* and S. *typhimurium* [15].

The most prevalent *salmonellae* recovered from Egyptian poultry farms are *Salmonella* enteritidis and *S.* typhimurium [16]. They may spread from farm to farm and from generation to generation by means of trans-ovarian infection [16]. Infected young hens, particularly those in their first few weeks of life, are more likely to show signs of paratyphoid. Depression, anorexia, and diarrhoea are the most prevalent signs of paratyphoid sickness in broilers, and the condition is most deadly in the first week of life. Although adults show no symptoms of illness, infected birds may spread the disease to other birds and humans via their eggs and meat.

The widespread use of antibiotics in poultry farms for treatment and growth promotion raises concerns about the emergence of antibiotic-resistant strains of *Salmonella* spp. serovars [17]. Two major advances in our understanding of the global epidemiology of nontyphoidal salmonellosis occurred in the latter part of the twentieth century [18]. The first is the emergence of *Salmonella* enteritidis as a major disease of poultry and eggs [19]. The second is the emergence of MDR *S.typhimurium* strains like *S. typhimurium* DT104.

This penicillin-binding protein 1 may be inhibited by the β-lactam antibiotic Amoxicillin. Bacteria often develop resistance to β-lactam antibiotics because they produce an enzyme known as a β-lactamase, which targets the β-lactam ring. The four basic ways that

prophylactic β-lactam resistance might emerge are as follows: first, by Gram-negative bacteria producing a β-lactamase enzyme; second, via decreased production of proteins on the outside of the cell membrane, modifications to the dynamic binding site of penicillin-binding proteins (PBPs), and lastly, active efflux [20]. Certain genes are linked to the ability to resist β-lactamase, including blaTEM-1, blaTEM-2, and blaSHV-1. In order for blaTEMβ-lactamases to function, they hydrolyze the β-lactam ring of penicillin [21]. Erythromycin binds to the 50S component of the ribosome and the bacterial cell membrane, preventing the bacteria from making their protein. Ribosomes in bacteria consist of two parts: a tiny 30S subunit and a big 50S subunit. This second kind has 23S rRNA and 30 proteins or more. The binding of erythromycin to the 50S subunit stops protein synthesis. An enzyme called Erythromycin ribosomal methylase changes the binding site of erythromycin on the 50S subunit of ribosomal proteins. The gene ermB encodes it. The target-binding affinity of erythromycin is significantly diminished due to the alteration gene [22]. By attaching to the exit tunnel of the 50S ribosomal subunit, Bacteria are unable to synthesize proteins when exposed to macrolides such as erythromycin. Their function is based on inhibiting peptidyl transferase, which is responsible for moving the growing peptide connected to tRNA to the amino acid that follows. Additionally, it stops bacteria from translating ribosomal proteins [23]. The phosphotransferases mphA and mphB are responsible for inactivating macrolides as well [24,25]. One possible cause of macrolide resistance is the erythromycin resistance esterase type I (ereA) gene [26]. The erythromycin esterase enzyme, which acts as a catalyst for the hydrolysis of the macrolactone ring, is encoded here [23].

The epidemiology of Salmonellosis is determined by serotyping which is a basic biomarker and is typically used to identify the cause of contamination during outbreaks [27]. For serotyping Salmonella spp., the procedure mentioned is the gold standard. Among the several benefits of serotyping *Salmonella* spp. is the information it provides about the severity of diseases, the pathogen's origin, and the resistance configuration. Different *Salmonella* strains have been identified using molecular characterization techniques. Some examples of these techniques include polymerase chain reaction (PCR), random Amplification of polymorphic DNA (RAPD), and pulsed-field gel electrophoresis (PFGE) [28].

This investigation aimed for isolation and molecular identification of *Salmonella* species from local chickens and their eggs.

Materials and Methods:

A total of four chicken flocks located inside Baghdad's urban limits provided the 100 cloacal swabs and 100 egg shells used to isolate *Salmonella* spp.

In order to culture *Salmonellae*, one must first do a non-selective pre-enrichment, then a selective enrichment, and then plating onto differential and selective agars. Biochemical and polymerase chain reaction confirmation of suspect colonies is performed [29]. While swabs were infected into 10 ml of nutritional broth and cultured at 37◦C for 18 hours, one gramme of solid material was added to 9 ml of soup [30,31].

We used the method described by Loongyai *et al*., [32] to make the egg shell and contents swabs. We introduced 1 ml of each sample type's enriched cultures to 9 ml of Selenite F broth after preenrichment and incubated it at 37°C for 18 hours.
A loop-full of Selenite F broth culture was streaked onto XLD plates. After 18 hours of incubation at 37°C, the plates were inspected for the formation of characteristic of

Salmonella spp. colonies [33].

The following biochemical tests were conducted in accordance with established protocols: indole, urase, lysine decarboxylase, triple sugar iron (TSI) slant reaction, ornithin decarboxylase, citrate utilisation, motility, and carbohydrates fermentation (lactose, xylose, sucrose, trehalose, arabinose,and rhamnose) [34]. Following the instructions on the API 20E test kit (bioMérieux, Inc., France), colonies that were suspected to be present on XLD agar were further identified by inoculating twenty mini-test tubes with saline solutions of the cultures. The colour responses were evaluated after 18 hours of incubation in a humidity room at 37°C, with the use of additional reagents provided by the kit, in some cases. Results indicating the presence of Salmonella with probability of 89% or higher were verified after analysing the data using the manufacturer's keys. The manufacturer of the bacterial extraction kit (Genaid, Korea) provided the instructions for extracting the bacterial DNA. In order to amplify the *rfbsg* and *rfbsp* genes of *S. gallinarum* as well as *Salmonella pullorum*, the PCR conditions were set according to *Shah et al* [35], and for flic, they were set according to Paiva *et al*., [36] (Table 2).

Table 1. genes used in this study

Gene name	Sequence	bp	Ref.
rfbsg	F GTA TGG TTA TTA GAC GTT GTT R TAT TCA CGA ATT GAT ATA CTC	187	36
rfbsp	F GTA TGG TTA TTA GAC GTT GTT R TAT TCA CGA ATT GAT ATA TCC	187	35
flic	F CTGGTGATGACGGTAATGGT R CAGAAAGTTTCGCACTCTCG	197	35

After confirming the presence of *Salmonella* spp., the susceptibility of all isolates to antibiotics was determined using the Kirby-Bauer disc diffusion method (16). The antibiotics that were included in the test were from Bioanalyse in Turkey and included the following: Trimethoprime and Sulphamethoxazole (25 µg), Azithromycin (15 µg), Ampicillin (10 µg), Gentamycin (10 µg), Florfenicol (30 µg), Ciprofloxacin (5 µg), and Tetracycline (30 g).

Statistical analysis:

Software for statistical analysis, SPSS version 23, was used.

Results and Discussion:

The current results showed that Salmonella spp. was isolated from cloacal swabs at 15% while from egg shell the percentage was 2% and the total percentages of isolation was 8.5% (table 2).

Table 2. Rate and percentages of isolation

Source of sample	No.of samples	No.	%
Cloacal swab	100	15	15%
Egg shell	100	2	2%
Total	200	17	8.5%

These findings corroborate those of a study conducted in Basrah city (37) that indicated a total prevalence of 9.2% for Salmonella spp. Along with that, Akbarmehr (38) discovered that 9.4% of Iranians lived in the west of the country and 8.2% in the south.

While *S. gallinarum* and *S. pullorum* infect

chickens in various ways, they are very similar. The somatic antigenic structure is identical in *S.* gallinarum and *S.* pullorum, two non-motile bacteria that cause chicken typhoid and pullorum disease, respectively [35]. From an epidemiological and preventative standpoint, it is extremely crucial to differentiate between *S.* gallinarum and Salmonella pullorum. Traditional serological techniques are unable to differentiate between them due to their high degree of similarity [35]. Additionally, it was reported in Rehman *et al*., [39] that serotyping could only identify Salmonella serovar gallinarum and not differentiate it into its pullorum and gallinarum biovars. Since the antigenic structures of Salmonella serotypes gallinarum, pullorum, and enteritidis are extremely similar, polymerase chain reaction (PCR) is a powerful technique for the quick and accurate identification of these serotypes in birds [36]. Serotyping had previously identified all isolates as Salmonella serovar gallinarum; PCR (rfbsg) confirmed by Gillespie et al [40]. These findings are consistent with Paiva *et al*., [36], which came to the same conclusion: serotype-specific detection of *S. gallinarum* was successfully accomplished by allele-specific PCR employing a *S. gallinarum* specific primer (*rfbsg*). The *flic* gene had been also found in all isolates (figure 1).

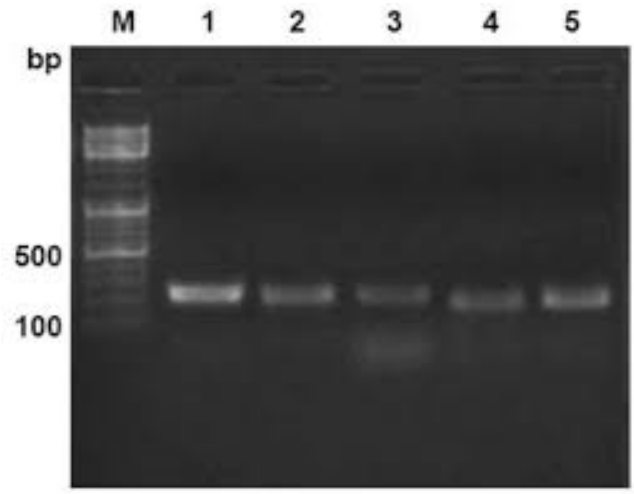

Figure 1. The 1.5% agarose gel, stained with ethidium bromide, was used to run the PCR amplification mixture. The results showed that the *Salmonella Spp*. gene, which is 197 bp long, was positive.

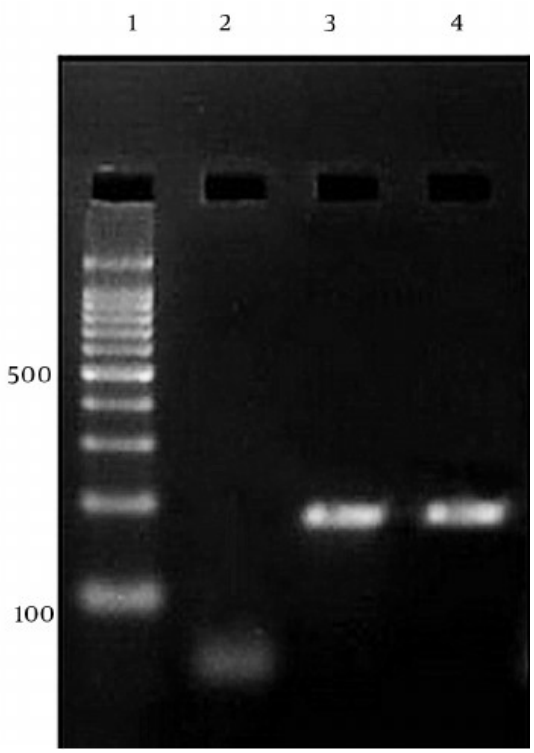

Figure 2. The *S. gallinarum rfbsg* gene is 187 base pairs long, and the PCR amplification mixture was carried out on a 1.5% agarose gel stained with ethidium bromide.

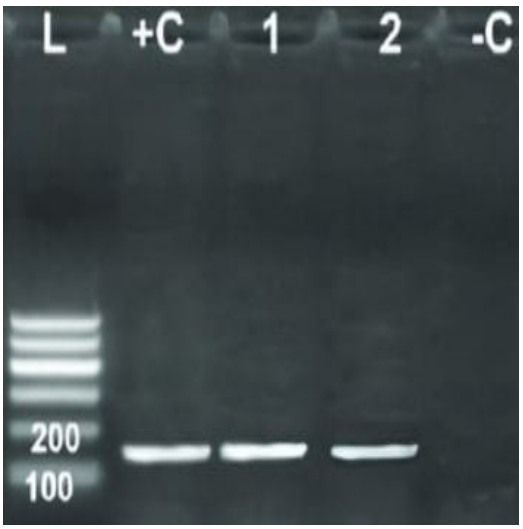

Figure3. The S. *gallinarum* is the *Rfbsp* gene 187 base pairs long, and the PCR amplification mixture was carried out on a 1.5% agarose gel

stained with ethidium bromide.

Although chromosomal mutations and the introduction of transposable genetic elements are two potential sources of Salmonella resistance [41], numerous researchers have pointed to antibiotic use in poultry for a variety of reasons, including growth promotion, prophylaxis, and therapeutics [42,43]. The investigation found that the following antibiotic resistance rates among the isolates: azithromycin (82.3%), florfenicol (70.6%), trimethoprime-sulphamethaxezole (70.6%), tetracycline (64.7%), ciprofloxacin (58.8%), ampicillin (52.3%), and gentamycin (35.3%), as shown in table (3). The outcomes of this investigation consistent with the discoveries of [44, 45].

It follows that multidrug-resistant Salmonella is common in animal-based foods, since our research shows that Salmonella isolates are resistant to many antibiotics. Our results are consistent with those of other studies [46,47].

Table 3. antibiotic sensitivity test for isolates

Antibiotic	Resistance No. (%)	Sensitivity No (%)
Ampicillin	9(52.3)	8(47.7)
Azithromycin	14(82.3)	3(17.7)
Ciprofloxacin	10(58.8)	7(41.2)
Florphenicol	12(70.6)	5(29.4)
Gentamicin	6(35.3)	11(64.7)
Tetracycline	11(64.7)	6(35.3)
Trimethoprime-sulphamethaxezole	12(70.6)	5(29.4)

Conclusion:

Salmonella spp. was isolated significantly from cloacal swabs than egg shell which were characterized by multidrug resistance chicken meat samplesand egg is prevailing in the areas of sampling due to poor hygienic conditions and also demonstrated the varied spectrum of antimicrobial resistance, including several multiple drug resistance phenotypes. Therefore, the present study emphasizes the need for continued surveillance of zoonotic foodborne pathogens including antimicrobial-resistant variants throughout the food production chain

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Conflict of interest: the authors declare that there was no conflict of interest.

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