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# Virulence Genes and Pathogenicity Assessment of *Salmonella* Isolated From Diseased Chickens

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**Abstract**

THIS study emphasizes Salmonella infections in broiler farms to monitor and notify the growing threat of antimicrobial resistance and its impact on public health. During the first half of 2024, 14% (14/100) of broiler farms were positive for Salmonella. Five serotypes were identified; the most prevalent were *S. enteritidis* (35%) and *S. typhimurium* (28%), followed by *S.* Kentucky (21%), and less commonly *S.* Infantis and *S. virchow* (7% each). Antibiotic susceptibility testing revealed multidrug resistance (MDR) across isolates, showing high resistance to lincomycin (85.7%) and significant resistance to β-lactams (Ampicillin: 42.8%, Ceftriaxone: 28.5%). Colistin sulfate demonstrated the highest efficacy with sensitivity percent 85.7%.The pathogenicity index (PI) evaluated for different serotyped found that there are four serotypes (*S. enteritidis, S. typhimurium, S. kentucky, and S. virchow*) which classified as intermediately pathogenic, while *S.* Infantis exhibited as low pathogenicity index. PCR analysis detected three virulence genes (*inv*A, *spv*C, *avr*A) in all isolates, with percentage 100%. This study reinforces the critical function of virulence factors and MDR in Salmonella-associated pathogenicity and underscores the importance of robust biosecurity measures, prudent antibiotic use, and continued surveillance to mitigate risks in poultry production and public health.

**Keywords:** Salmonella infections, antimicrobial resistance, Salmonella serotypes, pathogenicity index,

## Introduction

Salmonella infects bird, human, mice and livestock [1,2]. Salmonella causes the dangerous disease called salmonellosis. Salmonella comes in two species, including *Salmonella enterica* and *Salmonella bongori*. Salmonella is a motile bacterium except *S. Gallinarium and S. Pullorum* [3,4]. In poultry, there are different serotypes that make significant problems economically and in public health. *S. Pullorum* causes pullorum disease; *S. Gallinarum* leads to fowl typhoid, *S. enterica* subsp. *arizonae* results in arizonosis, and other serotypes cause salmonellosis known as paratyphoid infection [5].

In the poultry, pullorum disease infects birds for up to 21 days and making death in the shell. fowl typhoid infects adult birds. Acute cases occur with sudden death by septicemia, while chronic infection makes lesions as necrosis in the liver [5,6]. Salmonellosis occurs in birds at any age showing typhlitis and spreads to the liver, spleen, lung, and heart [7]. Salmonella make two main type of disease intestinal, enteritis or typhlitis, and extraintestinal [8]. It is transmitted horizontally and vertical [9]. Salmonella uses its invader related type 3 secretion system (T3SS). Salmonella destroy the integrity of intestinal epithelial cell spreading to other organ [2]. Systemic infection caused by salmonella decrease growth performance [10,11].

On public health, there is a significant threat to humans from infections caused by *Salmonella enterica* serovar Enteritidis (S. Enteritidis), while in poultry being the primary reservoir host [12]. Similarly, *Salmonella enterica* serovar Typhimurium

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(*S. Typhimurium*) poses a major risk [1,2]. Poultry can additionally be identified as a reservoir for *Salmonella enterica* serovar Infantis (*S. Infantis*), which has been associated with outbreaks and multidrug resistance [13]. The issue of *Salmonella enterica* serovar Virchow (*S. Virchow*) was tracked using the Rapid Alert System for Food and Feed (RASFF), first launched by France on 11 January 2023. The strains were traced to chicken from French farms and turkey meat supplied by German companies to restaurants [14].

This study gives us a comprehensive view of poultry farms with clinical signs and necropsy of salmonellosis confirmed by bacteriological examination and antimicrobial pattern. Serotype isolates assessed with pathogenicity in vivo. Five different serotypes analyzed by PCR to detect virulence genes.

## Material and Methods

*Sampling*

The research was conducted between December 2023 and June 2024 at the Reference Laboratory for Veterinary Quality Control in Poultry Production. Internal organ samples were aseptically collected from suspected broiler chickens from 100 broiler flocks located in the El Sharkia governorates. These birds exhibited clinical signs such as white diarrhea, clustering under heaters, and central nervous system (CNS) symptoms, including laying down with extended legs, ataxia, and occasional tremors. The ages of the affected birds ranged from 1 to 35 days.

*Isolation and identification of salmonella spp.*

The isolation and identification of Salmonella spp. were performed following ISO 6579–1:2017/Amd1:2020 standards.

Samples were pre-enriched in buffered peptone water (Oxoid®) and incubated at 37°C for 16–18 hours. A 0.1 mL aliquot of the pre-enrichment broth was inoculated onto Modified Semisolid Rappaport-Vassiliadis (MSRV) medium (Oxoid®) and incubated at 41.5°C for 24 hours. Simultaneously, 1 mL of the pre-enrichment broth was transferred to Muller-Kauffmann Tetrathionate (MKTTn) broth (Oxoid®) and incubated aerobically at 37°C for 24 hours. Followed by, Selective Plating: Enriched samples were streaked onto Xylose Lysine Deoxycholate (XLD, Oxoid®) agar and Hektoen Enteric (HE, Liofilchem®) agar. Plates were incubated aerobically at 37°C for 24 hours. Colonies with typical *Salmonella* morphology (pink colonies with or without black center) were confirmed using biochemical tests, including Urea agar, Triple Sugar Iron (TSI) agar, and Lysine Iron agar (LabM, Oxoid®, and Liofilchem®).

Serotyping of *Salmonella* isolates was performed according to ISO 6579–3:2014 [15] using the slide agglutination method using (O) antisera and (H) antisera and interpretation according to the Kauffman–White scheme [16] using *Salmonella* antiserum (Sifin Co., Germany).

*Antimicrobial Sensitivity Test for Salmonella Strains*

The antimicrobial susceptibility of *Salmonella* isolates was assessed using the CLSI 2021 [17].

The isolates were tested against 16 antibiotics from Oxoid® (UK), including: Beta-lactams **(**Cefotaxime, aztreonam, ceftazidime, ceftriaxone, ampicillin, and carbapenem). Aminoglycosides **(**Gentamicin, neomycin, and streptomycin). Tetracyclines **(**Tetracycline and doxycycline). Fluroquanilone (ciprofloxacin, norfloxacin, levofloxacin), Other Classes **(**Fosfomycin, colistin sulfate, lincomycin, chloramphenicol,). Zone diameters were interpreted based on CLSI 2021 [17] guidelines to determine sensitivity, intermediate, and resistance patterns.

*Experimental design to detect pathogenicity*

The protocol number for the experiment is IACUC protocol number ARC-AHRI-20-25. Six groups of ten chicks each were created from seventy one-day-old broiler chicks [18]. Ten chicks chosen at random for examination were found to be salmonella-free. ten 1-day-old chicks were infected with each strain, whereas ten chicks were used as controls and mock-inoculated. 200 µL of a solution containing around $2 \times 10^8$ colony-forming units (CFU) of one of the following (*S. kentucky, S. infantis, S. virchow, S. typhimurium, and S. enteritidis*) was administered intraperitoneally to each chick separately.200 µL of sterile 0.85% saline solution was used as an intraperitoneal inoculation for the control group. Salmonella isolates were confirmed as invA gene-positive using PCR [19]. Three colonies were inoculated into brain heart infusion broth and incubated for 24 hours. Mortality was recorded every 12 hours for seven consecutive days. Survivor chicks were euthanized, and liver and spleen samples were collected. Lesions were noted during necropsy, and the Pathogenicity Index was calculated. the formula: PI=(TD×5)+(Aerosaculitis (A)+Pericarditis (Pc)+Perihepatitis (Ph)+Peritonitis (Pt)+Omphalitis (O)+Cellulitis (C) [20]. Where TD (Time of Death) values were assigned as follows (Table 1). Re-isolation of *Salmonella* from Liver and Spleen according to ISO 6579–1:2017/Amd1:2020 standards [15].

*Molecular detection of virulence genes (Oliveira et al., [19]*

*DNA extraction*

Polymerase Chain Reaction (PCR) test was carried out for five Salmonella samples in PCR unit in Animal Health Research Institute, AHRI for detection of virulence genes . DNA extraction from

samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 O C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

*Oligonucleotide Primer.*

The primers used were supplied by Metabion (Germany) and are listed in Table (2). The primer design was based on the gene bank ID Numbers: invA gene (M90846.1), spvC gene (FJ460230), and avrA gene (NC_003197.2).

*PCR amplification.*

Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler.

*Analysis of the PCR Products.*

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE (Tris-Borate-EDTA) buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

## Results

This study showed the importance of monitoring of *Salmonella* infections in broiler farms . during the first half of 2024, laboratory diagnosis was conducted on samples collected from 100 broiler poultry farm suspected of salmonellosis . Out of these, 14 farms (14%) tested positive for *Salmonella* Photo 1.

Serotyping of *Salmonella* isolates demonstrated the presence of five distinct serotypes with varying prevalence rates. *Salmonella* Enteritidis was the most frequently identified serotype, accounting for 35% of the isolates. This was followed by *Salmonella* Typhimurium, which represented 28% of the isolates, highlighting its significant contribution to salmonellosis in broiler farms. *Salmonella* Kentucky was also identified, comprising 21% of the isolates, indicating its notable presence among poultry populations. Meanwhile, *Salmonella* Infantis and *Salmonella* Virchow were less prevalent, each accounting for 7% of the total isolates.

Antibiotic susceptibility testing was conducted on 14 Salmonella isolates, showing that different results of sensitivity, intermediated, and resistant across 16 antibiotics belonging to 5 families are cleared in Fig. 1, Table 3. The β-lactam antibiotics, cefotaxime, ceftazidime, and ceftriaxone revealed resistance rates of 35%, 21.4%, and 28.5%, respectively, with sensitivity rates ranging from 57.1% to 64.2%. Ampicillin showed the highest resistance among this group (42.8%), with only 50% sensitivity. Similarly, Fosfomycin exhibited 35% resistance and 57.1% sensitivity.

Colistin sulfate proved to be the most effective antibiotic, with 85.7% of isolates showing sensitivity and only 7% resistance. Conversely, lincomycin had the highest resistance rate (85.7%) with no sensitive isolates detected. Aminoglycosides, including gentamicin, neomycin, and streptomycin, displayed resistance rates between 21.4% and 35%, with sensitivity reaching 50%.

Tetracycline and doxycycline exhibited similar resistance patterns (35%), while their sensitivity rates were 50% and 57.1%, respectively. Chloramphenicol displayed moderate resistance (28.5%) and sensitivity (35%), with 35% of isolates showing an intermediate response. For fluoroquinolones, ciprofloxacin, norfloxacin, and levofloxacin demonstrated resistance rates ranging from 35% to 42.8%, with sensitivity varying between 42.8% and 50%.

In this study, ten chicks were randomly selected from each group and examined for *Salmonella* isolation to ensure they were free from infection prior to the experiment. The pathogenicity index (PI) was determined using standard parameters, including time of death and lesion scoring.The results revealed that four strains (*S. Enteritidis*, *S. Typhimurium*, *S. Virchow*, and *S. Kentucky*) exhibited intermediate pathogenicity, On the other hand, *S. Infantis* exhibited a low pathogenicity index, as shown in Fig 2.

The polymerase chain reaction (PCR) technique, known for its high specificity and sensitivity, was employed to detect three key virulence genes (*inva*, *spvC*, and *avrA*) in five different multidrug-resistant *Salmonella* serotypes. All five strains tested positive for all three virulence genes as shown in Fig 3. with *inva* being crucial for pathogenesis and identification, *avrA* involved in inducing programmed cell death and the inflammatory response, and *spvC* associated with the virulence plasmid, used for detecting strains linked to non-typhoidal bacteremia.

## Discussion

This study found that 14% of broiler poultry farms tested positive for *Salmonella*. Among the isolates, *Salmonella* Typhimurium accounted for

35%, followed by *Salmonella* Enteritidis at 28%. It was also found that *Salmonella* Kentucky made up 21% of the isolates. *S.* Infantis and *S.* Virchow, which made up only 7% of the isolates each, were less common. These findings align with those of [21], which also reported a high prevalence of *S. Enteritidis* and *S. Typhimurium*. In china found that range of salmonella isolation ,in poultry farms, was (12.6 %-45.2%) depending on geographical region [22]. on the other hand the ratio of *salmonella* isolation in northwestern Spanish (1.02%) [23], and EU (1.89%) [24].

These serovars are classified as non-typhoidal *Salmonella* and pose significant risks to poultry production. They not only reduce growth performance, high morbidity, and around 20% mortality in poultry but also cause intestinal, extraintestinal infection and bacteremia in humans [25-29]. The standard biosecurity is very important to decrease spreading of *salmonella* infection horizontally and vertically.

The *Salmonella* serotypes identified in this study are non-typhoidal and recognized as significant foodborne pathogens. The study examined 14 *Salmonella* serotypes against 16 antibiotics from six families: β-lactams, tetracyclines, fluoroquinolones, aminoglycosides, colistin, and fosfomycin.

The increasing antimicrobial resistance (AMR) in *Salmonella* highlights the critical importance of continuous monitoring. This study demonstrated that colistin showed the highest sensitivity, followed by ceftazidime, cefotaxime, and ceftriaxone. In contrast, *Salmonella* exhibited high resistance to lincomycin, followed by ciprofloxacin and ampicillin. This resistance is attributed to the misuse of antibiotics, including their overuse as preventive measures without conducting antimicrobial susceptibility testing (AST). These results are consistent with the findings of [22], who reported an increasing resistance to fluoroquinolones in China, as they are the primary choice for treating *Salmonella* infections.

Cephalosporins are the primary antibiotics used to treat *Salmonella* infections in humans. However, the increasing resistance to cephalosporins, exceeding 20%, raises a significant alarm. In Egypt, regulations prohibit the utilize of third and fourth generation cephalosporins in veterinary pharmaceutical to help control resistance. Notably, most *Salmonella* strains in this study were multidrug-resistant (MDR), which poses a serious concern for AMR, particularly in foodborne pathogens like *Salmonella*.

The study investigated the pathogenicity index and three virulence genes (*invA*, *spvC*, and *avrA*) of five multidrug-resistant *Salmonella* serotypes (*S. Enteritidis, S. Typhimurium, S. Kentucky, S. Infantis,* and *S. Virchow*). All serotypes exhibited intermediate pathogenicity, except for *S. Infantis*, which demonstrated low pathogenicity.

The *invA* and *avrA* genes were consistently present in 100% of the isolates, aligning with previous findings [30]. The *invA* gene, in particular, is a target gene used in PCR for the rapid detection of *Salmonella spp.*.The prevalence of the *avrA* gene has been reported to vary significantly, from 100% to 17% across studies [31]. High prevalence rates of this virulence genes have been considered a critical factor contributing to *Salmonella*-associated pathogenicity [30,32].

In the present study, *spvC* was detected in 100% of the isolates, indicating a high prevalence compared to previous research. For instance, [30,33] reported only 15.1% of isolates positive for *spvC*, with most belonging to the *S. Enteritidis* serotype and originating from eggs or their environment. Other studies also noted that *spvC* presence likely does not influence caecum colonization or liver and spleen invasion. Moreover, spvC is portion of the spv operon preserved in virulence plasmids over several *Salmonella* serotypes that cause systemic infections However, findings from cattle trials [30,34] demonstrated that virulence plasmids, including *spvC*, are not essential for enteric infection or bacterial dissemination but may enhance bacterial persistence in systemic sites. The 100% prevalence of *spvC* in this study contrasts with previous findings, emphasizing its potential significance in the isolates studied and suggesting that its role in pathogenicity might vary depending on the strain or host.

## Conclusion

This study underscored that Salmonella infections are of high prevalence in broiler farms, in particular, *Salmonella* Enteritidis and Typhimurium. It also found that many of these bacteria are resistant to important antibiotics, which is a concern. Ongoing monitoring, antibiotic stewardship, and biosecurity are important to control these infections and reduce the risk of drug-resistant Salmonella.

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*Declaration of Conflict of Interest*

The authors declare that there is no conflict of interest.

*Ethical of approval*

Not applicable.

**TABLE 1. Calculation of time of death according to day of death.**

| Day of death | 1 | 2 | 3 | 4 | 5 | 6 | 7 | s |
|---|---|---|---|---|---|---|---|---|
| Time death (TD) | 1 | .86 | .72 | .58 | .44 | .30 | .16 | 0 |

**TABLE 2. Primers sequences, target genes, amplicon sizes and cycling conditions.**

| Target gene | Primers sequences | Amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | | | Final extension | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | Secondary denaturation | Annealing | Extension | | |
| *invA* | GTGAAATTATCGCCACGTTCGGGCAA<br>TCATCGCACCGTCAAAGGAACC | 284 | 94˚C 5 min. | 94˚C 30 sec. | 55˚C 30 sec. | 72˚C 30 sec. | 72˚C 10 min. | Oliveira *et al*., 2003 |
| *spvC* | ACCAGAGACATTGCCTTCC<br>TTCTGATCGCCGCTATTCG | 467 | 94˚C 5 min. | 94˚C 30 sec. | 58˚C 30 sec. | 72˚C 30 sec. | 35 | Huehn *et al*. 2010 |
| *avrA* | CCTGTATTGTTGAGCGTCTGG<br>AGAAGAGCTTCGTTGAATGTCC | 422 | | | | | | |

**TABLE 3. Antibiotic sensitivity pattern for 14 salmonella isolates across 17 antibiotics.**

| Antibiotic Disc | Resistant no. (%) | Intermediated no. (%) | Sensitivity no. (%) |
|---|---|---|---|
| **Cefotaxime** | 5(35%) | 1 (7%) | 8(57.1%) |
| **Ceftazidime** | 3 (21.4%) | 2 (14.2%) | 9 (64.2%) |
| **Ceftriaxone** | 4 (28.5%) | 2 (14.2%) | 8 (57.1%) |
| **Ampicillin** | 6 (42.8%) | 1 (7%) | 7 (50%) |
| **Fosfomycin** | 5 (35%) | 1 (7%) | 8 (57.1%) |
| **Colistin Sulfate** | 1 (7%) | 1 (7%) | 12 (85.7%) |
| **Gentamicin** | 5 (35%) | 2 (14.2%) | 7 (50%) |
| **Neomycin** | 3 (21.4%) | 4 (28.5%) | 7 (50%) |
| **Streptomycin** | 4 (28.5%) | 3 (21.4%) | 7 (50%) |
| **Tetracycline** | 5 (35%) | 2 (14.2%) | 7 (50%) |
| **Doxycycline** | 5 (35%) | 1 (7%) | 8 (57.1%) |
| **Lincomycin** | 12 (85.7%) | 2 (14.2%) | 0 (0%) |
| **Chloramphenicol** | 4 (28.5%) | 5 (35%) | 5 (35%) |
| **Ciprofloxacin** | 6 (42.8%) | 2 (14.2%) | 6 (42.8%) |
| **Norfloxacin** | 5 (35%) | 3 (21.4%) | 6 (42.8%) |
| **Levofloxacin** | 5 (35%) | 2 (14.2%) | 7 (50%) |

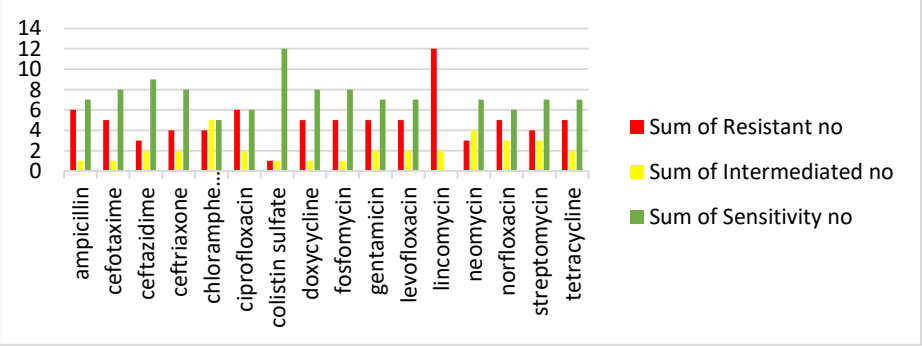


**Fig. 1. Antibiotic sensitivity pattern of 14 salmonella isolates.**


**Fig. 2. Pathogenicity index of five different multidrug-resistant Salmonella serotypes.**

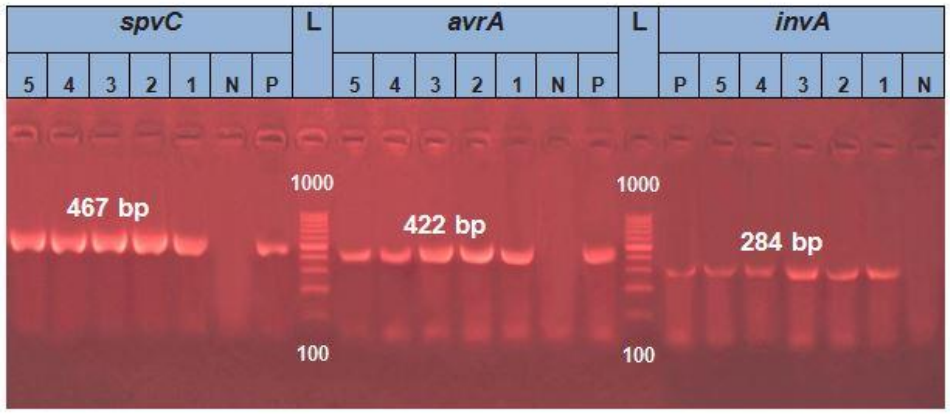


**Fig. 3. Examination of virulence genes (invA, spvC, and avrA) in five Salmonella serotypes.**

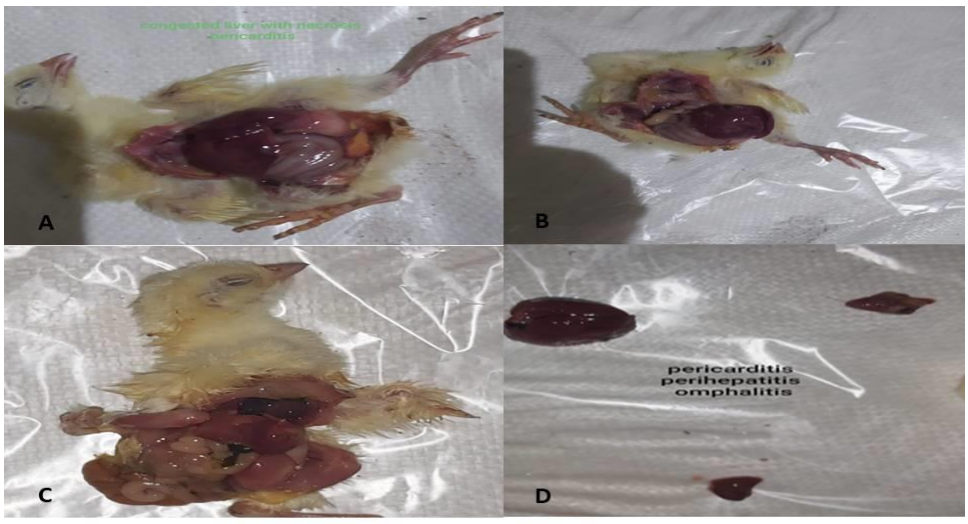


**Photo 1: (A) Congested liver, necrotic liver, and pericarditis observed in broiler chicks infected with Salmonella. "(B) Congested liver, necrotic liver, and pericarditis on day 4 after inoculation with *Salmonella entertidis* . (C) Clogged heart, pericarditis chickens infected with *Salmonella kentucky*. (D) Pericarditis is triggered by Salmonella infection in broiler chicks.**

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## جينات الضراوة وتقييم الإمراضية لـلسالمونيلا المعزولة من دجاج مصاب

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**الملخص**

هذه الدراسة تركز علي حالات عدوي السالمونيلا في مزارع دجاج التسمين . التهديد المتنامي للسالمونيلا ليس فقط لكونها تؤثر علي صناعة الدواجن ولكن للمقاومة المتزايدة للمضادات الحيوية . خلال النصف الاول من عام 2024 تم فحص 100 مزرعة و كانت 14% (100/14) من مزارع دجاج التسمين إيجابية لـلسالمونيلا .تم تحديد خمسة أنماط مصلية، وكان الأكثر انتشارًا *S.* Enteritidis (35%)و*S.* Typhimurium (28%)، *S.* Kentucky (21%)، في حين كانت *S.* Infantisو *S.* Virchowالأقل شيوعًا بنسبة 7% لكل منهما. تم عمل اختبار حساسية لجميع المعزولات 14 للتاكد من درجة الحساسية والمقاومة لعدد 16 مضاد حيوي وكانت النتائج ان الكولستين سلفات اعلي حساسية بنسبة 85.7% وان اللينكومايسن اعلي مقاومة بنسبة 85.7 % . كما ظهر تزايد المقاومة لمضادات *lactams*(الأمبيسلين: 42.8%، السيفترياكسون: 28.5%). تم تقييم مؤشر الإمراضية (*PI*) للأنماط المصلية المختلفة، حيث صُنفت أربعة أنماط (*S.* Enteritidis، *S.* Typhimurium، *S.* Kentucky، و *S.* Virchow) على أنها متوسطة الإمراضية، بينما أظهر *S.* Infantisمؤشر إمراضية منخفضًا. باستخدام تقنية تفاعل البلمرة المتسلسل PCR تم الكشف عن وجود ثلاثة جينات في المعزولات المفحوصة بنسبة 100% . هذه الدراسة تكشف الدور الحاسم لعوامل الضراوة والمقاومة المتعددة للمضادات الحيوية (MDR ) ومؤشر الامراضية (*PI*) في توضيح خطورة عترات السالمونيلا وتؤكد على أهمية تدابير الأمن الحيوي الصارمة، والاستخدام الرشيد للمضادات الحيوية، واستمرار الدراسات للكشف المبكر عن المخاطر في إنتاج الدواجن وحماية الصحة العامة.

**الكلمات الدالة:** السالمونيلا، المقاومة للمضادات الحيوية، اختبار الحساسية، مؤشر الإمراضية، تفاعل البلمرة المتسلسل.