

A Comparative Analysis of the Conventional Versus Molecular Detection of *Salmonella spp.* Infection, with Particular Emphasis on the Histopathologic Changes Observed in Layer Hens

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Abstract

This research was carried out to investigate the presence of *Salmonella* SPP in layer poultry farms through various microbiological tests . A total of 585 samples (from 150 suspected birds) included (liver (150), ceca (50), heart (48), spleen (37), oviduct (150), ovary (150) were obtained from flocks in Diyala. Determination of *salmonella spp* was carried out via PCR technique, biochemical tests, serotyping by O antigen and H antigen, API 20 and Vitek. Liver samples were examined microscopically to detect any changes. The results revealed that the highest number of *Salmonella spp.* isolates was seen in liver (34) (25 *Salmonella ohio*, 4 *Salmonella Kentucky* and 5 *Salmonella enteritidis*). and the rate of infection reach to (22.66%) followed by ovary (15) (10%)(13 *Salmonella ohio* and 2 *Salmonella enteritidis*), oviduct (6) (4%)(5 *Salmonella ohio* and 1 *Salmonella enteritidis*). The histological examination of the liver showed necrosis of hepatocytes, hemorrhage, aggregation of inflammatory cells.

In conclusion, *S. ohio*, *S. enteritidis*, and *S.Kentucky* were the main causative agent for Salmonellosis in laying hens at Diyala governorate .

Keywords: *Salmonella*, histological, chicken, PCR, liver, API 20, Vitek

Introduction

Among many diseases in poultry such as influenza, Gumboro, Marek, Newcastle, E- Coli, the *Salmonella* also consider as main

cause of mortality and egg reduction in layers which lead to economic losses (1). *Salmonella* according to (2), Salmon and Smith were the first to isolate *Salmonella*

from pigs in 1885. A significant genus within the Eenterobacteriaceae family is Salmonella. The genus's Salmonella members live in both humans and animals' digestive tracts and are facultative anaerobes that are Gram-negative (3&4). They can be recovered from a variety of hosts, including humans, pigs, poultry, and food sources. Salmonella genus members can be harmful to wild or Humans with domesticated animals(5). Food-borne outbreaks have been linked to this disease, which is significant to the food sector. Enteric fever, gastroenteritis, and septicemia are among the human pathogenic conditions caused by Salmonella (6).

Salmonellosis in poultry results in significant financial loss because of mortality and low manufacturing (7). Salmonellosis has grown to be a major issue around the world as poultry farming has expanded (8). The fact that the disease's causative agents are passed vertically from parent to child makes it especially important. The natural hosts of *S. pullorum* and *S. gallinarum* are chickens (9). Pullo-rum illness often strikes between two and three weeks of age and

can occasionally affect adults (10). It's common knowledge that fowl typhoid affects adult birds, but reports of significant mortality rates in young chicks also exist . Farms with laying hens of varying ages, housing arrangements, and flock size are risk factors linked to salmonellosis infection in laying hens (11).

It is typically advised to use conventional bacteriological techniques to identify Salmonella spp. from various references (12). Salmonella spp. surveillance has been made possible by the development of molecular tools like PCR (13). The internal and external poisoning of eggs by Salmonella spp. during laying hen rearing is a serious problem. Therefore, it is exceedingly challenging to implement appropriate salmonellosis management methods (14). Egg contamination can happen through either a vertical or horizontal path. Horizontal transmission results from exterior egg shell contamination and may enter when the egg shell cracks, while vertical transmission is caused by bacterial agent colonization in the female reproductive system and oviduct prior to egg creation of shells (15)

Salmonellosis in multilayer chicks as well as hens need to be assessed in order to carry out effi-

Materials and Methods

Geographical distribution of the study regions:

The experiment included the following areas of Diyala Governorate: Al-Hawish, Al-Jadida, Al-Khalis, Al-Muqdadiya, Al-Sindiya, Baqubah, Baladruz, Kan Bani Saad, Kanaan, and Mandali.

Collections of samples

Five hundred and eighty-five samples were collected from laying chicken in Diyala Governorate based on clinical signs, including samples of various organs (liver, oviduct, ovary, ceca, spleen, heart) in order to identify *Salmonella spp*, for the period from October 2023 to April 2024

Bacterial isolation and culturing

- **Pre-enrichment and enrichment**

All specimens were processed in a sterile manner. One gram of each specimen was placed onto (9 ml) non-selective broth (nutrient broth) then underwent for one day/ at degree of 37 to incubation. Following this, one ml of enriched sample was transferred into nine ml of selective media (F Selenite). They

cient disease control strategies (16).

were then subjected to the same incubation time and temperature as before used (17).

- **Culturing**

Salmonella-shigella agar (SS agar), agar of xylose lysine deoxycholate (XLD), and agar of MacConkey had streaked with a loop-full of enriching broth (with selenite F soup). The media left for 24-hour for incubation at 37°C, then examined to detected the growth of salmonella as colonies

Traditional biochemical examinations

Applying Urase test, Oxidase test, Catalase test, Triple sugar iron test (TSI), Simmons citrate test (citrate utilization), Indole test, Ornithine-decarboxylase test, Carbohydrates fermentation test.

API 20E System.

The system of this test composed of twenty-five strips, and each one of them was contained twenty microtubules that formed of (lower tube and upper cupule). Every microtubule was acting as biochemical test and contained substrate of dehydrated material.

The API 20E test was done based on the manufacturer's directions (BioMerieux, France).

Vitek2 System

The Vitek2 system is a fully automatic system, with its identification card was used to recognize bacterial colonies suspected of being Salmonella. Sample was planted on XLD agar, then incubating for one day at 37 C. In later day, and by using of sterile inoculate loop, a clear colony was picked up and mixed into (a tube approved by the company) with normal saline. The Vitek-2 (DensiChek) spectrophotometer was used to measuring the density of the mixture in the tube (the standard turbidity of device is 0.5 McFarland).

Molecular detection

After incubating the colonies for 24 hours, the DNA of bacteria was

Serological tests (Serotyping)

All samples which appeared positive result for salmonella spp. by previous techniques as (biochemical, APi 20 , PCR), were transmitted on TSI media to the laboratories of the central public health/ Ministry of health, Baghdad, Iraq (done Slide agglutination

extracted. The needed primers were prepared depending on instruction of manufacturing. Preparing all requirements of PCR as reaction mixture and amplification conditions. The invA gene was investigated and searched for in all positive isolates of Salmonella bacteria.

Conventional PCR

The conventional (uniplex) PCR process include the following; Reaction mixture (A tube of PCR was a prepared to fill with 20µl of reaction mixture), Amplification condition (The condition of PCR is includes the temperature, time and cycle for each step from denaturation to extension), and the electrophoresis and preparation of gel agarose.

test and serotyping diagnostic technique was made using the Anti-Salmonella test reagents according manufactures of (Sifin diagnostic gmbh, Germany).

Histological examination

The histological procedures were performed according to steps followed by (18).

Specimens preparation : The chicken in this experiment were sacrificed by dislocating of cervical vertebrae. The abdomen of each bird was opened anatomically (19), and the liver was examined in situ. Then the liver removed from the abdomen for preparation of its specimens to histological examination through applying tissue processing routinely. The Hematoxylin and Eosin stain was used to staining the sections of tissues of the liver and examined under light microscope (Olympus) and pictures were taking via a digital camera (20).

Results

As shown in Table (1), which displays the samples and the number of suspected birds from layer chicken in Diyala province decenter. After being grown on chromogenic agar specifically designed for Salmonella, the colonies showed variations in size, rounded shape, and violet color (Figure 1).

The morphological features of the Bacteria:

Isolates were viewed as gram-negative, tiny rod-shaped things that were grouped in lone or cou-

pending on the clinical signs and necropsy findings.

Salmonella spp. isolation and characterization

Morphology of the Colonies:

Different Salmonella species produced different qualities in different media. The broths used for enrichment and pre-enrichment initially had cloudy or opaque. Salmonella colonies on solid medium had a black core and appeared small, flat, globular, and clear on SS agar due to the production of hydrogen sulphide. All of Salmonella's colonies on MacConkey agar plates appear translucent and colorless because the bacteria are unable to digest lactose. The colonies on XLD agar appeared smooth, with a black

pled under a compound light microscope Figure (2).

Biochemical and serological results

Every isolate was catalase positive and oxidase negative. Further, its positive for Simmon's citrate, lysine, and ornithine decarboxylase, but negative for urease and indole Figure (3).

Api 20E results:

This examination revealed the occurrence of a color change in the test strips, which is represented here by the numerical appearance (6120010), which indicates that the isolate is *Salmonella* spp (Figure 4).

Molecular identification (PCR) result

The target band of 284 bp was formed in the current study's invasion gene screening by PCR using primer (*invA*). The PCR result showed a distinct band on all positive samples, indicating the band a coupled with this salmonella gene. Figure (6) *Salmonella* could be found in the liver, oviduct, and ovary in each of the tissues that were analyzed.

Vitek system result

The findings of Vitek system show positive result for salmonella spp. (figure 5). the Identification of gave positive results that showed *Salmonella* partially resistant to Gentamycin, Ciprofloxacin and Levofloxacin, and sensitive to Amikacins and Imipenem for antibiotics used in Vitek.

Serotyping results

The results of serotyping tests that conducted at the Ministry of

Health/Central Public Health Laboratory showed that the diagnosed bacteria were *Salmonella ohio*, *Salmonella kentucky*, and *Salmonella enteritidis*.

Histological Results

Histological findings of the liver of chicken

A. Control chicken

The histological examination of the liver showed that its parenchyma consists of hepatocytes that arranged in cords or chain radiated from the central vein and form liver lobules (Fig. 7). Numerous sinusoids found in between the hepatic cords. Each liver cells were taken polyhedral shape.

B. Salmonella infected chicken

The microscopic observation of liver revealed necrosis of hepatocytes that formed spaces in liver parenchyma associated with presence of hemorrhage in affected area (Fig.8). It was detected aggregation of inflammatory cells in blood vessel lumen. However, some of these inflammatory cells were penetrated the blood vessel wall toward adjacent hepatocytes. The Infiltration of inflammatory cells (mostly mononuclear cells) found near central vein as well as aggre-

gated as cluster to fill the space of necrotic hepatocyte (Fig.9)

Table (1): The regions and samples of suspected layer affected with salmonella spp.

| Regions | No. of samples | Suspected birds |
|--------------|----------------|-----------------|
| Al Huwesh | 50 | 21 |
| Al Jududa | 50 | 24 |
| Al khalis | 50 | 15 |
| Al mugadia | 50 | 10 |
| Al sindia | 50 | 19 |
| Baqubah | 50 | 15 |
| Buldroz | 50 | 13 |
| Kan beni sad | 50 | 14 |
| Kanaan | 50 | 11 |
| Mindlei | 50 | 8 |
| Total | 500 | 150 |

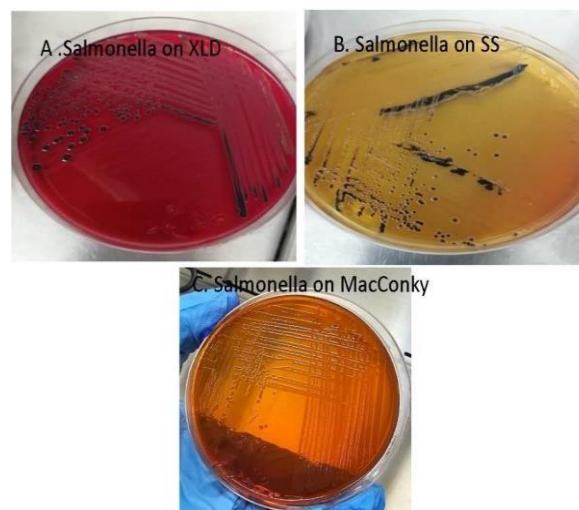


Figure (1) A: Salmonella on XLD agar; **B:** Salmonella on SS agar; **C:** Salmonella on MacConkey agar

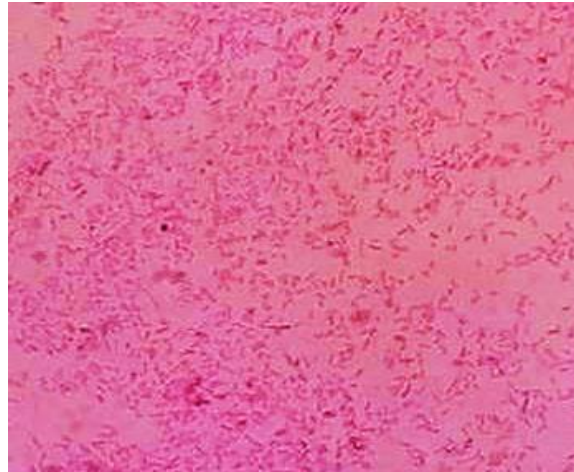


Figure (2): rod shaped Salmonella which appear in pink color.

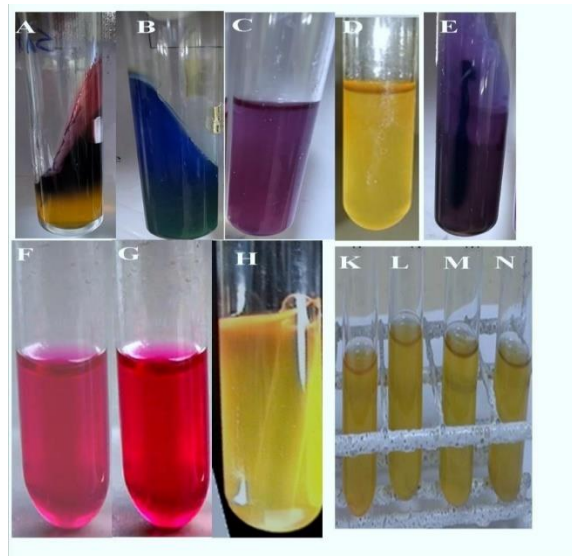


Figure (3) A: TSI ; B: Simmons citrate test ; C:ornithine decarboxylation ; D: indole test ; E: lysine decarboxylation ; F: lactose fermentation; G: sucrose fermentation; H: rhamnose fermentation; K: glucose fermentation; L: maltose fermentation M: mannitol fermentation ; N: dulcitol fermentation



Figure (4): A &B show the Api 20 E Test positive for Salmonella

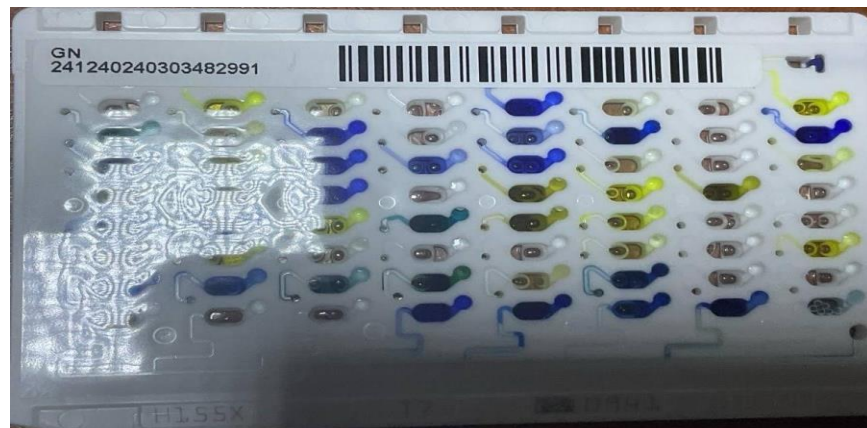


Figure (5): Vitek Test positive for Salmonella

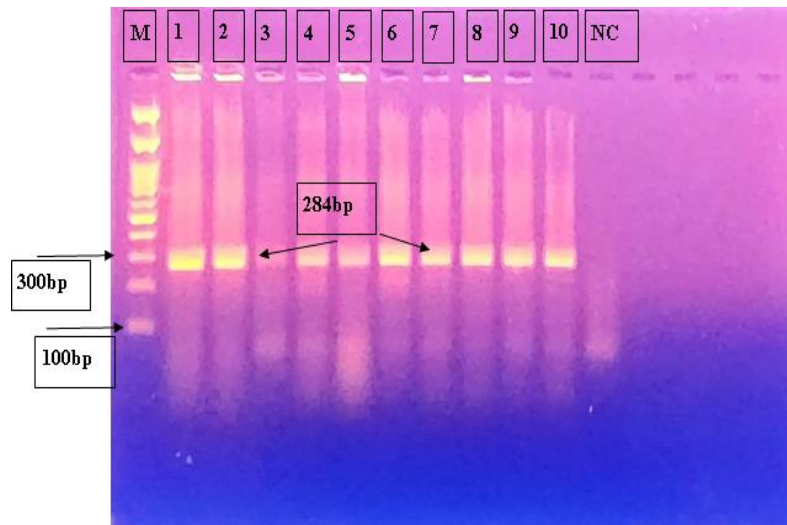


Figure (6): The isolated genomic DNA samples were subjected to gel-electrophoresis using 1% agarose and 3vol/cm in TBA buffer. By using the primer (invA) for PCR screening of the invasion gene, a 284 bp target band was obtained. Samples in Lanes 1 through 11 had positive results (*Salmonella* spp.). PCR markers (1-3 oviducts), (4-6 ovaries), and (7-10 liver) Lane were seen in.

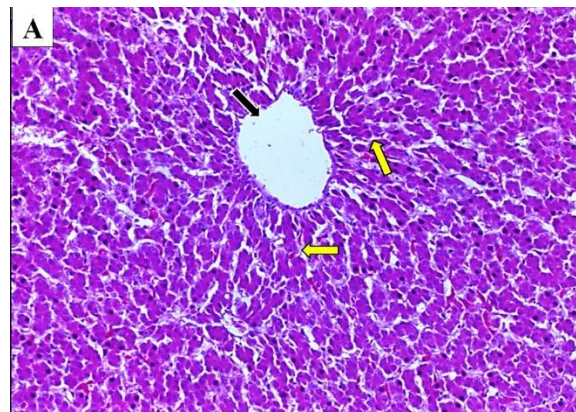


Figure (7) A : Liver photomicrograph in control group chicken showed: Normal hepatic histological architectures. Note central vein (black arrow) and hepatocyte chains (yellow arrow) that surrounded the central vein. (100x) stain H&E.

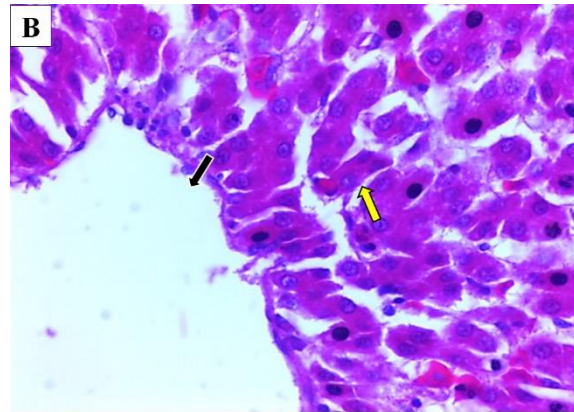


Figure (7) B : Liver photomicrograph in control group chicken showed: Normal hepatic histological architectures. Note central vein (black arrow) and hepatocyte chains (yellow arrow) that surrounded the central vein. (400x) stain H&E.

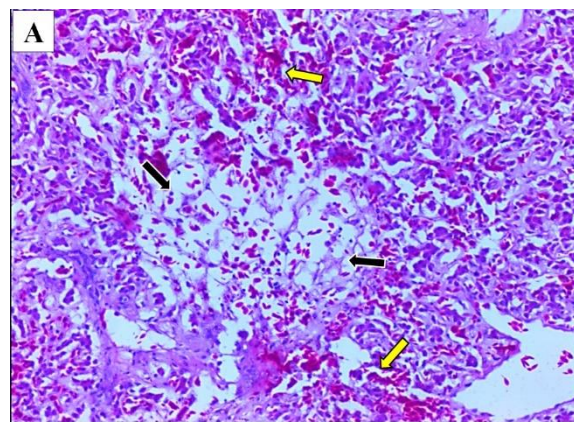


Figure 8: Liver photomicrograph in Salmonella infected chicken showed: Necrosis of hepatocytes that formed spaces (black arrow) in liver parenchyma was observed with presence of hemorrhage in affected area (yellow arrow). (100x), stain H&E.

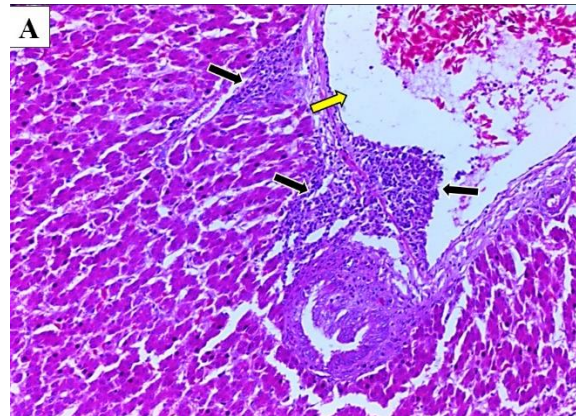


Figure 9: Liver photomicrograph in *Salmonella* infected chicken showed: Aggregation of inflammatory cells (black arrow) in blood vessel lumen (yellow arrow), however some of these inflammatory cells were penetrated the blood vessel wall toward adjacent hepatocytes (black arrow). H&E. A: 100x

Discussion

At the present study, All of *Salmonella*'s colonies on MacConkey agar plates appear translucent and colorless because the bacteria are unable to digest lactose. The colonies on XLD agar appeared smooth, with a black center. After being grown on chromogenic agar specifically designed for *Salmonella*, the colonies showed variations in size, rounded shape, and violet color. These findings corresponding to description of (21 & 22). The result of Biochemical and serological tests that including catalase, oxidase, Simon's citrate, lysine, and ornithine decarboxylase positive, but nega-

tive for urease and indole in current experiment were comparable to that data detected by (23) for salmonella characterization. The positive result of Api 20E test for isolates in the current experiment was support that suggestion of (24) who stated highest value result may associated with using of API 20E . Further, the findings The findings of Vitek system show positive result for salmonella spp. in present study which also showed the *Salmonella* partially resistant to Gentamycin, Ciprofloxacin and Levofloxacin, and sensitive to Amikacins and Imipenem for antibiotics were similar to finding of (25) through using of Vitek

2 system. The PCR result showed a distinct band on all positive samples, indicating the band a coupled with this salmonella gene. Similar finding by (26) was detected in chicken samples when examined by PCR technique which proven to presence of salmonella bacteria. In addition, (27) reported that poultry products as meat showed positive reaction for gene *invA* which refer to presence of salmonella when used of the technique of PCR. As well as, (28) reported in Saudi Arabia, that *invA* gene which amplifies 284 bp fragments was a target to identify all the examined *Salmonella* serovars, while negative results were given in all non-*Salmonella* serovars. These results were in agreement with the results of this study. At previous study on chicken carcasses by (29) which detected 40 *Salmonella* isolates, then all these strains were subjected to *invA* gene and clear the product of 284 bp DNA fragment, and these results were in same to that observed in present study

By using of analysis of PCR, (30) recorded at 270 sample of pork about 37 *Salmonella* isolates which were displayed positive reaction to gene *invA* gene. These findings were corresponding to

these detected in this study. The results of present research were in disagreement with the results that reported by (31) who show 5 isolate out of 8 isolate of salmonella were contain the gene of *invA* in captive wildlife. *Salmonella*'s usual characteristics, which were in line with findings from previous study (32). The conventional approach of detecting *Salmonella gallinarum* was found to have rapid and definite fidelity and discriminatory capacity, as demonstrated by the molecular identification of the isolates using a serotype-specific PCR assay (33). The results of serotyping tests that conducted at the ministry of health/central public health laboratory showed that the diagnosed bacteria were *Salmonella ohio*,

Salmonella kentucky, and *Salmonella enteritidis*. This test confirmed that the bacterial isolate was *Salmonella* genus by causing clear agglutination when adding polyvalent antisera H and O separately. These finding were confirmed what that (34) said about the outer surface of bacteria contain O and H antigen. Depending on the Kauffmann-White scheme, H and O antigens serve as the basis

For immunological identification and classification methods for *Salmonella* (35 & 36). In the present examination, the liver in control chicken showed the normal architectures and arrangement of hepatocytes, cells shape, and the liver lobules. These finding is corresponding with that reported of other authors (37 & 38). The histo-

logical lesions that observed in the liver of chickens infected with salmonella were corresponded with the that mentioned by (39, 40 & 41) in chickens.

In conclusion, *S. ohio*, *S. enteritidis*, and *S. Kentucky* were the main causative agent for Salmonellosis in laying hens at Diyala governorate.

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