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Detection of *Salmonella* Enteritidis (*sef*A) gene, isolated from internal organs of broiler chickens.

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ABSTRACT

Salmonella is one of the genus under the family Enterobacteriaceae and is recorded as an important zoonotic pathogen. Salmonella infection (salmonellosis) is a serious problem for chicken farms in different areas because the broiler represents its important host. *Salmonella* Enteritidis (*S.* Enteritidis) strain affected mainly the internal organs of the chicken, resulting in elevation of the morbidity and mortality rates, consequently a high economic loss in broiler production. The aim of this work was the detection of the *S.* Enteritidis (*sef*A) gene isolated from internal organs of healthy slaughtered broiler chickens. The total samples, 129, were determined as follows: (Liver, kidneys, small intestine, gizzard, and heart blood) are (20, 19, 37, 30, 23) respectively. The samples were collected under microbiological examination. The incidence of Salmonella Species was tabulated as flow: (20%, 10.52%, 37.83%, 6.66%, and 8.69%) from the liver, kidneys, small intestine, gizzard, and heart blood, respectively. Only eight isolates from 24 isolated strains were undergone serological tests, recording one serotype of *S.* Enteritidis. The high prevalence occurred in the small intestine and liver of apparently healthy broilers, and the lower prevalence occurred in the heart blood, kidneys, and gizzard of internal organs of broilers. The data resulted from antimicrobial sensitivity test application, high resistance rate to vancomycin (64.6%), gentamicin (64.6%); tetracycline (92.8%); chloramphenicol (85.7%); ciprofloxacin (35.7%); levofloxacin, (64.28%); amoxicillin + clavulanic, (100%); streptomycin, (92.85); trimethoprim + sulfamethoxaole (85.7%). While the sensitivity to florfenicol was 100%. Confirmatory PCR technique for detection of (*sef*A) gene amplification at (310 bp) in 8 isolated strains.

1. INTRODUCTION

Salmonella spp. is a Gram-negative rod-shaped, facultative anaerobe, non-spore-forming, usually motile by flagella. Salmonella spp. is a heterogeneous bacterium and is one of the most common infectious agents in the tropics, especially in areas of low hygienic measures (Bell et al., 2016). The infection with Salmonella spp. is known as salmonellosis. The source of infection is the contaminated drinking water and food by feces or urine of infected humans and animals; besides the infected fish, flies, and dust, which act as intermediaries for Salmonellosis. *Salmonella* Enteritidis (*S.* Enteritidis) is one of the most important chicken farms pathogenic agents and member of foodborne diseases (Li et al., 2016). The Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) reported that 75% of human cases of Salmonellosis annually result from eating contaminated poultry and poultry byproducts. In conclusion, poultry and poultry byproducts are considered the main sources of Salmonellosis (Loharikar et al., 2013). The ingested Pathogenic microorganism (salmonella) with the contaminated food can overcome the gastric acidity barrier and then attack the mucosal membranes of the small and large intestine, associated with the production of toxins. The inflammatory reaction resulted from the entrance of the pathogenic microorganism to the epithelial lining, accompanied by the elimination of pro-inflammatory cytokines (Zha et al., 2019). The determination of Salmonella infection in the laboratory by the ordinary traditional methods of bacterial isolation, identification, and confirmation by (PCR) (Hendriksen, 2003). The PCR technique is of high accuracy, mainly in isolates that haven't O-antigen, which is known as rough isolates (Roy et al., 2002). Many trials were carried out to reduce the dissemination of Salmonella infection in the poultry industry as an application of good hygienic and sanitation programs in the poultry farm; the use of suitable and specific antibiotics, which was determined according to antibiotic sensitivity agar diffusion test, also the antibiotic drug of choice must be of corresponding concentration and course time of treatment (Raji et al., 2021). The current work was aimed at the determination of the *S.* Enteritidis *sef*A gene isolated from the internal organs of healthy slaughtered broiler chickens. The *sef*A gene is the only specific gene for indicating and serotyping of *S.* Enteritidis (Borges et al., 2013). Therefore, *sef*A gene is reported as a target, marker, and restrict gene for *S.* Enteritidis serovars (O'Regan et al., 2008; Amini et al., 2010, and Borges et al., 2013).

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2. MATERIAL AND METHODS

2.1. Sampling:
The samples were collected from Giza province- Egypt from healthy slaughtered broiler chicken aged (35-40) days. Samples were 129 internal organs from broiler, liver n= (20), kidneys n= (19), small intestine n= (37), gizzard n= (30), heart blood n= (23). Samples were collected under aseptic condition on nutrient broth then sent to the laboratory in ice bags.

2.2. Laboratory detection of Salmonella spp:
Collected samples on the Nutrient broth were put in Rapaport vassiliadis soya (RVS) broth (oxoid, UK) incubated at 37 ºC/24h then cultured on the Salmonella shigella agar (SSA) (oxoid, UK), Salmonella Chromogenic (S.C.) agar (Oxoid, UK) and XLD agar (xylose lysine deoxycholate agar) (oxoid, UK) at 37ºC/24h according to Clinical Laboratory standard institute (CLSI2018). Then the suspected colonies undergo gram stain (Krieg et al. 1984).

2.3. Biochemical identification:
The isolated suspected strains of Salmonella were subjected to biochemical tests as (urea hydrolysis), triple sugar iron agar (TSI), (lactose fermentation), (catalase), and (oxidase) tests (Barrow and Feltham, 1993).

2.4. Sero-diagnosis of the suspected colonies:
The suspected colonies were previously determined using biochemical tests as Salmonella, were sent to the animal health institute at the serological unit for serological identification, according to Kauffmann-White scheme (2003).

2.5. Antibiotic sensitivity test:
The antimicrobial sensitivity test doing by using Mueller Hinton agar (oxoid) according to CLSI (2018) with diffusion discs of antibiotics of the florphenicol (flc 25µg), ciprofloxacin (cip 5µg), enrofloxacin (NOR 10µg), levofloxacin (LE 5µg), amoxicillin + clavulanic (Amc 20/10µg), Vancomycin (VA 30µg), gentamicin (Gen 10µg), streptomycin (S 10µg), tetracycline (TE 30µg) and trimethoprim-sulfamrthoxazole (Sxt 1/19 µg) according to Panzenhagen et al., (2016).

2.6. Detection of sefA gene by PCR technique:
The Mini Kit Catalogue no.51304 of QIAamp DNA which is already manufactured provides silica-membrane-based nucleic acid purification for all types of tested samples. GT PCR master mix (2x premix) in addition to PCR grade water. For detection of sefA gene, the sefA primer sequencing F / GCAGCGGTTACTATTGCAGC and R /TGTGACAGGGACATTTAGCG was used. The amplification of this gene at (310 BP) according to Akbarmehr et al., (2010). The total reaction volume 25 µl that consist of PCR master mix (2x premix) 12.5µl, PCR grade water 5.5 µl., Forward primer (20 pmol) 1 µl, Reverse primer (20 pmol) 1µl, and Template DNA 5µl. The used PCR program was as follow: Primary denaturation at 94˚C for 5 minutes., Secondary denaturation at 94˚C for 30 seconds, annealing at 52˚C for 30 sec, Extension 72 ºC for 30sec., Number of cycles were 35 and Final extension 72 ºC 7 min. according to Sambrook et al., (1989) for electrophoresis 20 µl of PCR product for every sample, in addition to the negative and positive control. The ladder or marker of graduation 100, 200, 300...etc. The volt power was 1-5 volts/cm the length of tank. The voltage run was stopped after about 30 minutes then transferring the gel to UV cabinet. The gel was photographed using the gel documentation system. The analysis of data was carried out by computer software.

3. RESULTS

The collected samples on the Nutrient broth (oxoid) were inoculated in RVS and incubated at 37ºC/24hours till appearance of turbidity, then it was cultured on the solid media at 37 ºC/24hrs. the colonies of salmonella appeared of smooth surfaces with opaque shadow and colorless on SS agar, while Salmonella strains that produce H_2S which causing the black-center of the isolated colonies these colonies are of pink to rose-red color, due to Lactose-fermentation that usually associated with a precipitate. The growing colonies on SC were of blue green to blue in color, plus black center. Therefore, the majority of colonies appeared either of large glossy black centers or completely black in color. On the other hand, the negative H_2S cultured colonies were of blue green to blue in color, with the absence of black centers. The colonies on XLD agar appeared with black centers. The red colored of translucent zone as a result of the variation in the indicator color. Then the suspected colonies undergo gram stain which give negative bacilli to gram stain.

Biochemical tests
TSI test giving AKL / ACID with H_2S, Glucose, Mannitol L-arabinose Sorbitol giving positive for Sugar fermented, Oxidase Test negative, Indole test negative, Ureases test Negative, Simmon Citrate Slant Test Negative and Salmonella positive for H_2S production.
From the results in table (1) Salmonella were isolated from (liver, kidneys, small intestine, gizzard and heart blood) in a rate of (20%, 10.52%, 37.83%, 6.66% and 8.69%) respectively.

Table 1 Prevalence of *Salmonella* in collected samples from broilers internal organs.

Type of sample	Positive sample no.	Results
Liver	4	%20
Kidneys	2	%10.52
small intestine	14	%37.83
Gizzard	2	%6.66
Heart blood	2	%8.69
Total	24	% 18.60

The molecular Characters of *S.* Enteritidis strains (8 isolates) were tested using the PCR technique, in order to the detection of *sef*A gene, and were recorded as all isolates have the *sef*A gene. The amplification of the marker gene for *S.* Enteritidis at 310bp figure (1).

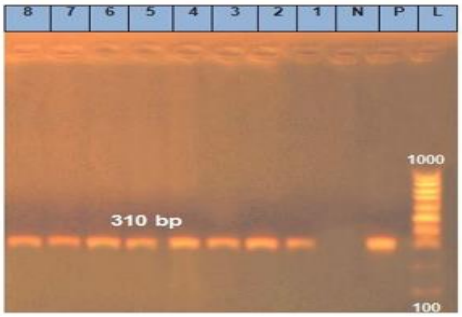

Figure 1 Agarose electrophoresis sefA gene of S. Enteritidis at 310 bp, L (Ladder) or marker 100- 1000. P (positive control) and N (negative control). Samples 1-8.

The data resulted from antimicrobial sensitivity test application; high resistant rate to (vancomycin 64.6%; gentamicin 64.6%; tetracycline 92.8%; chloramphenicol, 85.7%; ciprofloxacin 35.7%; levofloxacin, 64.28%; amoxicillin+clavulanic, 100%; streptomycin, 92.85; trimethoprim + sulfamethoxaole 85.7%). while the sensitivity to florfenicol was 100%.

4. DISCUSSION

The incidence rate of salmonellosis in broiler chicken farms differed among different localities of countries. In India, *S.* Typhimurium and *S.* Gallinarum with *S.* Enteritidis were reported as the major common strains of salmonellae. High-rate recording about 96.2% of isolates (Kumar et al., 2019). India Salmonella incidence rate was Partially similar in Egyptian poultry farms; *S.* Enteritidis with *S.* Typhimurium were more prevalent strains that were recognized from broilers and retail shops (Elkenany Elsayed et al., 2019). The main route of infection dissemination is the adult carrier chickens without any clinical signs of salmonellosis; the distribution of salmonellosis was carried out by horizontal and vertical transmission. Consequently, the microbe persists in the apparently healthy chickens, as the pathogenic effect was poor in infected chickens. The colonization of *S.* Enteritidis in small and large intestines of broiler chickens, in contrast, may be causing human gastroenteritis. Which has become one of the most important recorded sources of infection, leading to foodborne disease outbreaks (Paiva et al., 2011). The rate of *S.* Enteritidis isolation was (18.60%) from the total collected samples (24/129), The rate of isolation accepted with that calculated by (Elkenany et al., 2019) (11.4 %) and (Wang et al., 2020) (16.6%). On the over mined, the high percentage of *S.* Enteritidis isolation was determined by (Tan et al., 2022) and (Shen et al., 2023), which was (39.7%) and (43.52%) respectively. The prevalence of salmonellosis in liver was (20%). Which is nearly approaching those of (Oscar, 2021) and (Abd El-Mohsen et al., 2022) were (15%) and (13.33%) respectively. In contrast, the highest rate was recorded (59.4%) by Jung et al. (2019). while this result was more than that of (El-Morsi, 1998) and (Abdelaziz et al., 2020), where they reported (2.66%) and (7.4%) respectively. The rate of *S.* Enteritidis isolated from the small intestine was (37.83%) which disagrees with that of Abd Elkader et al. (2021) and (Raji et al., 2021), reporting (1.66%) and (6.6%) respectively. while Temelli et al. (2010) recorded 50% of the layer chicken intestine culture were positive to *S.* Enteritidis. On the other hand, the rate of *S.* Enteritidis isolation from the kidney was 10.52%, less than that recorded by Ramya et al., (2012) (30%) while it was higher than obtained by Abdelaziz et al. (2020) reported (1.1%). In the case of the heart blood was (8.7%), which agrees with that reported by Abdelaziz et al. (2020) of (9.6%). Finally, the incidence rate detected in the gizzard was (6.7%) in this study; that parallel with that of Abdel-Aziz (2016) was (6.6%) which was higher than that recorded by (Raji et al., 2020) (2%). Generally, the frequency of *S.* Enteritidis serovar isolation was varied in different localities due to variations in the scales of management and hygienic measures in addition to the environmental and individual differences (Kim et al., 1991). The isolated strain of *S.* Enteritidis showed a high resistance rate against ciprofloxacin (cip five µg), enrofloxacin (NOR 10µg), levofloxacin (LE 5µg), amoxicillin+clavulanic (Amc 20/10µg), vancomycin (VA 30µg), gentamicin (Gen 10µg), streptomycin (S 10µg), tetracycline (TE 30µg) and trimethoprim-sulfamethoxazole (Sxt 1/19 µg). The high resistance rate to vancomycin (64.6%), gentamycin (64.6%), tetracycline (92.8%); chloramphenicol (85.7%); ciprofloxacin (35.7%); levofloxacin, (64.28%); amoxicillin+clavulanic, (100%); streptomycin, (92.85); trimethoprim+sulfamethoxaole (85.7%). While the sensitivity to florfenicol was (100%). The resistant rate in the case of the strains isolated from broiler Chickens or poultry products in the US was detected as (85%) and (35%) against ampicillin and tetracycline, respectively (Gad et al., 2018). On the other hand, in Malaysia, (89.5%) and (85.1%) are against ampicillin and tetracycline, respectively (Chuah et al., 2018). But in Egypt (86.7%) and (40%) for ampicillin and tetracycline, respectively (Moawad et al., 2017). While the isolated *S.* Enteritidis appears highly sensitive against florfenicol (FLC 25µg). From the above statement, the isolated *S.*Enteritidis serovar is mainly multidrug resistance microbes (MDR). MDR phenomena lead to limitations in the choices of antibiotic drugs for the treatment of *S.* Enteritidis infection in chicken farms. In this study, the isolated strains of Salmonella spp. achieved *S.* Enteritidis from internal organs of broiler chickens at a percentage of (18.60%). The high rate of *S.* Enteritidis was tabulated through the butchering of broiler chickens because of the carriage of healthy-looking chickens, representing a high percent of *S.* Enteritidis able to contaminate the food processing stages, indicating a public health threat. In addition, the treatment regime of *S.* Enteritidis infected broiler farms, using improper antibiotic drugs by uncorrected protocol, resulting in antimicrobial resistant strains, reliable the dangerous factors in the treatment of poultry diseases, representing a danger threat to public health. Serological identification of the Salmonella isolated strains depended mainly on the Somatic (O) or flagellar (H) antigens with polyvalent and monovalent Salmonella antisera. That reported eight isolates of *sefA* gene, which represented fingerprint and marker specific for *S.* Enteritidis (Hendriksen, 2003).

5. CONCLUSIONS

Gene determination of the *S.* Enteritidis *sef*A, as a fingerprint and the guide for serological identification and serotyping of *S.* Enteritidis. Most of the internal organs show a higher incidence of *S.* Enteritidis in the liver and small intestine, other than the kidney, gizzard, and heart blood, respectively. Salmonella isolates were highly sensitivity to florfenicol, which was used in the proper regime of treatment.

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