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Salmonella Species Threats in Duck Meat in Egypt: Prevalence and Correlation Between Antimicrobial Resistance and Biofilm Production

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SALMONELLA is a foodborne, Gram-negative bacterium able to stand the absence of oxygen and cause enteric disease in animals and humans. In addition, it is the main causative agent of most gastrointestinal diseases all over the world. In this study, we aimed to investigate the prevalence of *Salmonella spp* among duck meat isolated from retail shops in Mansoura city in Egypt in addition to evaluating their antimicrobial sensitivity. A total of 170 duck meat samples were sampled in different areas through Mansoura city in Egypt to identify *Salmonella spp* by standard isolation method. PCR gene amplification for the *inva* targeting gene was used to confirm suspected isolates. All the confirmed isolates were assessed for their antimicrobial resistance using a traditional disc diffusion test. Results confirmed ten isolates encoding the *inva* gene. The ten confirmed *Salmonella spp* confer 90% against the B-lactam and lincosamide family (amoxicillin and clindamycin); 40% resistance to aminoglycoside (gentamicin) and 30% was exhibited against fluoroquinolone, tetracycline, and sulphonamide (ciprofloxacin, tetracycline, and trimethoprim-sulfamethoxazole). Meanwhile, chloramphenicol was the lowest represented in our study only one strain was found resistant against it. Biofilm-forming *Salmonella* strains were characterized in more than half of our isolates. In conclusion, the emergence of multidrug-resistant *Salmonella* strains in addition to their ability to produce biofilm require urgent monitoring measures and new eradication techniques; Furthermore, it confirms the ability of duck to act as a vehicle for *Salmonella* strains in human besides the hazard of acquiring antimicrobial resistance via food.

Keywords: *Salmonella*, Duck, Multidrug, Resistance, Biofilm.

Introduction

Salmonellosis, a frequently recovered foodborne disease, is accounted to be one of the most significant pathogens causing public health hazards in most countries [1]. The world health organization (WHO) surveillance program for control of foodborne infection and intoxication has noticed the rise in the occurrence of salmonellosis [2] and reported that it is the causative agent for most outbreaks and human cases in the US [3].

Salmonella spp is considered a major public health hazard that causes economic losses widely [4]. *Salmonella spp* is characterized by causing gastroenteritis, bacteremia, and enteric fever [5]; the severity of *Salmonella* infections in humans varies depending on the type of serotype involved and the health status of the human host. The immunocompromised, elderly, and infants are more supposed to *Salmonella* infection.

Poultry and dairy food products are considered the most common source of *Salmonella* pathogens

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[6]. *Salmonella* was first known by Theobald Smith in 1855 from the infected pig intestines [**7**]. Thus, the intestinal tract of animals such as swine, poultry, and cattle are considered reservoirs for *Salmonella* infections; they are involved in the transfer of the pathogen especially through contamination of uncooked animal-derived food products. Contamination by *Salmonella* can occur due to unhygienic techniques during the slaughtering or during the cooking process [8-10]. Poultry is the main reservoir of salmonellosis dominating other food animals and the main responsible for 23% of human cases 17% of these cases were caused due to chicken meat consumption [11]. During the poultry production cycle, the possibility of *Salmonella* contamination can occur at several points at the abattoirs or the poultry processing equipment.

The antibiotic-resistant *Salmonella* strains have emerged which led to public health difficulties [12]. The first isolated resistant *Salmonella* strain was against the antimicrobial agent chloramphenicol [13]. Since then, the incidence of resistant *Salmonella* strains was extensively reported worldwide [14]. The first line of antibiotics used for salmonellosis treatment, ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole, *Salmonella* spp was found to gain resistance against them. Recently, scientists have been using fluoroquinolones and extended-spectrum cephalosporins as substitutes for first-line antibiotics, and resistant strains have emerged [15]. Moreover, the incidence of MDR *Salmonella* strains has been exaggerated [16]. For years, reports illustrated that the increased rate of MDR strains may be attributed to usage of the antibiotics as growth promoters in animal diets or the inappropriate usage of antibiotics in treatment through veterinary practice [17].

Microbial society is divided into pathogens, which are capable to produce biofilm, and others that stay in their planktonic form [18]. Bacteria produce biofilm in response to stress conditions to enable them to survive urgent unfavored conditions ([19]. Biofilm production helps bacteria escape host defense mechanisms, and antimicrobial agents [20,21]. Previous studies have compared the survival rate of the biofilm-producer bacteria and its planktonic form, the data revealed that the biofilm-producer bacteria can resist antibiotics from 10 to 1000 times more than its planktonic form [22-24]. Extensive studies were performed to discuss the role of biofilm formation in *Salmonella* during the infection and transmission cycle [25-27]. Therefore, evaluating the role of biofilm production in resisting antimicrobial resistance should be assessed.

Studies were conducted to evaluate the emergence of *Salmonella* strains in Egypt [28, 29]. The necessity of continuous assessment of the frequency of *Salmonella* and their multidrug resistance is needed in addition to biofilm production. Therefore, we aim to evaluate the occurrence of *Salmonella spp* in raw duck meat in duck meat samples recovered from retail markets in Mansoura city, Egypt. Moreover, the determination of their antimicrobial resistance and biofilm production and analysis of the correlation between the ability of *Salmonella* strain biofilm production and antimicrobial resistance to assist the risk of infection.

Material and Methods

Ethical statement

Our study was approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt under Protocol code number: M/67.

Sampling

The period between November and January 2020, 170 samples were collected from duck meat and were gathered in random manner from most of retail shops represented in supermarkets, and street markets in Mansoura city, Egypt. All the samples were allocated and stored in sterile one-time used containers and shipped in an icebox to the Laboratory of Bacteriology, Mycology, and Immunology Department at the Faculty of Veterinary Medicine, Mansoura University for examination within 6 hours.

Isolation and identification of Salmonella spp

Isolation of Salmonella spp

The standard method ISO6579:2002 (International Organization for Standardization, 2002) was followed in culturing the duck meat samples to isolate *Salmonella* isolates. Pre-Enrichment of samples was performed by roughly mixing 25 grams of duck meat with 225ml buffered peptone water (BPW; Oxoid, UK) and was incubated at 37°C for 18-24 hr. Then, 1 ml of the pre-enriched 24hrs BPW broth was added to 9 ml Rappaport Vassiliadis broth (RV; Oxoid, UK) at 42°C for 18 h for a second enrichment. The selective Xylose Lysine Deoxycholate agar (XLD; Oxoid, UK) was used as a selective media for *Salmonella spp*. Briefly, loopful from the 18-

hr enriched RV broth was streaked on XLD and incubated at 37 °C for 24 hr. All the suspected *Salmonella* isolates were further tested for their biochemical activity and all the biochemically confirmed *Salmonella* isolates were stored at 80°C at 70% glycerol for further diagnosis.

Identification of Salmonella spp strains

Molecular conformation of Salmonella spp

DNA sample extraction

All the biochemically confirmed to be *Salmonella* isolates were subjected to extraction of DNA samples via the boiling technique [30]. The suspected isolates were cultured overnight and single to two colonies were selected and mixed in Eppendorf contain 100 ul deionized free water. The Eppendorf was boiled in water bath for 10 minutes. All heated samples were centrifuged at maximum for 3 to 5 minutes and were followed by transferring all the supernatant in a sterile Eppendorf tube. All the suspected DNA samples were stored at -20 °C for molecular characterization.

Molecular characterization of Salmonella spp strains using PCR

The suspected isolates were screened for the *Salmonella* species-specific gene *inv*A via polymerase chain reaction (PCR), using the primer sequence F: *GTGAAATTATCGCCACGTTCGGGCAA* and R: *TCATCGCACCGTCAAAGGAACCC* which amplified at 284 bp as described earlier [31]. The cycling conditions began with initial amplification for 2 min at 95°C then 35 cycles of amplification for 1 min at 95°C, annealing at 62°C for 30 sec, and extension for 30 sec at 72°C; and as a final step, a cycle of 10 min at 72°C was applied. The PCR products were visualized by gel electrophoresis on 1% agarose gels stained by ethidium bromide and gel documentation. Positive control was obtained from previous studies [28, 29].

Antimicrobial susceptibility testing for Salmonella strains

The Kirby–Bauer disc diffusion method was used to assess the antimicrobial susceptibility of *Salmonella* strains on Mueller–Hinton agar plates (MHA; Oxoid, UK) after 24 h of incubation at 37°C, as discussed before by the Clinical and Laboratory Standard Institute [32] recommendations. Seven antibiotics belonging to seven antimicrobial classes were used: amoxicillin (AM, 10 µg), ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), and clindamycin (DA, 2 µg). The antimicrobial susceptibility results were interpreted as mentioned before by CLSI [32]. Resistance against three or more antimicrobial classes was recognized as multidrug resistance (MDR) [33]. The multiple antibiotic resistance (MAR) index was calculated by dividing the total number of antimicrobial resistances for each isolate by the total number of antimicrobials tested [34].

Biofilm production assessment

Salmonella strains were evaluated against biofilm production by the crystal violet glass tube method [35]. An overnight culture for 10 hr at 28°C of *Salmonella* strains in sterile glass tubes containing Tryptone Soya Broth (TSB; Oxoid, UK) was performed followed by discarding all the supernatant in a hygienic manner. Then, the glass tubes were stained in a standing position with 1% crystal violet stain for 15 min. After that, all the stain was discarded in a hygienic manner and washed using distilled water two or three times. An uncultured TSB tube was used as a negative control. The trial was held in triplicate. All data were interrupted in four results according to the density of the visible film underlying the tubes stained (strong positive, positive, weak positive, and negative).

Statistical methods

A correlation analysis was done to determine the relationship between phenotypic antimicrobial resistance agents and biofilm production of *Salmonella strains* in our study as discussed before [36, 37]. The results were interrupted according to Jiang *et al*. [38].

Results

The total viable bacterial count in poultry meat and egg samples ranged between $4.25x10^6$ and $1.165x10^8$ CFU/ml (Table 1).

Prevalence of Salmonella spp isolates in duck meat.

Our study examined an estimated number of 170 sampled duck meat, only nineteen samples were tested and characterized by red colonies with a black center on XLD agar, and the further examination by biochemical methods excluded them to eleven isolates. Meanwhile, using PCR gene amplification of the *inv*A gene, only 10 isolates (5.9% of the total samples) encoded the *inv*A gene and were confirmed to be *Salmonella* strains (Figures 1, 2).

TABLE 1. Viable plate count and arithmetic mean of Salmonella spp from different samples.

Sample	Cfu/ml	Log value
I	$1.51x10^{7}$	7.17
II	$5.8x10^{6}$	6.16
III	$7.4x10^{7}$	7.86
IV	$6.25x10^{6}$	6.76
V	$2.325x10^{7}$	7.366
VI	$1.24x10^{7}$	7.093
VII	$1.165x1^{0}8$	8.066
VIII	$1.7x10^{7}$	7.230
IX	$6x10^{6}$	6.77
X	$4.25x10^{6}$	6.62

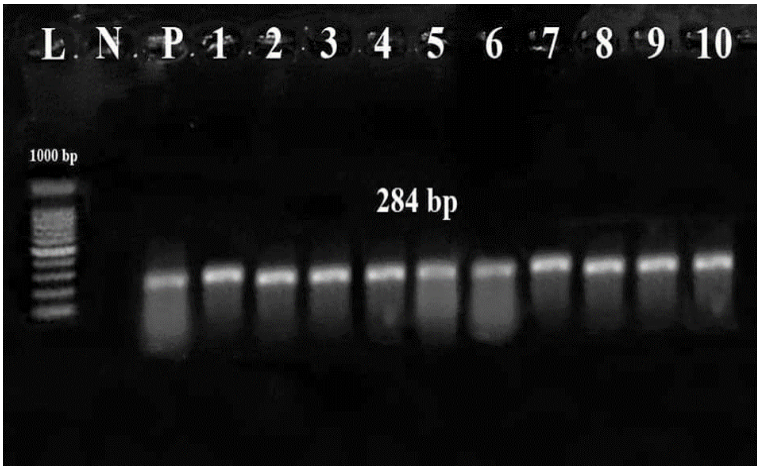

Fig. 1. Illustration of amplification of the *inv*A gene at 284 bp in *Salmonella* strains isolated from duck meat. L: ladder 100bp;Lane1; negative control (water sample) at Lane 2; positive control at Lane 3 ; *Salmonella* isolates from Lane 4 to 13.

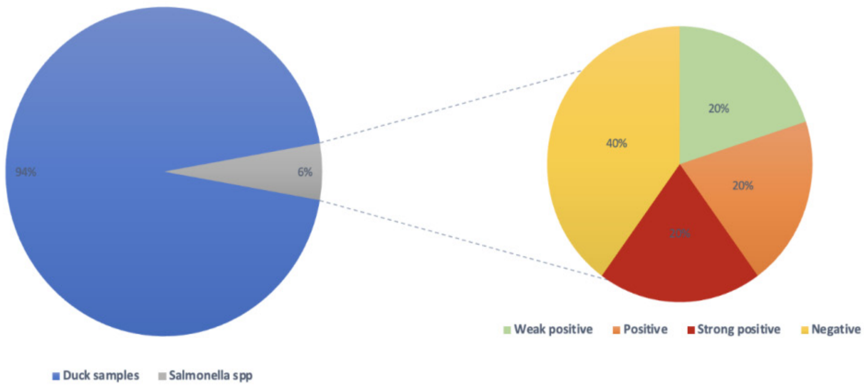

Fig. 2. Prevalence and biofilm production of *Salmonella spp strains* in duck samples using PCR assay and Crystal violet glass tube test, respectively.

Antimicrobial susceptibility testing of Salmonella strains in raw duck meat

Phenotypic antimicrobial susceptibility of *Salmonella* strains recovered from duck meat against 7 antimicrobial agents belonging to seven antimicrobial classes was investigated against the ten identified *Salmonella* isolates. The highest antimicrobial resistance was observed against amoxicillin and clindamycin (90% for each), followed by gentamicin (40%), and ciprofloxacin, tetracycline, and trimethoprim-sulfamethoxazole (30% for each). The lowest frequency of antimicrobial resistance was observed against chloramphenicol (10%) (Table 2). Six *Salmonella* strains (60%) showed MDR against three or more antibiotic classes, while the other strains were sensitive against the tested antibiotics. The most prevalent antimicrobial-resistant pattern was AM, CIP, CN, SXT, DA which was observed in two strains. The MAR index ranged between 0.14 to 0.7 (Tables 3).

Biofilm production

In our study, the biofilm-producer *Salmonella* strains exhibited a higher percentage than the non-biofilm-producer strains. A total of 6 isolates (60%) were positive for biofilm production ranging from strong positive, positive, and weakly positive in 20% (2/10) or each. Four strains (40%) were found negative for biofilm production using crystal violet tube test.

TABLE 2. Percentage of antimicrobial susceptibility for *Salmonella* isolates (n=10).

Antimicrobial agent	Family	Disc code	CPD	Salmonellae					
				Resistance		Intermediate		Sensitive	
				No	%	No	%	No	%
Amoxicillin	β-lactam	AM	10µg	9	90	1	10	-	
Ciprofloxacin	Fluoroquinolone	CIP	5µg	3	30	3	30	4	40
Gentamicin	Aminoglycoside	CN	10µg	4	40	3	30	3	30
Tetracycline	Tetracycline	TE	30µg	3	30	4	40	3	30
Chloramphenicol	Inhibit protein synthesis	C	30µg	1	10	2	20	7	70
Trimethoprim-sulfamethoxazole	Sulphonamide	SXT	25µg	3	30	3	30	4	40
Clindamycin	Lincosamide	DA	2µg	9	90	-	-	1	10

TABLE 3. Antibiogram and multiple antimicrobial resistance (MAR) for *Salmonella spp*.

Antibiotypes	Resistance pattern	MDR	MAR Index	Isolates No (%)
I	AM	1/7	0.14	1
II	DA	1/7	0.14	1
III	AM-DA	2/7	0.29	2
IV	AM, TE, SXT, DA	4/7	0.57	1
V	AM, CN, TE, DA	4/7	0.57	1
VI	AM, CN, C, DA	4/7	0.57	1
VII	AM, CIP, TE, DA	4/7	0.57	1
VIII	AM, CIP, CN, SXT, DA	5/7	0.7	2

AM, Amoxicillin; DA, Clindamycin; TE, Tetracycline; SXT, Trimethoprim-sulfamethoxazole; CN, Gentamicin; C, Chloramphenicol; CIP, Ciprofloxacin.

Analysis of the correlation of antimicrobial resistance phenotypes and biofilm production in Salmonella strains in duck meat

In our study, we analysed the correlation between antimicrobial resistance phenotypes and biofilm production for *Salmonella* strains. Interrupted results had shown a moderate positive significant correlation between biofilm production and the antimicrobial resistance phenotype CIP, SXT DA, and AM (r= 0.53, 0.53, 0.408, and 0.408, respectively). The other resistance phenotypes expressed weak significance (r) ranged from 0.27 to 0.08. While the antimicrobial resistance phenotypes belonging to different antimicrobial classes showed moderate positive significance in CIP and SXT (r=0.52) and CN and C (r=0.408). Non-significant relation was represented between AM and DA, CIP and TE, CIP and C, CN and TE, TE and C, and C and SXT which showed r< 0.05. Moreover, all the antimicrobial resistance phenotypes showing r from o.35 to 0.089 were considered weak positive significant (Figure 4).

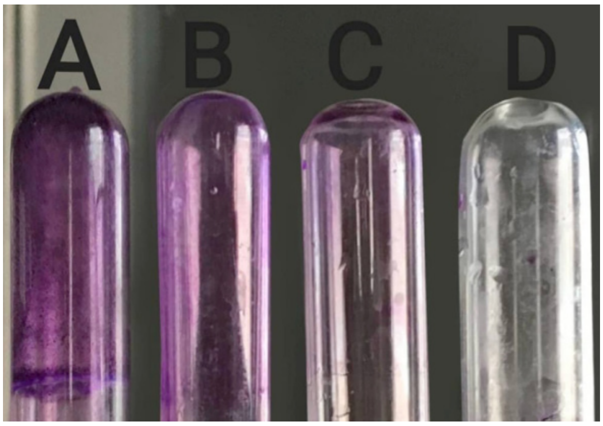

Fig. 3. Biofilm producing Salmonella detected using crystal violet tube test; Salmonella biofilm production was illustrated as following: A represented strong positive producer; B represented positive producer: C represented weak producer; D represented no biofilm production.

	AM	CIP	CN	TE	C	SXT	DA	Biofilm production	Color scale
AM	1								1
CIP	0.21821789	1							0.6
CN	0.272165527	0.35634832	1						
TE	0.21821789	0.04761905	-0.08908708	1					
C	0.111111111	-0.21821789	0.40824829	-0.21821789	1				
SXT	0.21821789	0.52380952	0.356348323	0.04761905	-0.21821789	1			0.2
DA	-0.11111111	0.21821789	0.272165527	0.21821789	0.11111111	0.21821789	1		
Biofilm production	0.40824829	0.53452248	0.25	0.08908708	0.27216553	0.53452248	0.4082483	1	-0.3

Fig. 4. Diagram showing the correlation between antimicrobial resistance, and biofilm production in *Salmonella* strains recovered from duck samples. Red and blue colors of boxes indicate positive and negative correlation, respectively. The density of the colors and numbers corresponds to the correlation coefficient (*r*).

Discussion

Salmonella infection has a public health concern as it is the major cause of gastrointestinal diseases worldwide. It inhibits the animal intestinal tract; Salmonellosis can occur by ingestion of food contaminated with animal feces [39,40]. Recently, *Salmonella* outbreaks were reported as the third foodborne-related outbreaks after *Norovirus* and enteropathogenic *Escherichia coli* (EPEC) [41] and the second reported cause of death in the United States [42]. Salmonellosis occurs through the consumption of different food sources unless the main cause is attributed to poultry consumption such as Chicken, duck, and eggs [43, 44].

In our study, the incidence of *Salmonella spp* in one hundred seventy duck meat collected from different retail markets (major supermarkets and retail shops) distributed in different areas in Mansoura city, Egypt were =10 (5.8%) *Salmonella* strains. Previous studied reported 1.9% (5/270) *Salmonella* strains in poultry samples [45]. Other reports observed a high prevalence of *Salmonella* strains in commercial markets by 54.7% [46], while in broiler farms nearly 19.2% of samples were reported positive for *Salmonella* [47]. The difference in isolation rate between different production stages indicates the importance of hygienic processes taken throughout the poultry supply chain from slaughtering to consumption. Therefore, it is critical to evaluate and take all the control measures at every stage of the production cycle. In addition, ensuring the heat treatment of food during the stage consumption is enough to stop the *Salmonella* infection cycle. According to the CDC, about the third quarter of *Salmonella* outbreaks; occur due to consumption of raw or insufficiently cooked poultry meat or eggs [48].

In recent years, multidrug-resistant *Salmonella* strains have emerged, and special concern should be given against them in the poultry industry which requires vigorous and continuous mentoring locally and widely. Our study reviled that a total of 60% (=6) strains were multidrug resistant against the tested antibiotics amoxicillin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, and clindamycin. The resistance against these antibiotics confirms previous reports [49, 50]. The excessive usage of antimicrobial resistance in the poultry industry in way of medication against disease or as growth promotors may be the cause for the emerged resistance [51- 53], besides, reports about antimicrobial resistance acquisition by natural gene transfer have been discussed extensively in the last days and was reported as a major cause of increasing the antimicrobial resistance worldwide [54]. In regarding this, governmental efforts must be done to stop the unconscious usage of these antibiotics in the animal production cycle. This will give place to new measures to be taken in order to take a step in eliminating the emergence of multidrug resistance *Salmonella* strains and the emergence of new antibiotic resistance. Following a new alternative technique for medication should take place in our hygiene.

Salmonella biofilm producer strains have significant importance, especially in agriculture, industry, and food processing. It has the ability to stay in a dormant state until engulfed by the host and causing enteric diseases [55, 55, and 57]. Food products such as Poultry, meat, fruits, and vegetables were discussed to be a viable vehicle for *Salmonella* forming biofilm [58, 56, 59]. Human bacterial infections were implicated by biofilm counted to be more than 65% [60]. Our study showed the isolation rate of biofilm producer *Salmonella*; a total of six isolates were biofilm producers by different degrees while four isolates were non-biofilm producers. The significant correlation between antimicrobial resistance phenotypes and biofilm production gives us insight about the threats of biofilm in host health due to its ability to resist antibiotics. The high recovery rate of biofilm-forming *Salmonella* requires taking different hygiene measures besides improving the periodical monitoring measures taken through slaughterhouses, industry, retail shops, and even privet kitchens. Studies showed that performed to produce powerful disinfection and eradication methods capable to eliminate and stop the biofilm persistence in the food cycle.

Conclusion

Poultry meat is mainly infected through cross-contamination during the production process. The incidence rate of duck meat still low, but it has dangerous hazard because only one *Salmonella* colony can cause infection, however, the concept that Egyptian culture in food preparation depends on the consumption of fully cooked poultry is a relief. Although, the risk of consumption of food contaminated by *Salmonella* is still present due to the possibility of contamination of food through contact between raw and cooked food. In addition, the recovery of multidrug-resistant *Salmonella* strains and biofilm forming *Salmonella* is an alarming concern that needs urgent measures; especially due to the significant relation between antimicrobial phenotypes and biofilm production reported. Our study was limited to a single district of Dakahlia provenance (Mansoura city) in Egypt; thus, a recommendation is given to include different areas from Egypt to perform a surveillance study in addition including different food samples.

Authors' contributions

AS and GY conceived and designed the study. RA, and AS performed the sampling and the experiments. RA and AS analyzed the data. RA and AS wrote the original draft. AS and GY reviewed and edited the manuscript. All authors contributed to the article and approved the submitted manuscript.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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ميكروب السالمونيلا فى لحوم الدجاج فى مصر انذار بالخطر: انتشارها والعلاقة بين مقاومة المضادات الحيوية ووجود الاغشية الخلوية

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يعتبر ميكروب السالمونيلا من الميكروبات التى تستطيع الانتقال عبر الغذاء ، وهي بكتيريا سالبة الجرام قادرة على تحمل نقص الأكسجين وقادرة على التسبب بأمراضًا معوية في الحيوانات والبشر. بالإضافة إلى ذلك ، فهى العامل الرئيسي المسبب لمعظم أمراض الجهاز الهضمي في جميع أنحاء العالم. هدفت هذه الدراسة إلى تقصي مدى انتشار السالمونيلا بين لحوم الدجاج المعزولة من محلات البيع بالتجزئة في مدينة المنصورة بمصر بالإضافة إلى تقييم حساسيتها لمضادات الميكروبات. تم تحليل 170 عينة لحوم دجاج من مناطق مختلفة فى مدينة المنصورة بمصر للتعرف على بكتيريا السالمونيلا والتأكيد على وجودها وتحليل جميع العزلات المؤكدة لمقاومتها لمضادات الميكروبات باستخدام اختبار انتشار القرص التقليدي.

أكدت النتائج وجود عشر عزلات من ميكروب السالمونيلا. تمتلك السالمونيلا المعزولة مقاومة عالية ضد المضادات الحيوية عائلة البيتالكتام ، تم عرض مقاومة معتدلة للأمينوغليكوزيد (جنتاميسين) ومقاومة أقل في الفلوروكينولون والتتراسيكلين والسلفوناميد (سيبروفلوكساسين ، التتراسيكلين ، وتريميثوبريم- سلفاميثوكسازول). وفي خلال ذلك، كان الكلورامفينيكول هو الأقل تمثيلًا في دراستنا ، ووجدت سلالة واحدة فقط مقاومة له. في الختام ، تم تأكيد ظهور سلالات السالمونيلا المقاومة للأدوية المتعددة وكذلك عترات سالمونيلا قادرة على انتاج اغشية خلوية التي تؤكد قدرة الدجاج على العمل كوسيلة لسلالات السالمونيلا في الإنسان إلى جانب خطر اكتساب مقاومة مضادات الميكروبات عن طريق الغذاء.