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STAPHYLOCOCCUS AUREUS, ENTEROTOXINS GENES AND *SALMONELLA TYPHYIMURIUM* IN CHICKEN MEAT AND ORGANS

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ABSTRACT

This study investigated presence of *Staphylococcus aureus* and *Salmonella typhimurium* and some of their enterotoxins and virulence genes in live chicken and chicken meat in New Valley, Egypt. 200 broilers samples were collected as following; Liver and blood of 100 clinically diseased, 6 weeks old chicken were obtained from five broilers farms in New Valley governorate for surveillance of the microorganisms in poultry while, 100 freshly slaughtered broilers chicken which apparent healthy were collected from public live bird markets. The samples were meat specimens from the breast muscle, thigh muscle, liver, and blood (100 of each). The identified strains were screened for enterotoxin genes of *Staph. aureus* (SEA to SAE) genes. Prevalence of *S. aureus* in live chicken was higher than *S. typhimurium* which was 76% and 18%, respectively which was higher in blood than liver samples. On the other hand, the incidence of *S. aureus* was higher than *S. typhimurium* in apparently healthy and symptomatically diseased broilers. In chicken meat was with an overall incidence of 56.67%. The total prevalence of *S. aureus* and *S. typhimurium* was 51.33% and 5.33% respectively the highest microbial load was in liver samples followed by breast muscle then thigh muscle samples. *S. aureus* SAB gene was the only detected enterotoxins gene among chicken samples. Raw poultry meat available for consumers in Egypt often contaminated with pathogenic zoonotic bacterial agents.

Key words: Chicken, food poisoning, PCR. *Salmonella typhimurium*, *Staphylococcus aureus*.

INTRODUCTION

Staphylococcosis and salmonellosis infection causing great importance problems hazarding chicken industry and consumers' public health (Ezzat *et al.*, 2014; Butcher *et al.*, 2015). *S. aureus* infected live chicken appear as abscesses on the foot bottom called "bumble foot", which leading to painful walking on the foot for the bird. *S. typhimurium* like many *Salmonella* species are normally inhabitant in the digestive tract of fowl and excreted in the feces of infected animals and people, people become infected usually by eating undercooked eggs or chicken meat. *Salmonella* organisms can infect the ovaries of hens and thus infect the egg before the egg laid or by contaminated food by animal feces or by infected food handlers who do not adequately wash their hands after using a toilet. *S. aureus*, and *S. typhimurium* are the main predominant food poisoning bacterial agents transmitted by handling and improper sanitation which causing foodborne disease in humans worldwide. The lethal enterotoxin produced by *S. aureus* is a common cause of food poisoning. The most common symptoms of food poisoning are fever, pneumonia, nausea, wound infections and gastrointestinal disturbances (EFSA 2010; Aydin *et al.*, 2011).

In Egypt, lack of enough poultry slaughterhouses, insufficient marketing infrastructure and traditional preference of consumers of freshly slaughtered poultry consumption, chicken, and other domestic poultry led consumers to buy live birds from markets and slaughtering in the same area or at home. Live chicken markets are an important chain to promote spreading and maintenance of livestock zoonosis pathogens. There are two types of poultry markets in Egypt: retail shops and traditional live bird markets where never or minimal, veterinary inspection is applied (Khalafalla *et al.*, 2015).

Food poisoning is an important issue overall worldwide consumer's health due to its fast contagiousness and lethality. Contaminated raw or improper cooked chicken meat is one of the major sources of food-borne pathogens (Shafizi *et al.*, 2016). Many of food-borne zoonotic diseases commonly found in the intestines of healthy food-producing animals and birds (Al-Bahry *et al.*, 2014).

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S. aureus is one of the most ordinary found pathogenic bacteria with difficulty of its elimination from the human environment. It is the main causative agent of food intoxication by its diversity of enterotoxins (Iandolo, 1989). Twenty different types of staphylococcal enterotoxin, including SEA through SEE, SEG through SER, and SEU have already identified, however, only a few of the toxin serotypes are frequently associated with food poisoning outbreaks (Martin *et al.,* 2004). Staphylococcosis infections affect almost all the avian species. Over 36 Staphylococcus of normal birds' skin inhabitants may infect them after wound entrance to the bird body or after trimming of beak or toenails, inflammation, vaccinations, or chronic infection and impairment of the bird immunity.

Poultry meat is one of the major vehicles of *S. typhimurium* infections is considered one of the major zoonotic food-borne pathogens representing an important public health problem worldwide. It can cause a variety of clinical manifestations ranging from mild gastroenteritis to bacteremia and extra intestinal localized infections involving many organs (Rabie *et al.*, 2012). It is necessary to know the extent to which the poultry industry and the public health exposure to zoonotic diseases via poultry and poultry meat as observed in poultry slaughtered in markets. The main objectives of this study were to assess the role of fresh chicken meat sold in the New Valley governorate as one of Egyptian live bird markets in the transmission of entero-pathogenic strains of *S. aureus* and *S. typhimurium*.

MATERIALS AND METHODS

The study area: This study was conducted in Elkharga, the capital of New Valley Governorate.

Samples collection:

- *Live broilers:* 100 clinically diseased chicken 6 weeks old were collected from five broilers farms in New Valley. The samples were liver and blood of each bird.
- *Chicken meat and offal samples:* 100 freshly slaughtered broilers chickens apparently healthy were collected from public live bird markets. The samples included breast and thigh muscles, liver, and blood (100 of each) which were analyzed for poultry bacterial burden using specific and selective nutrient media and molecular diagnosis. All data was recorded and samples were transported with minimal delay in an ice-box to the laboratory for microbiological and molecular examinations. The identified strains were screened for *S. aureus* (SEA to SAE) enterotoxin genes.

Samples preparation: Samples were prepared following the protocols of APHA, (1992) as the following: Ten grams of each sample were weighted under complete aseptic conditions, and transferred into sterile polyethylene bag containing 90 ml of sterile 0.1% peptone water (Oxoid®). Samples blended in a stomacher (lab-blender, 400) for one minute to provide 10^{-1} dilution from which serial tenfold dilutions were prepared.

Microbiological examination:

1 - *Isolation of S. aureus was performed following the protocols of Valls et al. (2000):* The enriched broth was plated on Baird-Parker Agar (Oxoid®, CM 275) supplemented with Egg Yolk-Tellurite Emulsion (Oxoid®, SR 54).

2 - *Isolation of S. typhimurium was performed according to USDA and FSIS, (2004)* protocols as following: 1ml of diluted sample suspension was enriched in 9 ml of Rappaport-Vassiliadis broth (RV; Oxoid®), incubated at 43°C/24 hr., followed by streaking a loopful of selective enrichment broth on Xylose lysine deoxycholate (XLD®) agar (Merk®) and Salmonella Shigella (SS) agar media and incubated at 37°C/24 hr.

All the isolates, preserved on nutrient agar (Oxoid®), were examined microscopically by Gram's stain to reveal morphological arrangement and staining reaction and biochemically identified according to Quinn *et al.* (1994). Ten pure positive *S. aureus* isolates were used for further enterotoxins and pathogenicity gene identification by PCR.

PCR detection: genomic DNA extracted from the selected *S. aureus, and S. typhimurium* isolates from chicken samples using the QIAamp DNA Mini kit (Qiagen®, Germany, GmbH). Briefly, 200 µl of the sample suspension incubated with 10 µl of proteinase K and 200 µl of lysis at 56°C/10 min. After incubation, 200 µl of 100% ethanol added to the lysate. The sample washed and centrifuged. Nucleic acid eluted with 100 µl of elution buffer provided in the kit. Primers used supplied from (Biobasic®, Canada) as listed in the Table 1. Primers were utilized in a 25µl PCR reaction containing 12.5 µl of 2X DreamTaq Green Mastermix kit (Fermentas®, Germany), 1 µl of each primer of 10 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reactions performed in applied biosystem 2720 thermal cycler as initial denaturation at 94°C/4 min, followed by 35 cycles of 94°C denaturation for 30 sec, annealing (temperature, mentioned in table 1)/30 sec, and extension at 72°C/45 sec. Followed by the cycling of final extension at 72°C/5 min. The PCR products were separated by electrophoresis on 1.2% agarose gel (Applichem, Germany, GmbH) in 1X TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 10 µl of PCR products loaded in each gel slot and 100 bp plus DNA Ladders (Qiagen, Germany, GmbH®) used to determine fragment sizes. Control positive and control negative were included in each reaction. The gel photographed by a gel documentation system (Alpha Innotech, Biometra®).

Table 1: Primers sequences, target genes, amplicon sizes and annealing temperatures of PCR reactions.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Annealing	Reference
S. aureus	*16rRNA*	5-GTAGGTGGCAAGCGTTATCC-3	228	60	**Monday and Bohach, (1999)**
		5-CGC ACATCAGCGTCAG-3			
	Sea	GGTTATCAATGTGCGGGTGG	102	50˚C .	**Mehrotra *et al*, (2000)**
		CGGCACTTTTTTCTCTTCGG			
	Seb	GTATGGTGGTGTAACTGAGC	164		
		CCAAATAGTGACGAGTTAGG			
	Sec	AGATGAAGTAGTTGATGTGTATGG	451		
		CACACTTTTAGAATCAACCG			
	Sed	CCAATAATAGGAGAAAATAAAAG	278		
		ATTGGTATTTTTTTTCGTTC			
	See	AGGTTTTTTCACAGGTCATCC	209		
		CTTTTTTTTCTTCGGTCAATC			
Salmonella spp.	*Gene-specific*	ATCGCTGACTTATGCAATCG	204	50˚C	**Alvarez *et al.*, (2004)**
		CGGGTTGCGTTATAGGTCTG			
	Species-specific	TTGTTCACTTTTTACCCCTGA A	401		
		CCCTGACAGCCGTTAGATATT			

RESULTS

1- *Prevalence of S. aureus, and S. typhimurium in the examined diseased and apparent healthy broilers chicken samples:* The prevalence of *S. aureus* and *S. typhimurium* bacteria from life symptomatically diseased chicken liver and blood samples are presented in Table 2; Results revealed that the total prevalence of *S. aureus* and *S. typhimurium* was 152/200 (76%) and 36/200 (18%) respectively. *S. aureus* could be recovered from 72 and 80% of the examined liver and blood samples respectively. Regarding, *S. typhimurium* 20% and 16% from blood and liver samples respectively proved to be positive. Comparison between infection percent of *S. aureus*, and *S. typhimurium* in the examined broilers chicken which was apparent healthy from life bird markets and symptomatically diseased from broilers farms reveled that; the incidence of *S. aureus* was higher than *S. typhimurium* in both cases as following; the incidence of *S. aureus* infection was about 51.33% and 80% in apparently healthy and symptomatically diseased broilers respectively while the *S. typhimurium* incidence was 5.33% and 20% respectively in apparently healthy and symptomatically diseased broilers (Figure 1).

2- *The prevalence of S. aureus and S. typhimurium in chicken meat and liver samples:* The overall prevalence of *S. aureus* and *S. typhimurium* in different chicken meat and liver samples presented in Table 3 and Figure 2 was 170/300 (56.67%). The total prevalence of *S. aureus*, and *S. typhimurium* was 154/300 (51.33%) and 16/300 (5.33%) respectively. *S. aureus* could be detected in 56 (56%), 53 (53%) and 45 (45%) of the examined liver, breast muscle, and thigh muscle, respectively. *S. typhimurium* isolates were 6 (6%), for liver and only 5 (5%) for each thigh and pectoral meat samples. However, *S. aureus* was the highest prevalence rate in chicken samples while, *S. typhimurium* recorded the lowest incidence rate in all samples.

3- *The prevalence of S. aureus 5 main enterotoxins* (A, B, C, D and E) evaluated from chicken meat and liver samples illustrated in Table 4 and Figure 3: Enterotoxin (B) was the only detected enterotoxin, its highest concentration was reported in 3 liver (30%) followed by 2 of each pectoral and thigh samples 20% for each.

Table 2: Prevalence of *S. aureus*, and *S. typhimurium* in the examined life diseased broilers chicken samples (n. = 100 each)

Samples	*S. aureus*	*S. typhimurium*
	No (%)	**No (%)**
Liver	72 (72)	16 (16)
Blood Samples	80 (80)	20 (20)
Total (No=200)	**152 (76)**	**36 (18)**

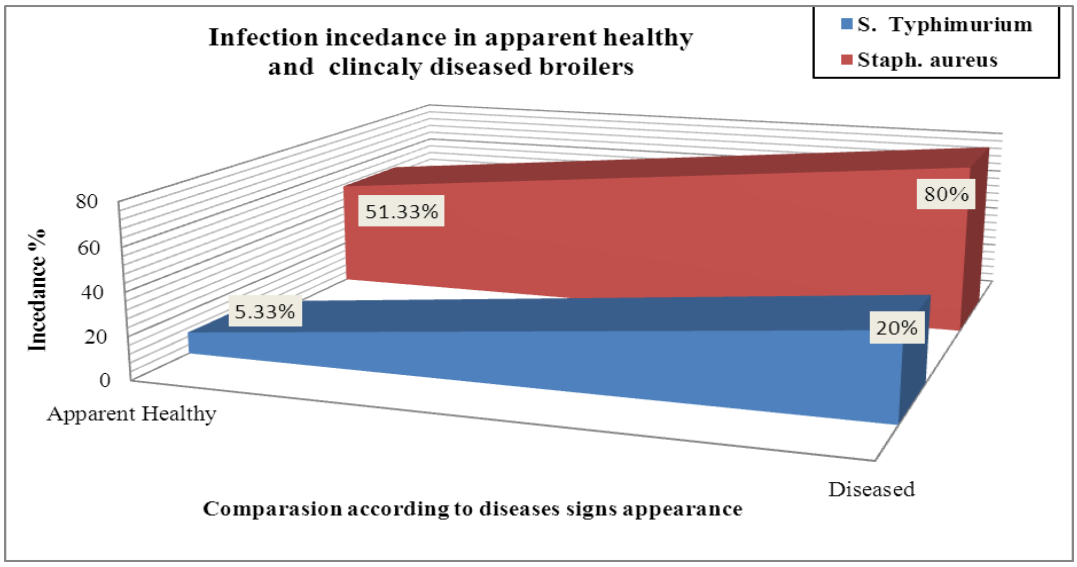

Figure 1: Comparison between infection percent of *S. aureus*, and *S. typhimurium* in the examined broilers chicken samples according to clinical diseases

Table 3: Prevalence of *S. aureus*, and *S. typhimurium* in the examined chicken samples (n. = 100 of each)

Samples	*S. aureus*	*S. typhimurium*	Total No= 300
	No (%)	**No (%)**	**No (%)**
Breast muscle	53 (53)	5 (5)	**58 (58)**
Thigh muscle	45 (45)	5 (5)	**50 (50)**
Liver	56 (56)	6(6)	**62 (62)**
Total (No=300)	**154 (51.33)**	**16 (5.33)**	**170 (56.67)**

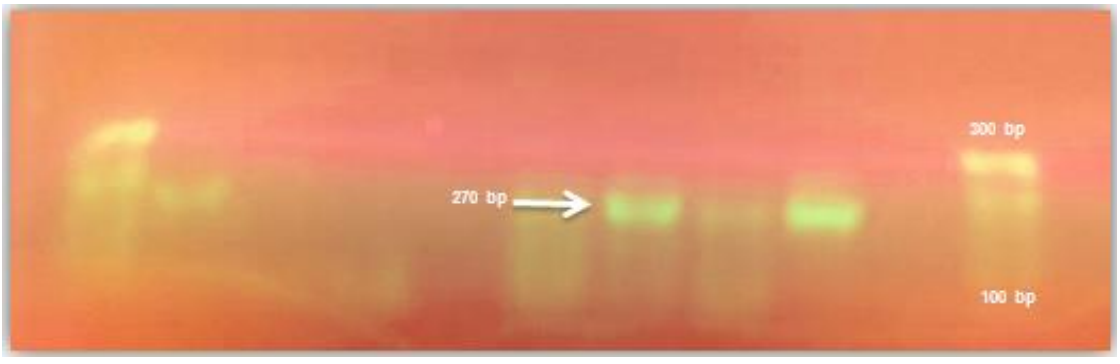

Figure 2: Agarose gel electrophoresis of specific dose-dependent amplification of *Staph. aureus* PCR amplification 270 bp products of DNA extracted from *Staph. aureus* chicken samples respectively.

Table 4: Prevalence of enterotoxigenic *S. aureus* isolated from the collected chicken samples evaluated by PCR.

Samples	Enterotoxigenic genes of *Staph. aureus*				
	Sea *No (%)*	**Seb** *No (%)*	**Sec** *No (%)*	**Sed** *No (%)*	**Sae** *No (%)*
Breast muscle	ND	2 (20%)	*ND	ND	ND
Thigh muscle	ND	2 (20%)	*ND	ND	ND
Chicken liver	ND	3 (30%)	*ND	ND	ND
Total No =30	**0**	**7 (23.33%)**	**0**	**0**	**0**

*ND: Not Detected

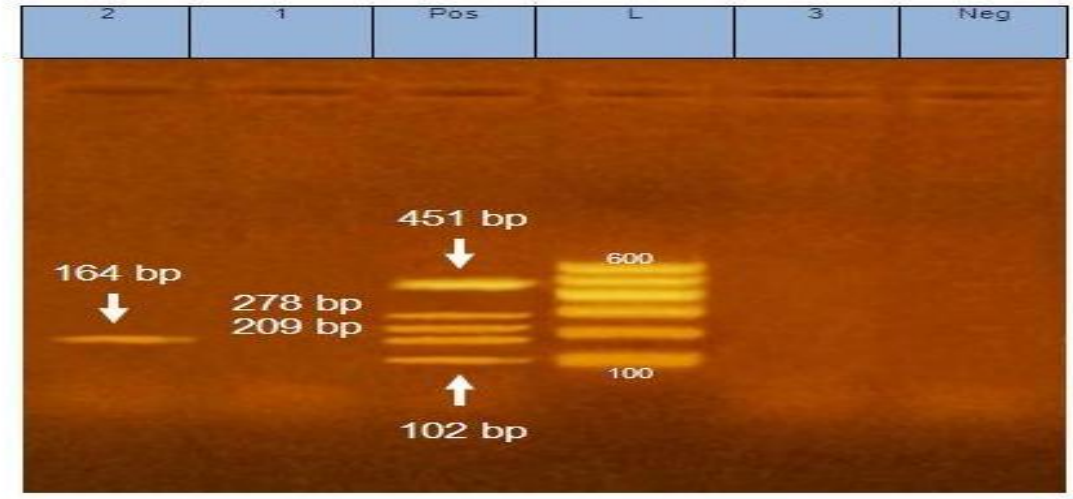

Figure 3: Agarose gel electrophoresis of specific dose-dependent amplification of *S. aureus* pathogenic gene *(Seb)*
PCR amplification of the enterotoxins (*Seb*) 164 bp products of DNA extracted from ***S. aureus*** chicken samples respectively

DISCUSSION

S. aureus, and *S. typhimurium* transmitted in between chicken to the contact of live birds or through consumption of contaminated food (e.g. chicken meat) directly by the microorganisms or through their enterotoxins (Niyonzima *et al.,* 2016). Causing different signs in infected birds such as; diarrhea, decrease production, acute septicemia to chronic osteomyelitis and chicks' mortalities (Awan and Matsumoto, 1998; Ezzat *et al.,* 2014). In addition to food poisoning, which ranged from mild to severe include fever, stomach pain, nausea, abdominal cramps, diarrhea, vomiting and dehydration (Abd El-Malek1 *et al.,* 2010). The finding from this study found that the prevalence of *S. aureus* (76%) was higher than *S. typhimurium* (16%) which was higher in blood than liver, samples, also, the incidence of *S. aureus* was higher than *S. typhimurium* in apparently healthy and symptomatically diseased broilers. These results agrees with that obtained by Ezzat *et al.* (2014) who reported 18.5% salmonellosis in Egyptian broilers and were Kudaka *et al.* (2006) who recorded an incidence of 18% Japanese broilers. However, higher results were reported by Fofana *et al.* (2006) who recorded 62.5% salmonellosis in Sengal broilers. Salmonellosis was reported (21.99%) earlier in Bangladesh by Rahman *et al.* (2004) while in China, 52.2% was recorded by Yang *et al.* (2011). In Saudi Arabia, *S. typhimurium* reported about 22.22% in chicken (Moussa *et al.,* 2010). In Nigeria, salmonellosis prevalence in apparently healthy chicken, clinically sick chicken was 6.6% and 11.6% respectively by Nwiyi *et al.* (2015) while, also in Nigeria, Dashe *et al.* (2013) found *S. aureus* in about 20.5% of clinically sick birds and 2.3% from apparently healthy chickens. On the other hand, lower results recorded in Brazil (2.7% salmonellosis) was reported by Medeiros *et al.* (2011). Staphylococcosis prevalence reached to 44% in chicken in Qena, Egypt (Karmi, 2013). While in Turkey Citak and Duman (2011) recorded 47.2% Staphylococcosis in chicken.

Staphylococcosis in chicken varies from acute ascites, septicemia, and mortalities to chronic osteomyelitis but infection commonly appears as a systemic disease proceeded on hock joint as osteomyelitis, synovitis and/or bumble foot, which look like a large swollen

abscess on the foot bottom, which resulted in restriction of the affected bird movement. Chicken salmonellosis is deadly in young chicks, transmitted to the chick through the egg from infected hens or from chick to another in the incubator. In the chicken, it spread through direct contact between birds, using contaminated water containers and feeders from feces resulting in inflammation of the intestine, which appears as white diarrhea (Skeeles 1997; EI-Jakee *et al.,* 2013; Ezzat *et al.,* 2014).

Chicken staphylococcosis and salmonellosis can be transmitted to humans by eating infected eggs, using unsanitary practices. Staphylococcal food poisoning is one of the most common causes of gastroenteritis worldwide. Symptoms have a rapid onset of food poisoning signs within 2-4 hours after ingestion of thermostable Staphylococcal enterotoxins (SEs). Since SEs are more stable than *S. aureus* bacteria, it is possible to test food product and obtain negative *S. aureus* culture results and positive SEs tests (EI-Jakee *et al.,* 2013). In the present study, the total *S. aureus* prevalence in chicken meat and liver was 51.33%, which was higher than the previously reported from Egypt by Osman *et al.* (2015) who detected 15% *S. aureus* from chicken meat. Kumar *et al.* (2011) recoded 10.8% of *S. aureus* enterotoxins in South Asian. However, higher results were reported by Khalifa *et al.* (2014) who reported about 63% while Gwida and Elgohary (2015) found only about 22% *S. aureus* from Mansoura, Egypt chicken meat. Mathenge *et al.* (2015) detected 37.4% of meat products contaminated with *S. aureus* in Kenya.

Salmonellosis is one of the most important zoonotic bacterial pathogens of food-borne infection all around the world. In the present, study the total prevalence of *S. typhimurium* was 5.0% in chicken meat and liver. Controversially, Mohamed and Aly (1998) failed to detect *Salmonella* spp. in 30 chicken collected from different localities in Assiut city, Egypt. These differences in the prevalence of *Salmonella* in chicken referred to many factors, such as isolation methods, sample type, size, and seasonal variations and geographical location.

Regarding the site of contamination of the examined bacteria, the total contamination rate of the two recovered bacteria was highest in liver samples (62.0%), followed by breast muscles (58.0%) and thigh muscles (50.0%).

Egyptians chicken consumers usually buy live chicken from public live bird markets and slaughter it immediately after selection in the markets using the same primitive manual equipment's in slaughtering, feathering and evisceration that considered excellent sources to spreading microbial contamination to the slaughtered bird (Oladele-Bukola and Odetokun, 2014; Khalafalla *et al.,* 2015). Other reasons for relying on live bird sales are that 'on-the-spot' butchering means product substitution is less likely. Slaughter, plucking, and evisceration lead to carcass contamination will have high levels of zoonotic bacteria when a positive flock slaughtered. Unless all the necessary precautions taken along the poultry production, marketing, and processing chains, contaminated poultry meat could be harmful to humans. The Centers for Disease Control and Prevention (CDC) stated that 25% of healthy worker's skin or nostrils carry *S. aureus* and transmitted to the chicken meat during improper personal hygiene by handling and processing (Gwida and El-Gohary, 2015).

Egyptian Organization Specification (2005) and European regulations (2005) prevent consumption of any food containing any amount of *S. typhimurium*. In the present study, the detection of *S. aureus* and *S. typhimurium* with their poultry impacts and zoonotic importance can only control by exceeding the standards public health hazards control systems in poultry farms, raises the needs for proper implementations of preventative programs through sanitation and sound management, disinfection and proper use of antibacterial agents and regular surveillance.

From public health perspectives, SEs causes about 95% of humans' food poisoning. Enterotoxins increase the potential consumer's risk in the absence of strict hygienic measures (Clarisse *et al.,* 2013; Al-Jumaily *et al.,* 2014; Mathenge *et al.,* 2015). *S. aureus* can contaminate foods through contact with contaminated hands, materials, and surfaces. Considering this hazard, meat and meat products should not subject to unnecessary contamination and they should be free from such serious pathogen to ensure a maximum margin of consumer safety. In this study, SEB was prevalent in chicken meat isolates. However, the enterotoxin genes not uniformly distributed among all *S. aureus* strains (Dinges *et al.,* 2000). It is known that 59% of staphylococcal food poisoning outbreaks are caused by SEA to SEE (Bergdoll, 1989).

In conclusion, this survey revealed that raw poultry meat available for consumers in Egypt often contaminated with zoonotic bacterial agents. Furthermore, several strains were positive for the several putative virulence marker genes as well as enterotoxin genes. All these findings suggest that the consumption of undercooked meat or food cross-contaminated with zoonotic bacteria may pose a serious threat to consumer health. Therefore, there is a need for enhanced efforts to avoid foodborne pathogens. Adequate cooking of meat, the personal and equipment cleanliness and chemical disinfectants, hygienic handling, storage and effectively processing reduce bacterial infection. Therefore, shutting down live poultry markets is extremely effective in preventing human cases. However, even temporary

shutdowns create economic problems but should consider.

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جينات سموم البكتيريا العنقودية والسالمونيلا تيفي ميوريم في لحوم واحشاء الدواجن

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تعتبر اصابات البكتيريا العنقودية والسالمونيلا وسمومها من اهم المخاطر التي تهدد صناعة الدواجن وصحة مستهلكيها. لذلك اهتمت هذه الدراسة بالتقصي عن مدى انتشار البكتيريا العنقودية والسالمونيلا تيفي ميوريم وسمومها وجينات الضراوة في الدجاج المتداول في اسواق الوادي الجديد قبل وبعد ذبحه. وذلك بفحص عدد ٢٠٠ دجاجة منها ١٠٠ دجاجة بعمر ٦ اسابيع وتظهر عليها اعراض مرضية تم جمعها من ٥ مزارع داجنة في الوادي الجديد. حيث تم اخذ (اكبادها وعينات دم) من كل منها. كذلك تم جمع ١٠٠ عينة من لحوم الدجاج المتداول في اسواق الوادي الجدي والظاهر عليه علامات الصحة التامة فور ذبحه. حيث كانت العينات هي لحوم الصدر والفخذ بالاضافة لكبد لكل دجاجة مذبوحة من العينات محل الدراسة ثم تم اجراء الفحص البكتري المعملي للكشف عن البكتريا العنقودية والسالمونيلا تيفي ميوريم ثم الفحص الجيني للكشف عن انواع السموم في البكتريا العنقودية. وبالفحص وجد ان معدلات انتشار البكتريا العنقودية اعلى من السالمونيلا تيفي ميوريم في الدجاج الحي حيث كان ٧٦% و ١٨% على التوالي. كذلك سجلت الاصابة بالبكتريا العنقودية نسب اعلى في لحوم الدجاج المتداول في الوادي الجديد ٥١.٣٣% و ٥.٣٣% على الترتيب. كما اظهرت الدراسة معدلات اصابة اعلى في اكباد الدجاج يليه لحوم الصدر ثم الافخاذ. اما بالنسبة لسموم البكتريا العنقودية فتم الكشف عن وجود جينات نوع واحد فقط من السموم وهو النوع (ب). مما سبق استخلصت الدراسة وجود نسب عالية من البكتريا الممرضة وسمومها في لحوم واحشاء الدجاج المتداول في اسواق الوادي الجديد.