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Molecular detection of some virulence genes in multidrug resistant *Salmonella* species isolated from chicken meat products and raw milk

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ABSTRACT

*Salmonella still has a serious foodborne outbreak with public health risk. Chicken meat, chicken meat products, and raw milk are important reservoir for Salmonella. In the current study, a total of 120 samples of chicken meat and chicken meat products (breast, thigh, giblets, frozen thigh, nuggets, burger, shish and luncheon, 15 of each) and 50 raw cow milk samples were tested for prevalence, serotyping, virulence genes, and antibiotic resistance profiles of Salmonella spp.. The prevalence of Salmonella spp. was 11.67% in chicken meat samples with the following incidence on each sample group; 13.33, 20, 26.67, 0, 6.67, 6.67, 13.33 and 6.67%, respectively. Raw milk samples overall occurrence of Salmonella spp. was 6%. Serological identification of the isolated Salmonella revealed presence of five different serotypes including S. kentukay, S. entritides, S. typhimurium, S. lindenberg and **S**. bassa. All isolated Salmonella spp. har-bored stn gene; while S. kentukay, S. entritides and S. typhimurium harbored mgtC gene but invA was found in S. entritides, S. typhimurium, S. lindenberg and S. bassa. Also, sopB was detected in S. kentukay, S. entritides, S. typhimurium and S. lindenberg. The isolated Salmonella spp. was resistant to sulphamethoxazol trimethoprim, chloramphenicol, and penicillin (100%). Meanwhile, the sensitivity was 70.6% % to ampicillin, enrofloxacin and amoxycillin clavulanic. The results confirm the importance of application of strict hygienic measures in food industry and proper use of antibiotics for meat and milk producing animals.*

***Keywords**: Chicken meat, Raw milk, Salmonella spp., Virulence genes, Antibiotic resistance*

1. Introduction

Despite the high nutritional value of chicken meat and milk, they could be incriminated with many health hazards and food poisoning outbreaks to the consumers. Chicken meat and raw milk are associated with *Salmonella* outbreaks around the world (CDC, 2018). Chicken meat and its products can be contaminated from different sources starting from de-feathering, evisceration, and the subsequent during processing in plant (Houf et al., 2002; Yar et al., 2020). Raw milk could be contaminated with *Salmonella* from feces of infected dairy animal and milkers, infected udder, milking equipment, air, and animal insects ((Ponce et al., 2008).

The Centre for Disease Prevention and Control (CDC, 2021) reported *in* the United States about 1.35 million infections with 26,500 hospitalizations, and 420 deaths by *Salmonella* every year and food are the main source most of these illnesses.

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The clinical symptoms of *Salmonella* infection include typhoid fever, enteritis, and bacteremia (Santos et al., 2001). Non-typhoid Salmonellosis has been linked with acute gastroenteritis with unpleasant effects on the surrounding organs (Su et al., 2004).

Salmonella Pathogenicity Island 1 (SPI1) is found in all Salmo-nella spp., it is a genetic element on the chromosome which contains the virulence genes, encoding for factors responsible for invasion of the epithe-lial cells (Hensel, 2004) as well as adhesions, intracellular survival, antimi-crobial resistance, systemic infections, toxin production, and iron and magnesium uptake (Aydin et al., 2011).

The continuous and uncontrolled usage of antimicrobials during livestock production had led to the development of the drug resistance phenomenon among the originated foodborne pathogens including *Salmonella* (Darwish et al., 2013). The current study was conducted to evaluate the level of contamination with multidrug resistant *Salmonella* spp. contained virulence genes in chicken products and raw milk.

2. Materials and Methods

2.1. Collection of samples

A total of 120 chicken meat products (breast, thigh, giblets, frozen thigh, nuggets, burger, shish and luncheon, 15 of each) and 50 raw cow milk samples were randomly collected from different local groceries and street vendors at Zagazig, Sharkia Governorate and Damanhour, El-Behira Governorate, Egypt, respectively. The samples were aseptically transferred as soon as possible in an ice box to laboratory to be examined bacteriologically.

2.2. Isolation of Salmonella spp. in chicken meat product and raw milk samples (APHA, 2001):

25 g or 25mL of each sample was mixed with 225mL of buffered peptone water and incubated at 37 ºC for 18 ±2 hrs. Pre-enrichment of 1 ml of previously incubated homogenate was transferred to 10 mL of Rappaport Vassiliadis with soya (RVS broth) and incubated at 41.5 C^{o}±1C^{o} for 24 ±3 hrs (Vassiliadis *et al.*, 1978). Then, a loopful was streaked on the plates of (XLD) agar, incubated at 37C^{o}±1C^{o} for 24 ±3 hrs. Morphologically typical colonies (pink to red colonies with black center) were recorded, picked up and were identified biochemically according to Quinn et al., (2002)

2.3. Serological identification of Salmonella:

Determination of both Somatic (O) and flagellar (H) antigens was done by *Salmonella* antiserum (DENKA SEIKEN Co., Japan) according to Kauffman (1974).

2.4. Molecular identification of Salmonella virulence genes:

DNA extraction was conducted by QIA amp Kit according to the manufacturing instructions. Oligonucleotide primer sequences were illustrated in (Table 1). PCR assay was done according to Sambrook et al. (1989).

2.5. Antibiogram of the isolated Salmonella spp.

Antimicrobial susceptibility was tested by the single diffusion assay according to NCCLS (2001). The tested strains were evaluated as susceptible, intermediate, and resistant. Multiple drug resistance (MDR) index for each strain was determined according to Singh et al. (2010). MDR index= Number of resistance (Isolates classified as intermediate were considered sensitive for MDR index) / total Number of tested antibiotics.

Table 1: Oligonucleotide primers sequences for *Salmonella* spp. PCR

Gene	Primer Sequence (5'-3')	PCR conditions	PCR product	Reference
sopB	TCA GAA GRC GTC TAA CCA CTC TAC CGT CCT CAT GCA CAC TC	94˚C 10 min, 94˚C 45 sec, 49˚C 45 sec, 72˚C 45 sec for 35 cycles.	517 bp	Huehn et al. (2010)
mgtC	TGA CTA TCA ATG CTC CAG TGA AT ATT TAC TGG CCG CTA TGC TGT TG	94˚C 5 min, 94˚C 30 sec, 58˚C 45 sec, 72˚C 45 sec for 35 cycles.	677 bp	Huehn et al. (2010)
Stn	CTT TGG TCG TAA AAT AAG GCG TGC CCA AAG CAG AGA GAT TC	95˚C 15 min, 94˚C 1 min, 55˚C 1 min, 72˚C 1 min for 35 cycles.	260 bp	Makino et al. (1999)
***inv*A**	GTG AAA TTA TCG CCA CGT TCG GGC AA TCA TCG CAC CGT CAA AGG AAC C	94˚C 1 min, 94˚C 60 sec, 64˚C 30 sec, 72˚C 30 sec for 35 cycles.	248	Kumar et al. (2008)

Table 2: Prevalence and serological identification of isolated *Salmonella* in chicken meat products

Samples (n=15)	*S. lindenberg*	*S. kentukay*	*S. typhimurium*	*S. bassa*	*S. entritides*	*Total*
Breast	1	0	1	0	0	2(13.33%)
Thigh	0	2	0	0	1	3(20%)
Giblets	0	1	1	0	2	4(26.67%)
Frozen thigh	0	0	0	0	0	0
Nuggets	0	1	0	0	0	1(6.67%)
Burger	0	1	0	0	0	1(6.67%)
Shish	0	1	0	0	1	2(13.33%)
Luncheon	0	0	0	1	0	1(6.67%)
Total	1(7.14%)	6(42.86%)	2(14.29%)	1(7.14%)	4(28.57%)	14(11.67%)

2.6. Statistical analysis:

Statistical analysis of data was done by using the statistical package for social sciences (SPSS-16.; Chicago, IL, USA) software and one way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Isolation and identification of Salmonella spp.

Salmonella is an important microorganism which most frequently associated with food-born outbreaks. As illustrated in Table (2,3), the prevalence of *Salmonella spp.* in the examined chicken meat products and raw milk was 14/120(11.67%) and 3/50(6%), respectively. *Salmonella* was detected in 2(13.33%), 3(20%), 4(26.67%), 0, 1(6.67%), 1(6.67%), 2(13.33%) and 1(6.67%) of the examined chicken breast, thigh, giblets, frozen thigh, nuggets, burger, shish and luncheon, respectively. The highest incidence of *Salmonella spp*. was in chicken giblets, while *Salmonella spp*. failed to be detected in frozen chicken thigh samples. The obtained results nearly agree with Nawar (2007) and Ruban and Fairoze (2011) who isolated *Salmonella spp.* from 11.11 and 71.43 % of chicken thigh, respectively; Rady et al. (2011) who isolated *Salmonella spp*. from 16% of chicken breast; Samaha et al. (2012) who isolated *Salmonella spp.* from 8% of nuggets and Hassanin et al, (2017) who isolated *Salmonella spp.* from 30% of giblets. Meanwhile, *Salmonella spp*. not detected in chicken meat products (Gad, 2004; Khalifa and Abd El- Shaheed 2005).

According to Egyptian Organization for Standardization and Quality control (EOS 1651, 2005) chicken meat and chicken meat products should be free from *Salmonella spp.*. The results revealed that 14(11.67%) of examined chicken meat and its products were incompatible with Egyptian standard. Only, examined frozen chicken thigh samples were compatible this standard. The variation of results may attribute to the differences in manufacturing, handling and the effectiveness of hygienic practices applied during the production process. Also, this variation may be due to different localities of isolation either cities or shops which have different levels of sanitation.

For raw milk samples, previous studies reported variable prevalence of *Salmonella* in raw milk as determined by Jayarao et al. (2006) (6%); Tesfaw et al. (2013) (1.6%) and Omar et al. (2018) (52%). While other studies did not report *Salmonella* from raw milk samples **(**Mhone et al., 2012; Zeinhom and Abdel-Latef, 2014, Elafify et al., 2019). EOS 154-1(2005) stated the raw milk must be free from *Salmonella*, In the current study 6% of examined raw milk samples were incompatible with Egyptian standard. The variable incidence rate of *Salmonella* in raw milk could be associated with different source of samples, sampling and isolation methods, geographical distribution, seasonal variation, and farm husbandry practices (Oliver et al., 2005).

Serological identification of the isolated *Salmonella* strains revealed that the chicken meat products were contaminated by five different serotypes including *S. kentukay*, *S. entritides*, *S. typhimurium*, *S. lindenberg* and *S. bassa* with a prevalence of 6(42.86%), 4(28.57%), 2(14.29%), 1(7.14%) and 1(7.14%), respectively (Table 2). *S. entritides* (4%) and *S. typhimurium* (2%) were serologically identified in raw milk samples (Table 3).

Table 3: Prevalence and serological identification of isolated *Salmonella* in raw milk

Samples	*S. entritides*	*S. typhimurium*	Total
Raw milk	2/50 (4 %)	1/50 (2 %)	3/50 (6 %)

(n=50)

3.2. Molecular identification of Salmonella spp. virulence genes:

The *Sop*B is involved in invasion of intestinal cells and membrane ruffling (Rahman, 2006; Bourgeois et al., 2021). The *mgtC* virulence gene is required for growth in medium low in Mg^{2+} and for survival inside the macrophage. Also, it may be involved in activating the Na+/K+-ATPase to regulate the membrane potential (Thi et al., 2020). The *Salmonella* inner membrane contains protein coded by *invA*, that is important for invasion into epithelial cells (Salehi et al., 2005); it enables the bacterial cell to invade the intestine and cause gastroenteritis (Hu et al. 2008; Ekwanzala et al. 2017; Lan et al. 2018), in addition to survival in macrophages (Goodman et al., 2017).Furthermore, the *stn* gene was reported as a suitable PCR target for detection of *Salmonella* strains (Elkenany et al., 2019).

As shown in (Table 4) and (photo 1), all the isolated *Salmonella spp.* in this study harbored *stn* gene; while *S. kentukay*, *S. entritides* and *S. typhimurium* harbored *mgtC* gene but invasive (*inv*A) was found in *S. entritides*, *S. typhimurium*, *S. lindenberg* and *S. bassa.* Also, *Salmonella* effector proteins (*sopB*) was detected in *S. kentukay*, *S. entritides. S. typhimurium* and *S. lindenberg*. These detected virulence genes agree with findings of Rahman (2006); Lan et al. (2018); Omar et al. (2018).; Thi et al. (2020) and Bourgeois et al. (2021) who identified *sopB; invA,sopB, stn*, and *spvC; invA,stn* and *avrA; sopB, mgtC, rhuM, spvRBC, sopE pipB,gipA* and *sodCI,* ; *SopB* and *SopE2* virulence genes from isolated *Salmonella spp*. in their studies.

3.3. Antibiogram of the isolated Salmonella strains:

Antibiotics are used in poultry and dairy farms for many purposes as growth promoters, prophylaxis, and therapeutics. However, the misuse of antibiotics caused increased bacterial resistance (Abdellah et al., 2009; Suleiman et al., 2013).

Table 4: Distribution of virulence genes among *Salmonella* serotypes

Serotypes	*Stn*	*sopB*	*mgtC*	*Inv*A
S. entukay	+*ve*	+ve	+ve	-*ve*
S. entritides.	+ve	+*ve*	+ve	+ve
S. bassa	+ve	-*ve*	-*ve*	+ve
S. lindenberg	+*ve*	+*ve*	-*ve*	+*ve*
S. typhimurm	+ve	+ve	+ve	+ve

As recorded in Table (5); hundred percent of the isolated *Salmonella spp.* was resistant to chloramphenicol, sulphamethoxazol trimethoprim, and penicillin. Furthermore, the resistance was 88.2% to oxytetracyclin and kanamycin, and 64.7% to cefadroxil and doxycyclin. Meanwhile, the sensitivity was 70.6% to ampicillin, enrofloxacin and amoxycillin clavulanic acid but it was 58.8% and 47% to gentamicin and neomycin, respectively with different MDR value for each isolate (Table 5,6). Saad et al. (2015) and Almashhadany, (2019) were reported similar resistance rate of *Salmonella*. Antibiotic resistant of *Salmonella* is linked with the misuse of antimicrobial agents for food producing animals; *Salmonella* resistant strains can be transmitted to consumers through food (Nygard et al., 2008) constituting public health hazards and affects the efficacy of drug treatment in humans (Abdellah et al., 2009).

Table 5: Antimicrobial susceptibility of the isolated *Salmonella* spp.

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Chloramphenicol	0	0	0	0	17	100
Cefadroxil	0	0	6	35.3	11	64.7
Sulphamethoxazol, Trimethoprim	0	0	0	0	17	100
Kanamycin	2	11.7	0	0	15	88.2
Doxycyclin	6	35.3	0	0	11	64.7
Oxytetracyclin	2	11.8	0	0	15	88.2
Gentamicin	10	58.8	0	0	7	41.2
Penicillin	0	0	0	0	17	100
Enrofloxacin	12	70.6	0	0	5	29.4
Amoxycillin, Clavulanic acid	12	70.6	0	0	3	17.6
Neomycin	8	47	6	35.3	1	5.9
Ampicillin	12	70.6	3	17.6	0	0

(n=17)

Table (6): Multiple drug resistance (MDR) index of the isolated *Salmonella* spp.

***Salmonell* spp. (n=17)**	**Antimicrobial resistance profile**	**MDR index**
S. typhimurium	C, CFR, SXT, K, DO, T, CN, P, ENR, AMC	0.83
S. entritides	C, CFR, SXT, K, DO, T, CN, P, ENR	0.75
S. typhimurium	C, CFR, SXT, K, DO, T, CN, P, AMC	0.75
S. entritides	C, CFR, SXT, K, DO, T, CN, P	0.67
S. entritides	C, CFR, SXT, K, DO, T, P	0.58
S. kentukay	C, CFR, SXT, K, DO, T, P	0.58
S. kentukay	C, CFR, SXT, K, T, P	0.50
S. lindenberg	C, CFR, SXT, K, T, P	0.50
S. entritides	C, SXT, K, DO, P	0.42
S. kentukay	C, SXT, K, DO, P	0.42
S. kentukay	C, SXT, k, T, P	0.42
S. kentukay	C, SXT, k. T, P	0.42
S. kentukay	C, SXT, T, P	0.33
S. bassa	C, SXT, T, P	0.33
S. typhimurium	C, CFR, SXT, K, DO, T, CN, P, ENR, AMC, N	0.92
S. entritides	C, CFR, SXT, K, DO, T, CN, P, ENR, N	0.83
S. entritides	C, CFR, SXT, K, DO, T, CN, P, ENR	0.75

Conclusion

Chicken meat products and raw milk samples were contaminated with *Salmonella spp.*, that harbored virulence genes with multi drug resistant properties. Lack of hygiene in handling and production process, inadequate storage and post-process contamination would be the main causes of this contamination. Implementation of hazard analysis and critical control point system (HACCP) as well as food safety and inspection service (FSIS) in all meat and dairy processing units is effective for controlling food poisoning bacteria. Customers should provide adequate heat treatment of chicken products and raw milk to kill *Salmonella spp.* with proper refrigeration during storage.

Conflict of interests

The authors have not declared any conflict of interests.

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