

Occurrence of *Salmonella* infection and antimicrobial susceptibility for local *Salmonella* isolates from different sources in a cross-sectional study

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Abstract

Salmonellosis is a considerable public health problem worldwide, with high economic importance in developed countries. The main purpose of this study was to determine the prevalence of *Salmonella* infection and antibiogram analysis of isolated strains in a cross-sectional study in Egypt 2016-2017. The study investigated twenty-eight *Salmonella* isolates from different areas in Egypt and different types of samples, such as human stool (9.3%), Egyptian cattle egrets and storks (28.5%) and grilled chicken from electric grills (36.6%). No isolates were detected from grilled chicken from charcoal grills or drinking water. The main *Salmonella* serotype detected in the isolates was *S. typhimurium* (86.5%). Molecular characterization of the *invA* gene by PCR was carried out and then confirmed by sequencing, and the results were submitted

to GenBank. Antibiogram analysis of Egyptian isolates carried out on 9 antimicrobial discs reported that the routine regimes of treatment were not yet effective for recent new *Salmonella* generations in 2016-2017. The new isolates could be treated with levofloxacin, cefaperazone/sulbactam, chloramphenicol, imipenem or meropenem.

Introduction

Salmonella is a gram-negative, facultative anaerobic bacteria belonging to the family Enterobacteriaceae (Barlow and Hall, 2002). It has been associated with a wide spectrum of infectious diseases, including typhoid salmonellosis and non-typhoid salmonellosis (NTS). Salmonellosis is a considerable public health problem worldwide, contributing to the economic loss of both industrialized and underdeveloped countries due to the costs associated with control and treatment of the disease (Crump *et al.*, 2004). The most common manifestation of *Salmonella* infection is gastroenteritis followed by bacteraemia and fever. *Salmonella* has the ability to adapt within a variety of animal hosts, including humans (Allerberger *et al.*, 2003; Majowicz *et al.*, 2010). *Salmonella* is the most important foodborne pathogen and is mostly found in poultry, egg and dairy products; furthermore, fresh fruits and vegetables are a very important source of food involved in *Salmonella* transmission (Pui *et al.*, 2011; Silva *et al.*, 2011). Uncooked animal products from poultry, cattle and pigs are another important source of infection. Antibiotic resistance is a substantial problem related to *Salmonella* and increases the public health importance of *Salmonella* because of the high virulence and increasing morbidity rates of infected patients (Chiu *et al.*, 2002).

In Egypt, the spread of *Salmonella* infection depends on socioeconomic factors, including educational level, sanitation, poor habits and misuse of antibiotics, which all help in the spread and persistence of *Salmonella* infection in urban, suburban and rural areas. Our work aimed to study the occurrence of *Salmonella* strains isolated and identified from different sources in different areas in Egypt. Quesna and Menoufia were used as rural area models, and Cairo and Giza were used as urban area models from 2016-2017. Molecular identification of isolated *Salmonella* strains was performed using PCR and sequencing of the *invA* gene. Antibiogram analysis of recent *Salmonella* isolates is very important for the evaluation of currently used antibiotics in

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the treatment of salmonellosis. After checking the treatment strategies in the study locations, we found that approximately 90% of physicians treat adults >18 years with ciprofloxacin (CIP), patients <18 years are treated with cefotaxime (CTX) for 3 days, and the course is completed with azithromycin (AZM). Evaluation of these treatment strategies and selection of a highly effective antibiotic for isolated *Salmonella* strains are very important targets. Studying the occurrence of *Salmonella* infection in the aforementioned areas in Egypt can help emphasize the main points involved in disease control.

Materials and Methods

Samples

Human samples: Seventy-five stool samples were collected from clinically affected persons showing different clinical signs, such as fever, enteritis and diarrhoea. Serological examination of all blood samples was performed using the Widal test (*Salmonella* Ag O and H). Of the 75 samples, 25 were human stool samples from

Quesna and Menoufia, and 50 were human stool samples from Giza and Cairo.

Carriers: Thirty-five samples were collected from storks and cattle egret mainly in rural areas. The liver and gallbladder were obtained for cultivation.

Foodstuffs: This study used 50 grilled chickens from different restaurants in Cairo, Giza and Menoufia, 20 grilled chickens from charcoal grills and 30 grilled chickens from multilayer electric grills.

Drinking water: Fifteen drinking water samples were collected with a volume of 5 litres each. The 5 litres were filtered, and the filter that contained the filtered bacterial load was cultivated.

Isolation of *Salmonella* spp.

Non-selective pre-enrichment: Twenty-five grams of human stool or grilled chicken were weighed and added to 225 mL of buffered peptone water (Sigma Aldrich, USA). The liver and gallbladder of carriers and the filter that contained bacterial load in the water were also inoculated and incubated at 37°C overnight (ISO, 1993; Post, 1997).

Selective pre-enrichment: A total of 100 µL of cultured buffered peptone water was transferred into 10 mL of Rappaport Vassiliadis soy peptone broth (RVS) and incubated at 41.5°C for 24 hrs in a shaker incubator.

Streaking on selective media: A loopful of RVS broth was streaked on XLD and Hektoen enteric (HE) agar and incubated at 37°C for 24 hrs.

Biochemical identification of isolated *Salmonella*

Single colonies of the isolates on selective media were selected and subcultured on nutrient agar medium and then incubated at 37°C for 24 hrs. Single colonies from nutrient agar were selected and cultivated into triple sugar iron (TSI) slants, citrate slants and urea hydrolysis test slants, which were incubated at 37°C for 24 hrs (ISO, 1993).

Serological identification of *Salmonella* isolates

The *Salmonella* isolates were preliminarily identified biochemically by serological identification according to Kauffman (1961) in the Microbiological Laboratory of the Ministry of Health.

Molecular diagnosis by PCR

All *Salmonella* isolates were tested by *invA* gene PCR (Rahn *et al.*, 1992).

DNA extraction: The local *Salmonella* isolates were grown on nutrient agar medium, and the bacterial cells were harvested from the culture plates by a sterile cotton

swab and placed into Eppendorf tubes containing 1X phosphate buffered saline (PBS) and mixed well. Then, centrifugation was performed at 9000 × g for 5 min (washing was repeated in PBS three times), and the sample was washed once with sterile deionized distilled water. Centrifugation was then performed at 9000 × g for 5 min, and the supernatant was removed. Then, the bacterial pellet was suspended in 100 µL of sterile deionized distilled water. The suspension was heated in a heat block (Grant-Bio, England) at 100°C for 5 min and then immediately cooled in ice water. Then, the samples were centrifuged, and the supernatant (DNA) was removed (Wang *et al.*, 1996).

Primers: As indicated in Table 1, *invA* forward and reverse primers designed by Rahn *et al.* (1992) were used.

PCR amplification and electrophoresis: PCR amplification was carried out in a 25 µL reaction mixture containing 12.5 µL of 2X Green Master Mix (EmeraldAmpGT PCR 2X Master Mix, A2201-1, Takara), 0.5 µL (25 pmol) of each primer (*invA* F and R), 2 µL of extracted DNA and 9.5 µL of nuclease free water (Thermo Scientific, #R0581). The PCR cycling parameters consisted of denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 64°C for 40 s and 72°C for 30 s. A final extension step was then carried out at 72°C for 7 min (Rahn *et al.*, 1992). All reaction products were analysed by electrophoresis using 1.5% (w/v) agarose gels in 1X TAE running buffer at 100 V for 30 min and imaged using a Spectroline UV transilluminator (Slimline Series, USA).

Invasive protein A (*invA*) gene products sequencing and bioinformatics analysis

PCR products generated from the *invA* gene in selected isolates of *Salmonella* were sent to be sequenced at the Animal Health Research Institute (AHRI) in Dokki, El-Giza. The PCR products were purified using the GeneJET PCR Purification Kit (Thermo) and sequenced on a DNA sequencer. The sequencing step was conducted with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained sequences were determined by BLAST analysis with

sequences available in GenBank (NCBI). Phylogenetic tree analysis was used to assess the relatedness of the gene sequences recovered from different sources. BioEdit software was used for Clustal W multiple sequence alignment and sequence identity matrix, and MEGA7 software was used for the construction of phylogenetic trees by the neighbour-joining method.

Antibiogram of isolated *Salmonella* strains

Antimicrobial susceptibility testing was performed using the disc diffusion method. The result was interpreted as per the recommendation of the National Committee for Clinical Laboratory Standards Institute (CLSI) (CLSI, 2016; Abdi *et al.*, 2017). The isolated *Salmonella* strains were subcultured on nutrient agar medium, and then single colonies were transferred into tubes containing 5 mL of sterile saline. The bacterial density was matched with 0.5 McFarland turbidity standard, which was equal to 1.5×10⁸ bacterial cells/mL. A sterile cotton swab was used to spread the inoculum uniformly over the surface of the Muller Hinton agar plate by the spreading method. The plates were held at room temperature for 30 min to allow for drying and then tested for gentamicin (CN) 10 µg, CIP 5 µg, chloramphenicol (C) 30 µg, CTX 30 µg, cefoperazone/sulbactam (CES) 75/30 µg, AZM (30 µg), meropenem (MEM) 10 µg, imipenem (IPM) 10 µg and levofloxacin (LEV) 5 µg. The plates were incubated at 37°C for 24 hrs. The diameter of the zones of inhibition was classified as resistant, intermediate, or susceptible according to the CLSI (2016). All antibiotic discs were manufactured at Oxoid Ltd., England, and the expiry dates were 2018.

Results

Isolation and characterization of local Egyptian *Salmonella* strains

Twenty-eight local Egyptian *Salmonella* isolates were recovered. Seven isolates were from human stool, 10 were from carriers (Egyptian cattle egret and stork) and 11 isolates were from grilled chickens from multilayer electric grills. No isolates were recovered from grilled chick-

Table 1. The set of *invA* gene primers for diagnosis of *Salmonella* isolates.

Gene	Sequence	Product	Ref.
<i>invA</i> F	5- GTG AAA TTA TCG CCA CGT TCG GGC AA -3	281 bp	Rahn <i>et al.</i> , 1992
<i>invA</i> R	5- TCA TCG CAC CGT CAA AGG AAC C -3		

ens on charcoal grills or from drinking water. The *Salmonella* isolates gave the typical colonial morphology on the selective media, which was colourless colonies with a black centre on XLD agar medium and transparent and black colonies on HE agar medium. The biochemical identification revealed that all local *Salmonella* strains were TSI K/A-positive with H₂S production, negative for the urea hydrolysis test, and citrate-positive.

Serological identification of *Salmonella* isolates

The serotyping analysis of Egyptian *Salmonella* strains showed that all *Salmonella* isolates from carriers (10 isolates) and grilled chickens from multilayer electric grills (11 isolates) were *S. typhimurium* (100%). The human strains varied between *S. typhimurium* (42.8%), *S. enteritidis* (14.3%), *S. virchow* (14.3%), *S. haifa* (14.3%) and *S. kentucky* (14.3%). Of the 28 total *Salmonella* isolates in the present study, *S. typhimurium* represented 86%, and the other serotypes each represented 3.5% of the total isolates (Table 2).

Molecular diagnosis by *invA* gene PCR

All 28 local Egyptian isolates were PCR-positive for the *invA* gene, which was

detected by electrophoresis of the target band at 281 bp of the *invA* gene product (Figure 1).

Invasive protein A (*invA*) gene product sequencing and bioinformatics analysis

Representative sequences from this study were deposited into the GenBank database under accession numbers MF953387, MF953388 and MF953389. The *invA* gene nucleotide sequence data

available at GenBank were selected based on the criteria of the information available regarding the isolation source, location and year (Table 3). Three amplified PCR products of the *invA* gene were sequenced and aligned with the other related *invA* gene sequences obtained from GenBank NCBI-BLAST. The $\leq 99\%$ sequence identity to the *invA* gene of *S. enterica* strains showed that the set of primers described in the present study exclusively amplifies the 281 bp fragment of the target gene. Phylogenetic anal-

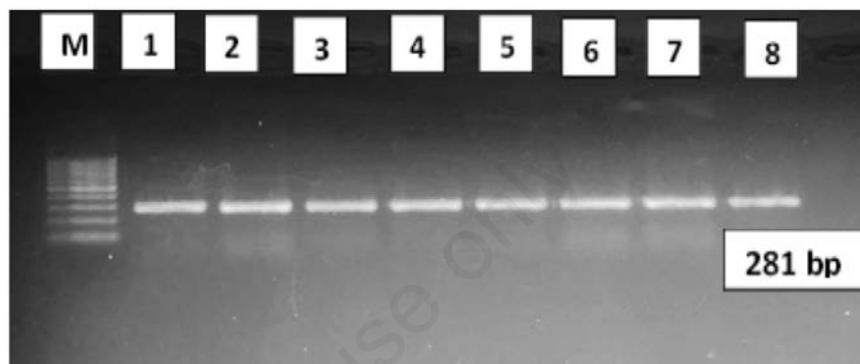


Figure 1. Molecular detection of *Salmonella* isolates by *invA* gene PCR for detection of 281 bp gene product in *Salmonella* isolate genome. (M) 100 bp DNA ladder. (1 to 8) *Salmonella* isolate genome.

Table 2. Serotyping analysis of the 28 *Salmonella* Egyptian isolates from human stool, stork carriers and grilled chickens from multi-layer electric grills.

Salmonella serotype	Type of sample			Total no. (%)
	Humans (7), (%)	Storks (10)	Grilled chickens (11)	
<i>S. Typhimurium</i>	3 (42.8)	All 10 isolates (100%)	All 11 isolates (100%)	24 (86)
<i>S. enteritidis</i>	1 (14.3)	-	-	1 (3.5)
<i>S. virchow</i>	1 (14.3)	-	-	1 (3.5)
<i>S. haifa</i>	1 (14.3)	-	-	1 (3.5)
<i>S. kentucky</i>	1 (14.3)	-	-	1 (3.5)

Table 3. GenBank accession no. and epidemiological information of nucleotide sequences used in phylogenetic analysis encoding the *invA* protein.

Sample no.	Serotype	Isolation source	Location	Sample collection date	Accession no.
1	<i>Salmonella</i> Heidelberg	Broilers	Egypt	—	ARS88237
2	<i>Salmonella</i> enterica	Broilers	Egypt	—	AID54915
3	<i>Salmonella</i> cerro	Cattle faecal sample	USA	2006	ETC76711
4	<i>Salmonella</i> enterica	Chicken	China	—	AHA92023
5	<i>Salmonella</i> Enteritidis	Ground turkey	USA	—	ELL58393
6	<i>Salmonella</i> Typhimurium	Food sample	Portugal	2011	KNB31714
7	<i>Salmonella</i> Cubana	Desiccated coconut	Philippines	1972	ESJ52477
8	<i>Salmonella</i> enterica	Human stool	India	2015	KYN92226
9*	<i>Salmonella</i> Typhimurium	Stork (wild bird)	Egypt	2016	MF953387
10*	<i>Salmonella</i> Typhimurium	Grilled chicken	Egypt	2016	MF953388
11*	<i>Salmonella</i> Typhimurium	Human stool	Egypt	2017	MF953389

*Gene sequences enrolled in the present study.

ysis results of 3 *S. enterica* isolates based on *invA* gene sequences are shown in Figure 2. The *invA* gene sequences of these isolates were initially compared to those of *S. enterica* strains reported in the GenBank database (Table 3).

Occurrence of Salmonella isolates in Egypt

One hundred seventy-five samples were collected from different sources from different areas in Egypt in 2016-2017. The occurrence rate of Salmonella isolates found in Table 4 shows a 12.6% occurrence rate in rural areas and a 19.3% occurrence rate in urban areas with no significance, with a P value of 0.235 ($P < 0.05$). The occurrence rate according to sample type showed that 9.3% of samples were from human stool, 28.5% were from carriers, 36.6% were from grilled chickens from multilayer electric grills, and 0% were from grilled chickens from charcoal grills and drinking water, with a highly significant P value of 0.0002 ($P < 0.05$).

Antibiogram of Salmonella isolates

All *Salmonella* isolates were intermediate resistant (I) for CIP-I (100%), twenty isolates were intermediate resistant (I) for AZM-I (71.4%), sixteen isolates were CN resistant (57%), and four isolates were multidrug resistant (MDR) (resistant for ≥ 3 antimicrobial drugs) for CTX, AZM and CN, which were isolated from grilled chickens. All isolates were susceptible to C, LEV, CES, IPM and MEM (Table 5).

Phenotypic pattern of antimicrobial resistance

Approximately 14.6% of *Salmonella* isolates were MDR, and their pattern was CTX*AZM*CN*CIP-I. They were isolated from chickens in multilayer electric grills (*S. typhimurium*). These isolates were resistant to all types of antimicrobial drugs used

in routine treatment of salmonellosis at different ages. Nine isolates (32%) (3 isolates from grilled chickens and 6 from humans) had the CN*CIP-I*AZM-I pattern. Approximately 39.2% of *Salmonella* isolates had the CIP-I*AZM-I pattern (3 isolates from grilled chickens, 7 from carriers and one from humans). Three carriers of *S.*

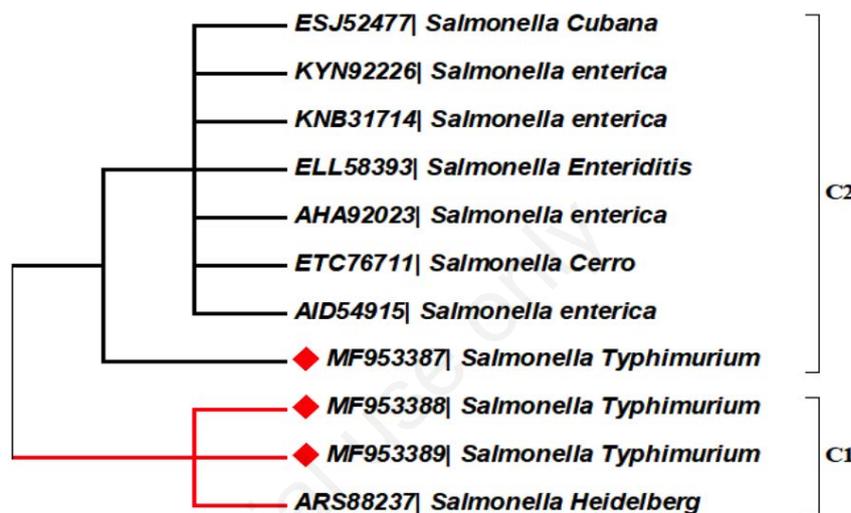


Figure 2. Neighbour-joining phylogenetic tree based on *invA* gene sequences showing the relationship between the present study sequences and 8 representative sequences retrieved from GenBank. The accession numbers of sequences are shown on the branches. The isolate sequenced in this study is indicated by red diamond.

Table 4. Occurrence rate of Salmonella isolates from different sources in different areas in Egypt.

Risk factor		No. of samples	No. of positive	Prevalence rate (%)	P value (<0.05)
Area of study	Rural (Qwesna)	87	11	12.6	0.235 (No significance)
	Urban (Giza, Cairo)	88	17	19.3	
Type of sample	Human	75	7	9.3	0.0002 (High significance)
	Carrier stork	35	10	28.5	
	Electric grilled chicken	30	11	36.6	
	Charcoal grilled chicken	20	0	0	
	Drinking water	15	0	0	

Table 5. Antibiogram analysis of Salmonella isolates from grilled chickens, human stool and carriers towards nine antibiotic discs.

Salmonella isolates	Antibiotic discs								
	CTX	CIP	AZM	CN	CES	C	MEM	IPM	LEV
Chicken Salmonella isolates	S	IR	IR	S	S	S	S	S	S
	S	IR	IR	R	S	S	S	S	S
	S	IR	S	IR	S	S	S	S	S
	R	IR	R	R	S	S	S	S	S
Human Salmonella isolates	S	IR	IR	S	S	S	S	S	S
	S	IR	IR	R	S	S	S	S	S
Salmonella isolates from carriers	S	IR	S	R	S	S	S	S	S
	S	IR	IR	S	S	S	S	S	S
	S	IR	IR	S	S	S	S	S	S

typhimurium isolates had the CN*CIP-I pattern (10.7%), and one *S. typhimurium* strain from grilled chickens had the CN-I*CIP-I pattern (3.5%) (Table 6).

Discussion

Salmonella infection is widespread, especially in developed countries (Ramadan *et al.*, 1992; Schutze *et al.*, 1997). Salmonellosis is underdiagnosed because samples are not obtained for culturing; only the serum is taken for the Widal test, which depends only on circulating blood antibodies against typhoidal Salmonella O/H antigens. The Widal test has many limitations, including that the result of a single test has no diagnostic significance in an endemic region according to WHO recommendations. This is due to the difficulty in establishing the baseline titre of Widal agglutination, which limits the usefulness of the test as a reliable diagnostic indicator of the disease process (Baired-Parker, 1990; Keusch, 1998). During 2016-2017, recurrent salmonellosis increased due to poor socio-economic factors, poor hygiene, and misuse of antibiotics, which led to the spread of antimicrobial-resistant (AMR) Salmonella strains.

In the present study, 28 obtained Salmonella isolates were identified culturally and biochemically. Serological characterization revealed that the main serotype was *S. typhimurium*, which was found in approximately 86% of the total isolates; 100% of the isolates were recovered from carriers and grilled chickens from multilayer electric grills (the main risk factor for typhoidal salmonellosis). Human isolates varied between *S. typhimurium* (42.8%), with the highest prevalence, *S. enteritidis* (14.3%), *S. virchow* (14.3%), *S. haifa* (14.3%) and *S. kentucky* (14.3%). Molecular characterization by *invA* gene PCR was considered the most common diagnostic marker until now (O'Regan *et al.*, 2008; ElSharkawy *et al.*, 2017). The invasive protein A (*invA*) gene is a critical virulence determinant of Salmonella because of its role in invasion of the host epithelial cell layer.

DNA sequencing and bioinformatics analyses were performed to confirm the PCR results in our study and compared with related *invA* gene sequences retrieved from the GenBank NCBI-BLAST database. Regarding the present study sequences, we found $\leq 99\%$ sequence similarity to *invA* gene sequences of *S. enterica* strains with different serotypes available in the GenBank database, which has been confirmed by others to be the *invA* gene, which contains sequences unique to Salmonella; this gene is a suitable PCR target. Phylogenetic analysis of *S. enterica* serotypes (Figure 2) using the sequences of the invasion protein A (*invA*) gene showed that the sequences recovered from 3 isolates in this study had accession numbers of MF953387, MF953388 and MF953389, and 8 isolates retrieved from GenBank (Table 3) were grouped into two clusters (C1&C2). C1 showed that our study sequence isolated from carriers (wild bird) (MF953387) was in a close relationship with other sequences retrieved from GenBank (Figure 2), which include different hosts, isolation source, locality and isolation dates. This finding highlights the public health hazard of the spread of some wild birds, such as the Egyptian cattle egret and stork. The second cluster (C2) showed that our study sequences isolated from grilled chickens (MF953388) and humans (MF953389) are closely related to the sequence (ARS88237) retrieved from GenBank isolated from broilers. This relationship poses the greatest risk to public health and the human incidence of salmonellosis transmitted indirectly through foodstuffs (Wegener *et al.*, 2003). From the phylogenetic tree analysis, it is clear that there is a high degree of convergence between grilled chicken and human sequences, which confirms what was previously discussed, as negligence and mishandling during the preparation of grilled chicken may act as potential sources for cross contamination. Consistent with the aforementioned data, poor personal hygiene practices of food handlers, improper food processing, and inadequacy of both education of food handlers and information directed to consumers all make foodborne diseases still dominant as the most impor-

tant public health problems in most countries (Ulaya, 2013).

There was no significance regarding area of study in the Salmonella infection occurrence, but sample type showed high significance regarding the Salmonella infection occurrence, which is considered an important risk factor (Table 4).

From the aforementioned data, the Salmonella occurrence scenarios could be explained as follows. First, in rural areas, the expected route of Salmonella infection is eating of food contaminated vegetables and fruits. The agricultural lands are irrigated with sewage, and the Egyptian cattle egret and stork stand in both sewage and agricultural lands, which facilitates Salmonella contamination and the spread of the infection to different locations. Then, the contaminated vegetables and fruits are eaten fresh, without washing, by farmers, sellers and clients in vegetable and fruit markets. Second, in urban areas, the expected route of infection is eating of contaminated fast food, such as grilled chicken in electric multilayer grills (36.6% prevalence rate) in contrast to grilled chickens on charcoal grills (0% occurrence). The cooks in most restaurants handle cooked and uncooked food at the same time, which helps spread the infection to consumers. Most restaurants use electric multilayer grills, which cook high amounts of chicken and other meat. In these types of grills, the upper layer is cooked faster than the lower one, and then the cooks exchange the raw chicken with cooked ones, which allows for contamination of the cooked chickens by the water droplets that fall from the uncooked ones. The cooks take the contaminated cooked grilled chicken to the clients, who acquire the infection and are now at risk. Furthermore, tracing the AMR patterns of Salmonella isolates is extremely important. In the study locations, after checking the most common physician advice for patients, we found that salmonellosis is treated mainly by the following regimens: patients >18 years are treated with CIP, and patients <18 years are treated with CTX for 3 days, and the regimen is completed with AZM. The high recurrence rate of infection, improper habits of consumers, poor hygiene practices and misuse of antibiotics are all reasons we need to evaluate the treatment protocols for salmonellosis and test the antimicrobial susceptibility to other available antimicrobial drugs. As mentioned in Table 5, CIP, which is most advisable for salmonellosis treatment, is not sufficiently effective on Salmonella isolates. CTX, the most effective drug for salmonellosis in young patients, began to lose its effectiveness because a new generation of MDR

Table 6. AMR patterns of all Salmonella isolates.

AMR pattern	Number of AMR isolates	Number of isolates	Prevalence rate %
CIP-I*AZM-I	0(CIP-I, AZM-I)	11	39.2
CN*CIP-I*AZM-I	1(CIP-I, AZM-I)	9	32
CN-I*CIP-I	0(CN-I, CIP-I)	1	3.5
CTX*AZM*CN*CIP-I	3(CIP-I)	4	14.6
CN*CIP-I	1(CIP-I)	3	10.7

Salmonella strains that are resistant to CTX, AZM and CN appeared (Table 6). AZM also lost its effectiveness on most Salmonella isolates in the current study. Thus, the consequences of the misuse of antibiotics are the development of AMR Salmonella strains and the ineffectiveness of routine treatment regimens for salmonellosis. Antimicrobial susceptibility testing of Salmonella isolates (shown in Table 5) showed that all Salmonella isolates could be treated with LEV, CES, C, IPM and MEM.

Conclusions

Salmonella bacteria are highly dangerous foodborne pathogens that cause considerable hazards to public health in our country. The socioeconomic factors of poor habits of consumers, poor hygiene practices, misuse of antibiotics and improper lifestyle are the main factors in the generation of new MDR strains of Salmonella. Therefore, informing the public by authorized communities is very important.

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