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Molecular Characterization of ESBL and Carbapenenemase Producing *Salmonella* spp. Isolated from Chicken and its Public Health Importance

Mona Kadry, Sara Mohamed Nader*, Esraa A. Elshafiee and Zeinab S. Ahmed
Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

ABSTRACT

Antimicrobial resistance has become one of the most severe worldwide dangers to human and veterinary Medicine. β-lactam resistant Salmonellae are of great concern as they are becoming multi-drug resistant. This study describes the role of chicken in harboring and environmental spread of ESBL and carbapenemase - producing Salmonella spp, which could pose a potential hazard to human and animal health. A total of 334 chicken meat samples, 197 eggshells and 160 human stool specimens were included in this study. The presence of salmonellae spp. was examined using bacteriological isolation and serological identification. PCR amplification of the ESBL and carbapenemase-encoding genes was performed. The bacteriological examination of the samples showed that 6 Salmonella strains [*S.* Typhimurium (3), *S.* enteritidis (2) and *S.* Infantis (1)] were isolated from chicken meat and eggshell surface samples. In human, it was found that 6 salmonella strains (*S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, *S.* Virchow, *S.* Haifa, *S.* Kentucky) were isolated. The results of multiplex PCR showed that ESBL-producing salmonellae and Carbapenem-resistant salmonellae occurred in four strains from chicken meat samples while not from egg, also in human five strains carried the β-lactamase-producing genes while no strain was positive to carbapenemases. The detection of ESBL and carbapenemase- producing salmonellae from chicken in Egypt is confirmed and represents a major public health problem.

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Authors' Contribution
MK, SMN, EAE and ZSA presented the concept. MK and SMN wrote the manuscript. ZSA prepared the samples and applied bacteriological examination and PCR assay. EAE helped in laboratory work

Key words
Chicken meat, Salmonellae, ESBL, Carbapenem, Egypt

INTRODUCTION

Antibiotics are used as veterinary and human medicines for treatment, control and prevention of infectious diseases. However, their recurrent use can have unexpected adverse effects, including the development of antibiotic resistance which is the one of the greatest challenges in modern medicine (Doi *et al*., 2017). The prevalence of multidrug resistant organisms, a major threat to public health, continues to rise globally and is associated with significant morbidity and mortality (Logan and Weinstein, 2017). These trends are highlighted in Enterobacteriaceae, a family of gram-negative bacteria responsible for a variety of infections acquired from the community and the health care.

Salmonella is the most important foodborne pathogen which mostly found in poultry and egg. The majority of *Salmonella* infections cause self-limiting diarrhea and do not require antimicrobial treatment. However, in certain cases, *Salmonella* may lead to some complication, especially when it spread via blood stream. In such cases, fluoroquinolones and cephalosporins are the drugs of choices (Miriagou *et al*., 2004). Resistance to these agents would compromise the efficacy of empiric treatment of suspected Gram-negative infections and limit the therapeutic options for their definitive treatment as well (Doi *et al*., 2017).

Many animal species, in particular chickens are potential reservoirs for this bacterium (Sanchez *et al*., 2002). Humans can get the infection through the food chain (Majowicz *et al*., 2010).

Today, treatment of bacterial infections in human and animals is facing several problems due to increased antimicrobial resistance (AMR) in bacteria against the most of the antibacterial agents. One of the most important AMR mechanisms in Enterobacteriaceae family is the production of extended-spectrum β-lactamase (ESBL) and metallo β-lactamases (MBLs) enzymes (carbapenem -resistant) (Lee and Ko, 2012).

According to Ambler classification, ESBL is divided into four main groups from A to D (Jacoby and Munoz-Price, 2005). ESBL enzymes TEM, SHV, and CTX-M from Group A have been widely reported to be produced by bacteria. These enzymes can hydrolyze ampicillin, carbenicillin, oxacillin, and an extended spectrum of cephalosporins such as ceftazidime and cefotaxime (Paterson and Bonomo, 2005).

Infections due to ESBL-producing Enterobacteriaceae

* Corresponding author: Dr.saranader@yahoo.com
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are concerned for many reasons including increased hospital costs, length of stay, and mortality rates in addition to they are often treated by carbapenems (e.g., ertapenem, imipenem, meropenem, and doripenem) (Legese *et al*., 2017). Recently, the efficacy of carbapenems has been threatened worldwide by the emergence of carbapenem-resistant bacteria (Woodford *et al*., 2014) but to date no information is available about the occurrence of such problem in poultry which is an important source of protein produced in Egypt owing to their high feed–meat conversion and fast growth.

Carbapenems are broad-spectrum B-lactam antibiotics of critical importance in human medicine (Nordmann, 2014) and resistance of Enterobacteriaceae to carbapenems involves multiple mechanisms, the most remarkable of which is the production of enzymes called carbapenemases that are capable of hydrolyzing the carbapenems and loss of outer membrane proteins and the most remarkable of which are the big five enzymes KPC, NDM, IMP, VIM and OXA (Munoz-Price *et al*., 2013) KPC, OXA and NDM comprise three of the so-called 'big five' carbapenemases that have been associated with nosocomial infections (Cantón and Ruiz-Garbajosa, 2011).

The most of human enteric illness caused by bacterial pathogens originate from animals and can be transmitted directly from animals to humans or indirectly through foods of animal origin especially poultry derived items which are the main sources of infection by *Salmonella* spp. (Greig and Ravel, 2009). There are multiple links between human, animal and environmental compartments that allow not only the transfer of the bacteria, but also of mobile genetic elements and the drugs themselves (Woolhouse and Ward, 2013).

Therefore, this study was conducted to investigate the occurrence of ESBL and carbapenemase producing salmonellae in chicken meat, egg and human clinical samples suffering with diarrhea from different localities in Egypt.

MATERIALS AND METHODS

A total of 334 fresh chicken meat samples were collected from randomly selected poultry shops and 197 eggshells collected from poultry farms. In addition, 160 stool swab specimens selected from hospital lab suffering with diarrhea during the period from February to June 2018 in different localities in Egypt.

The Fresh chicken meat samples were transferred in boxes containing ice cubes and immediately processed in the laboratory for bacterial isolation where 25 g of the meat samples were obtained and processed with 225 ml buffered peptone water (Singh *et al*., 2010).

A sterile cotton swab, soaked in sterilized normal saline, was swabbed on egg shell surface and immersed in 10 ml normal saline solution followed by transmission to 90 ml of buffered peptone water then incubated at 37°C for 18 h (Singh *et al*., 2010). For human stool samples: About 2 to 10g stool was collected into a sterile leakproof container (without using any preservatives). The sterile cotton swab was coated thoroughly with the fecal material and then aseptically inoculated into tubes containing buffer peptone water.

For identification of *Salmonella* one ml of pre-enriched sample was added in 10 ml Rappaport-Vassiliadis (RV) medium vortexed and incubated for 24 ± 2 h at 42°C, then loopfull of each RV tube was cultured onto the surface of XLD agar for 24 h at 37ºC. Typical *Salmonella* colonies were subjected to a series of biochemical, serological and molecular tests for identification of *Salmonella* spp.

Suspected colonies were identified by using chemical tests, including Gram staining, indole, methyl red, Voges Proskauer, citrate utilization, triple sugar iron and lysine decarboxylation (Atek *et al*., 2017).

Serotyping of isolates was performed in serogroup level with a standard agglutination test using O and H antisera (Difco, USA) in the central laboratories of the Ministry of Health, Giza, Egypt.

For molecular detection of carbapenemase and ESBL encoding genes, the DNA of *Salmonella* strains was extracted by using the conventional boiling method (Murugkar *et al*., 2003).

For multiplex PCR**,** specific oligonucleotide primers for *ESBL* encoding Genes (*bla* CTX-M, *bla SHV* and *bla TEM)* and carbapenemase-encoding gene (KPC, OXA and NDM) (Table I).

The PCR reaction conditions consisted of initial denaturation cycle for 10 min, followed by 35 amplification cycles for carbapenemase and ESBL encoding genes using the following conditions: Denaturation for 60 s at 95 °C, annealing for 60 s at 58 °C, extension for 1 min at 72 °C and final extension for 10 min at 72 °C.

RESULT AND DISCUSSION

A total of six isolates was isolated from 334 chicken meat and 197 eggshell surface samples (Table II). The serotyping of these isolates was *S*. Typhimurium (3), *S*. Enteritidis (2) S. Infantis (1) from chicken meat and one *Salmonella* isolates [*S*. Enteritidis] from eggshell surface samples. In addition, of 160 stool specimens collected from human, six *Salmonella* strains (*S*. Typhimurium, *S*. Enteritidis, *S*. Infantis, *S*. Virchow, *S*. Haifa, *S*. Kentucky) were isolated. Among the total six strains of salmonella (Tables II, III) the overall prevalence of ESBL-producing salmonellae was four strains of chicken meat samples

Table I. Primer sequences used for PCR amplification of *ESBL* and carbapenemase encoding genes.

Target gene	Primer sequence (5′-3′)	Amplified length (bp)	Reference
*bla*CTX-M	GCGATGGGCAGTACCAGTAA (F) TTACCCAGCGTCAGATTCCG (R)	392	Hamza *et al.,* 2020
blaSHV	TCAGCGAAAAACACCTTG (F) TCCCGCAGATAAATCACCA (R)	472	Hamza *et al.,* 2020
blaTEM	ATGAGTATTCAACATTTCCG (F) TTACCAATGCTTAATCAGTGAG (R)	861	Hamza *et al.,* 2020
KPC	ATG TCA CTG TAT CGC CGT CT (F) TTT TCA GAG CCT TAC TGC CC (R)	882	(Li *et al.,* 2012)
OXA	TTG GTG GCA TCG ATT ATC GG (F) GAG CAC TTC TTT TGT GAT GGC (R)	743	(Li *et al.,* 2012)
NDM	GGT TTG GCG ATC TGG TTTTC (F) CGG AAT GGC TCA TCA CGA TC (R)	621	(Li *et al.,* 2012)

Table II. Serotyping of *Salmonella* strains and distribution of ESBL and carbapenemase producers and non producers.

Isolation source	Samples (n)	*Salmonella* isolates	ESBL-producer	Non- ESBL producer	CARB producer	Non-CARB producer
Chicken meat	334	6 *S.* Typhimurium(3), *S.* Enteritidis(1), *S.* Infantis(1)	4	2	4	2
Egg	197	1 (*S.* Enteritidis)	0	1	0	1
Human stool	160	6 *S.* Typhimurium (1), *S.* Enteritidis (1), *S.* Infantis (1), *S.* Virchow(1), *S.* Haifa (1), *S.* Kentucky (1)	5	1	0	6

Table III. Distribution of carbapenemase determinants, ESBL determinants genes in Salmonella strains.

Salmonella isolates (n)	Resistance genes							
	Carbapenemase determinants				ESBL determinants			
	No.	NDM	OXA	KPC	No.	SHV	TEM	CTX
Chicken meat (6)	4	2	2	0	4	2	4	0
Egg (1)	0	0	0	0	0	0	0	0
Human (6)	0	0	0	0	5	5	0	3

carrying β-lactamase-producing genes, 2 of which carried bla SHV and 4 carried bla TEM, while no strain in egg. This result agrees with (Pieskus *et al*., 2006) who mentioned that *S.* Enteritidis and *S.* Typhimurium were the dominant serotypes in both chickens and humans. The appearance of *S.* Enteritidis in human isolates was reflected by an increase in such serotype in chicken samples (van Duijkeren *et al*., 2002). *S.* Infantis is still of public health concern and it is the most commonly identified pathogenic serotype in the chickens, eggs and humans (Fearnley *et al*., 2011).

The carbapenem-resistant salmonellae were detected in four strains isolated from chicken meat samples; two carried the resistance genetic determinants bla_{NDM} and two carried bla_{OXA}.

Among the total six *Salmonella* strains isolated from human (Tables II, III) five stains carried the β-lactamase producing genes where all of them were bla_{CTX} and three strains carried bla_{SHV} , while no strain was positive to carbapenemases. The inappropriate administration of antimicrobial agents in empiric therapies, lack of effective infection control strategies that could trigger a change in the prevalence of resistant organisms in the population, and selective pressure for the use of third-generation cephalosporins have been established as the most important factors in the emergence of ESBL-producing strains.

The genus *Salmonella* has recognized as the potential reservoir for different genes which encode antimicrobial resistance (Trudel *et al*., 2016). Although ESBL production by *Salmonella* spp. isolated from animals has been reported (Jiang *et al*., 2014), the reports on ESBL from animal origin are less frequent (Egervärn *et al*., 2014). The main driving force of resistance is the presence of β-lactamases (Bush, 2016). Moreover, many of these organisms carry additional plasmid-borne genes that are active against other antibiotic classes, thus making bacteria resistant to multiple drugs (Bush and Fisher, 2011). This result is in accordance to (Olesen *et al*., 2004) who reported that in Denmark the major recorded ESBL was the TEM group. However, (Djeffal *et al*., 2017) isolated two strain carried bla_{TEM} genes from poultry and no bla_{SHV} was detected in Algeria.

At the same time, four Carbapenem resistant *Sa*lmonellae were recorded. carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL-producing organisms. Unfortunately, the use of carbapenems has been associated with the emergence of

carbapenem-resistant bacteria species such as salmonellae. Carbapenem-resistant Salmonellae determinants showed that two genotypes of bla_{NDM} and two genotypes of bla_{OXA} were carried on the isolated strains while no determinants are detected in the egg.

The result of ESBL and carbapenem resistance genes in human was revealed that among the total 6 *Salmonella* strains; 5 stains carried the β-lactamase-producing genes, including 3 carried bla_{CTX} and 5 carried bla_{SHV} while no strain positive to carbapenemase. Although in general antimicrobial treatment is not recommended for human salmonellosis, treatment can be necessary in cases of extraintestinal development of the illness, and immunologically compromised. Young and immunocompromised patients are the most susceptible to dangerous complications commonly treated with fluoroquinolones and extended-spectrum cephalosporins, which are widely used in veterinary medicine (Le Hello *et al*., 2011).

The most prevalent forms of ESBL in human are TEM, SHV, and CTX-M. The rates of CTX-M producing bacteria have increased worldwide compared to TEM and SHV over the last decade. This situation is made more complicated as these enzymes confer co-resistance to other drug classes. The bla_{CTX} gene and most antimicrobial resistance genes were found in a large conjugative plasmid. This plasmid has numerous genes that encode heavy metal ion resistance (Cantón and Coque, 2006). It is well known that the location of both metals and antibiotic resistance genes on the same mobile element plays a major role in the survival, selection and spread of antibiotic-resistant bacteria in anthropogenic environments heavily polluted with detergents, heavy metals and other antimicrobials (Laffite *et al*., 2016).

As well as, in developing countries, due to intense unregulated urbanization, rivers, lakes and lagoons are frequently polluted with untreated hospital and industrial effluents and often with urban storm-water containing anthropogenic contaminants. In addition, the release of antibiotics used in human and veterinary medicine into sewage and manure distribution systems can be associated with sewage sludge, contamination of rivers, liquid manure and farm soil (Tello *et al*., 2012).

CONCLUSION

The ESBL and carbapenem resistant genes can be transmitted from humans to animals, and from animals to humans through the food chain. Continuous surveillance of resistance to these "last resort" antibiotics is required to establish possible links between reservoirs and to limit the bidirectional transfer of the encoding genes.

Statement of conflict of interest

The authors have declared no conflict of interest.

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