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Molecular characterization of quinolone resistant salmonellae isolated from poultry.

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A B S T R A C T

In the last few years fluoroquinolone treatment failure has been steadily increased in *Salmonella* spp. infection . Both chromosomal and plasmid-mediated quinolone-resistance mechanisms have been reported. The aim of this study was to identify the prevalence of these mechanisms in a total of 81 *Salmonella* spp. isolates of poultry origin. The antimicrobial susceptibility to nalidixic acid, enrofloxacin, norfloxacin and ciprofloxacin were determined by antibiotic sensivity discs.All isolates showed nalidixic acid resistance while 22.2 %, 56.7 % and 37.3 % of the isolates were resistant to ciprofloxacin, norfloxacin and enrofloxacin respectively.By using PCR techniques *qnrA, qnrB, qnrS* and *qepA* genes were detected in 13.5%, 11.1% , 14.8 % and 7.4% of the isolates respectively while *aac(6)-Ib-cr* gene was absent. DNA sequencing of *gyrA* gene showed substitutions in the A.A Serine 83and Aspartate 87 while *parC* gene showed substitutions in the A.A Serine 57, Serine 80 and Isoleucine 153 . Mutations in the quinolones resistance determination region (*gyrA* and *parC* genes) were critical for fluoroquinolone resistance while the plasmid-mediated quinolone resistance (PMQR) did not seem to play a major role.

Keywords: PMQR, DNA, quinolone, poultry.

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INTRODUCTION

In both human and in veterinary medicine the quinolones and fluoroquinolones considered the widely and extensively used antimicrobial agents for the treatment of bacterial infections. (Kehrenberg *et al.,* 2006, Hopkins *et al.,* 2005).

Salmonella entriditis and S. typhimurium are the main accused microbial agents for food-borne gastroenteritis in human worldwide in the last few years. (Yang *et al.,* 2002).Quinolones destructs bacterial cell through interacting with the complexes formed between the DNA and topoisomerase II or topisomerase IV leading to inhibition of bacterial growth. (Androle 2005) The point mutation in quinolones resistance determinant region (QRDR) is the main mechanism in quinolones resistance especially the mutation in genes encoding topoisomerase II (*gyrA, gyrB*) and/or topoisomerase IV (*parC,parE*) increasing the resistance level of bacteria to the quinolones. (Fabrega *et al.,* 2009) There are a group of genes like *qnr,qep and aac-(6)-lb-cr* which contribute with a pivotal role in reducing the susceptibility of the microorganism to the quiolones named plasmid mediated quinoloes resistance (PMQR) genes .(strahilevitz *et al.,* 2009) .*QnrA* gene was considered as the first plasmid-mediated gene that conferred resistance to quinolones such as nalidixic acid and increased MICs of FQs. *QnrA* was reported in *Klebsiella pneumoniae* in USA. Other groups of *qnr* genes, *qnrB* and *qnrS*, as well as their variants have been reported. (Tran and Jacoby 2002) *Qnr* gene encode to pentapeptide repeat family and mimic DNA fragments bound to the DNA topoisomerases preventing quinolones from binding to DNA topoisomerases.. (Tran and Jacoby 2002).*AAC(6')-Ib-cr,* is aminoglycoside acetyltransferase which is capable for modifying ciprofloxacin and reducing its activity providing low-level of quinolone resistance and facilitating the emergence of higher-level of resistance in the presence of quinolones at therapeutic levels. (Robicsek *et al.,* 2006) *Qep* gene is a plasmid-mediated resistance gene which was associated with over expression or decreased expression of outer membrane porins, contribute to decreased susceptibility to fluoroquinolone (Yamane *et al.,* 2007)

MATERIALS AND METHODS

Bacterial isolates

A total of 81 clinical isolates of different Salmonella strains were recovered during October 2013 to March 2016 from poultry origin in Egypt.

Salmonella serovars	NO	Salmonella serovars	NO	Salmonella serovars	NO
S. Kentucky	19	*S. Infantis*	6	*S. Heidlberg*	1
S. Enteritidis	16	*S. uganda*	3	*S. Tamale*	1
S. Typhimurium	12	*S. Larochelle*	2	*S. Nigeria*	1
S. Virchow	8	*S. Molade*	2	*S. Salami*	1
S. Blegdam	8	*S. Gallinarum*	1	*Total*	81

Bacteriological examination

All collected samples were inoculated in buffer peptone water; the procedures for isolation of *Salmonella* from food and animal feces given in this protocol follow the ISO-6579:2014

Serological identification of *salmonellae*

Typing of *Salmonella* isolates was performed according to ISO- 6579 (2014)

Antibiogram study for *salmonellae*

Antimicrobial agents

Norfloxacin**,** ciprofloxacin, enerofloxacin, and nalidixic acid were employed for inhibition tests. The panel of antibiotic disks (Becton, Dickinson and Company, Maryland, USA) used in panel screens belonged to quinolone class.

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Antimicrobial susceptibility tests

Antimicrobial susceptibility test was assayed by the disc diffusion method on Mueller Hinton agar (Oxoid) plates following the clinical and laboratory standards institute guidelines NCCLS (2008), and Performance standards for antimicrobial susceptibility testing (Twentieth Informational supplement, Gan *et al*., 2011). The zones of inhibition were measured and the manufacturer's instructions were followed to assess resistance or susceptibility. Multi-drug resistance (MDR) isolate is defined as that isolate resistance to two or more antibiotics belonging to different quinolone types. Susceptibility and resistance were determined according to the interpretation criteria to *E. coli* (ATCC No. 25922) established by Clinical Laboratory Standards Institute (CLSI) standard.

Detection of quinolone resistance genes in isolated *salmonella* strains

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH)

Oligonucleotide Primer. Primers used were supplied from **Metabion (Germany)** are listed in table (2).

Table (2): Primers sequences, target genes, amplicon sizes

Gene	Primer (5' to 3')	Temperature (C)	Product size (bp)	Reference
QnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	55	516	Robicsek *et al*.2006
QnrB	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	55	469	
QnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	55	417	
aac(6')-Ib-cr	CCCGCTTTCTCGTAGCA TTAGGCATCACTGCGTCTTC	55	112	Lunn *et al*., 2010
qepA	CGTGTTGCTGGAGTTCTTC CTGCAGGTACTGCGTCATG	59	403	Cattoir *et al*., 2007
gyrA	AAATCTGCCCGTGTCGTTGGT GCCATACCTACTGCGATACC	58	365	Fàbrega *et al*., 2009
parC	AAGCCGGTACAGCGCCGCATC GTGGTGCCGTTCAGCAGG	57	460	

PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of template. The reaction was performed in a Biometra thermal cycler

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1-1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A Gelpilot100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Sequencing of the *gyrA* and *parC* gene:

DNA sequencing for *gyrA* gene was done for 21 representative. PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) and the phylogenetic tree was created by the MegAlign module of Lasergene DNAStar.]

RESULTS

Antimicrobial susceptibility

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Table (2): Antimicrobial susceptibility of Salmonella and the prevalence of PMQR genes

Strain	NO.	Quinolones susceptability testing				Plasmid mediated quinolones resistance genes				
		Nalidixic acid	Norfloxacin	Enrofloxacin	Ciprofloxacin	QnrA	QnrB	QnrS	QepA	aa(6)Ib
S. Kentucky	19	19	13	3	7	4	3	1	1	0
S. Enteritidis	16	16	9	7	8	2	1	4	4	0
S. Typhimurium	12	12	7	3	6	1	0	2	0	0
S. Virchow	8	8	4	1	3	0	1	2	0	0
S. Blegdam	8	8	4	1	1	1	1	0	0	0
S. Infantis	6	6	2	0	1	0	2	2	0	0
S. Ingnada	3	3	2	2	1	0	0	0	0	0
S. Larochelle	2	2	0	0	0	0	0	1	0	0
S. Molade	2	2	2	1	2	1	0	0	1	0
S. Gallinarum	1	1	1	0	0	1	1	0	0	0
S. Heidlberg	1	1	1	0	0	0	0	0	0	0
S. Tamale	1	1	0	0	0	1	0	0	0	0
S. Nigeria	1	1	0	0	1	0	0	0	0	0
S. Salami	1	1	1	0	0	0	0	0	0	0
Total	81	81 (100%)	46 (56.7%)	18 (22.2%)	30 (37%)	11 (13.5%)	9 (11.1%)	12 (14.8%)	6 (7.4%)	0 (0 %)

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Mutation of quniolones resistance determination region(QRDR)

Table (8): Amino acids changes in the *gyrA* and *parc* proteins of *Salmonella* serovars

Strain no.	Accession no.	Serovar	GyrA protein sequance			ParC protein sequance		
			Ser-83-->Phe	Ser-83-->Tyr	Asp87-->Gly	Ser-57-->Thr	Ser-80-->Ile	Glu153-->Gly
1	KX859447	***S. Enteritidis***	NO	NO	NO	NO	NO	NO
10	KX859448	***S. Virchow***	NO	NO	NO	NO	NO	NO
15	KX859449	***S. Typhimurium***	Present	NO	NO	NO	NO	NO
19	KX859450	***S. Kentucky***	NO	NO	NO	Present	NO	NO
26	KX859451	***S. Kentucky***	NO	NO	NO	Present	NO	NO
27	KX859452	***S. Typhimurium***	NO	NO	NO	NO	NO	NO
31	KX859453	***S. Enteritidis***	NO	NO	NO	NO	NO	NO
32	KX859454	***S. Kentucky***	NO	NO	NO	NO	NO	NO
33	KX859455	***S. Blegdam***	Present	NO	Present	NO	Present	NO
37	KX859456	***S. Typhimurium***	Present	NO	NO	NO	Present	NO
44	KX859457	***S. Typhimurium***	Present	NO	NO	NO	NO	NO
45	KX859458	***S. Enteritidis***	Present	NO	NO	NO	NO	NO
49	KX859459	***S. Virchow***	NO	Present	NO	NO	NO	NO
51	KX859460	***S. Kentucky***	Present	NO	Present	NO	Present	NO
54	KX859461	***S. Gallinarum***	NO	NO	Present	NO	NO	NO
55	KX859462	***S. Blegdam***	NO	NO	Present	NO	Present	NO
63	KX859463	***S. Typhimurium***	NO	Present	NO	NO	NO	NO
66	KX859464	***S. Enteritidis***	NO	NO	Present	Present	NO	NO
70	KX859465	***S. Typhimurium***	Present	NO	Present	NO	NO	Present
73	KX859466	***S. Enteritidis***	Present	NO	NO	NO	NO	NO
76	KX859467	***S. Infantis***	Present	NO	Present	NO	NO	NO

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DISSCUSSON

In this study antibiotic susceptibility testing applied for 81 salmonella isolates against nalidixic acid, ciprofloxacin, enrofloxacin and norfloxacin .The result revealed that 100% of the isolates showed resistance to nalidixic acid ,This results agrees with Boscan *et al.* (2007 and Lu *et al.* (2015) with percentages (96.1% and 100%) respectively. The result is higher from that obtained from Soto *et al.* (2003), Souza *et al.* (2010) and Tamang *et al.* (2011) with percentage 22%, 45%and 47.7% respectively.

Karunakaran *et al.* (2014) reported that 24 salmonella isolates of 75 (32%) showed resistance to ciprofloxacin. with percentage 32%. Our result revealed that 37% showed resistance to ciprofloxacin. Which is very close to previously mentioned and higher than that obtained from Lee *et al* (2003), Gopal *et al.* (2014) and Bai *et al.* (2015) with percentage 10.9%, 12.5 % and 8.6 % respectively and lower than that obtained by Yu F *et al.* (2011) and Lu *et al.* (2015) with percentage 64.5 % and 82.7% respectively.

Also the result revealed that 22.2% of isolates showed resistance to enrofloxacin sensivity testing showing 22.2% of isolates showing resistance which was higher than 6.5% that was reported by Lee *et al* (2003) and lower than 65.4 % and 73.1% that gained by Lu *et al.* (2011) and Lu *et al.* (2015) respectively.

Our result revealed that 56.7% of isolates showed resistance to norfloxacin closely enough to 52.5% and 58% which reported with Lee *et al.* (2003) and Boscan *et al.* (2007) respectively and lower than Lu *et al.* (2011) and Lu *et al.* (2015) with percentage 78.9 % and 71.2% respectively.

Our study revealed that the nalidixic acid has the Lion's share in the resistance followed by norfloxacin then ciprofloxacin finally enrofloxacin with percentage 100%, 56.7%, 37% and 22.2% respectively. Strongly agree with Lee *et al* (2003) and Boscan *et al.* (2007).

Fluoroquinolones exert their antibacterial effects by inhibition of certain bacterial topoisomerase enzymes, namely DNA gyrase (bacterial topoisomerase II) and topoisomerase IV. These essential bacterial enzymes alter the topology of double-stranded DNA (dsDNA) within the cell. DNA gyrase and topoisomerase IV are heterotetrameric proteins composed of two subunits, designated A and B. Mechanisms of bacterial resistance to quinolones as described by Hooper (2001). Mutations in the bacterial genes encoding DNA gyrase and topoisomerase IV may confer resistance to quinolones and it has been shown that altered structures of these enzymes prevent binding of quinolones. Michael *et al.* (2006). The study investigates the mutation in quinolones resistance determination region (QRDR) in 21 of salmonella isolates with different degree of resistance.The analysis for the sequence data of the GyrA subunit of DNA gyrase gene by sequencing revealed that three pattern. Substitution of phenylalanine for serine at position 83, Substitution of tyrosine for serine at position 83 and Substitution of glycine for aspartate at position 87. The finding was confirmed by Reyna *et al.* (1995) which was (Ser83Phe) and (Ser83Tyr) also Piddock (2002) (Ser83Phe) and (Asp87-->Gly or Tyr). , also Nair *et al.* (2006) (Ser83-->Phe and(Asp87-->Gly). and Zhang *et al.* (2014) (Ser83Phe/Asp87Gly). The analysis for the sequence data of ParC subunit of topoisomerase IV gene showing three patterns. Substitution of threonine for serine at position 57 agreeing with Ling *et al.* (2003) , Tamang *et al.* (2011) and Bai *et al.* 2015 . Substitution of Isoleucine for serine at position 80 agreeing with Weigel (2002) and Cui *et al.* (2009) differing with Ling *et al.* (2003) ,Eaves *et al.* (2004), Nair *et al.*(2006) and Bai *et al.* (2015) whom results were Substitution of arginine for serine at position 80. Substitution of glycine for glutamate at position 80. This mutation is anovel.Our finding is differing with Piddock *et al.* (1998), Piddock (2002), Hirose *et al.* (2002), Stevenson *et al.* (2007) and Kozoderovic *et al.* (2012) whom results revealed that no mutation in parC reported.

Three major mechanisms of PMQR have been reported in enterobacterial species and associated with the acquisition of *qnr* (topoisomerase protection), *aac(6)-Ib-cr* (quinolone and aminoglycoside acetylation), and/or *qepA* (quinolone efflux pump) genes Poirel *et al.,* (2008).Dissemination of plasmid-mediated quinolone resistance among pathogenic bacteria is a concern for public health because of decreased sensitivity to fluoroquinolones and increased potentials to develop high fluoroquinolone resistance. Akiyama and Khan (2012) .

In an earlier study Lunn *et al*., (2010) and Zhang *et al.* (2014) reported detection of qepA gene with percentage 2.4% and 1.3% respectively.

our study showed that presence of qepA gene in 7.4% of the isolates which was slightly higher than previously reported. While Yu F *et al.* (2011) and lu *et al.* 2015 reported that absence of qepA gene.

Our results revealed that absence of aac(6) lb agreeing with Asai *et al.* (2010) and Velhner *et al.* (2014) and very much lower than that obtained by Yu F *et al.* (2011) and Zhang *et al.* (2014) with percentage 37.1% and 23.2% respectively .

Qnr genes testing revealed that presence of qnrA , qnrB and qnrS genes with percentage 13.5%,11.1% and 14.8% respectively. The results were convergent with whom obtained with and Zhang *et al.* (2014) who reported that presence of qnrA , qnrB and qnrS genes with percentage 11.3%,13.9% and 2.4% respectively while Taguchi *et al.* (2009) and Velhner *et al.* (2014) reported that absence of qnr genes.

CONCLUSION

The wide and extensive use of quinolones and fluoroquinolones antimicrobial agents for the treatment of salmonella infections in both human and in veterinary medicine Reflected on inreasing the Prevalence of resistance in salmonella spp.This study demonstrates the emergence of quinolones resistance in salmonalla strain of avian origin confers that the classical quinolone resistance pathyway (topoisomerase mutations) is very common and considers the man cause for resistance. Also the nonclassical quinolone resistance pathyway (PMQR) is notable but did not seem to play a major role in resistance.

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