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Detection of Some resistance genes of Salmonella enterica subsp. Salamae and Salmonella enterica serotype Kentucky isolated from Turkey

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ABSTRACT

The aim of this study was to determine the serotyping and antimicrobial resistance of isolated Salmonella from the apparently healthy turkey. A total 150 of cloacal samples from apparently healthy turkey were screened bacteriologically for the occurrence of Salmonella. A total of 4% (6/150) of the Salmonella isolates were recovered. Serotyping revealed two different serotypes; *Salmonella enterica* subsp. Salamae (33.33%) and *Salmonella enterica* serotype Kentucky (66.67%). The isolated Salmonella were highly resistant to ampicillin, cefaclor, cefotaxime, ceftazidime, amoxicillin/clavulanic acid (100%) followed by chloramphenicol and ciprofloxacin (83.3%) then gentamicin (66.67%) and azithromycin (33.3%). All isolates showed a high sensitivity for imipenem. All strains are multidrug-resistance (MDR). Polymerase chain reaction (PCR) was applied to Salmonella isolates to detect resistance genes. Antibacterial resistance genes *bla*$_{TEM}$, *bla*$_{OXA}$, *flo*R, *aad*B and *qnr*A were detected in (100%), (0%), (100%), (100%) and (0%) of tested Salmonella respectively. A combination of genotypic and phenotypic markers can be useful in studying genetic variation among salmonellae populations in turkey farms and delineating possible transmission pathways. In conclusion apparently healthy turkeys could be a reservoir for Salmonella resistant to multiple antimicrobials and poses a serious public health threat.

1. INTRODUCTION

Antimicrobial resistance (AMR) is a global health threat, and as well as antimicrobial usage. AMR in animal production is one of its contributing sources. Poultry is one of the most widespread types of meat consumed worldwide (Nhung et al., 2017). *Salmonella* spp. and *Escherichia coli* are the two most important food-borne pathogens of public health interest incriminated in poultry meat worldwide (Adeyanju and Ishola 2014). The emergence and spread of resistant bacteria strain like *Escherichia coli,* salmonella from poultry products to consumers set humans at risk to new strains of bacteria that resist antibiotic treatment. Resistant bacteria inhibit antimicrobials by different mechanisms, as a synthesis of inactivating enzymes, alteration in configuration of the cell wall or ribosome and modification of membrane carrier systems (Apata et al., 2009). The development of antibiotic resistance is usually associated with genetic changes encoded by chromosomal and plasmid genes (Bennet et al., 2008).

Salmonella infection caused by a variety of *Salmonella* species and it is one of the most important bacterial diseases in poultry causing heavy economic losses through high mortality and decrease production (Haidar et al., 2004).

Salmonella isolates from turkeys associated with high levels of antimicrobial resistance. Some studies indicating that, resistance is more frequent in Salmonella isolates from turkeys than in other livestock species. Therefore, Salmonella in turkeys and turkey meat have an impact of great public health significance (Poppe et al., 2005; Zhao et al., 2007).

Salmonella spp. acquire antibiotic resistance by random chromosomal mutations, mutation of existing genes, and through mobile genetic elements, such as plasmids, transposons, and gene cassettes in integrons, which facilitates the acquisition and dissemination of resistance genes. The association of these integrons with plasmids that confer the extended-spectrum b-lactamase phenotype is an example (Fluit and Shmitz, 1999).

The present study was conducted to investigate the prevalence of Salmonella from apparently healthy turkey, the serotypes involved, the antimicrobial susceptibility patterns of Salmonella isolates and the detection of some resistance genes by PCR.

2. MATERIAL AND METHODS

2.1. Sample collection

A total of 150 cloacal samples collected from living apparently healthy turkeys (40 at 35 days old, 110 at 4 months old) from different farm in Gharbia Governate using sterile swabs. Samples were collected under aseptic

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condition as possible to prevent cross contamination in icebox and were then transferred to the laboratory.

2.2. Bacterial isolation and identification of Salmonella

The isolation method was done according to ISO method *(*ISO, 2007*).* This method was based on the pre-enrichment method in buffered peptone water at 37 °C for 18 hours. After overnight incubation, 0.1 ml of the incubated pre-enrichment was transferred to 10 ml of Rappaport-Vassilliadis enrichment broth (Oxoid) and incubated at 42 °C for 24 hours. After incubation, one loop of each selective enrichment broth was streaked onto xylose-lysine-deoxycholate agar (XLD) (Oxoid) and Salmonella-Shigella agar (SS); (Oxoid) at 37 °C for 24 hours. After incubation, colonies were observed. The colony with a black center in XLD and blackish growth in SS agar were considered as presumptive Salmonella positive. The suspected colonies were picked up and kept in semi-solid agar for morphological, biochemical, and serological identification.

2.3. Identification of Bacteria

Suspected colonies were identified using standard microbiological identification techniques including motility test, indole, triple sugar iron test, H2S production test, citrate utilization test, voges–proskauer test, Hydrolysis of urea and Methyl-red test (Cheesbrough, 2000).

2.4. Serological typing of Salmonellae

The isolates that were identified biochemically as Salmonella were subjected to serological identification according to the Kauffmann–White typing scheme (Popoff et al., 2004). The serotyping was applied at the Serology Unit, Animal Health Research Institute, Dokki, Egypt,

2.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility studies were applied according to the guide of Clinical Laboratory Standard Institute (CLSI, 2016) using the Kirby-Bauer disk diffusion method. The obtained bacterial isolates were tested in vitro for their susceptibility to the following antimicrobial discs; chloramphenicol (C) 30µg, azithromycin (AZR) 15µg, cefaclor (CEC) 3µg/disk, ceftzidime 30µg/disk (CAZ), imipenem(IPM) 10µg, ampicillin (AMP) 10µg, amoxicillin-clavulanic (AMC) 30µg , ciprofloxacin (CIP) 5µg , gentamicin (CN) 10µg, According to (Konemann et al., 1997) and the degree of sensitivity was interpreted According to NCCLS (2002) and NCCLS (2016).

2.6. Detection of resistance genes of Salmonella

DNA was extracted from the isolated Salmonella using QIAamp DNA mini kit. It was applied to 5 random isolates. PCR Master Mix and cycling conditions of the primers during PCR were prepared according to Emerald Amp GT PCR master mix (Takara) kit. Oligonucleotide primers used in PCR have specific sequence and amplify a specific product as shown in Table 1. DNA samples were amplified in a total of 25µl as follows: 12.5 µl of Emerald Amp GT PCR master mix, 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water and 6 µl of template DNA. The reaction was performed in a Biometra thermal cycler. The temperature and time conditions of the primers during PCR were applied**.** Aliquots of amplified PCR products were electrophoresed in 1.5 % agarose gel (ABgene) in 1x TBE buffer at room temperature. For gel analysis, 15 µl of PCR products were loaded in each gel slot. A100 bp DNA ladder (QIAGEN Inc, Valencia, CA, USA) was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Table. 1 PCR primers and amplicons size used for the detection of antimicrobial resistance genes

Antimicrobial	Target resistance gene	Primer Sequence (5'-3')	Amplicons size	Reference
CN	*aad*B	F-GAGCGAAATCTGCCGCTCTGG	319 bp	Frana *et al*., (2001)
		R-CTGTTACAACGGACTGGCCGC		
AMP	*bla*$_{TEM}$	F- ATCAGCAATAAACCAGC	516bp	Colom *et al*., (2003)
		R-CCCCGAAGAACGTTTTC		
AMP	*bla*$_{OXA}$	F-ATATCTCTACTGTTGCATCTCC	619 bp	
		R-AAACCCTTCAAACCATCC		
CIP	*qnr*A	F-ATTTCTCACGCCAGGATTTG	516 bp	Robicsek *et al*., (2006)
		R-GATCGGCAAAGGTTAGGTCA		
C	*floR*	F-TTTGGWCCGCTMTCRGAC	494 bp	Doublet *et al*., (2003)
		R-SGAGAARAAGACGAAGAAG		

PCR=Polymerase chain reaction, AMP=Ampicillin,CN=Gentamicin, CIP=Ciprofloxacin, C=Chloramohenicol

3. RESULTS

3.1. Salmonella isolation, identification and serogrouping.

From 150 cloacal samples, 6/150 (4%) Salmonella isolates were isolated. Four isolates belonged to the *Salmonella enterica* serotype Kentucky (66.67%) and two isolates to *Salmonella enterica* subsp. salamae (33.33%).

3.2. Antimicrobial susceptibility of the tested isolates:

Results of antibiotic sensitivity test showed that 100% of tested salmonella isolates exhibited resistance against ampicillin, cefaclor, ceftazidime, amoxicillin /clavulanic acid; 83.3 % for chloramphenicol and ciprofloxacin; 66.67% against gentamicin and 33.33 % against azithromycin. No resistance against imipenem detected.

3.3. Incidence of Antimicrobial Resistance Genes

The β-lactam resistance genes included *bla*$_{TEM}$ was detected (6/6) but *bla*$_{OXA}$ was not detected in this study. Chloramphenicol resistance genes(*flo*R) and gentamicin resistant gene (*aad*B) detected in all isolates of salmonella**.** Resistance gene of ciprofloxacin(*qnr*A*)* was failed for detection as shown in (Figure 1-3). Phenotypic resistance and resistance determinants found in Salmonella isolates were illustrated in table (2).

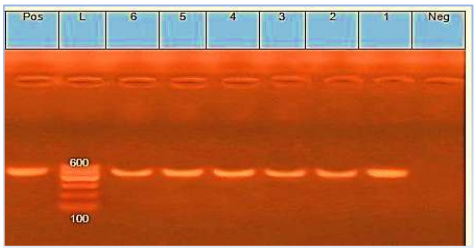

Fig. 1. Agarose gel electrophoresis for amplified PCR product of the B-lactams resistance gene (*bla*TEM) and Aminoglycoside resistance gene (*aad*B) in different Salmonella serotypes. L : DNA ladder 100 - 600 bp. Pos : positive control (tested and confirmed field isolates in R.L.Q.P), Neg: Negative control: Field isolate that were tested and confirmed to be negative by PCR for the related genes in R.L.Q.P lane 1,2,3, 4 : *Salmonella enterica* serotype Kentucky; lane 5,6 :S.Enterica subspp Salamae

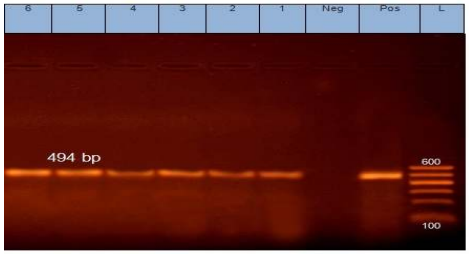

Fig. 2. Agarose gel electrophoresis for amplified PCR product of chloramphenicol resistance gene (*flo*R) in different *Salmonella* serotypes. L : DNA ladder 100 - 600 bp. Pos : positive control (tested and confirmed field isolates in R.L.Q.P).), Neg: Negative control: Field isolate that were tested and confirmed to be negative by PCR for the related genes in R.L.Q.P , lane 1,2,3, 4 : *Salmonella enterica* serotype Kentucky ; lane 5,6 :S.Enterica subspp Salamae

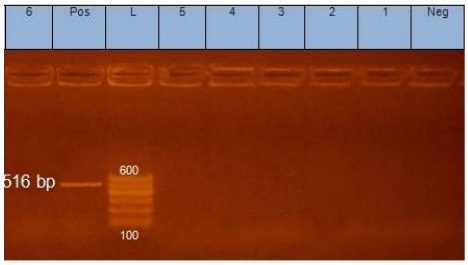

Fig. 3. Agarose gel electrophoresis for amplified PCR product of quinolone resistance genes (qnrS) and (*bla*OXA) in different Salmonella serotypes L : DNA ladder 100 - 600 bp. Pos : positive control (tested and confirmed field isolates in R.L.Q.P).), Neg: Negative control: Field isolate that were tested and confirmed to be negative by PCR for the related genes in R.L.Q.P, lane 1,2,3, 4 : *Salmonella enterica serotype Kentucky* ; lane 5,6 :S.Enterica subspp Salamae

Table 2 Phenotypic resistance and resistance determinants found in *Salmonella* isolates in this study

*S*almonella isolates Sample no.		Resistance phenotype	Resistance genes
1	*Salmonella*	AMP, CTZ, CEC, AMC, CIP, C, CN, AZM	*Bla*TEM, *flo*R, *aad*B
2	*enterica* serotype Kentucky	AMP, CTZ, CEC, AMC, CIP, C, CN	*bla*TEM, *flo*R, *aad*B
3		AMP, CTZ, CEC, AMC, CIP, C	*bla*TEM, *flo*R, *aad*B
4		AMP, CTZ, CEC, AMC, CIP, C, CN, AZM	*bla*TEM, *flo*R, *aad*B
5	S. Enterica subspp. Salamae	AMP, CTZ, CEC, AMC	*bla*TEM, *flo*R, *aad*B
6		AMP, CTZ, CEC, AMC, CIP, C, C	*bla*TEM, *flo*R, *aad*B

AMC-amoxycillin–clavulanic acid, AMP ampicillin, AZM Azithromycin, CEC- Cefaclor CAZ ceftazidime, CRO chloramphenicol, CIP-Ciprofloxacin, CN-Gentamycin

4. DISCUSSION

The incidence of Salmonella in the present study was (4%). These results very close to the results were obtained by Yeh et al. (2017) who isolated 11.9% from a turkey farm. Conversely, this result is lower than that obtained by Fakhr et al. (2006), who detected salmonella by (40.5%).

Salmonella isolates were serotyped using poly and monovalent "O" and"H" antisera and the result of this study revealed that 2 different serogroups were identified as *Salmonella enterica* subsp. Salamae (33.33%) and *Salmonella enterica* serotype Kentucky (66.67%) from turkeys. These results coincide with El Allaoui et al., (2017), who detected *Salmonella enterica* serotype Kentucky as the most prevalent serotype; Santos et al., (2007), who reported that *Salmonella enterica* serotype Kentucky was the most prevalent serotype.

Multidrug-resistant (MDR) due to Salmonella is known as a major public health problem around the world and there is increased use of antibiotics in human and animal settings (Hsu et al., 2013).

In the present study all isolated strains were resistant to at least four or more of the used antibiotics Among antibiogram, all isolated salmonella were resistant to ampicillin, cefaclor, ceftazidime, amoxicillin-clavulanic with 100% followed by chloramphenicol and ciprofloxacin with 83.3% then gentamicin with 66.67% and azithromycin with 33.33 % . Meanwhile, 100% of tested *Salmonella* isolate showed sensitivity against imipenem. Similar results were obtained by Beutlich et al. (2010) for ampicillin (82%) and gentamicin (78%); Yeh et al. (2017) for chloramphenicol (69.1%); Gad et al. (2018) for amoxicillin/clavulanic (96%) and cephalothin (81%). Conversely, these results disagreed with Yeh et al. (2017) for ciprofloxacin (0.8%) with Santos et al. (2007) for ampicillin, Fakhr et al. (2006) for gentamicin and Nisar et al. (2017) for ciprofloxacin and azithromycin(0%) for each.

The expanded use of antibiotics as supplements for growth promotion and prophylaxis and has advanced the selection of antimicrobial-resistant *Salmonella* strains at the farm during poultry production. Since salmonellosis is primarily transmitted through food, especially food of animal origin, the presence of antimicrobial resistant Salmonella in raw meat products has important public health hazard especially in developing countries, where there is widespread and uncontrolled use of antibiotics (Hart et al., 1998).

PCR has emerged as a highly sensitive and specific method for identifying pathogens (Lim et al., 2004). In this study, none of the examined samples harbored bla_{OXA}, *qnrA* while bla_{TEM} , *aadB* and *floR* detected in all isolates. This result agreed with Beutlich et al., (2010), who detected bla_{TEM}, *aadB* and bla_{OXA} by 100%; 98% and 0% respectively. Similar results were conducted by Yeh et al., (2017), who detected *flo*R gene and blaTEM with 63.8% and 42% respectively.

5. CONCULOSION

The current study revealed that the incidence of multidrug resistant Salmonella spp. in the cloacal swab samples of apparently healthy turkey flock could be a threat to public health. The results reinforce the need to develop monitoring strategies and to perform specific control procedure to reduce the use of antibiotics and subsequently the development of antimicrobial resistance by misuse /over of antibiotic agents.

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