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Genotypic characterization of multi drug resistant *E. coli* and *Salmonella* isolated from virally infected broilers

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ABSTRACT

Genotypic characterization of the multi-drug resistant (MDR) circulating bacterial pathogens among small broilers flocks helps in obtaining a clear picture of the MDR dilemma in the animal care sector and its impact on the safety of human and environment. The current study used ten *E. coli* and five *salmonella* isolates obtained from virally infected small broilers flocks at Giza and El Qalyubia province, Egypt in a previous study. All isolates were MDR against at least seven different antimicrobial agents. Polymerase chain reaction (PCR) was used to determine the prevalence of a pool resistance genes including antimicrobial and disinfectant resistance, and mobile genetic element genes (bla_{TEM}, bla_{SHV}, $bla_{OXA,}$ *aada1*, *qacEΔ1* and *intI1*) within the bacterial isolates. The results of inspection of bla_{TEM}, bla_{SHV}, bla_{OXA} genes in *E. coli* & *salmonella* isolates revealed 100%/80%, 90%/100%, 0%/0% prevalence in *E. coli*, *salmonella* isolates respectively. While the *aada1* gene was detected in 100% of *E. coli* and *salmonella* isolates also the *qacEΔ1* gene was detected in all the bacterial isolates which is considered as one of the quaternary ammonium compounds ((QACs)) resistance genes that is usually detected among the *Enterobacteriaceae* family members). Finally, *intI1* gene was detected in of *E. coli* and *salmonella* isolates as followed 100%, 80% respectively. These findings clearly showed that bla_{TEM}, $bla_{SHV,}$ *aada1*, *qacEΔ1*and *intI1* are highly prevalent among the MDR bacteria in Egypt.

1. INTRODUCTION

A Greek word consisting of three syllables anti means "against", mikros equal to "little" and bios meaning "life" developed into "anti-microbial" (Guardabassi *et al*., 2006). Used to describe any natural, synthetic, or semisynthetic substance that either inhibits bacterial growth (bacteriostatic) or kills the bacteria (bactericidal) without harm to the host cell (Demurtas and Perry, 2014).

Antimicrobial agents are widely used to control bacterial diseases since its first discovery, but now they are challenged by the antimicrobial resistance (the bacterial ability to survive and/or grow in the presence of antimicrobial agent that would normally kill or inhibit bacterial growth (Harbottle *et al*., 2006)). Antimicrobial resistance is usually explained by Genetic flexibility of bacteria allowing it to adapt, evolve and survive in their environment by developing different resistance mechanisms (Acar *et al*., 2012). Genotypic characterization of the bacterial pathogens is the corner stone in understanding the MDR pattern of the resistant bacteria.

Beta-lactam agents (e.g., Penicillin, cephalosporin and carbapenemases) are bactericidal by crossing bacterial outer membrane reaching their penicillin-binding protein targets to kill the bacterium cell (Alexander *et al*., 2009). The beta-lactam resistance through beta-lactamases enzymes (carbapenemases) which hydrolyze beta lactam rings into peri-plasmic fluid and beta-amino acids with no antimicrobial activity. These enzymes are encoded in several genes. The commonest genes responsible for beta lactam resistance in *Enterobacteriaceae* strains are bla_{TEM}, bla_{OXA} and bla_{SHV}.

Aminoglycosides have a bactericidal effect through protein synthesis inhibition. It is used alone or combined with other antibiotics especially β-lactams ensuring a broad-spectrum action against Gram-negative bacilli infections (Graham and Gould, 2002). It includes streptomycin gentamicin, neomycin, amikacin, and kanamycin (Gonzales and Spencer, 1998). Three genes control the genetic background for streptomycin resistance: *strA*, *strB* and *aada1*. The *aada1* gene encodes aminoglycoside adenyl transferase enzyme that inhibits the action of streptomycin, and it is found among *Enterobacteriaceae* strains.

Quaternary ammonium compounds (QAC) are active cationic surface detergents, non-corrosive, and non-irritating agents, with little toxicity and a high antimicrobial efficacy over a wide pH range, they are commonly used to control different microorganisms in the environment especially clinical facilities or industrial ones as well as surfaces disinfection (Ioannou *et al.*, 2007).

The last line of defense against microorganism in poultry production could be the use of disinfectants especially QACs that are frequently used in environments where antibiotics are used, this increases the concern of a

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relationship between QAC resistance and antibiotic resistance (Hegstad *et al*., 2010). Five QAC resistance genes [*qacE*, *qacEΔ1*, *qacF*, *qacG* and *sugE(p)*] are distinguished among Gram-negative bacteria (Kucken *et al*., 2000).

Gram-negative bacteria usually encode QAC resistance genes with b-lactam, aminoglycosides, chloramphenicol, sulfonamides, and trimethoprim resistance genes (Zhao *et al*., 2012). This close genetic relation between the antiseptic and antimicrobial resistance genes leads to the antiseptic resistant microorganism is less susceptible to antibiotic drug; this genetic relation and change in Outer membrane have been believed to be one of the mechanisms responsible for such increased non-specific cross-resistance (Russell *et al*., 1999).

Multidrug resistant (MDR) bacteria increase every day as a result of genetic mutation and antibiotic resistance genes transfer through vertical or horizontal transmission, by plasmids, transposons, bacteriophages and integron (mobile genetic elements). Integron was first reported in 1989 (Stokes and Hall, 1989). Integron characterized by the existence of an integrase gene (intI) and a proximal primary recombination site (attI) (Xu *et al*., 2011). The sequences of amino acid integrases (*IntI*) used for the classification of integrons into 'classes' as following: integrons that carries *intI1* defined as 'class 1', *intI2* as 'class 2', *intI3* as 'class 3', etc.

Class 1 integron was detected in 22 to 59% of the gram-negative bacteria of clinical importance, including *Acinetobacter, Aeromonas, Campylobacter, Citrobacter, Enterobacter, Escherichia, Klebsiella, Mycobacterium, Providencia, Pseudomonas, Salmonella, Serratia, Shigella, Stenotrophomonas*, and *Vibrio* (Xu *et al*., 2011).

Integrons class 1 usually associated with the resistance genes against most classes of antibiotics including all known β-lactams, all aminoglycosides, chloramphenicol, streptomycin, trimethoprim, Fosfomycin, lincomycin, rifampin, erythromycin, quinolones, and antiseptics of the quaternary ammonium-compound family (Cambray *et al*., 2010).

The significance of study is to understand the genetic background of the MDR bacteria that was isolated from virally infected flocks in Egypt.

2. MATERIAL AND METHODS

2.1. Samples:

Ten *E. coli* isolates and five *salmonella* isolates were isolated from small poultry flocks (small bird population varying from few hundreds up to few thousands) suffering from viral avian disease with high MDR pattern used in the study.

2.2. DNA extraction:

Total 15 bacterial isolates (10 *E. coli* and 5 *salmonella*) were harvested and extracted using QIAamp DNA Mini Kit (Catalogue no. 51304) in accordance with manufacture instruction after incubation with 10 µl of proteinase K and 200 µl of lysis buffer at 56 °C for 10 min.

2.3. Detection of β-lactam, streptomycin, quaternary ammonium compounds resistance and integron class I genes by (polymerase chain reaction) PCR:

A single PCR reaction was performed to amplify the following genes: *bla$_{TEM}$*, *bla$_{SHV}$*, *Bla$_{OXA-1}$*, *aada1*, *qacEΔ1*and *intI1*. Each PCR reaction was conducted in a 25 µl reaction mixture containing 12.5 µl of Emerald Amp GT, 5 µl of DNA template, PCR Master Mix (TAKARA BIO INC.™, Japan), 1 µl of reverse and forward primer each (20 pmol), and 5.5 µl of PCR grade water. The primers sequences, annealing temperatures, and size of the PCR product for the examined genes shown in table (1).

The reaction was done with applied bio system 2720 thermal cycler. Primary denaturation step done at 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec., 54°C for 40 sec for all genes except *intI1* was at 50 ˚C and *qacEΔ1* was at 58°C for 30 sec and 72°C for 45 sec. min. A final extension step was done at 72°C for 10 min. the results of the PCR are viewed by electrophoresis using 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature by gradients of 5V/cm. For gel analysis, 15 µl of the products loaded in each gel slot. The fragment sizes determined by gene ruler 100 bp DNA ladder (Fermentas, Thermo). The gel photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed using computer software (automatic image capture protein simple formerly cell bioscience, USA).

Table 1 The primers used in the genotypic characterization of the bacterial isolates

Target	Primer Sequence	Annealing	Amplified product	Reference
bla$_{TEM}$	F: ATCAGCAATAAACCAGC	54˚C	516 bp	Colom *et al*., 2003
	R: CCCCGAAGAACGTTTTC			
bla$_{SHV}$	F: AGGATTGACTGCCTTTTTG	54˚C	392 bp	Colom *et al*., 2003
	R: ATTTGCTGATTTCGCTCG			
bla$_{OXA}$	F: ATATCTCTACTGTTGCATCTCC	54˚C	619 bp	Colom *et al*., 2003
	R: AAACCCTTCAAACCATCC			
aada1	F: TATCAGAGGTAGTTGGCGTCAT	54˚C	484 bp	Randall *et al.* 2004
	R: GTTCCATAGCGTTAAGGTTTCATT			
intI1	F: CCTCCCGCACGATGATC	50 ˚C	280	Kashif *et al*., 2013
	R: TCCACGCATCGTCAGGC			
qacEΔ1	F: TAAGCCCTACACAAATTGGGAGATAT	58˚C	362	Chuanchuen *et al*., 2007
	R: GCCTCCGCAGCGACTTCCACG			

3. RESULTS

3.1. Prevalence of β-lactam resistance genes in E. coli and salmonella isolates isolated from virally infected broilers

All the E. coli and Salmonella isolates screened for the presence of β-lactam resistance (ESBLs) genes, bla$_{TEM}$, bla$_{SHV}$, and bla$_{OXA}$ were lacking bla$_{OXA}$ in any isolate, while bla$_{TEM}$ and bla$_{SHV}$ were found in nearly all the E. coli & salmonella isolates (Table 2).

Table 2 Detection of ESBLs resistance genes (*bla$_{TEM}$*, *bla$_{SHV}$* and *bla$_{OXA}$*) in *E. coli* and *salmonella* isolates.

Inspected gene	*E. coli* isolates		*salmonella* isolates	
	Positive/total	Percentage	Positive/total	Percentage
bla$_{TEM}$	10/10	100%	4/5	80%
bla$_{SHV}$	9/10	90%	5/5	100%
bla$_{OXA}$	Zero/10	Zero%	Zero/5	Zero%

3.2. Prevalence of aada1 (aminoglycosides resistance genes) in E. coli and salmonella isolates isolated from virally infected broilers:

There is an intimate relation between the ESBLs resistance genes and aminoglycosides resistance genes. Screening the

E. coli and *salmonella* isolates for presence of aada1 gene using PCR showed that aada1 was found in all the isolates (Table 3).

3.3. Prevalence of qacEΔ1 (quaternary ammonium compounds resistance) gene in E. coli and salmonella isolates isolated from virally infected broilers:

The quaternary ammonium compounds resistance genes are claimed to be in close relation with the MDR bacteria. In this study qacEΔ1 gene was confirmed in all isolates (Table 3).

3.4. Detection of integron class I gene (genetic mobile element) in E. coli and salmonella isolates isolated from virally infected broilers:

Detection of integron class I in the bacterial isolates under investigation by PCR revealed presence of intI1 in all isolates (Table 3).

Table 3 Result of prevalence of *aada1, qacEΔ1, and intI1* genes in *E. coli* and *salmonella* isolates.

inspected gene	*E. coli* isolates		*salmonella* isolates	
	Positive/total	percentage	Positive/total	percentage
aada1	10/10	100%	5/5	100%
qacEΔ1	10/10	100%	5/5	100%
intI1	10/10	100%	5/5	100%

4. DISUCCUSION

MDR bacteria is a serious problem not only for animal care sector, but also for human and environment. This triangle forms a closed circle for the bacterial existence. Normally proper disinfectant or antimicrobial agents kills or stop the bacterial growth. But MDR bacteria not affected by disinfectant or antimicrobial agents.

Genotypic characterization of the MRA bacteria is an important step in understanding this dilemma to reach a proper strategy for controlling this phenomenon and preventing its drawbacks.

Broad-spectrum beta lactams are widely used leading to induction of ESBLs production in bacteria in general especially *Enterobacteriaceae* by mutations in TEM (Temoneira) and SHV (sulfhydryl variable) genes (common plasmid mediates beta-lactams).

In this study, screening for β-lactam resistance genes as one of most common genes responsible for extended spectrum β-lactamases (ESBLs) in *E. coli* isolates revealed that bla_{TEM} gene founded in 100% of *E. coli* isolates agreeing with (Hardiati *et al*., 2021) who found the same percent after inspecting *E. coli* isolates from broilers farms in Sukabumi, Indonesia. In addition, Saad *et al*. (2019), who found that bla_{TEM} is the most predominant gene with a 100% detection rate in E. coli isolates isolated from chickens after a similar study here in Egypt.

The study also screened the *E. coli* isolates for other (ESBLs) genes: bla_{SHV} and bla_{OXA} revealing that bla_{SHV} found in 90% of them agreeing with (Mooljuntee *et al*., 2010). Who detected this high prevalence of bla_{SHV} in *E. coli* isolated from Thai broilers and disagreeing with (Saad *et al*., 2019) who detected bla_{SHV} in 22% E. coli isolates from different farms in Aswan governorate, Egypt. While bla_{OXA} was not detected in any isolates agreeing with Saad *et al*. (2019), who found that bla_{OXA} have the lowest prevalence among *E. coli* isolates isolated from different broilers farms in Aswan governorate.

This study also screened the salmonella isolates for encoding (ESBLs) genes revealing that 80% of them have bla_{TEM} gene agreeing with Alam *et al*. (2020) results of detecting bla_{TEM} in (82.85%) of MDR salmonella isolated from broiler chicken farms in Bangladesh. While the screening of bla_{SHV} and bla_{OXA} genes revealed that all isolates encode bla_{SHV} gene the results harmonize with Rady *et al*. (2020) results of detecting bla_{SHV} in all the salmonella isolates that were isolated from clinically affected broiler in Egypt. On the other hand, bla_{OXA} was not detected in any isolates revealing that bla_{OXA} is not encoded in the circulating pathogen in this area.

A further investigation on the MDR isolates is screening for *aada1* gene as one of the genes correlated with the resistance to aminoglycoside especially streptomycin, that are usually used with beta lactams antimicrobial agents in the broiler's farms. The results demonstrate that all isolates (*E. coli and salmonella*) encode *aada1* gene going along with findings of Enany *et al.* (2018) and disagreeing partially with Momtaz *et al*. (2012) findings, who did not detect *aada1* gene in *E. coli* from slaughtered broilers in Iran.

The intimate relation between the MDR and disinfectant resistance drove the necessity to inspect the isolates for encoding *qacEΔ1* as one of the genes responsible for quaternary ammonium compounds resistance and widely spread in Gram-negative bacteria, primarily in Enterobacteriaceae. The results showed that all the isolates (*E. coli and salmonella*) encode *qacEΔ1* gene emphasize the close relation between the drug resistance and disinfectant resistance agreeing with Enany *et al*. (2019) results after screening *E. coli* isolates isolated from diseased broiler chickens and environmental sources at large-scale poultry farms in Ismailia Governorate, Egypt. And (Yang *et al*., 2020) who found a close association between the ESBL resistance genes and *qacEΔ1*in both *E. coli* and *salmonella* isolates.

This genetic profile of encoding ESBL resistance genes (bla_{TEM} and bla_{SHV}) and aminoglycoside resistance gene *(aada1)* as well as quaternary ammonium compounds resistance gene (*qacEΔ1*), made it essential to inspect the isolates for encoding *intI1*as the gene responsible for presence of integron class 1. Usually, integron contribute to the spread of antimicrobial resistance and disinfectant resistance by gene transfer in a diversity of enteric bacteria (Maynard *et al*., 2003). The results showed that all isolates encode *intI1* gene, agreeing with the previous findings of Ali and Mohamed (2020), who find that all *salmonella* isolates which encodes *qacEΔ1* also encodes *intI1* gene.

These results signify that: the *E. coli* and *salmonella* circulating among the broiler farms harbor ESBL resistance genes (bla_{TEM} and bla_{SHV}) while bla_{OXA} is not encoded in these local isolates. They also encode aminoglycoside resistance gene *(aada1)* and quaternary ammonium compounds resistance gene (*qacEΔ1*), as well as *intI1* gene that will be further investigated to determine if these genes are encoded in its gene cassette.

5. CONCLUSION

Based on these findings, *E. coli* and *salmonella* with high level of drug resistance are credited to presence of pool of antibiotic- resistance genes and disinfectant resistance as well as mobile genetic element that may associate in the vertical dissemination of MDR and disinfectant resistance here in Egypt.

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