P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2020.033

Phenotypic and Genotypic Characteristics of Antimicrobial and Disinfectant Resistance of Gram-negative Bacteria Involved in Early Broiler Chick Mortality

Mohammed Iraqi[1], Soad A Nasef[1] and Mona El-Enbaawy[2]

[1]Animal Health Research Institute, Dokki, Egypt; [2]Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt
***Corresponding author:** dr_mona_1@hotmail.com

Article History: 20-213 Received: 08-Oct-20 Revised: 08-Dec-20 Accepted: 17-Dec-20

ABSTRACT

One of the most challenges facing the poultry industry in Egypt is the early mortality among broiler chicks during the first ten days of the rearing cycle, especially which caused by antimicrobial resistant Gram-negative bacteria .This study was conducted for the determination of the prevalence of Gram-negative bacteria involved in early mortality among broiler chicks. Antibiogram profile and antibiotic and disinfectant resistance genes were also performed. Five hundred samples (liver, yolk sac, cecum, spleen, and heart) from freshly dead affected chicks were cultured on different media for isolation of causative agents.by conventional and serological methods. PCR was used for detection of resistance genes. The Bacteriological examination revealed the presence of *Salmonella* spp., *E. coli*, and *P. aeruginosa* in the percentages of 23, 25 and 8%, respectively. Single and mixed infections were observed as 41, and 7%, respectively. 86.9% of *Salmonella* serovars were resistant to colistin sulphate, 48% of *E. coli* strains showed resistance against norfloxacin, and 87.5% of *P. aeruginosa* showed resistance against florfenicol. The *mcr1* gene was found in 86.9% of all *Salmonella* serovar, *qnrS* gene was detected in 16% of *E. coli*, and *floR* gene was present in 100% of *P. aeruginosa* isolates. PCR screening for *qacED1* revealed that all bacterial isolates under test were positive. It was concluded that results of current study assert the existence *mcr1*, *qnrS*, *floR*, and *qacED1* genes among (*Salmonella* spp., *E. coli*, and *P. aeruginosa*) which were isolated from early aged broiler dead chicks; that represents a high risk on the poultry industry in Egypt.

Key words: Dead broiler chicks *Salmonella serovars*, *E. coli serotypes*, *P. aeruginosa*, *mcr1*, *qnrS*, *floR*, *qacED1*.

INTRODUCTION

Early chick mortality is one of the most vital problems of the chicken industry. Flocks of poultry are often reared under strong hygienic environments with elevated levels of antimicrobials for disease prevention and diagnosis, as well as boosting growth. Pathogens which are resistant to antimicrobials can lead to treatment failure, this results in economic losses (Nhung et al. 2017). Infections due to antibiotic-resistant microorganisms are hard, and now and again not possible to treat and can cause mortality (Alaali and Thani 2020).

Omphalitis and yolk sac infection were considered as the major causes for the high mortalities during the first week of life in broiler farms. Several bacteria agents have been isolated and identified such as *E. coli*, *Salmonella*, *Staphylococcus*, *Proteus*, and *Pseudomonas* (Hassan et al. 2017). A widespread of antibiotics' resistance in *Salmonella* is of great concern (Cailhol et al. 2006; Dorgham et al. 2019). From day-old chicks and eggs, different percentages of bacterial (*E. coli* and *Pseudomonas* spp.) strains showed resistance against different classes of antibiotics (Nazer et al. 2006). Multidrug resistance *P. aeruginosa* is highly pathogenic to chicks of 1-10 days old (Kebede 2010).

Colistin represents one of the few available drugs for treating infections caused by resistant Enterobacteriaceae. As such, the spread of the colistin resistance gene mcr-1 poses a significant public health threat, requiring global monitoring and surveillance (Wang et al. 2018). Fluoroquinolones are considered as one of the most frequently applied antimicrobials' groups, particularly in poultry. Resistance towards fluoroquinolones came about because of the effects of some genes such as *gyr*A and *qnr*S which hinder the synthesis of bacterial DNA (Talukdar et al. 2013). Florfenicol is a by-product of

Cite This Article as: Iraqi M, Nasef SA and El-Enbaawy M, 2021. Phenotypic and genotypic characteristics of antimicrobial and disinfectant resistance gram-negative bacteria involved in early broiler chick mortality. International Journal of Veterinary Science 10(2): 129-134. https://doi.org/10.47278/journal.ijvs/2020.033

chloramphenicol which is prescribed mainly for the treatment of animal illnesses. the main gene that induces florfenicol resistance is floR which can distribute between bacteria of the same and specific species or genera via a horizontal gene spread (Lu et al. 2018).

The presence of bacterial resistance against many drugs emphasizes the need to prevent diseases in chicks by the hygiene. The remaining line of protection for the chicken production must probably be the usage of disinfectants as Quaternary Ammonium Compounds which is probably regularly utilized in environments where antibiotics are used (Hegstad et al. 2010). Many reviews reported a molecular relationship between QACs genes and antibiotic resistance in some pathogenic bacteria (Buffet-Bataillon et al. 2012).

Here we focus on the prevalence of antimicrobial-and disinfectant resistant-Gram-negative bacteria involved in early mortality in broiler chicks. Antibiogram profile and antibiotic and disinfectant resistance genes were also performed.

MATERIALS AND METHODS

A whole of five hundred samples (liver, yolk sac, cecum, spleen, and heart) 5 from each farm was collected aseptically. These samples were from 100 broiler farms suffered from weakness, depression, diarrhea, respiratory manifestation and omphalitis, during the first 10 days of the rearing period.

Microbiological Examination

One gram from each sample was inoculated in a tube containing 9ml of 1% buffered peptone water. and incubated at 37°C for 18-24 hours. The incubated samples were inoculated onto MacConkey agar plates. Then developed colonies depended on macroscopic and microscopic appearance, were subcultured on appropriate differential media.

Expected *Salmonella. P. aeruginosa* and *E. coli* colonies were streaked on brilliant green agar and xylose-lysine-deoxycholate (XLD) agar (Oxoid, Pseudomonas cetrimide agar medium (Oxoid) and E.M.B agar plates, respectively. And then aerobically incubated at 37°C for 18-24hrs. Suspected colonies were subjected to biochemical analysis as described by ISO 6579-1 (2007) and Quinn et al. (2013).

The biochemically identified *Salmonella* isolates were subjected to serological identification according to Grimont and Weill (2007). *E. coli* strains were serotyped by the usage of rapid diagnostic *E. coli* antisera Set 1 containing monovalent and polyvalent O antisera (Denka Seiken Company, LTD-Japan).

Antimicrobial Susceptibility Testing

An antimicrobial sensitivity testing was achieved by the disc diffusion technique, in steps with the standards and results interpretation defined with the aid of the National Committee for Clinical Laboratory Standards (NCCLS 2018). Eight antimicrobials discs (Oxoid, Hampshire, U okay) represented to six antimicrobial groups have been used as shown in Table 3.

PCR for Resistance Genes Detection

The DNA extraction from samples has been completed through the usage of the QIA-amp DNA Mini kit (Qiagen, GmbH, Germany). Oligonucleotide primers have specific sequences (Metabion, Germany), targeted genes and their amplified fragment sizes are shown in Table 1. These primers were applied in a 25µl reaction including 12.5µl of Emerald Amp Max PCR Mastermix (Takara, Japan), 1µl of separate primer of 20 picomole concentrations, 4.5µl of water and 6µl of the template DNA. Cycle conditions for different primers are shown in Table 2. In a Biometra thermal cycler, the PCR outcomes had been separated by way of electrophoresis in 1.5% agarose gel (ABgene). One hundred base pair and 100-600 base pair deoxyribonucleic-acid ladders (Qiagen, USA) decide the fragment sizes were used. The gel became pictured with the aid of a documentation device and the records became stored via a computer software program.

Statistical Analysis

Statistics evaluation had been carried out using Statistical Package for Social Sciences edition 22 for windows. A Chi-square test has been used to determine the presence of an association between antimicrobial resistance and the presence of resistance genes at $P \leq 0.05$ (IBM Corp. Released 2013).

RESULTS

Microbiological Examination and the Incidence of Bacterial Isolation

As shown in Table 3, the microbiological examination of the collected samples revealed Gram-negative bacteria (*Salmonella*, *E. coli* and *P. aeruginosa*) with different percentages.

Antibiogram Pattern of Isolated Gram-Negative Bacteria

Table 3 clarified the antibiogram pattern of Gram-negative bacteria isolates against 8 antibiotics related to 6 antibiotic groups.

PCR Results for Detection of Antibiotics and Quaternary Ammonium Compounds Resistance Genes

Genotypically, Table 4 and Fig. 1-6 showed the results of detection of resistance genes (*mcr1, qnrS*, and *floR*) in *Salmonella*, *E. coli*, and *P. aeruginosa* isolates, respectively as well as *qacED1* for all isolates under test.

DISCUSSION

Excessive use of antibiotics and disinfectants would lead to the elevation of antibiotic and disinfectant resistance (Eid et al. 2016). In order to study the phenotypic and genotypic characterization of some antibiotic-resistant Gram-negative bacteria responsible for early mortality in broiler chicks, this work was designated. In the present study the isolated bacteria were *Salmonella*, *E. coli*, and *P. aeruginosa* with 23, 25 and 8%, respectively. In Egypt, many studies (EL-Sawah et al. 2016; El-Tawab et al. 2017; Saad et al. 2017; Ibrahim et al. 2019) considered *E. coli* as the most common

Table 1: Oligonucleotide primers sequences

Isolates	Target gene	Sequences (5′ → 3′)	Amplified product size	References
Salmonella, E. coli, and P. aeruginosa	*Qac-ED1*	F: TAA GCC CTA CACAAA TTG GGA GAT AT R: GCC TCC GCA GCG ACT TCCACG	362 bp	Chuanchuen et al. (2007)
Salmonella Serovars	*mcr1*	F: CGGTCAGTCCGTTTGTTC R: CTTGGTCGGTCTGTAGGG	308 bp	Newton-Foot et al. (2017)
E. coli serotypes	*qnrS*	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	516 bp	(Robicsek et al. (2006)
P. aeruginosa	*floR*	F: TTTGGWCCGCTMTCRGAC R: SGAGAARAAGACGAAGAAG	494 bp	Doublet et al. (2003)

Table 2: Cycling conditions of the different primers

Isolates	Target gene	1ry denaturation	2ry denaturation	Annealing	Extension	No. of cycles	Final extension
Salmonella, E. coli, & P. aeruginosa	*QacED1*	94˚C 5min	94˚C 30s	58˚C 40s	72˚C 40s	35	72˚C 10min
Salmonella Serovars	*mcr1*	94˚C 5min	94˚C 30s	60˚C 30s	72˚C 30s	35	72˚C 7min
E. coli serotypes	*qnrS*	94˚C 5min	94˚C 30s	55˚C 40s	72˚C 45s	35	72˚C 10min
P. aeruginosa	*floR*	94˚C 5min	94˚C 30s	50˚C 40s	72˚C 45s	35	72˚C 10min

Table 3: Antibiogram sensitivity and resistance pattern of isolated Gram-negative bacteria

Antimicrobial group	Antimicrobial discs	*Salmonella serovars*		*E. coli*		*P. aeruginosa*	
		S %	R %	S %	R %	S %	R %
Aminopenicillins	AMC 30	91.3	4.3	76	12	50.0	25.0
	AMP 10	34.8	65.2	4	80	12.5	87.5
Aminoglycosides	S 10	34.8	13.0	12	48	37.5	25.0
	N 30	43.5	43.5	8	56	37.5	50.0
Polypeptide Abcs	CT 10	13.1	86.9	20	80	62.5	37.5
Florfenicol	FFO 30	78.3	21.7	48	52	12.5	87.5
Fluoroquinolones	NOR 10	65.2	26.1	44	48	75.0	12.5
Tetracyclines	DO 30	47.8	47.8	24	48	12.5	62.5

Delete % from all values: (Amoxicillin-clavulanic acid) AMP 10 (Ampicillin), S 10 (Streptomycin), N 30 (Neomycin), CT 10 (Colistin sulphate), FFO 30 (Florfenicol), NOR 10 (Norfloxacin), DO 30 (Doxycycline), S=Sensitive, R=Resistance.

Table 4: Distribution of antibiotics and QACs resistance genes by PCR

Target genes	*Salmonella* +*ve serovars*		*E. coli* +ve serotypes		+ve *P. aeruginosa*	
	No	%	No	%	No	%
QacED1	23/23	100	25/25	100	8/8	100
mcr1	20/23	86.9				
qnrS			4/25	16		
floR					8/8	100

contaminant of chicken yolk sacs. Other isolates from yolk sac were *P. aeruginosa*, *Escherichia coli*, *Salmonella* spp., *Coagulase Negative staphylococci* (CNS), *S. aureus*, *P. mirabilis*, *K. pneumonia*, *Streptococcus* spp. and *E. aerogenes.* On the other hand, Shahjada et al. (2017) isolated *E. coli*, *Salmonella*, and *Staphylococcus* with percentages of 28, 38 and 34%, respectively from broiler chicks suffered from omphalitis in Bangladesh.

Amare et al. (2013) find *E. coli* with the highest percentage followed by *S. aureus* and *P. mirabilis* from chicks in Ethiopia. On the other hand, Shahat et al. (2019) reported that the incidence of *Pseudomonas* species in broiler chicks (1-10 days) suffered from diarrhea, yellowish nasal secretion, ruffled feather, and conjunctivitis from different farms was 42%. Single and mixed infections were observed as the most predominant single infections were in *E. coli* (19%), followed by *Salmonella* (16%). The most predominant mixed infections were *Salmonella* and *E. coli* (5%). Other mixed infections were noticed in low percentages; *E. coli* and *Salmonella* in (1%) and *P. aeruginosa, E. coli,* and *Salmonella* (1%). EL-Sawah et al. (2016) stated that the diseased chicks, harbored bacterial strains with an incidence of 62.5 and 28.2% as single and mixed infections, respectively.

In the present study, the serotyping of *E. coli* isolates recovered from the dead chicks revealed 12 *E. coli* serotypes with the highest percentage for O78 (24%). Followed by O2, and O158 (each of 12%) and O128 and O1 were (each of 8%). On the other hand, the lowest serotypes percent were for O145, O144, O125, O119, O26, and O146 (each of 4%). Serotypes O78, and O2 constitute 80% of sickness-inflicting avian pathogenic *E. coli* (APEC) globally (Dziva and Stevens 2008). *E. coli* O146, O2 and O26 were recorded by EL-Sawah et al. (2016), Saad et al. (2017) and Ibrahim et al. (2019) as serogroups recovered from chicks suffered from omphalitis and newly hatched chicks in Egypt.

In our study, out of 23 *Salmonella* isolates recovered from diseased chicks *S.* Enteritidis possessed the highest percentage of isolation followed by *Salmonella Kentucky* and *Salmonella Typhimurium*. *S. Enteritidis, S. Typhimurium, S. Kingston, S. Senftenberg, S. Blegdam, S. Emek, S. Inganda, S. Molade,* and *S. Apyeme*

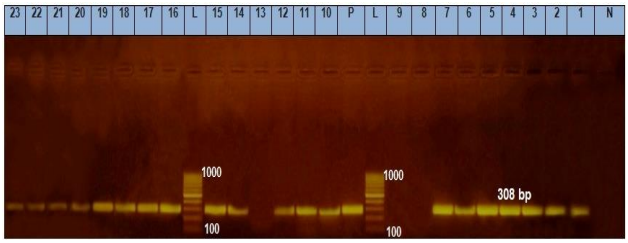

Fig. 1: Agarose gel electrophoresis of PCR: Amplification profile to monitor *mcr1* gene of 23 *Salmonella* isolates at 308 bp: Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control. Lane: 1-7, 10-12, 14, 15 and 16-23 were positive for *mcr*1 gene. Lane 1, 17: *S. tamale*; Lane 2, 6: *S. inganda;* Lane 3, 7, 12, 14, 18, 20: *S. enteritidis;* Lane 4: *S. molade;* Lane 5, 15, 19: *S. typhimurium;* Lane 22: *S. kentucky*; Lane 11: *S. labadi*; Lane16, 23: *S. takoradi*; Lane 21: *S. papuana*; Lane 10: Untypable.

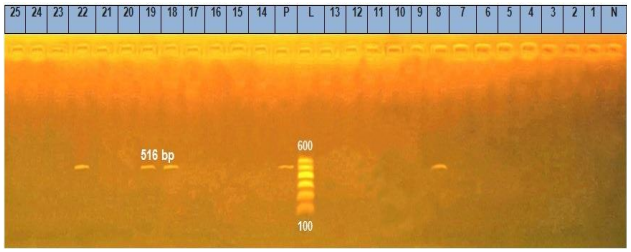

Fig. 2: Agarose gel electrophoresis of PCR: Amplification products (516 bp) to monitor *qnrS* gene in 25 of *E. coli* isolates. Lane L: 100-600 bp DNA ladder. N: Negative control, P: Positive control. Lane: 8,18,19, and 22 were positive for *qnrS* gene. Lane 8: O1: H7, Lane 18: O2: H6, Lane 19: O26, Lane 22: O146.

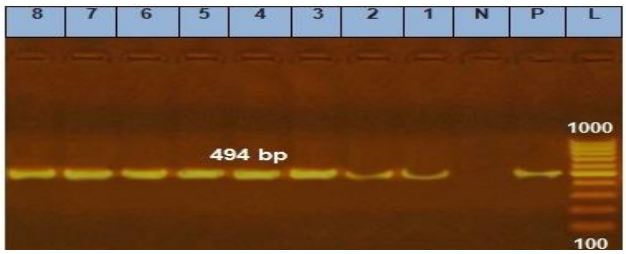

Fig. 3: Agarose gel electrophoresis of PCR: Amplification profile of *floR g*ene of 8 *P. aeruginosa* isolates at 494 bp: Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control. Lane: (1- 8) were positive for the *floR* gene.

were recovered by El-Sawah et al. (2016), El-sharkawy et al. (2017) and Hassan et al. (2017) from chicks suffering from omphalitis and from different organs of 1-day to1week old birds.

Antibiotics are profusely administered for therapeutic and prophylaxis purposes in the veterinary field (Dandachi et al. 2018). The resistance of a microorganism to numerous antibiotic groups outcomes in decrease within the medication efficacy of infectious illnesses resulting from those microorganisms (Alaali and Thani 2020).

In our study, as shown in Table 3, 86.9% of *Salmonella* were resistant to colistin sulphate while 48% of *E. coli* was resistant to norfloxacin and 87.5% of *P. aeruginosa* isolates showed resistance against florfenicol. In another report, *P. aeruginosa* strains isolated from 216 yolk sacs of baby chicks were resistant to florfenicol (El-Sawah et al. 2016). All *P. aeruginosa* isolates recovered

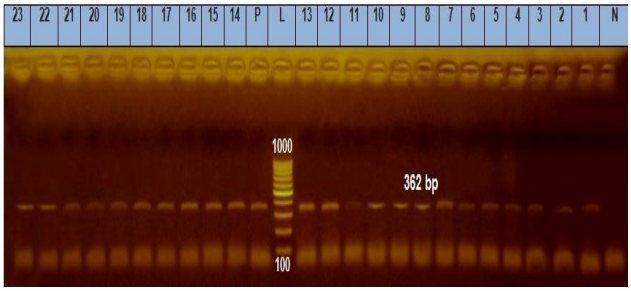

Fig. 4: Agarose gel electrophoresis of PCR: Amplification profile of *QacED1* gene at 362 bp for *Salmonella* isolates: Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control. Lane: (1-23) were positive for *QacED1* gene. Lane 1, 17: *S. tamale;* Lane 2, 6: *S. inganda;* Lane 3, 7, 12, 14, 18, 20: *S. enteritidis;* Lane 4: *S. molade;* Lane 5, 15, 19: *S. typhimurium;* Lane 8, 9, 13, 22: *S. kentucky*; Lane 11: *S. labadi*; Lane16, 23: *S. takoradi*; Lane 21: *S. papuana*; Lane 10: Untypable.

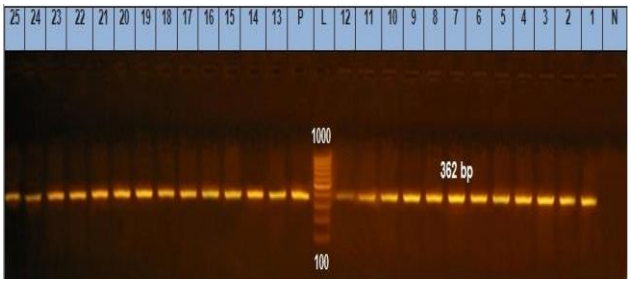

Fig. 5: Agarose gel electrophoresis of PCR: Amplification profile of *QacED1* gene at 362 bp for *E. coli.* isolates: Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control. Lane: (1 -25) were positive for *QacED1* gene. Lane 1, 18: O2:H6; Lane 2, 4, 9: O158; Lane 3: O145; Lane 5, 10, 15, 20, 21, 23: O78; Lane 6, 8: O1:H7, Lane 7: O1:H6, Lane 11: O144; Lane 12, 13, 25: O91; Lane 14: O125; Lane 16: O119; Lane 17, 24: O128; Lane 19: O26; Lane 22: O146.

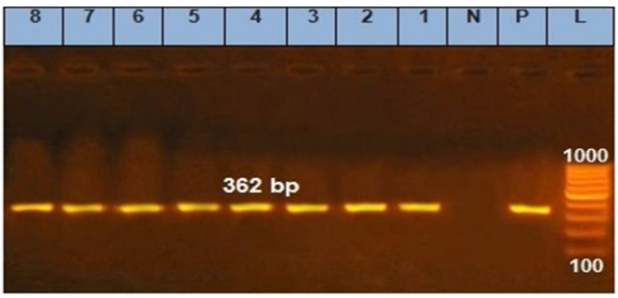

Fig. 6: Agarose: gel electrophoresis of PCR: Amplification profile of *QacED1* gene at 362 bp for *P. aeruginosa* isolates: Lane L: 100-1000 bp DNA ladder. N: Negative control, P: Positive control. Lane:(1 -8) were positive for *QacED1* gene.

from the liver, heart, and yolk sacs of 50 samples of baby chicks were 100% resistant to ampicillin (Shahat et al. 2019).

Antimicrobial-resistant microorganisms are basically a result of natural choice. Genetic differences in bacteria might also bring mutations, which expected to be effective for their viability even though the presence of the antimicrobials (Exner et al. 2017). Screening the presence of *mcr1*, *qnrS*, and *floR* genes in *Salmonella*, *E. coli*, and *P. aeruginosa* isolates, respectively by PCR technique revealed that *mcr*1 gene was found in 86.9% of all isolated *Salmonella* serovar except 3 serovars of *S.*

Kentucky (Fig. 1). The researches focusing on the *mcr*-1 gene-carrying *E. coli*, and *k. pneumonia* isolates are frequent, on the other hand, the researches on the emergence and molecular features of the *mcr*-1 gene in *Salmonella* spp. are still inadequate (Lu et al. 2019). The *mcr* genes have also been detected in *S.* Enterica, even though more scarcely than in *E. coli*. Many *mcr*-carrying *S.* Enterica strains display multidrug resistance forms, with several genes allowing resistance to tetracyclines, beta-lactams which include cephalosporins, streptomycin, sulfamethoxazole/ trimethoprim, and quinolones. Moreover, *S. typhimurium* is the maximum widespread serotype containing *mcr* genes (Borowiak et al. 2017). In the current work, it is interesting to note that other *Salmonella* serovars (*S. Tamale, S. Inganda, S. Molade, S. Labadi, S. Takoradi, S. Papuana, S. Kentucky*) also harbored the gene *mcr*1. Resistance towards norfloxacin is taken place commonly due to activities of genes products' such as *qnrS* (Talukdar et al. 2013). In our study, *qnrS* gene was detected in 4/25 (16%) of *E. coli* (O1, O2, 26 & O146).

In the present work, *floR* gene was found in 100% of *P. aeruginosa* isolates. The *floR* gene and its analogs have particularly been recognized in Gram-negative bacteria, while the different resistance genes have especially been found in Gram-positive microorganism (Lu et al. 2018).

Disinfectants have been used with a carelessness that leading to the adaptation of bacteria and augmenting the spread of resistant bacteria (Loughlin et al. 2002). Quaternary Ammonium Compounds also are supposed to destruct the outer membrane of Gram-negative microorganism, consequently enhancing their own uptake (McDonnell and Denver 1999). In our study, *qacED1* gene was found in all Gram-negative strains (*Salmonella*, *Escherichia coli*, and *Pseudomonas aeruginosa*) with the same 100% percentage, these results agreed with results reported by various workers (Nabil et al. 2019; Shahat et al. 2019) as they detected *qacED1* gene between *E. coli*, *Salmonella*, and *P. aeruginosa* isolates in 100% case. Between the Gram-negative bacteria, QACs resistance genes are frequently associated with plasmid-mediated class 1 integrons that possess a variety of antimicrobial resistance genes.

QACs resistance genes are combined decisively with genes that promote resistance to Sulphonamides, Trimethoprim Chloramphenicol, Aminoglycosides, and Beta- lactams (Zhao et al. 2012; Schill et al. 2017).

Conclusion

There is a direct link between illnesses due to antimicrobial-resistant Gram-negative microorganisms and early death in broiler chicks. It is fundamental to enhance the surveillance of antibiotic and disinfectant resistance levels in our chicken farms to stop the unfold of resistant microbes.

Author's Contribution: MEE and SAN designed the study and provided precious feedback and ideas throughout the course of the study; MI carried out the lab analyses, performed statistical analyses, and prepared the manuscript with the supervision and technical help of MEE. All authors approved the final version of the manuscript.

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