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ISOLATION AND CHARACTERIZATION OF SOME *ENTEROBACTERIACEAE* ISOLATED FROM EARLY MORTALITIES IN JAPANESE QUAIL CHICKS AT QENA GOVERNORATE, EGYPT

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ABSTRACT

Quail farms become widely spread in Egypt as a source of meat. Early mortality of quail chicks is one of quail breeding limiting factors. This study was performed to study role of some *Enterobacteriaceae* as a parable cause of early mortality in South Valley University quail farm, characterize isolates phenotypically and serologically, determine their antimicrobial sensitivity and to screen all isolates for presence of florfenicol resistance gene (*floR*), Quaternary ammonium compounds resistance gene (*QacA/B*) and class 1 integrons gene (*Int1*) and to screen *E. coli*, *Salmonella* and *K. pneumoniae* isolates for intimin encoding gene (*eaeA*), invasive encoding gene (*invA*) and mucoviscosity associated gene (*mag*A) respectively. Therefore, 80 freshly dead or moribund Japanese quail chicks under 12 days of age were collected from this farm during the period from December 2020 to April 2021 for clinical assessment and bacterial isolation. Totally, 14 *E. coli*, 6 *Salmonella* and 1 *K. pneumoniae* isolates were isolated and phenotypically identified from the internal organs of the examined quail chicks with percentage of (17.5%), (7.5%) and (1.25%) respectively. *E. coli* isolates belonged to 5 different O-serogroups comprising O_{86} (28.6%), O_{78} (21.4%), O_{26} (14.3%), O_{55} (14.3%) and O_{164} (7.1%) in addition to 2 nontypeable isolates (14.3%) while all *Salmonella* isolates were serologically identified as *S. Typhimurium* with antigenic formula 1,4,[5],12:i:1,2. All isolates were weak biofilm producer except (33.3%) of *S. Typhimurium* isolates and showed Congo red binding activity except (28.6%) of *E. coli* isolates while only (14.3%) of *E. coli* isolates had hemolytic activity. Antimicrobial susceptibility testing for the isolates to 10 different antibiotics revealed that all the isolates were sensitive only to chloramphenicol and oxytetracycline except (42.9%) and (85.7%) of *E. coli* isolates respectively and to azithromycin except (35.7%) of *E. coli* isolates and *K. pneumoniae*. PCR revealed that all the isolates harbor *Int1* and *floR* genes, (100%) and (33.3%) of *S. Typhimurium* isolates harbor *invA* and *QacA/B* genes respectively and only (21.4%) of *E. coli* isolates harbor *eaeA* and *QacA/B* genes. It was concluded that *E. coli* and *S. Typhimurium* are a major cause of early mortality of Japanese quail chicks.

Keywords: *Enterobacteriaceae*, mortalities, Japanese quail, Qena Governorate, Egypt.

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INTRODUCTION

Poultry industry is considered an important sector to meet the great demands of animal protein all over the world (Abd El-Ghany, 2019). Rearing of birds other than chickens as quails has been developed in the recent years worldwide to supply the human food requirements (Khoshbakht *et al*., 2017). Quails represent a hopeful source to cover the animal protein deficiency in the developing countries including Egypt (Farghaly *et al*., 2017).

Quails farming is increasing globally day by day as it requires low rearing investment and has fast financial returns. Moreover, quails is characterized by low feed intake, rapid growth rate, early sexual maturity, short generation interval, short incubation period, high nutritional value of meat, and less susceptibility to the infectious diseases compared to the other poultry species (Santos *et al*., 2011; Yusuf *et al*., 2016 and Hassan *et al*., 2017).

Egypt has fast-growing quail industry (Da Cunha, 2009). Nowadays, quails become widely distributed in Egypt as a source of meat production, since their meat has become highly popular among the Egyptian consumers (Mosaad *et al*., 2000).

The advancement in quail production is being obstructed by some managemental factors, infectious and non-infectious diseases (Barnes and Gross, 1997). Early mortality of quail chicks is one of the limiting factors in breeding of quails. Bacterial infection is one of the most important causes of quail chick's early mortality where various causative microorganisms are transmitted through the yolk sac. *Salmonella* and *E. coli* are the most important factors in yolk sac infection, other bacteria as *Proteus* and *Bacillus* can also cause yolk sac infection. Furthermor, *E. coli* bacteria are common in eggshell contamination. Poor management in breeder farm and hatchery hygiene are some of the predisposing factors to quail chick's early mortality (Boroomand *et al*., 2018).

E. coli and *Salmonella* are considered of the main causes of morbidity and mortality in poultry industry worldwide and they cause huge economic losses. *E. coli* causes different disease manifestations in poultry including omphalitis, septicemia, respiratory tract infection, enteritis, coli granuloma, swollen head syndrome, cellulitis, polyserositis, and salpingitis. On the other hand, *Salmonella* serotypes cause different acute and chronic diseases in poultry (Kabir, 2010).

There is a wide variation in *Salmonella* serovars commonly infecting poultry and the serovars may vary geographically (El-Demerdash *et al*., 2013). It is also known that a lot of *Salmonella* serotypes affect human, but some are more pathogenic for human (Hendriksen *et al*., 2011). Avian pathogenic *E. coli* (APEC) are very various, which are associated to the virulence factors and serotypes of them (Mellata *et al*., 2009). Antimicrobial resistance-associated and virulence-associated genes are responsible for the virulence potential of pathogenic bacteria (Capuano *et al*., 2013).

Quail play a significant role in spreading of many pathogens which have public health significance (Mosaad *et al*., 2000). *E. coli* and *Salmonella* are the most avian pathogens that cause food borne illnesses in most countries of the world (Kabir, 2010). It is widely accepted that design of any program for prevention of food borne diseases and/or spreading of the pathogens to the environment should be started from the farm (Farghaly *et al*., 2017). Disinfectants are important components of the biosecurity programs. QACs are vigorous detergents widely used in poultry farms and it is the disinfectant of choice for disinfection of the equipment as incubators and hatching trays (Haynes and Smith, 2003). Genes of QAC resistance are extremely existed among *E. coli* isolates and they were extremely associated with antimicrobial resistant isolates (Ibrahim *et al*., 2019).

Antimicrobial resistance is an increasingly significant problem in human and animals

(Jahantigh *et al*., 2013). The excessive use of antibiotics in the intensive animals production especially poultry and pork represent the cornerstone for emergence, permanence and spreading of the resistant bacteria (WHO, 2014). Development of multidrug resistant (MDR) pathogens makes prevention and control of the bacterial diseases of great difficulty affecting not only poultry industry but also human through transmission of such pathogens via consumption of the contaminated products (Darwish *et al*., 2013).

Resistance genes acquisition particularly by the mobile genetic elements is considered the main factor in the wide spreading of the antimicrobial resistance (Odumosu *et al*., 2013). Antibiotic resistance gene cassettes found most in association with class 1 or class 2 integrons (Dawes *et al*., 2010). Capture and spreading of the antibiotic resistance genes via integrons stimulate the rapid evolution of multidrug resistances among Gram-negative bacteria (Rowe-Magnus *et al*., 2002).

There are limited researches about the early mortality in the Egyptian quail farms, role of some *Enterobacteriaceae* members as a probable cause of this problem and about the antibiotic resistance of *Enterobacteriaceae* members isolated from quails. Appropriate knowledge about the diseases affecting quails is necessary for the suitable control and preventive programs in quail production. Furthermore, studying prevalence of the zoonotic microorganisms which are transmitted to human through foods of animal origin and their antibiotic resistance represent a major concern for public health. Therefore, the present study aimed to investigate role of some *Enterobacteriaceae* members as a probable cause of early mortality in South Valley University quail farm, characterize the isolates phenotypically and serologically, determine their antimicrobial sensitivity and to screen them for presence of some virulence genes, antibiotic and disinfectant resistance genes in addition to *Int1* gene.

MATERIALS AND METHODS

1- Sampling and clinical examination:

During the period from December 2020 to April 2021, 80 freshly dead and moribund Japanese quail chicks under 12 days of age from South Valley University quail farm were received at Department of Poultry Diseases, Faculty of Veterinary Medicine, South Valley University, Egypt for clinical assessment and diagnosis. Moribund quail chicks were killed humanely by neck dislocation. The collected quail chicks' were subjected to clinical, post mortem and bacteriological examinations. Under aseptic conditions, samples were collected from liver, spleen, kidney, yolk sac, lung and heart.

2- Bacterial isolation and biochemical identification:

The samples were inoculated into tryptone soya broth (TSB) (Oxoid, England) and incubated under aerobic condition at 37°C for 24 hrs. then streaked onto MacConkey agar (Oxoid, England) and incubated at 37°C for 24 hrs. The pink colonies were sub-cultured on eosin methylene blue (EMB) agar (Oxoid, England) and the pale colonies were sub-cultured on Xylose Lysine Deoxycholate (XLD) media (Oxoid, England). The isolates were preserved at -80°C in TSB (Oxoid, England) supplemented with 15% glycerol till the further identification. The suspected isolates were identified through assessment of their morphological characteristics, Gram-staining, catalase and oxidase test using the standard laboratory methods described by Holt *et al*. (1994) and API 20E system (bioMerieux, France) according to the manufacturer's instructions. The isolates were identified as *E. coli, Salmonella* and *K. pneumoniae* according to criteria of Mahon and Lehman (2019).

3 - Serotyping of *E. coli* and *Salmonella* isolates:

Serotyping of *E. coli* and *Salmonella* isolates was carried out by slide agglutination test using diagnostic polyvalent and monovalent *E. coli* antisera and diagnostic O and H *Salmonella* antisera obtained from Sifin diagnostics GmbH,

Germany. *E. coli* isolates were serotyped according to Edwards and Ewing (1972) while *Salmonella* isolates were serologically identified following Kauffman-White Scheme (Grimont and Weill, 2007).

4- Determination some virulence factors of the isolates:

4.1- Biofilm formation by Microtiter plate (MTP) assay:

The ability of bacterial isolates to form biofilm was assessed by microtiter plate method according to Melo *et al*. (2013) with slight modification. Briefly, overnight cultures of the tested strains were diluted by 1: 100 in trypticase soy broth contain 0.5% glucose. 200µl of the formerly prepared suspensions were added to the wells of a polystyrene plate (Costar, New York, USA). Each strain was tested in triplicate and three wells were used as a negative control (contained 200 µl of TSB+0.5% glucose only). After 24hrs. of incubation at 37°C, bacterial suspension was removed and washed by phosphate buffer saline three times then staining with crystal violet (0.1%) was done for all wells for 15 min, after that the dye was removed, the biofilm-bound dye was then eluted with 95% ethanol, The optical densities (OD) of the stained adherent films were determined with microplate reader (BioTek ELX800, USA) at 620 nm. Results were interpreted according to (Stepanović *et al*., 2007).

4.2- Congo red binding assay:

Bacterial isolates were grown at 37°C for 24 hrs. on tryptic soy agar (Oxoid, UK) supplemented with 0.02% Congo red (Sigma, USA) and 0.15% bile salt (Difico, USA). The isolates that produced red colonies were recorded as Congo red positive while those that produced grayish-white colonies were recorded as Congo red negative according (Roy *et al*., 2006).

4.3- Hemolytic activity:

Overnight bacterial cultures of the isolates were streaked on blood agar base (Oxoid, UK) containing 10% citrated sheep blood and then incubated at 37°C for 24 hrs. Hemolysis is indicated by appearance of erythrocytes lysis according to (Roy *et al*., 2006).

5- Antimicrobial Susceptibility Testing:

The antimicrobial susceptibility of the isolates was determined by Kirby-Bauer disc diffusion method using the antibiotics disks (HiMedia, India) illustrated in Table (1). Each isolate was streaked onto Mueller-Hinton agar (Oxoid, UK), antibiotic disks were dispensed and the inoculated plate was incubated at 37°C for 24 hrs. Inhibition zones diameters were measured and interpreted according to the CLSI (2006). Resistant to more than 3 antibiotics was considered as MDR (Magiorakos *et al*., 2012).

Table 1: Antibiotics used in antimicrobial susceptibility testing with discs concentration.

Antibiotic	Disc concentration	Antibiotic	Disc concentration
Ampicillin (AMP)	30µg	Amoxicillin/clavulanic acid (AMC)	30µg
Cefazolin (CZ)	30µg	Cefotaxime (CTX)	30µg
Kanamycin (K)	30µg	Azithromycin (AZM)	15µg
Clindamycin (DA)	2µg	Oxytetracycline (T)	30µg
Chloramphenicol (C)	30µg	Nitrofurantoin (F)	300µg

6 - Detection of some genes in the isolates by PCR:

In this study, all the isolates were screened by the conventional PCR for presence of *floR*, *QacA/B* and *Int1* genes. Furthermore, *E. coli*, *S. Typhimurium* and *K. pneumoniae* isolates were screened for presence of *eaeA*, *invA* and *mag*A genes respectively. The used oligonucleotide primers are illustrated in Table (2) and they were obained from Metabion (Germany).

6.1-DNA extraction:

Total bacterial DNA was extracted from an overnight subculture on TSB using GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Baltics UAB, Lithuania) according to the manufacturer's instructions. Concentration of the extracted DNA from each isolate was measured by Nano Drop™ Lite spectrometer (Thermo scientific, Germany) then it was preserved at -20°C till be used.

6.2-PCR amplification:

DNA was amplified using Emerald Amp Max PCR Master Mix (Takara, Japan) in a thermocycler (Applied biosystem 2720, USA) and under PCR conditions illustrated for each target gene in Table (2). According to manufacturer's instructions of mastermix, the reaction mixture was prepared in 25 µl containing 12.5 µl of Master Mix, 1 µl from each of forward and reverse primers, 5 µl from extracted DNA and 5.5 µl of nuclease-free water.

6.3-Analysis of the PCR products:

Products of PCR were electrophoresed on 1% agarose gel (Applichem GmbH, Germany) in 1x TBE buffer at room temperature using gradients of 5V/cm. To determine the fragment sizes, Generuler 100 bp DNA ladder (Thermo scientific, Germany) was used. Then, the gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Table 2: The target genes in the study, oligonucleotide primers and PCR conditions used.

Target gene	Primers sequences (5`- 3`)	Product Size (bp)	Primary denaturation	PCR conditions (35 cycles)			Final extension	Reference
				Denaturation	Annealing	Extension		
invA of *Salmonella*	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 7 min.	**Oliveira *et al.* (2003)**
eaeA of E. coli	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	95˚C 3 min.	95˚C 30 sec.	55˚C 30 sec.	72˚C 72 sec.	72˚C 7 min.	**Bisi-Johnson *et al.* (2011)**
*mag*A of *Klebsiella*	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1282	94˚C 5 min.	94˚C 45 sec.	55˚C 60 sec.	72˚C 72 sec.	72˚C 7 min.	**Yeh *et al.* (2007)**
floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Doublet *et al.* (2003)**
QacA/B	GCAGAAAGTGCAGAGTTCG CCAGTCCAATCATGCCTG	361	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 40 sec.	72˚C 7 min.	**Noguchi *et al.* (2005)**
Int1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	94˚C 10 min.	94˚C 60 sec.	54˚C 60 sec.	72˚C 2 min.	72˚C 10 min.	**Zhao *et al.* (2001)**

RESULTS

1- Results of clinical and post-mortem examination:

Clinically, the affected quail chicks showed depression, huddling together, ruffling feathers, some showed pasty vent. At necropsy, general congestion in internal organs (lung, liver, spleen and intestine), unabsorbed yolk sac, some showed inflammation in the pericardium and air sacs, typhlitis, urolithiasis and distension of ureters with urate.

2- Results of bacterial isolation and phenotypic identification:

Based on the morphological and biochemical characteristics of the isolates, 14 *E. coli* isolates (E1-E14), 6 *Salmonella* isolates (S1-S6) and 1 *K. pneumoniae* isolate were isolated and identified phenotypically from liver, spleen, kidney, yolk sac, lung and heart of the examined quail chicks with percentage of (17.5%), (7.5%) and (1.25%) respectively.

E. coli isolates produced pink colonies on MacConkey agar and dark colonies with green metallic sheen on EMB agar and they were oxidase negative, catalase positive, indole positive, methyl red (MR) positive, Voges-Proskauer (VP) negative and negative in citrate utilization test. *K. pneumoniae* isolate produced pink mucoid colonies on MacConkey agar and pink mucoid colonies on EMB and it was oxidase negative, catalase positive, indole negative, MR negative, VP positive and positive in citrate utilization test. While, *Salmonella* isolates produced pale colonies on MacConkey agar and pink colonies with dark center on XLD agar and they were oxidase negative, catalase positive, indole negative, MR positive, VP negative, negative in citrate utilization test and produced H_2S.

3- Results of serotyping of *E. coli* and *Salmonella* isolates:

Serotyping demonstrated that *E. coli* isolates belonged to 5 different O-serogroups comprising O_{86}, O_{78}, O_{26}, O_{55} and O_{164} in addition to 2 nontypeable isolates as illustrated in Table (3) while all *Salmonella* isolates were serologically identified as *S. Typhimurium* with the antigenic formula 1,4,[5],12:i:1,2.

Table 3: O-Serogroups of *E. coli* isolates.

O-Serogroup	O_{86}	O_{78}	O_{26}	O_{55}	O_{164}	Nontypeable
Number of isolates	4	3	2	2	1	2
%	28.6%	21.4%	14.3%	14.3%	7.1%	14.3%
Isolates belonged to this Serogroup	E8, E10, E12 and E13	E2, E7 and E11	E5 and E9	E1 and E4	E14	E3 and E6

4- Results of determination some virulence factors of the isolates:

It was found that all *E. coli* isolates were weak biofilm producer, (71.4%) of them showed Congo red binding activity and only (14.3%) of them had hemolytic activity (Table 4). On the other hand, All *S. Typhimurium* isolates showed Congo red binding activity while hadn't hemolytic activity and (66.7%) of them were weak biofilm producer (S1 and S5 weren't biofilm producer). *K. pneumoniae* isolate was weak biofilm producer, showed Congo red binding activity but hadn't hemolytic activity.

Table 4: Some virulence factors of *E. coli* isolates.

E. coli isolate	O-Serogroup	Biofilm formation	Congo red binding activity	Hemolytic activity
E1	O_{55}	Weak producer	+	-
E2	O_{78}	Weak producer	+	-
E3	Nontypeable	Weak producer	-	+
E4	O_{55}	Weak producer	+	-
E5	O_{26}	Weak producer	+	-
E6	Nontypeable	Weak producer	+	-
E7	O_{78}	Weak producer	+	-
E8	O_{86}	Weak producer	-	-
E9	O_{26}	Weak producer	-	-
E10	O_{86}	Weak producer	+	+
E11	O_{78}	Weak producer	-	-
E12	O_{86}	Weak producer	+	-
E13	O_{86}	Weak producer	+	-
E14	O_{164}	Weak producer	+	-
Number of positive isolates		14	10	2
%		100%	71.4%	14.3%

5- Results of antimicrobial susceptibility testing:

Results of evaluation susceptibility of the isolates to the tested antibiotics in this study are presented in Tables (5) and (6). It was found that all the isolates were resistant to 7 of the tested antibiotics namely ampicillin, amoxicillin/clavulanic acid, cefazolin, cefotaxime kanamycin, clindamycin and nitrofurantoin. Concerning the other three antibiotics used, all the isolates were sensitive to chloramphenicol and oxytetracycline except (42.9%) and (85.7%) of *E. coli* isolates respectively and to azithromycin except (35.7%) of *E. coli* isolates and *K. pneumoniae* isolate.

Table 5: Results of antimicrobial susceptibility of *E. coli* isolates.

E. coli isolate	AMP	AMC	CZ	CTX	K	AZM	DA	T	C	F	MDR
E1	R	R	R	R	R	R	R	R	R	R	10
E2	R	R	R	R	R	S	R	R	S	R	8
E3	R	R	R	R	R	S	R	R	R	R	9
E4	R	R	R	R	R	R	R	R	R	R	10
E5	R	R	R	R	R	S	R	R	I	R	8
E6	R	R	R	R	R	R	R	R	R	R	10
E7	R	R	R	R	R	R	R	R	R	R	10
E8	R	R	R	R	R	S	R	I	S	R	7
E9	R	R	R	R	R	S	R	R	I	R	8
E10	R	R	R	R	R	S	R	R	S	R	8
E11	R	R	R	R	R	R	R	R	S	R	9
E12	R	R	R	R	R	S	R	I	S	R	7
E13	R	R	R	R	R	S	R	R	I	R	8
E14	R	R	R	R	R	S	R	R	R	R	9
Number of sensitive isolates	0	0	0	0	0	9	0	2	8	0	
%	0	0	0	0	0	64.3	0	14.3	57.1	0	

R= resistant, S = sensitive and I= intermediate.

Table 6: Results of antimicrobial susceptibility of *S. Typhimurium* isolates.

S. Typhimurium isolate	AMP	AMC	CZ	CTX	K	AZM	DA	T	C	F	MDR
S1	R	R	R	R	R	S	R	S	S	R	7
S2	R	R	R	R	R	S	R	S	S	R	7
S3	R	R	R	R	R	S	R	S	S	R	7
S4	R	R	R	R	R	S	R	I	S	R	7
S5	R	R	R	R	R	S	R	I	S	R	7
S6	R	R	R	R	R	S	R	I	S	R	7
Number of sensitive isolates	0	0	0	0	0	6	0	6	6	0	
%	0	0	0	0	0	100	0	100	100	0	

R= resistant, S = sensitive and I= intermediate.

6- Results of isolates investigation for some genes by PCR:

In this study, screening *E. coli*, *S. Typhimurium* and *K. pneumoniae* isolates by conventional PCR for presence of *eaeA*, *invA* and *mag*A genes respectively revealed that (21.4%) of *E. coli* isolates harbor *eaeA* gene (Fig. 1) and all *S. Typhimurium* isolates harbor *invA* gene (Fig. 2) while *K. pneumoniae* isolate didn't harbor *mag*A gene (Fig. 3). On the other hand, screening these isolates by the same technique for presence of *Int1*, *floR* and *QacA/B* genes revealed that all the isolates harbor *Int1* gene (Fig. 4) and *floR* gene (Fig. 5) while only (21.4%) and (33.3%) of *E. coli* and *S. Typhimurium* isolates harbor *QacA/B* genes (Fig. 6) respectively. PCR results for *E. coli* and *S. Typhimurium* isolates were summarized in Tables (7) and (8) respectively.

Table 7: Prevalence of *eaeA*, *Int1*, *floR* and *QacA/B* genes among *E. coli* isolates by PCR.

E. coli isolate	*eaeA* gene	*Int1* gene	*floR* gene	*QacA/B* gene
E1	-	+	+	-
E2	+	+	+	-
E3	+	+	+	-
E4	-	+	+	+
E5	-	+	+	-
E6	-	+	+	-
E7	-	+	+	-
E8	-	+	+	-
E9	-	+	+	-
E10	-	+	+	+
E11	-	+	+	-
E12	+	+	+	+
E13	-	+	+	-
E14	-	+	+	-
Number of positive isolates	3	14	14	3
%	21.4%	100%	100%	21.4%

Table 8: Prevalence of *invA, Int1, floR* and *QacA/B* genes among *S. Typhimurium* isolates by PCR.

S. Typhimurium isolate	*invA* gene	*Int1* gene	*floR* gene	*QacA/B* gene
S1	+	+	+	-
S2	+	+	+	-
S3	+	+	+	+
S4	+	+	+	-
S5	+	+	+	+
S6	+	+	+	-
Number of positive isolates	6	6	6	2
%	100%	100%	100%	33.3%

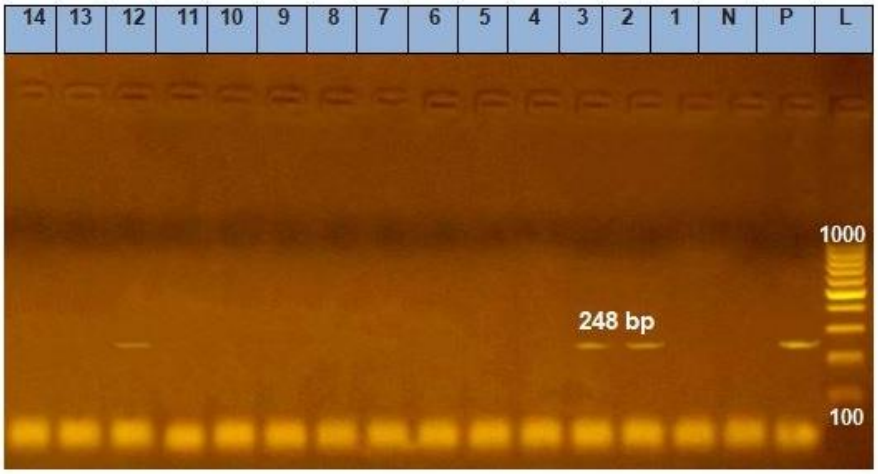

Fig. (1): Agar gel electrophoresis for products of PCR using specific primers targeting *eaeA* gene in *E. coli* isolates. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lanes 1-14: DNA extracted from *E. coli* isolates (E1:E14 respectively) showing positive bands at 248-bp in isolates E2, E3 and E12.

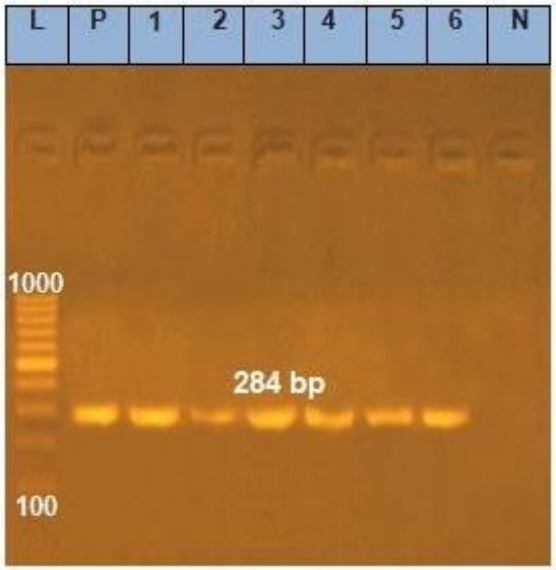

Fig. (2): Agar gel electrophoresis for products of PCR using specific primers targeting *invA* gene in *S. Typhimurium* isolates. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lanes 1-6: DNA extracted from *S. Typhimurium* isolates (S1:S6 respectively) showing positive bands at 284-bp in all the isolates.

Fig. (3): Agar gel electrophoresis for products of PCR using specific primers targeting *mag*A gene in *K. pneumoniae* isolate. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lane 1: DNA extracted from *K. pneumoniae* isolate showing no bands at 1282-bp (Negative).

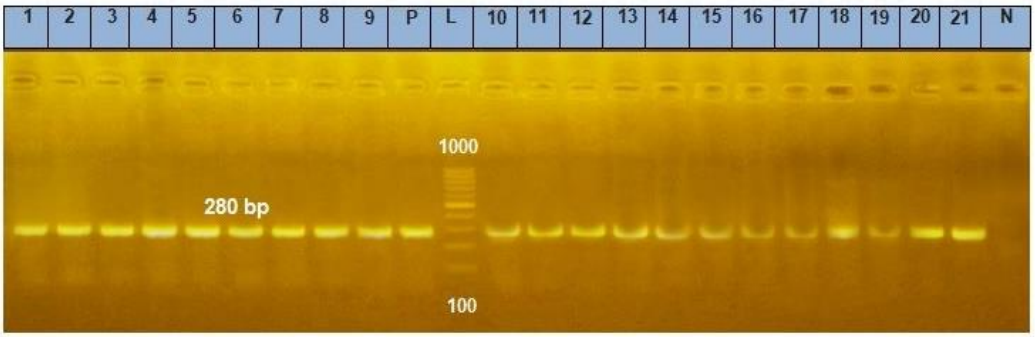

Fig. (4): Agar gel electrophoresis for products of PCR using specific primers targeting *Int1* gene in the isolates. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lane 1: DNA extracted from *K. pneumoniae* isolate, lanes 2-7: DNA extracted from *S. Typhimurium* isolates (S1:S6 respectively) and lanes 8-21: DNA extracted from *E. coli* isolates (E1:E14 respectively). All the isolates showing positive bands at 280-bp.

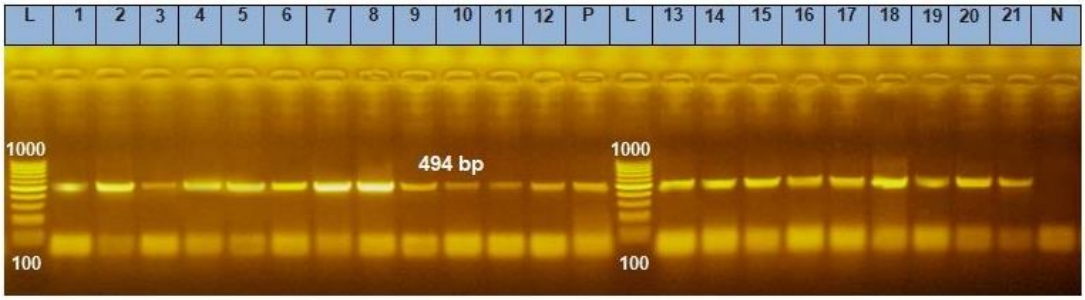

Fig. (5): Agar gel electrophoresis for products of PCR using specific primers targeting *floR* gene in the isolates. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lane 1: DNA extracted from *K. pneumoniae* isolate, lanes 2-7: DNA extracted from *S. Typhimurium* isolates (S1:S6 respectively) and lanes 8-21: DNA extracted from *E. coli* isolates (E1:E14 respectively). All the isolates showing positive bands at 494-bp.

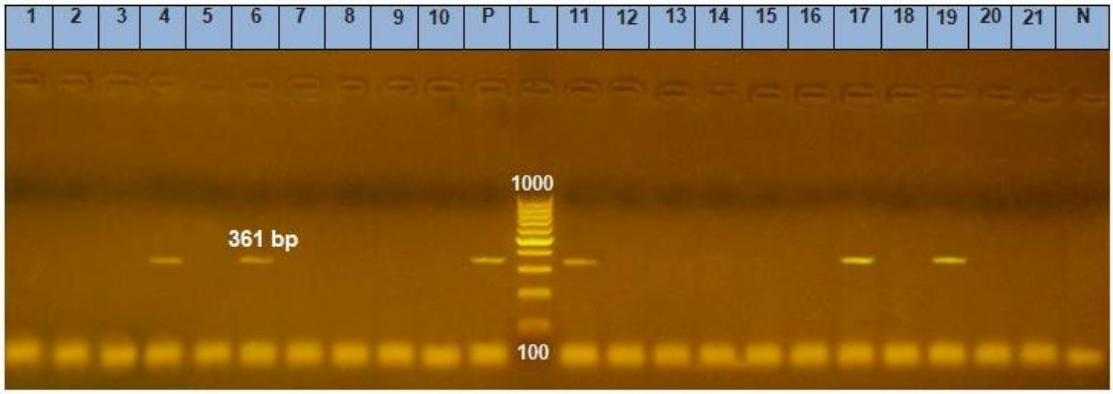

Fig. (6): Agar gel electrophoresis for products of PCR using specific primers targeting *QacA/B* gene in the isolates. Lane L: molecular weight marker 100 base pair, lane P: positive control, lane N: negative control and lane 1: DNA extracted from *K. pneumoniae* isolate showing no bands at 361-bp (Negaive), lanes 2-7: DNA extracted from *S. Typhimurium* isolates (S1:S6 respectively) showing positive bands at 361-bp in isolates S3 and S5 and lanes 8-21: DNA extracted from *E. coli* isolates (E1:E14 respectively) showing positive bands at 361-bp in isolates E4, E10 and E12.

DISCUSSION

Quail farming is considered as one of the most alternative sources for meat production to cover the deficiency in the animal protein in Egypt (Abd El-Dayem *et al*., 2020). Early mortality of quail chicks is one of the limiting factors in breeding of quails (Boroomand *et al*., 2018). Appropriate knowledge about diseases affecting quails is necessary for the suitable control and preventive programs in quail production. The present study identified the important role of some *Enterobacteriaceae* members as a cause of early mortality of quail chicks in Egypt through isolation and identification of *E. coli*, *S. Typhimurium* and *K. pneumoniae* isolates from the examined quail chicks with percentage of (17.5%), (7.5%) and (1.25%) respectively. Our results agreed with those of Abd El-Galil *et al*. (1993) who isolated *E.coli* and *K. pneumoniae* from the dead quails chicks at 1st ten day of life with percentage of (18%) and (2%) respectively. Also, our results agreed with results of El-Demerdash *et al*. (2013) and Boroomand *et al*. (2018) about isolation of the same bacterial species from early mortality investigations of quail chicks, but with higher rates of the isolates and which could be attributed to difference of the geographical areas, managemental practices and climate. El-Demerdash *et al*. (2013) isolated *E. coli*, *Salmonella* spp. and *Klebsiella* spp. from freshly dead and/or moribund Japanese quails chicks aged between 2 days and 3 weeks with percentage of (38.75%), (7.5%) and (6.25%) respectively. Boroomand *et al*. (2018) investigated *Enterobacteriaceae* responsible for early mortality in Japanese quail chicks and their results revealed isolation of *E. coli*, *Salmonella* spp. and *K. pneumoniae* with percentage of (44%), (8%) and (8%) respectively.

In this study, the infected quail chicks showed no significant clinical signs but Post-mortem lesions in dead and sacrificed chicks included congestion in the internal organs, pericarditis, abdominal air sacculitis, typhilitis and urolithiasis. Similar post-mortem lesions recorded by Ameh *et al*. (2011) and Kabir (2010).

In this study, serotyping of *E. coli* isolates revealed that *E. coli* isolates belonged to 5 different O-serogroups including O_{86}, O_{78}, O_{26}, O_{55} and O_{164} in addition to 2 nontypeable isolates as illustrated in Table (3). Consistent with our findings, Farghaly *et al*. (2017), Ibrahim (2019) and Abd El-Dayem *et al*. (2020) isolated *E. coli* O_{78} from the diseased quails in Egypt, Abd El-Dayem *et al*. (2020) also isolated *E. coli* O_{55} from such quail and

Dipineto *et al*. (2014) isolated *E. coli* O_{26} from common quails in Italy. On the other hand, all *Salmonella* isolates in this study were serologically identified as *S. Typhimurium*. Consistent with our findings, *S. Typhimurium* was isolated from quail chicks by Mosaad *et al*. (2000) and Boroomand *et al*. (2018). Isolation of *S. Typhimurium* only in this study with absence of the other *Salmonella* serovars may be attributed to either the geographic or temporal variances in *Salmonella* colonization of the birds. Furthermore, it has major public health consequences for consumers because of the lack of routine control of *Salmonella* serovars in the developing countries.

Many researchers advocated use of Congo red binding test for distinguishing between pathogenic and non-pathogenic microorganisms in APEC study (Saha *et al*., 2020). In this study, all the isolates except (28.6%) of *E. coli* isolates showed Congo red binding activity indicating their pathogenicity. Furthermore, virulence of our isolates was indicated from that all of them were weak biofilm producer except (33.3%) of *S. Typhimurium* isolates. Regarding to hemolysis on blood agar, only (14.3%) of *E. coli* isolates had hemolytic activity. A clear distinction between pathogenic and nonpathogenic bacteria couldn't be established based on hemolytic activity.

Antimicrobial agents are essential drugs for both human and animals, but some bacteria have showed complete or partial resistance to the different antibiotics in the recent years (Palanisamy and Bamaiyi, 2015). In the present study, all the isolates were resistant to 7 of the tested antibiotics (MDR) namely ampicillin, amoxicillin/clavulanic acid, cefazolin, cefotaxime kanamycin, clindamycin and nitrofurantoin. The high MDR to these antibiotics observed in this study could be related to their high indiscriminate use in poultry farming, more specifically in the quail breeding in area of study. Furthermore and according to our results, presence of class I integron gene in all these isolates could explain this MDR where it has found that MDR genes are clustered on individual mobile elements, most commonly on class 1 or class 2 integrons, which mean that their readily transferred and increase MDR bacterial population (Nikaido, 2009 and Dawes *et al*., 2010). Presence of such MDR bacteria in quails will result in a clear difficulty in prevention and control of the bacterial diseases affecting them and it also has epidemiological and public health implications represented in transfer of these MDR bacteria and the resistance genes to human and animals. Therefore, antibiotics use in quail farms must be prudent and supported by antibiogram tests before drug administration in addition to adoption of the strict hygienic measures. Concerning the other three tested antibiotics, all the isolates were sensitive to chloramphenicol and oxytetracycline except (42.9%) and (85.7%) of *E. coli* isolates respectively and to azithromycin except (35.7%) of *E. coli* isolates and *K. pneumoniae* isolate. Our results agreed with those of Boris *et al*. (2012) and Boroomand *et al*. (2018) who reported that all *Salmonella* isolates were sensitive to chloramphenicol and oxytetracycline respectively and also, nearly similar with those of Boroomand *et al*. (2018) who reported that (74.5%) of *E. coli* isolates were resistant to oxytetracycline.

The *eaeA* gene encodes intimin that enables the intimate attachment of *E. coli* to enterocytes (Ateba and Mbewe, 2014). In this study, *eaeA* gene was detected in (21.4%) of *E. coli* isolates and specifically in serotypes O_{78} (E2) and O_{86} (E13) as illustrated in Table (7) and Figure (1). Presence of *eaeA* gene in (21.4%) of *E. coli* isolates came in accordance with Abd El-Dayem *et al*. (2020) who found *eaeA* gene in (25%) of APEC isolates while it came incompatible with Yousef *et al*. (2015) who found *eaeA* gene in all APEC isolates and with Salehi and Ghanbarpour (2010) who didn't find *eaeA* gene in all APEC isolates. Concerning *E. coli* serotypes, Eid *et al*. (2016) detected *eaeA* in *E. coli* serotypes O_{78} while Abd El-Dayem *et al*. (2020) didn't detect *eaeA* gene in this serotype.

Intestinal epithelium cells invasion is one of the earliest steps in the pathogenic cycle of

Salmonella spp. (Galán *et al*., 1992). *InvA* gene encodes bacterial membrane protein essential for invasion of the host epithelial cells (Darwin and Miller, 1999). In this study, *invA* gene was detected in all *S. Typhimurium* isolates (100%) as illustrated in Table (8) and Figure (2) which is similar to that was reported by Ahmed *et al*. (2016) and Ammar *et al*. (2018).

The *mag*A gene contributes in bacterial virulence of *K. pneumoniae* (Fang *et al*., 2005). Presence of *mag*A and *rmp*A genes in *K. pneumoniae* serotypes K1 and K2 make them more invasive and resistant to the phagocytosis (Younis *et al*., 2016). Our isolate of *K. pneumoniae* was negative for *mag*A gene as illustrated Figure (3). Our finding is similar to that was reported by El Fertas-Aissani *et al*. (2013) who didn't find any positive isolate for *mag*A gene in the examined strains from different human clinical samples while came inconsistence with that was reported by Younis *et al*. (2016) who found that (53.33%) of the examined isolates were positive for *mag*A gene but this difference may be attributed to the low number of our *K. pneumoniae* isolates (n=1). Furthermore, absence of *mag*A gene wasn't expected finding where our *K. pneumoniae* isolate displayed a mucoid phenotype and *mag*A gene has hyperviscous phenotype (Struve *et al*., 2005), so further studies are needed for clarification the exact role of *mag*A in the hypermucoviscosity phenotype.

Bacteria can acquest the resistance genes via the mobile elements which help in dissemination and distribution of these genes among the different bacterial populations (Blair *et al*., 2015). Integrons are potent mobile genetic elements where their location on the plasmids, transposons and pathogenicity islands facilitate their transferring among the different bacteria (Firoozeh *et al*., 2019). Class 1 integrons have been frequently reported in gram negative bacterial isolates (Lima *et al*., 2014). In this study, it was found that all the isolates harbor Class 1 integrons gene as illustrated in Tables (7 and 8) and Fig. (4). In previous study, Antunes *et al*. (2006) reported lower prevalence of Class 1 integrons gene (75.0%) among *S. enterica* isolated from different sources in Portugal. On the other hand, Dotto *et al*. (2014) detected Class I integrons in (61.1%) of *E. coli* isolated from domestic and wild Lagomorphs in northern Italy.

Florfenicol is a derivative of chloramphenicol and it is only used for treatment of animals. Over the past decade, levels of the resistance to florfenicol and number of the resistant bacteria to it, have been increased due to the widespread use of florfenicol (Lu *et al*., 2018). As illustrated in Tables (7 and 8) and Fig. (5), screening our isolates by PCR for presence of *floR* gene that encodes florphenicol resistance revealed its presence in all the isolates. *FloR* gene was detected with somewhat lower prevalence in studies of Li *et al*. (2020) and El-Sharkawy *et al*. (2017) who found that (91.51%) of *E*. coli isolates and (80%) of *S. Typhimurium* isolates harbor *floR* gene respectively. In this study, it was found that all the isolates were sensitive to chloramphenicol except (42.9%) of *E. coli* isolates although they harbor *floR* gene that confers chloramphenicol resistance, this may be attributed to that this resistant gene is silent in vitro in these isolates or due to that chloramphenicol resistance may by expressed phenotypically through another resistance genes or another resistance factors aren't tested in this study.

In this study, it was found that only (21.4%) and (33.3%) of *E. coli* and *S. Typhimurium* isolates harbor *QacA/B* gene respectively as illustrated in Tables (7 and 8) and Fig. (6). Our results came in accordance nearly with Ibrahim *et al*. (2019) who found *QacA/B* gene in (14.7%) of *E. coli* isolated from chickens in Egypt while it came incompatible with Enany *et al*. (2019) who reported that all the tested *Salmonella* isolated from environmental and avian sources in Egypt harbor *QacA/B* gene and with Nabil and Yonis (2019) who can't detect *QacA*/*B* gene in *Salmonella* isolated from broiler chickens in Egypt. This difference may be attributed to the differences in farm management and geographic location.

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CONCLUSION

According to results of this study, *E. coli* and *S. Typhimurium* are one of the major causes of early mortality in Japanese quail chicks. *E. coli*, *S. Typhimurium* and *K. pneumoniae* isolated from quail chicks were pathogenic, harbor Class 1 integrons gene and they showed marked resistance to several antibiotics commonly used in poultry and human in Egypt (MDR). Therefore, more attention should be driven to the biosecurity measures in quail hatcheries and farms in Egypt and prudent use of antibiotics supported by antibiogram tests before drug administration in quail farms.

AUTHOR'S CONTRIBUTION

All authors contributed equally in this work. They read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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عزل وتوصيف بعض أنواع البكتيريا المعوية المعزولة من النفوق المبكر لكتاكيت السمان اليابانية بمحافظة قنا ، مصر

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أصبحت مزارع السمان واسعة الإنتشار فى مصر كمصدر للحوم. يعتبر النفوق المبكر لكتاكيت السمان أحد العوامل التي تعوق تربية السمان. أجريت هذه الدراسة لدراسة دور بعض أنواع البكتيريا المعوية كأحد أسباب النفوق المبكر لكتاكيت السمان في مزرعة السمان بجامعة جنوب الوادي ولتوصيف العزلات ظاهريا وسيرولوجيا ولتحديد حساسيتها للمضادات الحيوية ، وفحص جميع العزلات لوجود الجين المقاوم للفلورفينيكول (*floR*) والجين المقاوم لمركبات الأمونيوم الرباعية(QacA/B) وجين انتجرونات الدرجة الأولى (*Int1*) وكذلك فحص عزلات الإشريكية القولونية والسالمونيلا والكليبسيلا نيمونيا لجينات (*eaeA*) و (*invA*) و (*mag*A) على التوالى. لذلك فقد تم جمع ٨٠ صوص سمان ياباني نافق حديثًا أو محتضر من هذه المزرعة أعمارهم تحت سن ١٢ يوم خلال الفترة من ديسمبر ٢٠٢٠ إلى أبريل ٢٠٢١ للتقييم السريري والفحص البكتريولوجي. تم عزل وتعريف ١٤ عزلة من الإشريكية القولونية و ٦ عزلات من السالمونيلا وعزلة واحده من الكليبسيلا نيمونيا من الأعضاء الداخلية المختلفة لكتاكيت السمان المفحوصة بنسبة (١٧,٥٪) و (٧,٥٪) و (١,٢٥٪) على التوالي وقد أنتمت عزلات الإشريكية القولونية إلى ٥ مجموعات مصلية مختلفة هى O_{86} وO_{78} وO_{26} وO_{55} وO_{164} بنسبة (28.6%) و (21.4%) و (14.3%) و(14.3%) و(7.1%) على التوالى بالإضافة إلى عزلتين لم تصنف سيرولوجيا بينما عرفت جميع عزلات السالمونيلا سيرولوجيا على أنها سالمونيلا تيفيموريوم. أظهر إختبار الحساسية للمضادات الحيوية المختلفة المستخدمة فى هذه الدراسة أن جميع العزلات كانت حساسة فقط للكلورامفينيكول والأوكسي تتراسيكلين باستثناء (٤٢,٩٪) و (٨٥,٧٪) من عزلات الإشريكية القولونية على التوالي وأيضا للأزيثروميسين باستثناء (٣٥,٧٪) من عزلات الإشريكية القولونية وعزلة الكليبسيلا نيمونيا. أظهر إختبار تفاعل البلمرة المتسلسل أن جميع العزلات تحمل جينى (*Int1*) و (*floR*) وأن عزلات السالمونيلا تيفيموريوم تحمل جينى (*invA*) و(*QacA/B*) بنسبة (100%) و (٣٣,٣٪) على التوالي بينما تحمل عزلات الإشريكية القولونية جينى (*eaeA*) و(*QacA/B*) بنسبة (٢١,٤٪) فقط. وقد خلصت الدراسة إلى أن الإشريكية القولونية والسالمونيلا تيفيموريوم من الأسباب الرئيسية للنفوق المبكرة لكتاكيت السمان اليابانية.