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Original Research Article

THE LITTLE WHITE EGRET (*Egretta garzetta*) AS A POTENTIAL SOURCE OF MULTI-DRUG-RESISTANT AVIAN PATHOGENIC *E. coli*

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Abstract: Avian pathogenic *Escherichia coli* (APEC) is a significant threat that affect domesticated and wild birds. Studies related to the prevalence of APEC in the migratory and wild birds are relatively few compared with those related to other avian species. In particular, the role of the little white egret (*Egretta garzetta*) as a carrier and a reservoir for transmission of the APEC to other avian species had been neglected. Therefore, this work was done to study the occurrence of multidrug-resistant (MDR) APEC in the extraintestinal tissues of the little white egret. The overall isolation percentage of APEC was 20%. The highest isolation percentage was recorded in lungs followed by air sacs, heart blood, liver, and kidneys with percentages of 20%, 15%, 10%, 10%, and 5%, respectively. Sereotyping of *E. coli* isolates revealed, 4 (33.33%) strains of O26:K6, 3 (25%) strains of O78:K80, 2 (16.66%) strains of O114:K90, 2 (16.66%) strains of O2:K1, and 1(8.33%) strain of O127:K63. Virulence-associated genes including arginine succinyltransferase *(astA),* temperature sensitive hemagglutinin *(tsh),* putative avian hemolysin *(hlyF),* and iron outer membrane receptor *(iroN)* were screened using polymerase chain reaction (PCR) and all of tested genes were detected in *E. coli* serotype O78:K80. All *E. coli* isolates showed drug resistance to at least one of the 12 antimicrobials tested, with remarkable high resistance (100%) to ampicillin, nalidixic acid, and penicillin. In conclusion, the little white egret should be considered as a potential carrier for MDR APEC.

Key words: *E. coli*; little white egret; multidrug resistance; virulence attributes

Introduction

Escherichia coli (*E. coli*) is among the natural inhabitants of the gastrointestinal tracts of the living species. In birds, under certain conditions, *E. coli* may invade other tissues and cause localized or systemic infections, collectively is called colibacillosis (1, 2). Systemic avian colibacillosis is characterized by peritonitis, perihepatitis, pericarditis, and air sacculitis (3). Avian pathogenic *E. coli* (APEC) causes mass economic losses in the poultry industry worldwide. In addition, *E. coli* causes a significant decline in the numbers of wild birds (4, 5).

The abuse of the antimicrobials in the animals' intensive rearing programs had led to the development of multidrug resistance (MDR) phenomenon among pathogens in many countries (6). The spread of APEC in birds is facilitated by the development of their MDR (7, 8). Exposure to MDR *E. coli* represents a great hazard for birds, animals and public health (9, 10). Such pathogens can spread via proliferation in humans, birds and animals, including untargeted species (11).

The little white egret (*Egretta garzetta*) is a famous wild bird that is always seen nearby chicken farms and agricultural lands in the Middle-Eastern countries, particularly in Egypt. Multidrug-resistant APEC serovars were isolated from wild

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and migratory birds sampled at Pakistan (12), Alaska landfill (11), Mediterranian-black sea flyway (13), China (14), and Egypt (15). However, there is a clear lack of information about the prevalence of *E. coli* in the little white egret. Besides, there is no information available on the virulence-associated genes and the antimicrobial sensitivity testing in *E. coli* isolated from the little white egret. In addition, the possible contribution of the the little white egret as a carrier and a reservoir for the transmission of APEC to domesticated birds is poorly discussed.

Therefore, this study investigated the occurrence of APEC in different tissues of diseased little white egret captured nearby chicken farms in Egypt. Besides, detection of the virulence attributes in the recovered *E. coli* was done using PCR. Furthermore, antimicrobial sensitivity testing of the identified *E. coli* serotypes was examined.

Material and methods

Sampling

A total of one hundred samples of liver, lungs, air sacs, heart blood, and kidney (20 each) were aseptically collected from 20 diseased captured little white egret showing clinical signs (emaciation, respiratory signs, and diarrhea) nearby chicken farms in Sharkia Governorate, Egypt during August to October 2020. All samples were collected under full aseptic conditions and then quickly sent to the laboratory in an icebox for bacteriological examination.

Bacteriological Examination

The collected samples were plated on MacConkey agar plates (Difco, USA), followed by incubation at 37°C for 24 h. Pink colonies with lactose fermentation were inoculated onto Eosin Methylene Blue agar plates (Difco, USA). Colonies with metallic green shiny appearance were incubated in nutrient agar slants at 37°C for 24 h, then stored at 4°C for further identification. *E. coli* identification was done based on the standard staining and biochemical tests including Eijkman, catalase, oxidase, indole, methyl red, Vogus-Proscour, H_2S production, and gelatin liquefaction tests (16).

Serological identification of isolated E. coli

Serological identification of the biochemically-identified *E. coli* isolates was performed according to the method of Kok *et al.* (17) using diagnostic *E. coli* antisera sets (Hardy Diagnostics, Ohio, USA). Serological identification was conducted in Faculty of Veterinary Mediine, Benha University, Egypt.

DNA preparation

Purified *E. coli* isolates were prepared to obtain bacterial pellets (18). Tris-EDTA buffer was added to the pellet followed by three times heating-freezing cycles to obtain bacterial DNA. The DNA concentration and quality were confirmed using Nanodrop (ND-1000, Nanodrop Technologies, USA).

Detection of virulence-associated genes by PCR

E. coli were screened for the detection of virulence-associated genes including arginine succinyltransferase *(astA),* temperature sensitive hemagglutinin *(tsh),* putative avian hemolysin *(hlyF),* and iron outer membrane receptor *(iroN)* using PCR. The PCR procedures were done using the protocol of Boom *et al.* (18) using DNA (30 ng) as a tempelate. Primer sets used were displayed in Table 1 and they were prepared using Primer3Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The reference strains *E. coli* O157: H7 Sakai (positive control), and *E. coli* K12DH5α (negative control) were used.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the identified isolates was screened using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (19). The used antimicrobial sensitivity discs (Oxoid Limited, Basingstoke, Hampshire, UK) were ampicillin (10 μg; AM), cephalothin (30 μg; CET), chloramphenicol (30 μg; C), ciprofloxacin (5 μg; CIP), enrofloxacin (5 μg; ENR), erythromycin (15 μg; E), gentamicin (10 μg; GEN), kanamycin (30 μg; K), nalidixic acid (30 μg; NA), neomycin (30 μg; N), oxacillin (1 μg; OX), oxytetracycline (30 μg; OXY), penicillin (10 IU; P), and trimethoprim/sulfamethoxazole (25 μg; SXT).

Table 1: PCR primers used in the present study

No.	Virulent genes	Primer sequence (5'-3')	Size (bp)	GeneBank (Accession No.)	Annealing temperature (°C)
1	*tsh*	GCACAAGCAGGGAATGAGTAG TCATTCCGCCACGATATCCC	257	CP050998.1	60
2	*hlyF*	CCCGGAAAAGATGGCCTTCA CGTATAACGTTCACTCCGGGA	478	CP050998.1	60
3	*astA*	ATCAGTCCAGACCAGGAGCT AGCATCCAGTGGTCGGTTTT	317	CP050998.1	58
4	*iroN*	TCGGTATGGTTTGATTCC CAATGGCCGTACGTCCTA	120	CP076646.1	60

bp: base pair

Results

The captured little white egret had clinical signs of illness including emaciation, respiratory signs and diarrhea. At postmortem inspection, pericarditis, air sacculitis, caseation of air sacs, and necrotic foci in the liver were detected. Bacteriological examination revealed four positive birds for the isolation of *E. coli* with a percentage of 20%. *E. coli* was isolated from the different examined extraintestinal tissues. Lungs had the highest positivity rate at 20% (4 out of 20 samples), followed by air sacs at 15% (3 out of 20 samples), liver at 10% (2 out of 20 samples), heart blood at 10% (2 out of 20 samples), and kidneys at 5% (1 out of 20 samples) (Table 2).

Five serotypes of the isolated *E. coli* (n = 12) were detected in the present study including *E. coli* O2:K1, O26:K60, O78:K80, O114:K90, and O127:K63. *E. coli* O26:K60 had the highest prevalence at 33.33% (4 /12), followed by *E. coli* O2:K1 (16.66%; 2 / 12), *E. coli* O114:K90 (16.66%; 2 /12), *E. coli* O78:K80 (25.00%; 3 /12), and *E. coli* O127:K63 (8.33%; 1/ 12) (Table 2; Figure 1).

Using PCR, virulence-associated genes were detected in the identified *E. coli* serogroups. Only one isolate of *E. coli* O26:K60 had both *astA*, and *iroN*; while no virulence attributes were detected in *E. coli* O2:K1, and *E. coli* O114:K90. While, one isolate of *E. coli* O127:K63 expressed both *hlyF*, and *iroN*; all tested virulence-associated genes were detected in *E. coli* O78:K80 (Figure 2).

Regarding the antimicrobial sensitivity testing, the resistance profiles of the identified *E. coli* serogroups were presented in Table 2. *E. coli* O2:K1 was 100% resistant to penicillin, ampicillin, and nalidixic acid, and 50% resistant to cephalothin, kanamycin, nalidixic acid, oxacillin, oxytetracycline, and trimethoprim/sulfamethoxazole. While the isolates were sensitive to the other tested antimicrobials. *E. coli* O114:K90 had a resistance profile as follows: 100% to ampicillin, cephalothin, nalidixic acid, penicillin, oxytetracycline, and trimethoprim/sulfamethoxazole; 50% to chloramphenicol, ciprofloxacin, erythromycin, kanamycin, and oxytetracycline. The isolates of *E. coli* O78:K80 were resistant at 100% to penicillin, and nalidixic acid; while were less resistant to the other tested antimicrobials. The resistance profiles of *E. coli* O26:K60 were as following: 100% to ampicillin, nalidixic acid, and penicillin; 50% to each of oxacillin, oxytetracycline, and trimethoprim/sulfamethoxazole; 25% to both of cephalothin, and neomycin. *E. coli* O127:K63 showed 100% resistance to ampicillin, cephalothin, nalidixic acid, penicillin, and trimethoprim/sulfamethoxazole; while sensitive to the other tested antimicrobials (Table 2).

Table 2: Incidence (number and percentages) of different *E. coli* serotypes in the examined tissues of little white egret

	Positive samples		
Examined tissue	Number	%	Detected serotypes
Lungs	4	20	O2:K1, O26:K60, O78:K80, O114:K90
Air sacs	3	15	O2:K1, O127:K63, O78:K80
Liver	2	10	O26:K60, O78:K80
Heart blood	2	10	O26:K60, O114:K90
Kidneys	1	5	O26:K60

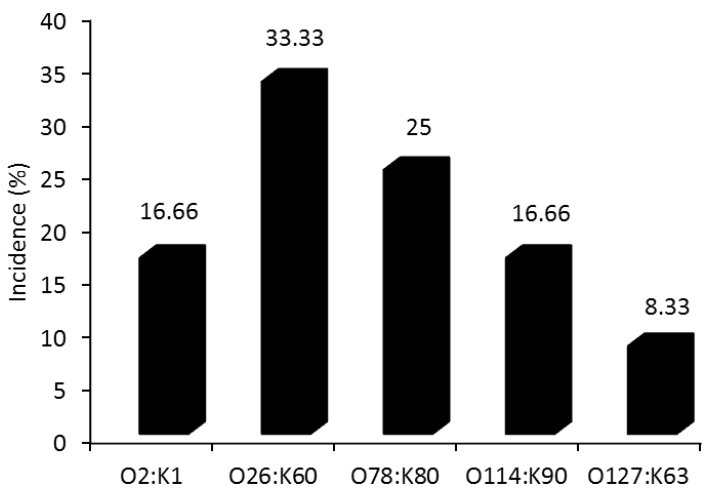

Figure 1: Incidence (%) of *E. coli* serotypes in the examined samples of little white egret

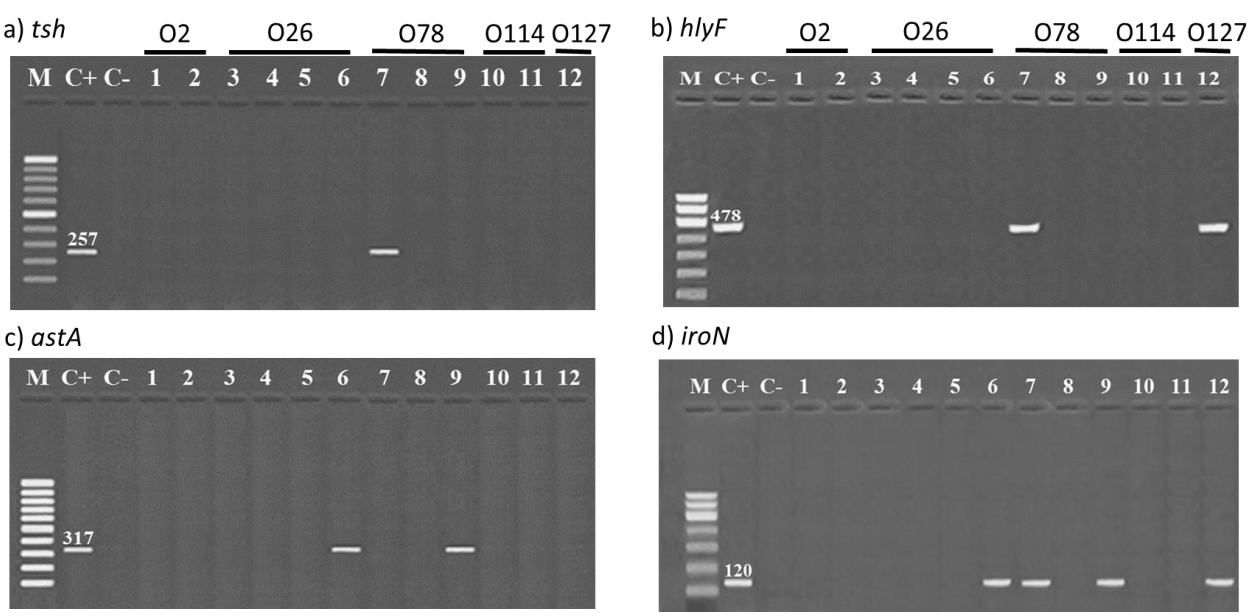

Figure 2: Agarose gel electrophoresis of virulence-coding genes in the recovered *E. coli* serotypes from the examined tissues of little white egret a) *tsh*, b) *hlyF*, c) *astA*, and d) *iroN*. M referes to a 100 base pairs DNA marker, C+ refers to a positive control, C- refers to a negative control

Table 2: Antimicrobial resistance (%) of extraintestinal *E. coli* serotypes isolated from little white egret

E. coli serotypes	O2:K1		O26:K60		O78:K80		O114:K90		O127:K63	
Antimicrobial	No.	%	No.	%	No.	%	No.	%	No.	%
AM	2	100	4	100	2	66.66	2	100	1	100
CET	1	50	1	25	0	0	2	100	1	100
C	0	0	0	0	0	0	1	50	0	0
CIP	0	0	0	0	0	0	1	50	0	0
ENR	0	0	0	0	1	33.33	0	0	0	0
E	0	0	0	0	1	33.33	1	50	0	0
GEN	0	0	0	0	1	33.33	0	0	0	0
K	1	50	0	0	1	33.33	1	50	0	0
NA	2	100	4	100	3	100	2	100	1	100
N	1	50	1	25	1	33.33	0	0	0	0
OXY	1	50	2	50	2	66.66	2	100	0	0
T	1	50	2	50	2	66.66	1	50	0	0
P	2	100	4	100	3	100	2	100	1	100
SXT	1	50	2	50	2	66.66	2	100	1	100

(No.) indicates the number of the resistant isolates in this serotype, (%) indicates the percentage of resistance among the identified isolates, AM: ampicillin, CET: cephalothin, C: chloramphenicol, CIP: ciprofloxacin, ENR: enrofloxacin, E: erythromycin, G: gentamicin, K: kanamycin, NA: nalidixic acid, N: neomycin, OX: oxacillin, OXY: oxytetracycline, P: penicillin, and SXT: trimethoprim/sulfamethoxazole.

Discussion

E. coli is considered as a significant threat affecting chicken industry leading to high morbidities and mortalities and massive economic losses worldwide (4). The little white egret is usually seen nearby poultry and large animal farms in Egypt. However, there is a scarce information about the prevalence of APEC in such wild migratory bird species. In the present study, *E. coli* serotypes were isolated and identified at 20% from the captured little white egret. The captured birds suffered from general signs of illness and emaciation. At postmortem inspection, such birds had pericarditis, perihepatitis and air sacculitis. *E. coli* were also isolated from cases of septicimia in the crested ibises (*Nipponia nippon*) in China. The postmortem lesions in the dead birds showed swelling in the liver; hemorrhagic pericarditis; miliary tubercles in lung (4). In agreement with our findings, *E. coli* was also isolated from other wild and migratory birds such sympatric gulls and bald eagles inhabiting a landfill habitat in Alaska (11), and migratory waterfowls in Egypt (15). *E. coli* was isolated from extraintestinal tissues, and this agreed with the postmortem findings and indicated dissemination of *E. coli* from intestine to other organs upon failure of the immune system in the affected birds (20). The tissue distribution of APEC in the current investigation agrees with that reported in ducks (21), turkeys (22), and quails from Egypt (23).

Five *E. coli* serotypes were identified in the present study including *E. coli* O2:K1, O26:K60, O78:K80, O114:K90, and O127:K63. Some *E. coli* serotypes are also classified as human *Enteropathogenic E. coli* (EPEC) which includes O114, O119, O26, O55, O86, O127, O128, O111, O125, O126, O142, and O158 (24). This indicates that little white egret is also of zoonotic importance in the transmission of EPEC serotypes as well as a reservoir for transmission of APEC to other avian species and farm animals. Likely, cattle egret was found to harbor shiga-toxin coding genes from *E. coli* isolates obtained from the intestinal contents of the birds sampled in Egypt

(25). Besides, zoonotic bacteria such as *E. coli* O157:H7 and *Salmonella* spp. were isolated from cattle erget cecal contents in a study conducted in USA (26). APEC strains had developed a set of virulence-associated genes that facilitate the spread of bacteria into different organs, colonization and resistance to antimicrobials (27). Temperature-sensitive hemagglutinin (*tsh*) was first identified in APEC O78 and it is a member of the autotransporter proteins. It is responsible for pericarditis, perihepatitis, air sacculitis, and peritonitis. *E. coli* strains harboring *tsh* have high pathogenicity and lethality (28). Hemolysin is an important determinant for enterotoxigenic strains of *E. coli*. An *E. coli* cellular locus, *hlyF*, is required for the synthesis and secretion of hemolysin (29). Interestingly, *hlyF* was detected in isolates of *E. coli* O78, and O127. Arginine is used as an energy source in *E. coli* and over expression of *astA* in bacteria has close association with their faster growth and rapid multiplication (30). In the current work, *astA* was expressed in *E. coli* O26 and O78. Likely, *astA* was highly detected in *E. coli* O78, O86 and O114 isolated from duck samples in Egypt (21). Iron outer membrane receptor (*iroN*) is a virulence-associated factor that facilitates biofilm formation, pathogenicity and iron uptake (31). In the current study, *iroN* was detected in *E. coli* O26, O78, and O127. Similarly, APEC strains (O26, O78, and O127) isolated from ducks and quails also had the *iron* gene (21, 23).

The unnecessary use of antimicrobials in animal and poultry farms led to the development of bacterial strains with antimicrobial resistance worldwide. Such drug-resistant bacterial strains can find their way into the environment and colonize in untargeted species and lead to spreading of such drug-resistant strains (6). Interestingly, in the present study, the identified *E. coli* serotypes showed marked resistance to several antimicrobials such as ampicillin, cephalothin, and trimethoprim/sulfamethoxazole. Multi-drug resistance was reported in several *E. coli* serotypes isolated from farmed ducks (32), and quails (23). Furthermore, multidrug resistant *E. coli* serovars were also isolated and identified in migratory birds. For instances, Ramey *et al.* (33) detected *E. coli* strains with antimicrobial resistance among migratory birds in Alaska. Furthermore, evidence for colistin resistance among *E. coli* isolated from migratory and resident birds was reported in Egypt (34). Besides, Wu et al. (35) reported that egret could transfer antibiotic resistance from a contaminated waterway (Jin River, Chengdu, China) into the surrounding environment (Wangjianglou Park). Therefore, it is suggested that little white egret is considered as a potential source for the spread of MDR *E. coli*.

Conclusion

The obtained results of the current study indicated the prevalence of APEC in little white egret sampled near poultry farms in Egypt. The identified *E. coli* serotypes were E. coli O2:K1, O26:K60, O78:K80, O114:K90, and O127:K63. Such pathotypes had virulence-coding genes including *tsh, hlyF, astA,* and *iroN*. The identified *E. coli* serotypes had multidrug resistance profiles. Therefore, as a practical application for the present study, little white egret should be considered as a reservoir for APEC and a potential source for transmission of the pathogenic *E. coli* serovars from *E. coli*-contaminated environment to other domesticated birds and farm animals.

Conflict of interest: None

Naser A. Al-Humam designed the study, funded the work, analyzed the data, and drafted the paper; Walaa Fathy Saad Eldin collected the specimens, conducted the experiments, analyzed the data, drafted and revised the paper. All authors agree to particicpate in this study. All authors agree to publish this study.

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