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Original Article

Detection of phenotypes, virulence genes and phylotypes of avian pathogenic and human diarrheagenic *Escherichia coli* in Egypt

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Abstract

Introduction: The purpose from this study was to determine phenotypes, intestinal virulence-associated genes, and phylotypic profiling of human diarrheagenic *E. coli* (DEC) and avian pathogenic *E. coli* (APEC).

Methodology: A total of 108 chicken visceral organs (liver, spleen, heart) from 36 diseased birds (three organs per each bird) and 78 human stool samples (50 diarrheic patients and 28 healthy persons) were randomly collected during the first half of 2015 in the district of Mansoura city, Egypt. Conventional culturing, serotyping, and molecular characterization of virulence genes and phylogroups were performed.

Results: Sixty-five (35%) biochemically identified *E. coli* isolates were detected from chicken visceral (29/108; 26.9%) and human stool samples (36/78; 46.2%). Serotypes O78, O2, and O1 were the most prevalent serotypes (62%) distinguished from APEC isolates, and only two similar serotypes (O119:H4 and O26:H11) were identified from both APEC and DEC isolates. By polymerase chain reaction (PCR), the respective percentages of 100 and 35 with *eae* and Shiga toxin genes were detected from APEC isolates while 50%, 27.8%, and 19.4% of human DEC isolates harbored *eae*, *stx1*, and *stx2* genes, respectively. Phylogrouping revealed a significantly higher occurrence of pathogenic phylogroups (D and B2) in APEC (19/29; 65.5%) than in human DEC isolates (8/36; 22.2%).

Conclusions: APEC isolates shared serotypes, virulence genes, and phylotypes with human DEC isolates, which is a subsequent potential public health concern. To the best of our knowledge, this is the first report in Egypt that determines virulence gene and phylogroup coexistence between APEC and DEC isolates.

Key words: APEC; DEC; *stx* gene; *eae* gene; phylotype; zoonoses.

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Introduction

Escherichia coli (*E. coli*) is a naturally occurring organism that forms part of the animal and human gut microbiota; however, some strains have the ability to produce pathogenicity in both [1]. Pathogenic strains have been divided into intestinal (diarrheagenic; DEC) and extra-intestinal (ExPEC) pathogenic *E. coli*. Avian pathogenic *E. coli* (APEC), a subdivision of ExPEC that produces a systemic disease in poultry, could serve as a potential zoonotic hazard to humans [2,3].

APEC colonizes the intestinal tract of the chicken and has the ability to disseminate systemically either through intestinal or respiratory mucosa in the presence of stressors such as inappropriate husbandry measures [4]. The conventional methods of slaughtering and evisceration that done manually, especially in developing countries, lead to overstate the incidence of carcass contamination with different bacterial pathogens [5] and accordingly a great value of the identification of these pathogens from chicken viscera.

The investigations of the zoonotic burden of chicken isolates depend on phenotypic characterization and assessment of the common serotypes that have been isolated from infected birds. However, the overlap between different serotypes makes virulence genotyping the valued method to categorize different *E. coli* pathotypes [6,7]. Nonetheless, the role of serotyping in distinguishing APEC and other *E. coli* cannot be absolutely ignored, as some serotypes such as O78, O1, O2, and O18 have been more commonly associated with APEC than other pathotypes [7-9].

The implementation of PCR methods to screen and identify the common virulence genes and phylogroups between different isolates is crucial [10]. The main

phylogenetic groups to which *E. coli* predominately belong are A, B1, B2, and D; the virulence genes have been mostly associated with phylogroups D and B2 rather than the other phylogroups. The simple and rapid triplex PCR method that determines *chuA* and *yjaA* and a DNA fragment, TspE4.C2, also provides a link between virulence genotyping and phylotyping by identifying potential pathogenic strains from commensal ones [11].

There is a paucity of information in Egypt about the possibility of diarrheagenic virulence genes sharing between APEC strains isolated from chicken viscera and human intestinal *E. coli* strains. Thus, the overall objective of this study was to investigate the presence of pathogenic *E. coli* in chicken viscera and human stool (diarrheic and healthy samples) by conventional isolation and serotyping, followed by the determination of the virulence genes *eae*, *stx1*, and *stx2* and the distribution of phylogroups in these isolates.

Methodology

Sample collection

A total of 108 chicken visceral organs (liver, spleen, and heart) from 36 diseased chickens (three organs per each bird) were collected randomly from different poultry farms (each farm contained 10,000–20,000 birds) located in the district of Mansoura city (latitude 31° north and longitude 31° east), Egypt, during the first quarter of 2015. Upon necropsy, the common lesions detected in these diseased birds were pericarditis, air sacculitis, perihepatitis, ascites, splenitis, and peritonitis. The visceral organs from each bird were individually packed in a polyethylene bag and immediately transferred in an ice tank to the laboratory of the Microbiology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Mansoura University, for bacteriological analysis under sterile conditions.

Seventy-eight human stool samples (50 from diarrheic patients and 28 from healthy persons) were included in this study. All the stool samples (1 sample per individual) were taken from a clinical pathology laboratory of a small charity hospital in the district of Meniet Sandoub, Mansoura, Egypt. When informed consent was received from those enrolled in this experiment, a detailed questionnaire was administered, which included information about age, health status, eating habits, and previous exposure to diarrheic episodes. Diarrheic patients admitted to the hospital presented symptoms of abdominal disturbances with foul-smelling diarrhea. All human stool samples were taken in sterile sample collection vials during the second quarter of 2015, transferred as fast as possible to the laboratory of Hygiene and Zoonoses Department, Faculty of Veterinary Medicine, Mansoura University, and tested for the presence of pathogenic *E. coli*.

Conventional identification of E. coli

From each human stool and chicken visceral organ, 2 g was directly enriched in tryptic soya broth (TSB; Becton Dickinson, Sparks, USA) and incubated at 37°C for 18 hours. Then, a loopful from the overnight enriched culture was streaked onto MaCconkey agar (Becton Dickinson, Sparks, USA) and incubated at 37°C aerobically for 18 to 24 hours. A single colony from each plate with the typical morphological pattern of *E. coli* on MacConkey was picked, streaked onto eosin methyelene blue (EMB; Becton Dickinson, Sparks, USA) and incubated overnight at 37°C. The identification of *E. coli* isolates depends upon the colony morphological criteria and biochemical testing [12]. *E. coli* isolates were stored in 25% glycerol-supplemented TSB at -80°C until used.

Serotyping

The biochemically identified *E. coli* isolates were subjected to serotyping as described by Kok *et al.* [13] at the Department of Food Hygiene Control, University of Benha, Egypt, by using rapid diagnostic *E. coli* antisera sets (Difco Laboratories, Detroit, USA).

DNA extraction

Three representative colonies of the same morphological type were picked from the slants of the previously isolated bacteria, transferred into a tube containing 3 mL of TSB, and incubated at 37°C for 18 hours. One milliliter of the overnight bacterial culture was centrifuged at 8,000 × g for 2 minutes and then sediment was washed with nuclease-free water, homogenized, and heated at 95°C for 15 minutes. The supernatants from boiled lysates were used as DNA template and transferred to the Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University, for the virulence gene and phylogroup identification.

Molecular identification of virulence genes

The primer pairs used (sequence, target gene, and PCR product) are summarized in Table 1 [14,15,11]. PCR was performed in a volume of 15 µL consisting of 7.5 µL of 2X PCR Master Mix (Promega, Madison, USA), 0.15 µL of each primer (100 µM each), and 2 µL DNA template. PCR program for both *stx1* and *stx2* genes was similar to that done by Paton and Paton [15],

with slight modifications, and started with an initial denaturation for 5 minutes at 95°C, 35 cycles (95°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds), and a final extension step at 72°C for 7 minutes. The PCR condition for the *eae* gene was similar to that described by Fagan *et al.* [16], with the annealing temperature at 64°C. DNA extracted from *E. coli* O157:H7 and *Salmonella* Typhimurium (personal unpublished data; isolated from cloacal swab of infected chicken, identified biochemically and serotyped) were used as positive and negative control in each PCR run, respectively.

Phylogenetic grouping of APEC and DEC isolates

The main *E. coli* phylogenetic lineages (A, B1, B2, and D) were determined by a triplex PCR for the amplification of gene specific markers *chuA*, *yjaA*, and the DNA fragment TspE4.C2 that yielded three characteristic amplicons (Table 2). PCR reaction was done using a Bio-Rad (Munich, Germany) thermal cycler with a condition similar to that done by Clermont *et al.* [11].

Statistical analysis

The association of phylogroup distribution between chicken and human isolates was determined by the Chi-square (χ^2) test performed with SPSS version 16.0 (SPSS Inc., Chicago, USA) software at a probability value $p < 0.05$.

Results

In this study, a total of 186 samples were tested using the conventional cultural methods mentioned to determine the occurrence of pathogenic *E. coli* from chicken visceral organs (n = 108) and human stool samples (n = 78; 50 diarrheic and 28 healthy subjects). Sixty-five isolates (35%) were biochemically identified to be *E. coli* from chicken visceral (29/108; 26.9%) and human stool samples (36/78; 46.2%). All the 29 biochemically identified *E. coli* isolates from chicken viscera were serogrouped and confined to 9 serotypes (Table 3). On the other hand, only 36.1% (13/36) of human *E. coli* isolates were identified and grouped into 8 serotypes (Table 4).

As shown in Table 3, O78:H$^-$ (11/29) was the most common serotype isolated from chicken viscera (37.9%), followed by O2:H6 (5/29; 17.2%); collectively, they represent 55.2% (16/29) of the isolated chicken serotypes. The distribution of *E. coli* isolates from human stool samples is shown in Table 4. A total of 29 strains (11 serotyped and 18 untypeable) and 7 strains (2 serotyped and 5 untypeable) were detected from diarrheic patients and healthy persons, respectively. Notably, only 2 similar serotypes (O119:H4 and O26:H11) from all 17 determined serotypes (11.8%) were detected in this study from both chicken and human isolates.

All 65 *E. coli* isolates from chicken viscera and human stool samples were subjected to uniplex PCR for the direct identification of *eae*, *stx1*, and *stx2* genes. The

Table 1. List of primers used for virulence genes identification and phylotyping of *E. coli* isolates.

Target gene	PCR product	Primer sequence	Reference
eae F R	890	5'GTGGCGAATACTGGCGAGACT-3' 5'-CCCCATTCTTTTTCACCGTCG-3'	[14]
stx1 F R	180	5'ATAAATCGCCATTCGTTGACTAC-3' 5'-AGAACGCCCACTGAGATCATC-3'	[15]
stx2 F R	255	5'-GGCACTGTCTGAAACTGCTCC-3' 5'-TCGCCAGTTATCTGACATTCTG-3'	[15]
chuA 1 2	279	5'GACGAACCAACGGTCAGGAT-3' 5'-TGCCGCCAGTACCAAAGACA-3'	[11]
yjaA 1 2	211	5'-TGAAGTGTCAGGAGACGCTG-3' 5'-ATGGAGAATGCGTTCCTCAAC-3'	[11]
TspE4C2 1 2	152	5'-GAGTAATGTCGGGGCATTCA-3' 5'-CGCGCCAACAAAGTATTACG-3'	[11]

Table 2. The key of *E. coli* phylotyping using triplex polymerase chain reaction of *chuA*, *yjaA*, and TspE4.C2 genes.

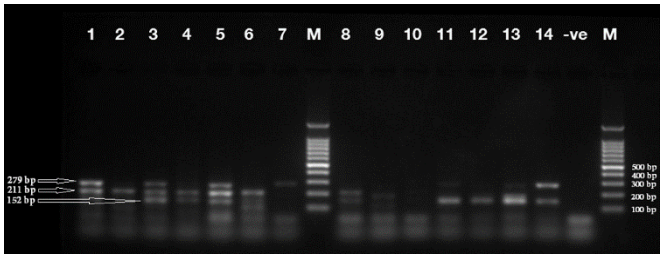
	Phylogroups							
Gene amplicons	A		B1		B2		D	
chuA (279 bp)	-	-	-	-	+	+	+	+
yjaA (211 bp)	+	-	-	+	+	+	-	-
TspE4.C2 (152 bp)	-	-	+	+	-	+	+	-

+ Presence of the target amplicon; - Absence of the target amplicon.

obtained results that determined the frequency of these virulence genes among the tested strains are summarized in Tables 3 and 4. All *E. coli* isolates from chicken viscera in this study carried the *eae* gene, and only 1 (3.4%) and 10 (34.5%) isolates harbored *stx1* and *stx2*, respectively. However, only half of the human *E. coli* isolates showed the specific amplified product with the *eae* gene at 890 bp, and the *eae* gene was more prevalent in diarrheic (15/29; 51.7%) than in healthy human isolates (3/7; 42.9%). The *stx1* and *stx2* amplicons (180 bp and 255 bp) were obtained from 10 (27.8%) and 7 (19.4%) human *E. coli* isolates, respectively.

The characterization of the four main phylogroups (Figure 1) among the 65 biochemically identified *E. coli* isolates revealed that distribution among the chicken *E. coli* isolates (Table 5) was as follows: D (15/29; 51.7%), A (7/29; 24.1%), B2 (4/29; 13.8%), and B1 (3/29; 10.3%). From a total of 36 human isolates (Table 5), most of the isolates were identified as commensal group (A and B1) (28/36; 77.8%), and only 8 (22.2%) isolates were typed as pathogenic (virulent, extra-intestinal) phylogroup (B2 and D). A Chi-square value (X^2) of 7.21 and one degree of freedom (d.f) was calculated from a contingency table with the occurrence of pathogenic phylogroups between chicken and human isolates where a significantly ($p < 0.05$) higher distribution was found among chicken isolates (19/29; 65.5%) than human isolates (8/36; 22.2%).

Figure 1. Phylogenetic typing of representative *E. coli* isolates by triplex PCR.



Lane M: 100 bp DNA ladder. Lane -ve: negative control. Lane 2, 6 and 9: phylogroup A (*chuA*$^-$, *yjaA*$^+$, TspE4.C2$^-$). Lane 10: phylogroup A (*chuA*$^-$, *yjaA*$^-$, TspE4.C2$^-$). Lane 4 and 8: phylogroup B1 (*chuA*$^-$, *yjaA*$^+$, TspE4.C2$^+$). Lane 12: phylogroup B1 (*chuA*$^-$, *yjaA*$^-$, TspE4.C2$^+$). Lane 3 and 5: phylogroup B2 (*chuA*$^+$, *yjaA*$^+$, TspE4.C2$^+$). Lane 1: Phylogroup B2 (*chuA*$^+$, *yjaA*$^+$,TspE4.C2$^-$). Lane 11, 13, and 14: phylogroup D (*chuA*$^+$, *yjaA*$^-$, TspE4.C2$^+$). Lane 7: phylogroup D (*chuA*$^+$, *yjaA*$^-$, TspE4.C2$^-$).

The distribution of extra-intestinal phylogroups (13/18; 72.2%) was higher in APEC serotypes (O78, O2, and O1) than that of commensal phylogroups (5/18; 27.8%). With regard to the coexistence of virulence genes and phylogroups, 70% (7/10) of the *stx*$^+$ chicken isolates belonged to pathogenic phylogroups. On the other hand, approximately 50% of the human isolates

Table 3. The distribution of virulence genes and phylogroups among chicken *E. coli* phenotypes.

Phenotypes	No. of isolates (n = 29)	*eae* +ve	*stx1* +ve	*stx2* +ve	Phylogroups			
					A	B1	B2	D
O26:H11	2	2 (100%)	0	1 (50%)	2	0	0	0
O78	11	11 (100%)	1 (9.1%)	4 (36.4%)	1	2	2	6
O2:H6	5	5 (100%)	0	3 (60%)	1	1	0	3
O124	2	2 (100%)	0	1 (50%)	1	0	1	0
O55:H7	2	2 (100%)	0	1 (50%)	2	0	0	0
O1:H7	2	2 (100%)	0	0	0	0	1	1
O119:H4	1	1 (100%)	0	0	0	0	0	1
O127:H6	3	3 (100%)	0	0	0	0	0	3
O126	1	1 (100%)	0	0	0	0	0	1

Table 4. The distribution of virulence genes and phylogroups among human *E. coli* phenotypes.

Phenotypes	(No. of isolates)		*eae* +ve	*stx1* +ve	*stx2* +ve	Phylogroups			
	Diarrheic (n = 29)	Healthy (n = 7)				A	B1	B2	D
O119:H4	2	0	2 (100%)	1 (50%)	1 (50%)	1	0	1	0
O26:H11	1	1	2 (100%)	1 (50%)	0	1	0	1	0
O111:H2	2	1	2 (66.7%)	2 (66.7%)	1 (33.3%)	1	0	2	0
O148	1	0	1 (100%)	0	0	1	0	0	0
O113:H7	1	0	1 (100%)	0	0	1	0	0	0
O125:H21	2	0	2 (100%)	0	1 (50%)	1	0	1	0
O124	1	0	0	0	0	1	0	0	0
O44:H18	1	0	1 (100%)	0	0	0	0	1	0
Untypeable	18**	5*	7 (30.4%)	6 (26.1%)	4 (17.4%)	15	6	1	1

*Healthy untypeable isolates belonged to group A; **Diarrheic untypeable isolates belonged to all phylogroups (A, 10; B1, 6; B2, 1 and D, 1).

Table 5. Phylogroups of *E. coli* isolates from chicken carcasses and human stool samples.

Phylogroups	Chicken isolates(n = 29)	Human isolates			Total (n = 65)
		Diarrheic (n = 29)	Healthy (n = 7)	Total (n = 36)	
A	**7** (24.1%)	17 (58.6%)	5 (71.4%)	**22** (61.1%)	**29** (44.6%)
B1	**3** (10.3%)	6 (20.7%)	0	**6** (16.7%)	**9** (13.8%)
B2	**4** (13.8%)*	5 (17.2%)#	2 (28.6%)#	**7** (19.4%)*	**11** (16.9%)
D	**15** (51.7%)*	1 (3.4%)#	0	**1** (2.8%)*	**16** (24.6%)

*A significant association of phylogroups (B2 and D) distribution between chicken and human isolates; #Non-significant association of phylogroups (B2 and D) distribution between human diarrheic and healthy isolates.

belonged to commensal phylogroups that harbored *eae* and *stx* genes.

Discussion

The overall occurrence of *E. coli* isolates (26.9%; 29/108) from chicken viscera in this study as well as the detected serotypes (predominantly O78:H$^-$, O2:H6 and O1:H7) was not far from those previously reported by other researchers [2,7-9]. It is difficult to compare between the incidences of pathogenic *E. coli* along with the distribution of its serotypes among different countries owing to the contribution of many conditions such as geographic area, seasonal variation, sampling techniques, and the conventional methods used for its isolation. However, it might be helpful in the prediction of certain outbreaks caused by these serotypes, especially in countries of the same geographical and climatic conditions, with a subsequent application of a suitable control regime.

Concerning human isolates, the occurrence of pathogenic *E. coli* was higher among diarrheic patients than healthy persons [17,18]. Different serotypes determined from the diarrheic isolates were mostly the classical non-O157 enterohemorrhagic *E. coli* (EHEC) serotypes such as O26:H11, O111:H2, O113:H7, O103:H2, and O145:H28 [19]. From healthy persons, only two serotypes were identified (O26:H11 and O111:H2); this finding is in agreement with many previous studies which verified that O26 is considered one of the most clinically serotypes that could be isolated from both diarrheic and healthy persons [20,21].

From the above findings, the predominant APEC serotypes O78, O1, and O2 were not recovered from human stool samples. This was in agreement with many previous studies that reported the genetic relatedness between APEC and human ExPEC isolates with an explanation of the origin similarity of both isolates along with the possession of common virulence and antimicrobial resistance genes [22-25]. Meanwhile, some other studies previously isolated O78 serotypes from stools of diarrheic patients [26-28]. Interestingly, the presence of the same serotypes in this study (O26:H11 and O119:H4) isolated from diseased chicken viscera and human stool increases the awareness about the presence of substantial overlap at the level of serogroups between chicken and human isolates.

The PCR assays in our study screened a subset of chicken- and human-derived *E. coli* isolates with serotypes that have been associated with diarrhea in human patients. Approximately 35% of APEC isolates possessed *stx* genes, similar to the findings of many investigations [29-31] that detected *stx* genes from APEC isolates. However, many previous reports [32-34] failed to genetically identify *stx* genes from chicken viscera and they asserted that chickens could not act as potential sources and reservoirs of Shiga toxin-producing *E. coli*.

Our findings identified that two serotypes (O113:H7 and O26:H11), which belong to the classical EHEC, isolated from diarrheic patients, were *eae* gene positive and *stx* genes negative. This was similar to the findings of Kozub-Witkowski *et al.* [35], who molecularly identified eight strains that belonged to classical EHEC serotypes and were *stx* genes negative. This is not surprising, as there is still a possibility that these strains might acquire *stx* genes by horizontal transfer with a subsequent disease burden to humans [36], or they may have originated as EHEC strains and then lost phage-encoded *stx* genes [37].

Concerning phylogrouping, higher frequencies of virulent phylogroups of D and B2 were found among APEC isolates (particularly serotypes O78, O2, and O1), confirming the previous results about the distribution of extra-intestinal pathogenic strains in these phylotypes [38,39]. Nonetheless, the presence of phylogroup A in approximately 25% of APEC isolates in our study, which is predominantly associated with commensal *E. coli*, could be ascribed to the origin of these strains as commensals that acquired virulence-related genes [40].

The phylotype distribution of human *E. coli* isolates was influenced by many factors such as host genetic

factors, dietary factors, use of medications, and geographical conditions [41,42]. In this study, there was an over-representation of commensal phylogroups A and B1 among human isolates, which is consistent with the reports of previous studies that DEC isolates were included in phylogroups A, B1, and D [43,44]. The molecular identification of *eae* and *stx* genes in 50% of the human commensal phylotypes in this study is in agreement with the explanation of Escobar-Paramo *et al.* [42], who reported that human DEC strains (which belong to the commensal phylogroups) might have the genetic precursors and virulence genes necessary for disease emergence.

Conclusions

This study concluded that there is an overlap between APEC and DEC serotypes, besides the association of intestinal virulence genes and phylogroups among these isolates. A PCR-based method for the identification of virulence genes and phylotypes with conventional phenotyping would be a valuable tool in the epidemiological surveillance that could identify the zoonotic potential sources and possible risks to humans. Nevertheless, our results recommend further investigations into these isolates using the sequence typing method to delineate their genetic diversity and clonal circulation between animal food sources and humans.

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Authors' contributions

The main contributor (HR) designed the experiment, collected human samples, carried out a part of the conventional culturing and molecular phylogrouping, analyzed data, wrote the paper, and corresponded with the journal. The second author (AA) participated in sample collection from birds, isolated and identified *E. coli* from birds, participated in DNA extraction, and wrote a part in the manuscript. The third author (AA) contributed in the PCR assays of virulence genes identification, wrote a part in the manuscript, and performed the statistical analysis. All authors approved the final version of the manuscript for publication.

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