Research Article

Molecular Studies on *Escherichia coli* Isolated from Diarrheic Dog

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ABSTRACT

The objective of the present study was the isolation and identification of *E.coli* strains from dogs, serological identification and the detection of the virulence gene characteristics of STEC and attaching-effacing *(*AE*) eaeA* strains and multidrug resistant gene (MDR). A total of 120 rectal swabs were examined bacteriologically for isolation of *Escherichia coli* from diarrheic dogs in Cairo and Giza governorates from private clinic and veterinary hospitals. Sixty eight isolates of *E. coli* were recovered in percentage of (56.6%), Serogrouping of (68) isolates of *E.coli* revealed "7" different "O" serogroups which were O_{157}, O_1, O_{44}, O_{128}, O_{26}, O_{146} and O_{55} and (20 strains) were untyped. Antibiogram pattern revealed that the most strains of *E.coli* were resistant to Oxytetracyclin, Ampicillin, Sulphamethazol, Cephalothin and Imipenem, while variable results were recorded with the remaining tested chemotherapeutic agents. Molecular Detection of Extended spectrum β-Lactamase (ESBL) gene and some virulence gene including *stx1, stx2* and eaeA gene were applied *E.coli* producing *sul1* are under serogroups O_{157}, O_1, O_{44}, O_{128} and O_{26} at 433bp. Also serogroups O_{55}, O_{146}, O_{26}, and O_{44} were producing tet (A) at 576 bp. While serogroups O_1, O_{128}, O_{26} and O_{146} were producing blaTEM gene at 516 bp. Mean while serogroups O_{44} and O_{128} producing bla CTX at 503 bp. The presence of *eaeA* gene among serogroups O_{44} and O_{55} were detected at rang 248bp.while *E.coli* isolates examined by PCR showed the presence *stx2* gene among serogroup O_{55} only. None of tested serogroups were produced *stx1* .

Keywords: *Escherichia coli*, ESBL, *Stx1*, *Stx2*, *eaeA* gene, diarrhea.

INTRODUCTION

Diarrhea remains one of the main sources of morbidity and mortality in today's world and a large proportion is caused by diarrheagenic strains in human and animals [1]. *Escherichia coli* are a normal commensal bacterial flora of most animals, including dogs and humans. *E.coli* can also cause a wide variety of intestinal and extra-intestinal diseases, such as diarrhea, urinary tract infections, septicaemia and neonatal meningitis [2], antimicrobial resistance in *E. coli* isolated from pet dogs can be considered a potential threat of infection for the human population. The commensal *E.coli* strains rarely cause disease where the normal gastrointestinal barriers are breached [3].

Pet dogs were shown to be a potential house hold source of multidrug resistant *E.coli* strains; they detected the simultaneous presence of multidrug-resistant *E.coli* in dogs and their owners, with different phenotypic profiles of antibiotic resistance, especially for ESBL. Furthermore, some strains from dogs and humans of the same household had similar resistance patterns, ESBL genes, identical phylogenetic groups and identical or closely related PFGE profiles. These data demonstrate high degrees of homology and therefore the possibility that resistant *E.coli* clones are circulating between individuals and animals in the same environment. Indeed some strains showed clonal relationships indicating within-household sharing [4]. Suggested that the prevalence of antimicrobial-resistant commensal *E.coli* is very similar in dogs and humans and there is no difference among the resistant *E.coli* isolated from dog owners and humans without pet dogs. ESBL are the major groups of enzymes that confer resistance to *β*-Lactamase antibiotic through hydrolyze the *β*-Lactamase ring in antibiotic.

The increasing prevalence of antimicrobial resistant *E.coli* isolates is a serious concern, particularly with respect to strains producing extended spectrum β-lactamases (ESBLs), both in humans and in animals [5]. ESBL-producing *E.coli* isolates from companion animals are of public health concern, due to potential transfer between animals and humans in the household environment [6].

The CTX-M-15 β-lactamase is an ESBL that has been disseminated among humans in hospitals, mainly associated with *E.coli* strain ST131-B2, although is less frequent in animals[7,8]. Plasmid-mediated AmpC β-lactamase (pAmpC)-producing *E.coli* strains are also an emerging problem in human and veterinary medicine because of the transferability of the resistance genes, that of *bla*CMY-2 being reported most frequently [9]. ESBL and pAmpC *E.coli* producers have been detected both in healthy and in sick dogs in different countries [10,11,12]. Nevertheless, no information do exist in healthy pets in Latin America [4] investigated the presence of cefotaxime-resistant (CTXR) *E.coli* strains in fecal samples of healthy dogs in Mexico, to characterize the mechanisms of

resistance and to perform molecular typing of selected isolates.

Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in the developing countries [13], much of the problem has been shown to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species [14]

Shiga toxin-producing *E.coli* (STEC) is among the most important causes of foodborne diseases. They are responsible for a range of human gastrointestinal diseases, including watery or bloody diarrhea. The common feature and main virulence factor of STEC is the production of Shiga toxin-1 (*stx-1*) and/or Shiga toxin-2 (*stx-2*) or its variants [16], they also added dogs and cats have also been found to carry STEC strains, but their role as bacterial reservoirs has not been completely elucidated. The prevalence of STEC strains among intestinal bacterial strains in pets has been shown to be of highly variable occurrence. The *E.coli* gene *eaeA* express intimin, which is required for the production of attaching and effacing (AE) lesions in epithelial cells [17].

MATERIALS AND METHODS

Samples and cultivation

A total of 120 rectal swabs were aseptically taken from diarrheic dogs in Cairo and Giza governorates from private clinic and veterinary hospitals. All rectal swabs were inoculated directly into MacConkey's agar and diluted on buffer peptone and sub cultured on EMB agar. The inoculated plates were incubated at 37°C for 24-48 hours. The isolation, purification and biochemical identification of the bacterial isolates were carried out as per standard protocols [18].

Serological identification

Antisera *Escherichia coli* were used for serological identification of somatic "O" sera using slide agglutination test (polyvalent sera, 8 vials monovalent sera, 43 vials). All were obtained from DENKA SEIKEN CO. LTD Tokyo, Japan. For somatic antigen was used and interpreted [19].

Antibiogram pattern

Antibiotic susceptibility tests were performed by disc diffusion method (Clinical laboratory standard institute, CLSI) [20], to different "O" serogroups of *E.coli* recovered from diarrheic dogs. The following antibiotics were used Cephalothin, Oxytetracyclin, Sulphamethazol, Cefpodoxime, Ampicillin, Cefotoxime, Ceftazidime, Impenem, Ceftriaxone and Cefeprime (Oxoid) for susceptibility testing. Zones of inhibition were measured after 24-hour incubation at 37°C. Interpretation of sensitivity andvresistance was based on EUCAST guidelines [21].

Molecular characterization of *E.coli*

PCR assay was carried out in Reference Laboratory for Veterinary Quality Control on Poultry Production as follows:

DNA extraction

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

- **Oligonucleotide Primer.** Primers used were supplied from Metabion (Germany) are listed in **table (1).**
- **PCR amplification. For uniplex PCR,** primers were utilized in a 25µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products were loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Bacteriological examination and Serological Identification

Out of 120 examined fecal swabs obtained from the diarrheic dogs were subjected to bacteriological examinations, 68 isolates of *E.coli* were recovered in percentage of (56.6%). Serogrouping of (68) isolates of *E.coli* recovered from diarrheic dogs revealed 7 different "O" serogroups and 20 strains were untyped as illustrated in table (2).

Antibiogram pattern

By comparing antibiotic susceptibility test rests, the majority of the test isolates of *E.coli* serogroups were resistant to Oxytetracyclin, Ampicillin, Sulphamethazol and Cephalothin. However, some strains were susceptible to Cefotaxime. Ceftazidime., Imipenem and Cefeprime (Table. 3).

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	[22]
Sul1	CGG CGT GGG CTA CCT GAA CG GCC GAT CGC GTG AAG TTC CG	433	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[23]
bla$_{TEM}$	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	[24]
blaCTX	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	45˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[25]
eaeA	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min	[26]
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[27]
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779						

RESULTS

Table 2: Serological identification of pathogenic strain of *E.coli* from diarrheic dogs.

E.coli Strains	No	%*
O_{157}	4	5.85
O_{1}	7	10.29
O_{44}	3	4.41
O_{128}	8	11.76
O_{26}	10	14.70
O_{146}	11	16.17
O_{55}	7	10.29
Untyped	20	29.41
Total	68	100

*The percentage % calculated according to total number of isolated strains of *E.coli* from diarrheic dogs

Table 3: Resistance pattern of *E.coli* pathogenic strains recovered from diarrheic dogs

E.Coli Strains	O_{157} (4)		O_{1} (7)		O_{44} (3)		O_{128} (8)		O_{26} (10)		O_{146} (11)		O_{55} (7)	
	R		R		R		R		R		R		R	
Chemotherapeutic agent	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Ampicillin	2	50	3	60	3	100	6	75	6	60	11	100	4	57.14
Cefepime	1	25	3	60	2	66.66	5	62.5	5	50	7	63.63	1	14.28
Cephalothin	2	50	3	60	2	66.66	7	86.25	7	70	9	81.81	4	57.14
Cefpodoxime	3	75	2	80	1	33.33	3	37.5	4	40	4	36.36	2	28.57
Ceftriaxone	2	50	3	60	2	66.66	4	50	6	60	8	72.72	3	42.85
Cefotoxime	2	50	3	60	2	66.66	5	62.5	7	70	8	72.72	3	42.85
Ceftazidime	1	25	2	40	1	33.337	4	50	5	50	7	63.63	2	28.57
Imipenem	1	25	4	80	2	66.66	5	62.5	6	60	9	81.81	2	28.57
Oxytetracyclin	2	50	5	100	3	100	3	37.50	9	90	10	90.90	7	100
Sulphamethazol	4	100	4	100	3	100	8	100	8	80	5	45.45	3	42.85

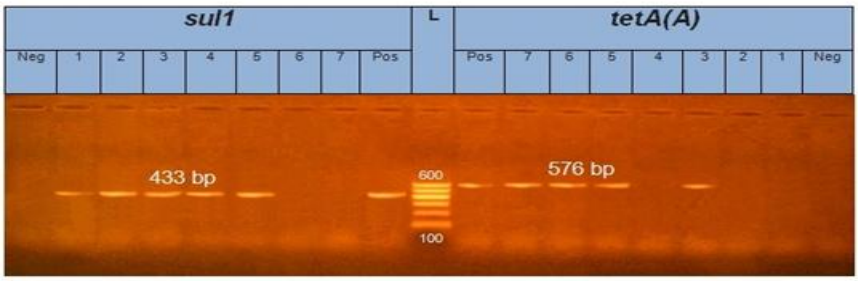

Figure 1: L. Pulsed gel electrophoresis for detection of sul 1 from different serogroups of *E.coli* were present in serogroups O_{157}, O_{1} , O_{44} ,O_{128} ,O_{26} at 433bp. R. Pulsed gel electrophorases for detection of tet (A) were present in serogroups O_{44}, O_{26} ,O_{146} and O_{55} at 576bp.

Table 4: Molecular analysis of ESBL gene and virulence gene including *SXT1 , SXT2* and *eaeA* gene.

Sample	Results						
	eaeA	*Stx1*	*Stx2*	*Sul1*	*TetA(A)*	*blaTEM*	*blaCTX*
1 (O_{157})	-	-	-	+	-	-	-
2(O_1)	-	-	-	+	-	+	-
3(O_{44})	+	-	-	+	+	-	+
4(O_{128})	-	-	-	+	-	+	+
5(O_{26})	-	-	-	+	+	+	-
6(O_{146})	-	-	-	-	+	+	-
7(O_{55})	+	-	+	-	+	-	-

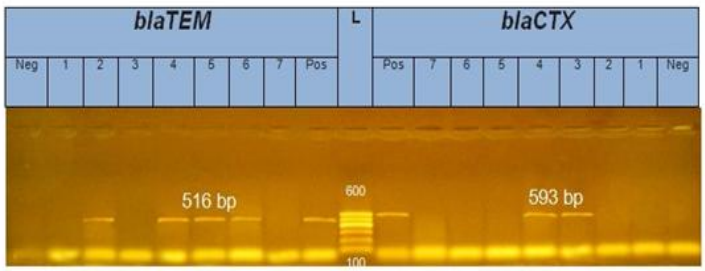

Figure 2: L. Pulsed gel electrophoresis for detection of bla TEM in different serogroups of *E.coli* were present in serogroups O_1, O_{128}, O26 and O_{146}. R. Pulsed gel electrophorases for detection of bla CTX were present in serogroups O_{44} and O_{128}

DISCUSSION

Colibacillosis is the major cause of morbidity and mortality and is responsible for significant economic losses worldwide [28]. Some serogroups of *E.coli* are known to play an important role as etiological agents of various diseases in dogs and cats, it was found to be associated with different diseases as diarrhea and urinary tract infection (UTI).

Also, Shiga toxin-producing *E.coli* (STEC) is responsible for a range of human gastrointestinal disease, including watery and/ or bloody diarrhea.

In this study, 68 *E.coli* strains were isolated from diarrheic dogs and the incidence rate was 56.6%. These results nearly lower than those reported [29] when 92 *E.coli* isolates from 35 diarrheic dogs. Ten isolates were characterized as extraintestinal pathogenic *E.coli* (EXPEC) strains [4] that isolated *E.coli* from diseased dogs in percentage of (31.3%) from fecal samples of dogs and owners.

In serological identification of isolated strains of *E.coli* revealed that 7 different "O" serogroups O_{157}, O_1, O_{44}, O_{128} , O_{26} , O_{146} and O_{55} and (20) strains were untyped as shown in table (2). In our results do contrast in part with the author who they did serogrouping of *E.coli* from canine feces and reported the serogroups of O_1, O_2, O_4, O_7, O_8 , O_{16} , O_{18} , O_{25} and O_{75} [30].

Although antimicrobial therapy is an important tool for the treatment of the infection, resistance to antimicrobials is widespread and constitutes a great concern in veterinary medicine. Indead a close relationship between the use of antimicrobial agents for the treatment of infection in animals and level of observed resistance was exists [14].

Table (3) illustrated that the antibiogram pattern of the isolated pathogens from diarrheic dogs. Multi-resistant against several antibiotics are recorded including Oxytetracyclines, Ampcillin, Sulphamethazol, Cephalothin and Impenem while variable results were recorded with the remaining tested chemotherapeutic agents. Dogs have therefore to be considering as an important reservoir for zoonotic *E.coli* strains, thus serving as substantial non-point source especially of strains capable of causing extra-intestinal diseases.

These result nearly conceded with those reported by many authors [31,29], who isolated *E.coli* from dogs and reported that these strains were resistance to Streptomycin, Nalidixic acid, Tetracycline, Cephalothine and Amoxicillin was most frequently observed also by another author, in the resistant pattern, determined MDR of *E.coli* using Streptomycin, Amoxicillin, Ciprofloxacine, Ceftriaxone , Amoxicillin , Clavulanic [32].

Cefotaxime resistant (CTX^R) *E.coli* strains were isolated from fecal samples of healthy dogs in Mexico in percentage of (17%). Isolates that are resistant to 3rd generation cephalosporins and ciprofloxacin are becoming more common in health care facilities and the general Multidrug-resistant (MDR) *E.coli* has a similar role in opportunistic infection in dogs[31]. Recently there has been an increased frequency of infections in veterinary

setting, possible because of greater use of intensive care facilities newer generation antimicrobials and longer hospitalization period [34].

Antimicrobial treatment selects for resistance in both pathogenic and commensal *Enterobacteriaceae*, and is considered the most important risk factor for acquiring extra intestinal infection with MDR strains [35].

Table (4),figure (1), (2) showed the results from our study suggest that tested strains of *E.coli* producing Sul1 are belonged under serogroups O_{157} ,O_1 , O_{44} ,O_{128} and O_{26} at 433bp. Also serogroups O_{55} ,O_{146} , O_{26} , and O_{44} were producing tet (A) at 576 bp., While serogroups O_1 , O_{128} ,O_{26} and O $_{146}$ were producing blaTEM gene at 516 bp. Meanwhile serogroups O_{44} and O_{128} producing bla CTX at 503 bp.

These results are similar to another study by Carvalho et al., and they reported that 28.5% of the *E.coli* isolates from dogs were resistant to at least one antibiotic belonging to the third or fourth generation of Cephalosporins and were positive for ESBL by disk-approximation test [4]. They reported that *beta*-lactamase resistance strains having both (bla TEM and bla CTX) genes from human and dog origin together. Additionally, they reported that in dog isolates, the blaTEM gene was present in 66.7% of the strains that tested positive for ESBL genes. The bla CTX-M gene was detected in 75% of these isolates, they mentioned that, in human strains the prevalent ESBL gene family was the bla TEM family. The bla CTX-M was present in similar proportions in strains of human and dog origin from the same household.

The detection of ESBL gene in isolates of pet dogs was published in 1988; other studies have also reported the presence of the bla genes in isolates from dogs, especially isolates of clinical origin. The presence of clinically important bla TEM gene in dogs and clinical isolates have been reported since 2002 [36,37]. However, the prevalence of these genes in dog is not well established sofar.

In addition, *E.coli* (CTX^R) Cefotaxime-resistant was recovered (17% of samples from healthy dogs and ESBL producing *E.coli* were detected 6% of samples). These samples carried the bla $_{CTX-M-15}$ gene and one isolate also carried the bla_{SHV-2} [34].

While the *E.coli* gene *eaeA* express initimin , which is required for the production of attaching and effacing (AE) lesions in epithelial cells [17]. The eae*A* genes were shown to be essential for intimate adherence and are present in most STEC strains included in Enterohaemorrhgic *E.coli* (EHEC) groups.

Our study revealed the presence of eae*A* gene among serogroups O_{44} and O_{55} and PCR products of this gene at 248bp. (Table 4, picture 3), while *E.coli* isolates showed the presence *stx2* gene among serogroup O_{55} only. None of the *E.coli* isolate examined by PCR showed that the presence of *stx1*.

These findings are similar with Kaper et al., and they stated that the major virulence factor and a defining characteristic of STEC is *stx* [17]. These potent cytotoxins not only inhibit protein synthesis in cells, but also induce the lytic characteristic of programmed cell death (apoptosis), while another author mentioned that no STEC strains were identified among the *E.coli* isolates from feces or urine [38].

The strains which carry only *stx2* are more virulent than those carry only *stx1* or even both *stx1* and *stx2* and more associated with cases of HUS in Humans [39,40] . The presence of eae*A* gene in STEc strains have been strongly correlated to their implication in severe illness resulting HUS [41]. While STEc strains lacking eae*A* gene have been reported to cause outbreaks [42]. Attaching effacing *E.coli* (AEEC) presenting with AE lesions has been associated with diarrheal illness in dogs [15], also AEEC strains resembling typical enteropathogenic *E.coli* (eae+) were isolated from cats in a small number(6.5%) of cases and pathogenic AEEC may transferred to humans by direct contact between animals and humans [43].

CONCLUSION AND RECOMMENDATIONS

In conclusion, *E.coli* one of most causes of diarrhea in dogs cross infection may occurred to human so diarrheic dogs must be well diagnosed and must be apply sensitivity test against isolated pathogens to correct treatment and prevent the transmission of multidrug resistant gene (MDR). Presence of viruence genes in *E.coli* from the pet animal are become serious public health concern .Because of possible transfer of these antibiotic resistant strains between companion animals and humans. Future studies are necessary to focus on risk factor for transmission of *β*-lactamase producing *E. coli* between pets and humans in household environment.

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