



## Characterization of Enteropathogenic *E. Coli* and Antibiotic Resistance Properties in Diarrheic Pets

Dalia H. Ali<sup>1</sup> and Ali Metwally<sup>2</sup>

<sup>1</sup>Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University

<sup>2</sup>Department of Internal Medicine, Faculty of Veterinary Medicine, Alexandria University

### Key Words:

*E.coli*, PCR, Antibiotic sensitivity.

### ABSTRACT:

The study was carried out to detect and characterize Enteropathogenic Escherichia coli isolated from diarrheic dogs and cats and its antibiotic resistance. To perform this aim :a total of 90 rectal swabs were collected from diarrheic dogs (n= 70) and cats (n= 20) from different localities at Alexandria and screened for the presence of *pathotypes of E.coli* using culture, biochemically, serological , molecular identification and *antibiotic sensitivity test*.a total of 33 (36.6%) *E. coli* isolated and identified {26 isolates from dogs (37.1%) and 7 isolates from cats (35%)}. Hemolytic activity revealed 8 (6 from dogs and 2 from cats) and 4 (3 from dogs and 1 from cats) isolates with Alpha ( $\alpha$ ) and Beta ( $\beta$ ) hemolysis respectively. Serotyping of these 12 isolates revealed 2 (16.6%) were positive poly 2 (O:127 K63 ), 1 (8% ) isolate was positive poly 3 ( O:164 K-) , 1 (8%) isolate was positive poly 1 (O:26 k 60) and 8 (66.6%) isolates were serotyped as positive poly one. Polymerase chain reactions (PCR) revealed the presence of attaching and effacing gene (*eaeA* ) in 4 (33.3%) isolates {3 from dogs (33.3%) and 1 from cat (33.3%)}. However, none of the isolates were positive for Heat-labile (LT) , heat-stable (STa) enterotoxin genes, shiga toxin one (Stx1) and shiga toxin two (Stx2) genes and bundle-forming pili (Bfp) gene. The highest rate of sensitivity was observed to Amikacin and Ofloxacin. And, the highest rate of resistance was observed to Cephalixin and Ampicillin sulbactam.

Corresponding author: Dalia A. Ali: [dalia\\_hussein28@hotmail.com](mailto:dalia_hussein28@hotmail.com)

## 1. INTRODUCTION

Diarrheagenic *E.coli* (DEC) strains in pets are classified into six categories: enteropathogenic (EPEC), enterotoxigenic (ETEC), Shiga toxin-producing (STEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffuse-adhering *E. coli* (DAEC) (Pun˜o-Sarmiento J et al., 2013). EPEC are a common cause of diarrhea in pets particularly young dogs (Beutin.,1999).And considered one of the majour causes of infantile diarrhea in developing countries (Hernandes et al ., 2009). EPEC can be classified into typical and atypical strains while both exhibit the majour virulence trait, the ability to cause attaching and effacing lesion (A/E) tEPEC strains carry the EPEC adherence factor (EAF) plasmid that encodes the bundle-forming pili (Bfp), while aEPEC strains are negative for both EAF plasmid and Bfp gene(Moura et al.,2009). (A/E) lesion are initiated by the attachment of bacteria to the enterocytes and disruption of brush border microvilli (Goffaux et al.,2000).This process mediated by intimin a bacterial outer membrane adhesion encoded by the *eae* gene (Almeida et

al.,2012).Increased prevalence of antimicrobials resistance of pathogenic bacteria occurs due to the extensive use of antimicrobial agents in pets(Paula C and marine ., 2009) and their close contact with humans especially children(Guardabassi et al. 2004)

## 2. MATERIAL AND METHODS

### 2.1 Sampling

A total of 90 rectal swabs were collected from diarrheic dogs (70) and cats (20) up to one year, immersed in nutrient broth and transferred in ice boxes with minimum of delay to the department of Microbiology, faculty of Veterinary Medicine, Alexandria University.

### 2.2 Bacterial culture and isolation (Mun˜nicha and Lubke. 2004):

Swabs immersed in nutrient broth were incubated aerobically at 37°C for 18 hrs,then a loopful subcultured onto Macconkey agar medium , pink colonies were picked up and sub cultured onto Eosine Methylene blue agar medium (Colonies of *Escherichia coli* usually have a characteristic

metallic sheen) and on blood agar media (colonies surrounded with a halo zone considered hemolytic).

**2.3 Identification of the isolates:**

A- Morphological characterization: Films were stained with Gram’s stain and examined microscopically for morphological characteristics of the isolates.

B- Cultural characteristics: The colonial morphology onto MacConkey’s agar and Eosine Methylene blue agar was studied.

C-Biochemical identifications were carried out according to (Koneman.,1988).

D-Serological identification

Serotyping of E. coli isolates was done at the department of clinical microbiology, central health laboratories Ministry of health, Cairo: Antisera intended for the serological identification of E. coli isolates were used acc to (Ewing .,1986).

**2.4. DNA extraction:**

According to ABIOpure Genomic DNA extraction kit instructions.

**2.5 Detection of virulence genes using Polymerase Chain Reaction(PCR)** Performed at Animal health research institute

**a.Primers and cycling conditions during PCR:** Oligonucleotide primers (Table:1) (Metabion, Germany).

Table (1): Oligonucleotide primers sequences

Target gene	Primers sequences	Amplified segment (bp)	Refernce
stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Dipineto et al. (2006)
stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	
eaeA	GACCCGGCACAAGCATAAGC	384	Wen-jie JIN et al. (2008)
STa	CCACCTGCAGCAACAAGAGG GAAACAACATGACGGGAGGT GCACAGGCAGGATTACAACA	229	Lee et al. (2008)
LT	GGTTTCTGCGTTAGGTGGAA GGGACTTCGACCTGAAATGT	605	
Bfp	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTAGT	300	Vidal et al ., (2004)

b.Polymerase chain reaction (PCR) protocol for Bfp and eaeA genes (Vidal et al ., 2004) and (Wen-jie JIN et al. 2008).

The reaction was included in a total volume of 25 µL in 0.5 ml eppendorf tube containing 6 µL templet DNA ,12.5 µL Emerald Amp GT PCR mastermix, 20 pmol (1 µL) of each primer, 4.5 µL PCR grade water.

c.Multiplex polymerase chain reaction protocol for (stx1, stx2) and(STa,LT) genes Dipineto et al. (2006) and Lee et al. (2008):

The reaction was included in a total volume of 50 µL in 0.5 ml eppendorf tube containing 12 µL templet DNA ,25 µL Emerald Amp GT PCR mastermix, 20 pmol (1 µL) of each primer, 9 µL PCR grade water .

**d- Programming thermal cycler**

**Programing thermal cycler for detection of eaeA gene-**

The thermal cycler was programmed as follow: one cycle at 94 °C for 5 min to denaturate template DNA,followed by 35 cycles of denatueration, annealing and extention at 94 °C for 45 sec , 54°C for 1 min and 72°C for 1 min ,the 35 cycles, followed by a final extension at 72 °C for 10 min.

**-Programing thermal cycler for detection of Bfp gene**

The thermal cycler was programmed as follow: one cycle at 94 °C for 5 min to denaturate template DNA,followed by 35 cycles of denatueration, annealing and extention at 94 °C for 1min , 60°C for 1.5 min and 72°C for 1,5 min ,the 35 cycles, followed by a final extension at 72 °C for 10 min.

**Programing thermal cycler for detection of STa and LT genes-**

The thermal cycler was programmed as follow: one cycle at 94 °C for 5 min to denaturate template DNA,followed by 35 cycles of denatueration,

annealing and extension at 94 °C for 1min , 57°C for 1 min and 72°C for 1 min ,the 35 cycles, followed by a final extension at 72 °C for 10 min.

**Programing thermal cycler for detection of stx1 and stx2 genes-**

The thermal cycler was programmed as follow: one cycle at 94 °C for 5 min to denature template DNA,followed by 35 cycles of denaturation, annealing and extension at 94 °C for 1min , 58°C for 1 min and 72°C for 1 min ,the 35 cycles, followed by a final extension at 72 °C for 10 min.

e.The PCR products were separated by electrophoresis in 1.5% agarose gel,stained with 0.5 µg/ml ethedium bromide, and visualized under UV light.

### 2.6 Antimicrobial susceptibility testing

The antimicrobial susceptibility was tested with disk diffusion method according to the guidelines established by (CCLS, 2012). The following antibiotic disks were tested: Amikacin 30 mcg(AK) , Neomycin 10 mcg (N) ,Gentamicin 10 mcg (CN), Doxycycline 10 mcg (DO) ceftriaxone 30 mcg (CRO), Cephalexin 30 mcg (CL) , Ampicillin sulbactam 10/10mcg (SAM), Ofloxacin 5 mcg (OFX).A strain was considered multidrug resistant (MDR) when demonstrating resistance to three or more antimicrobial classes (Schwarz.,2010).

## 3. RESULTS

3.1.Results of *E.coli* isolated from diarrheic pets at different ages:

*E.coli* isolated From dogs and cats with a percentage of 37.14% and 35% respectively With higher percentage in young age.

12 *E.coli* isolates were tested for detection of *eaeA* gene 4 isolates gave positive result.

3.2. Identification of suspected isolate:

3.2.1. morphological characteristics:

Gram negative,Rod sape ,non-spore forming and arranged singly.

3.2.2.Culture characteristics:

- Macconkey agar medium : lactose fermenting colonies,3-4 mm diameter.

-Eosine Methylene blue agar medium: Colonies of *Escherichia coli* usually have a characteristic metallic sheen.

-Blood agar media : Hemolytic activity revealed 8 (6 from dogs and 2 from cats) and 4 (3 from dogs and 1 from cat) isolates with Alpha ( $\alpha$ ) and Beta ( $\beta$ ) hemolysis respectively.

3.2.3. Biochemical identification :

Gave positive reaction for catalase test, indole and methyl red test.Meanwhile the isolates were negative for oxidase test, vogues proskature test, citrate test and urease test.

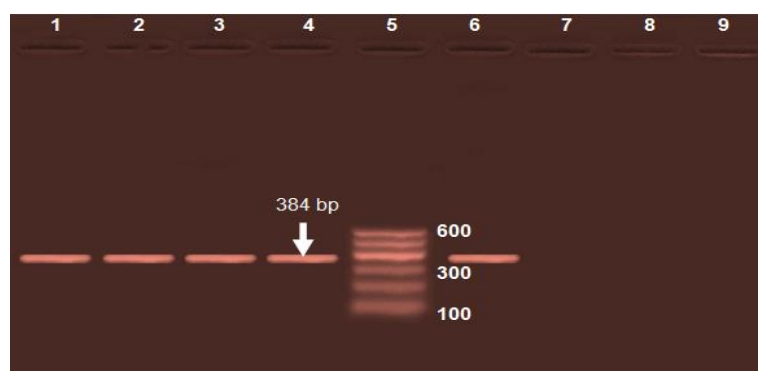
3.2.4Results of *E.coli* isolates serotyping:

12 *E.coli* isolates were serotyped using polyvalent & monovalent antisera .The results of serotyping in dogs were recorded as one isolate (8 %) was + ve poly 2 (O:127 K63 ), One isolate (8 %) was + ve poly 3( O:164 K-), one isolate (8 %) was +ve poly 1 (O:26 k 60) , 6 isolates (50%) were positive poly one.

The results of serotyping in cats were recorded as one isolate (8 %) were + ve poly 2 (O:127 K63 ), two isolates (16.6%) were positive poly one.

### 3.3. Results of PCR

#### 3.3.1. Result of PCR for detection of *eaeA* gene:



**Figure-1** Electrophoretic analysis of PCR amplified DNA of attaching and effacing gene (*eaeA*).

Lane (1), Lane (2), Lane (3), Lane (4): Positive results for *eaeA* gene.

Lane (5): DNA molecular weight ladder.

Lane (6): *E. coli* strain ATCC 25922 used as Positive control .

Lane (7), Lane (8), Lane (9): Negative results for *eaeA* gene.

### 3.3.2. Result of PCR for detection of Bfp gene:

12 E.coli isolates were tested for detection of Bfp gene gave negative results (Figure-2).

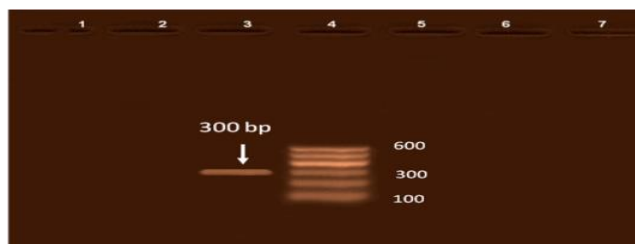


Figure (2): Electrophoretic analysis of PCR amplified DNA of Bundle forming bili gene (Bfp)

Lane (1), Lane (2), Lane (5),Lane (6), Lane (7): Negative results for Bfp gene.

Lane (4): DNA molecular weight ladder.

Lane (3): E. coli strain ATCC 25922 used as Positive control.

### 3.3.3.Result of PCR for detection of Stx1,Stx2,Lt and Sta genes:

12 E.coli isolates gave negative results (Figure-3).

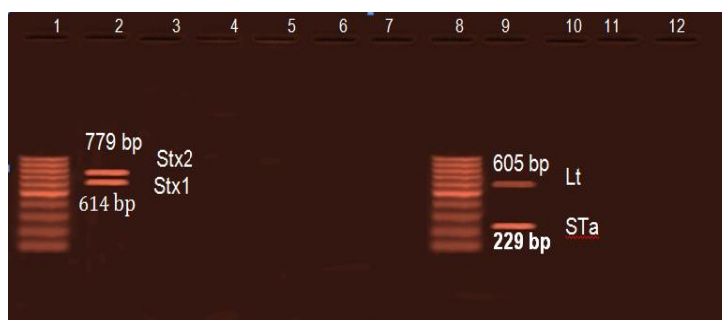


Figure (3): Electrophoretic analysis of PCR amplified DNA of Shiga toxin1, shiga toxin 2 , Labile toxin and Stable toxin genes .

### 3.4. Results of antibiogram :

The 12 E.coli isolates were tested for the resistance to antibiotics .The results were the antibiotic of high effect on isolated E.coli were Amikacin and Ofloxacin,Meanwhile

Neomycin and Doxycycline has moderate effect .Moreover antibiotics have less effect on the isolated E.coli were Cephalexin and Ampicillin sulbactam.

### 4- DISCUSSION

A trail was conducted to examine a number of fecal samples from diarrheic pets. Bacterial isolates were thoroughly studied for their Phenotypic, Genotypic characterization as well as antibiotic resistance. 33 E.coli isolates were recovered from 90 rectal swabs from diarrheic pets with a percentage of (36.6 %) , 12 were haemolytic (36.3%) with high prevalence in young age,our results agree with previous study represented by (Munnicha and Lubke.,2004) who found that high prevalence of E.coli isolates present in young age, As the age factor played an important role in the incidence of E.coli that's the intestinal epithelium of neonatal puppies appears to be more

permeable to E. coli than is the intestinal epithelium of older dogs. Adult dogs had a higher proportional morbidity of diarrhea (PMD) than adult cats,and younger animals in both species had higher PMDs than adult animals(Jones P et al.,2014).

The eaeA gene was found in 4 isolates (33.3 %) one of serotype O:26:k60 and three of serotype positive poly one , Similar result obtained by (Punõ-Sarmiento et al., 2013) who observed that 36% of the isolates contained the eaeA gene , higher result 48% of isolates were positive to eae gene recorded by (almeida et al .,2012) and lower values 13% of eae positive E. coli strains in dogs observed by (Nakazato et al.,2004) .

Bfp gene not detected in examined isolates this agrees with (almeida et al .,2012) who reported that all the eae-positive isolates recovered were aEPEC. Meanwhile (Nakazato et al.,2004) recorded the presence of only two bfpA positive isolates among 23 DEPEC isolates (8.7%) .

None of the isolates were positive for enterotoxins , Shiga toxins, this agree with (Nakazato et al.,2004) although genes for these toxins have been reported previously in diarrheic dogs (Turk et al.,1998 , Staats et al., 2003 Bentacour et al., 2007,) and Isolated E.coli were serotyped using polyvalent & monovalent antisera ,The results were recorded as 2 isolate were + ve poly 2 (O:127 K63 ) , One isolate was + ve poly 3( O:164 K-), one isolate was +ve poly 1 (O:26 k 60), 8 isolates positive poly one. The DEPEC isolates belonged to a wide variety of serotypes (Nakazato et al.,2004), Serotype is often not determined for DEPEC and only the serogroups of a limited number of isolates have been reported (Beutin and Zimmermann.,1999). The ONT serotype was predominant in most isolates and NT subtypes represent new subtypes that have not been described previously in dogs (Pun˜o-Sarmiento et al., 2013). Most strains demonstrated a high resistance to most of the antimicrobials tested, with high resistance for cephalexin and Ampicillin sulbactam that are commonly used in veterinary practice, High sensitivity for only amikacin and ofloxacin.This high antimicrobial resistance represents a public health hazard (Kroemer et al .,2014).

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