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Prevalence of resistance and virulence genes in *Escherichia coli* isolates from diarrheic dogs

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Abstract

The current study was designed to determine the prevalence of antimicrobial resistance and virulence genes in *Escherichia coli* isolated from canine diarrheal cases. Fecal samples were collected from 77 dogs with clinical diarrhea and 10 non-diarrheic dogs. Breed, age and sex, and clinical manifestations were recorded. Samples were plated on MacConkey and EMB agars, followed by standard isolation procedures and identification of *E. coli*. An antimicrobial susceptibility test using the disc diffusion method was performed against Ampicillin, Tetracycline, Trimethoprim, Gentamicin, and Streptomycin. PCR was used to determine if the isolates carry virulence intimin adherence protein (*eae*A) and antimicrobial resistance genes. Body temperature, respiratory, and heart rates in dogs with diarrhea were significantly higher than in non-diarrheic dogs. PCR detected the *eae*A virulence gene in 44(69.8%) of 63 isolates from diarrheic dogs. All isolates were resistant to ampicillin, and 55(87.3%), 47(74.6%), 29(46.0%), and 19(30.2%) of these were resistant to tetracycline, trimethoprim, gentamicin, and streptomycin, respectively. The frequency of antimicrobial resistance genes in the 63 isolates was 81.0, 52.4, 41.3, 33.3, 23.8 and 9.5% for *CITM*, *tet* (B), *dfrA1, aac (3)-IV*, *aadA1* and *tet* (A), respectively. Overall, 6(9.4%), 16(25.4%), and 41(65.1%) were positive for one, two, three, or four resistance genes, respectively. In conclusion, the high prevalence of virulence (69.8%) and resistance 9.5-81.0% genes in *E. coli* isolates could be responsible for the diarrhea episodes, which may have posed therapeutic implications in affected dogs.

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Introduction

Escherichia coli is a member of the family *Enterobacteriaceae,* a gastrointestinal microorganism capable of causing diseases in humans and animals (1,2). In dogs, it was shown that environments such as pet shops and kennels serve as sources of enteric canine colibacillosis (3). The diarrheagenic strains of *E. coli* include Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Necrotoxic *E. coli* (NTEC), Enterotoxigenic/ Shiga toxin-producing *E. coli* (STEC) and diffusely adherent *E. coli* (DAEC) pathotypes. Their pathogenicity depends on virulence factors and the ability of the bacteria to produce lesions (1,4). Some of these virulence factors include cytotoxic necrotizing factor (cnf) encoded by *cnf*1 and *cnf*2 gene, intimin encoded by locus of enterocyte effacement (LEE) (*eaeA*) gene, and Shiga toxin (*stx*) encoded by (stx_1 and stx_2) genes (5-7). These virulence factors are responsible for causing diarrhea in humans and

animals (5,8) and inducing a pyrogenic activity due to the action of the lipopolysaccharide (9). However, more complications for eradicating *E. coli* infection are added by the presence of ESCR *E. coli* (10). In the past decade, the global increase in antimicrobial resistance has posed health problems failing to treat infectious diseases (11). Resistance to antimicrobial agents is coded by plasmid and chromosomal genes sheltered by bacterial organisms. These genes may be naturally present or could be acquired via horizontal gen transfer, including transformation, conjugation and transduction mechanisms (12). *Escherichia coli* O157: H7 was recently isolated, and *the eaeA* gene was identified in buffalo calves (13), and this pathogen is also genotypically identified in lactating cows (14) that come into contact with domestic animals in north and south regions of Iraq.

However, there is limited information on the occurrence of antimicrobial resistance and virulence genes in dogs in Basra Province, Iraq. Therefore, this study was conducted to determine the prevalence of virulence and resistance genes in *E. coli* isolates recovered from diarrhea and non-diarrheic dogs and to relate the occurrence of these pathogens to host factors such as breed, age, and sex of tested animals.

Materials and methods

Ethical approve

The current study was designed according to the rules and guidelines of the College of Veterinary Medicine, University of Basra, Iraq.

Animals and study design

The present study was conducted on 87 dogs belonging to different breeds, ages, and sex between September 2016 and May 2017 in Basra Province, Iraq. The clinical status of each dog was determined, and 77 exhibited clinical signs of diarrhea, while 10 were healthy and served as controls. The general committee approved the current experiment on animal use and welfare/ University of Basrah, College of Veterinary Medicine, Basra state, Iraq.

Sampling and isolation of *E. coli*

Rectal swabs were collected from all dogs using a sterile swab. The isolation and identification of *E. coli* were conducted using standard methods (15). Samples were initially cultured on MacConkey agar followed by inoculation of Eosin Methylene blue (EMB) agar (Lab M, Lancashire, UK) and incubated aerobically at 37°C for 24-48 h. Colonies with the typical colors and appearance of *E. coli* on MacConkey agar were streaked on EMB agar. Colonies that showed green metallic sheen were considered *E. coli* and were selected and subjected to biochemical tests using standard methods. Positive *E. coli* colonies were confirmed by PCR amplifying the virulence gene *eaeA* (16).

Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed on all isolates identified as *E. coli* using the agar disk diffusion method (17). Five antimicrobial agents were used, namely ampicillin 10 µg, gentamicin 10 µg, streptomycin 10 µg, tetracycline 30 µg, and trimethoprim 5 µg. The isolates that exhibited multidrug resistance by the disc diffusion method were selected for antimicrobial gene amplification using PCR.

DNA extraction and PCR to detect resistance genes

DNA from a distinctive *E. coli* colony was extracted using bacteria DNA extraction kit (Promega, USA) following the manufacturer's instructions. Primers to detect antimicrobial resistance genes were selected based on published studies: tetracycline [*tet* (A), *tet* (B)] (18), streptomycin (*aadA1*), gentamicin [*aac (3)-IV*], Ampicillin [*CITM*] (19), and trimethoprim (*dfrA1*) (20), and virulence gene *eaeA* primers (16) (Table 1).

Table 1: The PCR primers used for the detection of virulence gene and antimicrobial resistance genes

No	Primers	Sequence 5'- 3'	Size (bp)	Temperature	Reference
1	*eaeA*	(F) GACCCGGCACAAGCATAAGC (R) CCACCTGCAGCAACAAGAGG	384	55°C	(12)
2	*tet*(A)	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	55°C	(14)
3	*tet*(B)	(F) CCTCAGCTTCTCAACGCGTG (R) GCACCTTGCTGATGACTCTT	634	55°C	(14)
4	*aadA1*	(F) TATCCAGCTAAGCGCGAACT (R) ATTTGCCGACTACCTTGGTC	477	55°C	(15)
5	*aac(3)-IV*	(F) CTTCAGGATGGCAAGTTGGT (R) TCATCTCGTTCTCCGCTCAT	286	55°C	(15)
6	*CITM*	(F) TGGCCAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	462	55°C	(15)
7	*dfrA1*	(F) GGAGTGCCAAAGGTGAACAGC (R) GAGGCGAAGTCTTGGGTAAAAAC	367	55°C	(16)

Primers were obtained from Alpha DNA (Montreal, Canada). All the PCR conditions for detection of antimicrobial resistance gene were performed in triplicate in a total 25 µL volume. It contained 12.5 µl of GoTaq® DNA polymerase in 2X Green GoTaq® Reaction Buffer (pH 8.5) [400 µM each of dATP, dGTP, dCTP, dTTP, and 3mM of MgCl2], 3 µl of forward and reversed primers at 30 µM concentrations of three primer pairs [able to amplify three genes], 3 µL of ~30 ng/µL genomic DNA, and 3.5 µl of nuclease-free water. First set for [*tet B*] 634 bp, Ampicillin [*CITM*] 462 bp, and Gentamicin [*aac(3)-IV*] 286 bp. The second set for [*tet A*] gene 577 bp, streptomycin [*aadA1*] 477 bp and trimethoprim [*dfrA1*] gene 367 bp. The PCR consists of an initial denaturation step at 94ºC for 5min, followed by 35 cycles of 30 s at 94ºC, 30 s at 55ºC, and 45 s at 72ºC; then 1 cycle of 72ºC for 5 min in Master Cycler Pro (Eppendorf, Hamburg, Germany).

A similar PCR master mix and cycling condition was used amplify virulence gene *eaeA*. The electrophoresis results were analyzed under UV light in 1.5% agarose gel stained with ethidium bromide.

Statistical analysis

All data were analyzed statistically using the SPSS, the level of statistical significance was set at P<0.05.

Results

Out of the total of 77 fecal samples tested from clinical cases, 63 dogs were confirmed to be shedding *E. coli* in their feces. Clinical assessment revealed that these dogs showed white and yellow watery diarrhea with or without blood. Moreover, animals were anorexic, weak, depressed, and showed signs of congested or icteric mucus membranes with different degrees of dehydration. The body temperature, respiratory rates, and heart rates in diarrheic dogs were significantly (P<0.05) higher than those parameters found in the ten non-diarrheic control dogs (Tables 2 and 3). The result of the antimicrobial susceptibility test using the disc diffusion method against five antimicrobial agents is shown in table 4. Resistance was observed in 63 (100%) for ampicillin. For the rest of the antimicrobial agents tested, resistance was observed for a total isolate of 55-(87.3%), 47-(74.6%), 2-(46.0%) and 19-(30.2%) for Tetracycline, Trimethoprim, Gentamicin, and Streptomycin, respectively. Despite 63 bacterial isolates being confirmed as *E. coli* by microbiology and biochemistry, 44 (69.8%) were found to express the 384 bp amplification fragment of the eaeA virulence gene by PCR (Figures 1 and 2). From the total of 63 *E. coli* isolates tested, 51 (81.0%), 33 (52.38%), 26 (41.3%), 21 (33.3%), 15 (23.8%), and 6 (9.5%) carried resistance genes for *CITM*, *tet* (B), *dfrA1, aac (3)-IV*, *aadA1* and *tet* (A) antibiotics, respectively (Table 5).

The overall analysis of the result of the antimicrobial resistance test revealed that 41 *E. coli* isolates from the total 63 tested carried multiple resistance genes for three or four antimicrobial agents (multidrug resistance), 16 isolates carried resistance genes for two antimicrobial agents, and 6 isolates carried antimicrobial resistance gene for only one antimicrobial agent.

Table 2: Body temperature, respiratory and heart rate of diseased dogs and controls

Parameters	Controls (n=10)	Diseased (n=77)
Body temperature (ºC)	38.2±0.531	40.7±0.484*
Heart rates (min)	124.4±3.83	176±8.25*
Respiratory rates (min)	19.6±2.66	41.52±6.04*

*P<0.05, values are mean ± standard error of mean.

Table 3: Clinical manifestations of diseased dogs (*E. coli* positive)

Clinical signs	No (%)*
Watery diarrhea	29 (46.03)
Yellow diarrhea	24 (38.09)
Bloody diarrhea	10 (15.87)
Anorexia	56 (88.88)
Weakness	51 (80.95)
Depression	48 (76.19)
Congested mucous membranes	44 (69.84)
Icteric mucous membranes	29 (46.03)
Mild and moderate dehydration	32 (50.79)
Severe dehydration	11 (17.46)

*Each doge carried one or multiple signs of illness at inspection time.

Table 4: Antimicrobial disk susceptibility test to *E coli* isolates from diarrheic animals

Antimicrobial	Susceptible	Intermediate	Resistance	Total No.
Ampicillin	0 (0%)	0 (100)	63 (100%)	
Tetracycline	0 (0%)	8 (12.70)	55 (87.30%)	
Trimethoprim	0 (0%)	16 (24.4%)	47 (74.6%)	63
Gentamycin	21 (33.37%)	13 (20.63%)	29 (46%)	
Streptomycin	38 (60.33%)	6 (9.52%)	19 (30.15%)	

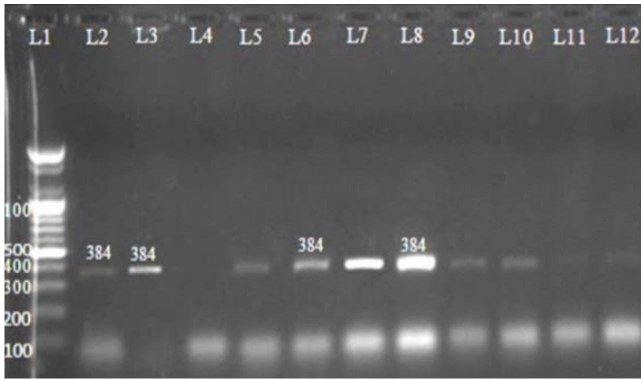

Figure 1: PCR detection of 384 bp amplicon of *E. coli* virulence gene *eaeA* visualized using agarose gel electrophoresis. Lane 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12 represent *E. coli* isolates that contain the *eaeA* virulence gene except lane 4.

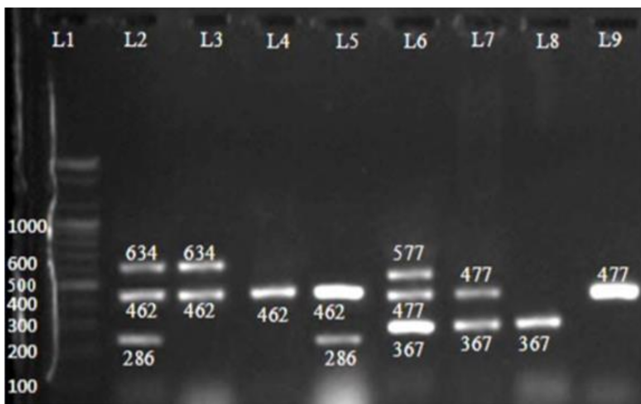

Figure 2: Multiplex PCR amplification mixture run on (1.2%) agarose gel stained with ethidium bromide. Lane 1 ladder; Lane 2 shows positive results for tetracycline [*tet B*] 634 bp and Ampicillin [*CITM*] (462 bp), and gentamicin [*aac(3)-IV*] 286 bp; Lane 3 shows positive results for tetracycline [*tet B*] 634 bp and Ampicillin [*CITM*] (462 bp) while negative to gentamicin [*aac(3)-IV*] 286 bp; Lane 4 shows positive results for Ampicillin [*CITM*] (462bp) while negative to tetracycline [*tet B*] 634 bp and gentamicin [*aac(3)-IV*] 286 bp; Lane 5 shows positive result for Ampicillin [*CITM*] (462 bp) and gentamicin [*aac(3)-IV*] 286 bp while negative to tetracycline [*tet B*] 634 bp; Lane 6 shows positive tetracycline [*tet A*] gene 577 bp, streptomycin [*aadA1*] 477 bp and trimethoprim [*dfrA1*] gene 367 bp; Lane 7 shows positive streptomycin [*aadA1*] 477 bp and trimethoprim [*dfrA1*] gene 367 bp while negative to tetracycline [*tet A*]gene 577 bp; Lane 8 shows positive to trimethoprim [*dfrA1*] gene 367 bp while negative to tetracycline [*tet A*] gene 577 bp and streptomycin [*aadA1*] 477 bp; Lane 9 shows positive streptomycin [*aadA1*] 477 bp while negative to tetracycline [*tet A*] gene 577 bp and trimethoprim [*dfrA1*] gene 367 bp.

Table 5: The multiplex polymerase chain reaction (PCR) for detecting the antimicrobial resistance genes

Resistance gene	Positive	Negative	Total number
CITM gene	51 (80.95%)	12 (19.15%)	63
tet (B)	33 (52.38%)	60 (47.62%)	
dfrA1	26 (41.26%)	37 (58.74%)	
aac (3)-IV	*21* (33.3%)	42 (66.7%)	
aadA1	15 (23.8%)	48 (86.2%)	
tet (A)	6 (9.5%)	57 (90.5%)	

Discussion

The clinical signs exhibited by dogs in the current study were similar to earlier reports by Torkan *et al.* (7), who described that *E. coli* infection is manifested by watery or mucoid diarrhea accompanied by slightly bloody feces, decreased appetite, and nausea. In addition, dogs exhibited dramatic weight loss and moderated to severe dehydration, fatigue, and lethargy (21). Secondary opportunistic infections by pathogenic *E. coli* following immune suppression and their subsequent discharge in diarrheic feces may occur (22). The pathophysiology of enteritis caused by *E. coli* in small animals resembles that of acute hemorrhagic enteritis in humans induced by certain enterotoxigenic strains of *E. coli* (23).

The results of the present study showed that the *eaeA* gene was detected in 69.8% of the *E. coli* isolates from diarrheic dogs, a finding in agreement with published reports (5,7). Others have reported a lower frequency of *eaeA* gene detection, such as 29% (24), 13% (25), and 7.2% (26). The differences in these frequencies may reflect the number of clinical cases of diarrhea tested, the location, laboratory techniques, and tools utilized (26). It is known that adherence is the first step in treating *E. coli* infection in animals, and *eaeA* (intimin) plays vital role in this stage (27). The *eae*A-positive isolates of *E. coli* can cause adherence and attach and effacing (AE) lesions in intestinal epithelial cells of both infected humans and animals. The *eaeA* gene was also found in *E. coli* strains that do not belong to the EPEC serotypes and are negative for Shiga-toxins. These strains also and effacing *E. coli* (AEEC) (28).

The current study showed the prevalence of the ampicillin *(CITM)* resistance gene at 81.0%, which could be due to the broad exposure to beta-lactam antibiotics such as ampicillin. They are extensively used in veterinary medicine for treating the diseases caused by *E. coli* in companion animals. The high frequency 81.0% of detection of the *CITM* gene in the current study is similar to the frequency of 68% reported by Toro *et al*. (19) and 52% reported by Yousefi and Torkan (29). The *CITM* gene coded AmpC β-lactamase, which hydrolyses β-lactams (19). The high prevalence of resistance to ampicillin prompted the decision to consider the

first-line therapeutic options such as cephalosporin or tetracycline for uncomplicated infections (30,31).

Bush (32) reported that the mechanism of antimicrobials resistance to ampicillin in *Enterobacteriaceae* is the enzymatic inactivation of penicillin and cephalosporins by plasmid-mediated extended-spectrum β-lactamases (ESBLs), such as the TEM, SHV, and CITM. The emergence of ESBL-producing *Enterobacteriaceae* in healthy and in diseased companion animals constitutes an increasing challenge to managing infection in veterinary medicine.

The present study shows that the prevalence of tetracycline *tetA* and *tetB* resistance genes in the *E. coli* isolates was 9.5% and 52.4%, respectively. Notably, these antibiotics are extensively used in veterinary medicine and aquaculture, and the detection frequencies were similar to those reported by other studies (19,33). Resistance to tetracycline is coded by *the tet* gene, which is involved in either active efflux of the drug, ribosomal protection, or enzymatic inactivation and drug target modification (34). The *tet*L gene includes 50 types, and they are governed by *tet* genes, the *tetK*, *tetL*, *tetM*, *tetO*, and *tetS*, and are significantly found in gram-positive bacteria, while the *tetA*, *tetB*, *tetD*, *tetE*, and *tetG* were reported in gram-negative bacteria (35).

Our data also showed a high prevalence of the *dfrA1* resistance gene in 41.3% of the total isolates. This could likely be due to the wide use of a combination of sulfonamide/trimethoprim in veterinary medicine. This prevalence is slightly higher than the 35.7% reported for diarrheic and non-diarrheic dogs in Iran (1). The standard resistance mechanisms are considered to be the acquisition of a trimethoprim-insensitive dihydrofolate reductase DHFR variant, encoded by *dfr* genes (33).

The results found a relatively higher prevalence of resistance genes in the isolates for gentamicin *aac (3)-IV* and streptomycin *(aadA1)*, which were 33.3% and 23.8% respectively. However, a study by Costa *et al*. (36) recorded the lower resistance rate of both gentamicin *aac (3)-IV* and streptomycin *(aadA1)* at 8.33 and 15%, respectively. Bacteria acquire different aminoglycoside resistance mechanism, including deceased affinity to its target via enzymatic modification. Aminoglycoside acetyltransferases catalyze the AcCoA-dependent N-acetylation of amino groups on aminoglycoside molecules (37).

The frequency of occurrence of multidrug resistance 65% in *E. coli* isolates found in the current study was similar to the frequency 66.8% reported in a Polish study (36). However, lower frequencies have been reported elsewhere, for example, 43.3% in Japan (38) and 28.9% in the USA (39).

In the present study, the high occurrence of multidrug resistance genes may be associated with treating animals with antimicrobials agents such as β-lactams, tetracycline, and gentamicin in most suspected cases of bacterial infection. This is a common practice by veterinary clinicians and non-veterinarians, especially in developing countries, where there are no strict regulations for the use of these drugs in animals. This lack of or limited regulation has led to an increased prevalence of antimicrobial-resistant pathogenic *E. coli*; this scenario is under other researchers who reported similar findings (7,12,40,41). Prolonged exposure to sub-therapeutic antimicrobials agents increased antimicrobial resistance amongpathogenic bacteria (41).

Despite this, dogs transmit *E. coli* carrying and spreading the infection (42). Moreover, antimicrobial resistance complicates strategies to prevent and control the spread of *E. coli* infection.

Conclusion

Multidrug resistance in *E. coli* probably occurs via the complex interaction of different mechanisms (drugs efflux, enzymatic inactivation, target protection), conferring simultaneous resistance to a wide range of older and/or new antimicrobial compounds or drug classes. The high prevalence of MDR detected should lead to the enforcement of regulations to facilitate prudent antimicrobial use in veterinary medicine and human medicine. The association of virulence genes and exhibition of resistance in *E. coli* isolates provides important clinical and therapeutic implications.

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Conflict of interest

The authors declare that there were no competing interests in the current study.

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انتشار جينات المقاومة والفوعة في عزلات الإيشريكيا القولونية من الكلاب المصابة بالإسهال

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الخلاصة

صممت الدراسة الحالية لتحديد مدى انتشار مقاومة مضادات الميكروبات وجينات الفوعة في عزلات الإيشريكيا القولونية من حالات الإسهال في الكلاب. جمعت عينات البراز من ٧٧ كلباً مصاباً بالإسهال السريري و ١٠ كلاب غير مصابة بالإسهال. تم تسجيل السلالة والعمر والجنس والعلامات السريرية. تم زرع العينات على أكار الماكونكي وأكار الأيوسين والمثلين الأزرق، متبوعة بالإجراءات القياسية للعزل، وتحديد الإيشريكيا القولونية. تم إجراء اختبار الحساسية لمضادات الميكروبات باستخدام طريقة الانتشار القرصي ضد الأمبيسيلين والتتراسيكلين والتريميثوبريم والجنتاميسين والستربتومايسين. تم استخدام تقنية تفاعل السلسلة المتبلمرة لتحديد ما إذا كانت العزلات تحمل بروتين الالتصاق الضار (eaeA) والجينات المقاومة لمضادات الميكروبات. كانت درجات حرارة الجسم والجهاز التنفسي والقلب في الكلاب المصابة بالإسهال أعلى معنويا من الكلاب غير المصابة بالإسهال. كشف تفاعل البوليميراز المتسلسل عن جين ضراوة (*eaeA*) في ٤٤ (٦٩,٨٪) من إجمالي ٦٣ عزلة من الكلاب المصابة بالإسهال. كانت جميع العزلات مقاومة للأمبيسيلين، و ٥٥ (٨٧,٣٪)، ٤٧ (٧٤,٦٪)، ٢٩ (٤٦,٠٪) ١٩ (٣٠,٢٪) كانت مقاومة لمضادات التتراسيكلين، التريميثوبريم، الجنتاميسين والستربتومايسين على التوالي. كان تواتر جينات مقاومة مضادات الميكروبات في ٦٣ عزلة ٨١,٠٪، ٥٢,٤٪، ٤١,٣٪، ٣٣,٣٪، ٢٣,٨٪ و ٩,٥٪ الى *CITM*، *tet (B)*، *dfrA1*، *AAC (3) -IV*، *aadA1* و *tet (A)*، على التوالي. بشكل عام، كانت ٦ (٩,٤٪) و ١٦ (٢٥,٤٪) و ٤١ (٦٥,١٪) موجبة لواحد أو اثنين أو ثلاثة أو أربعة جينات مقاومة على التوالي. في الختام، قد يكون معدل انتشار الضراوة ٦٩,٨٪ والمقاومة ٩,٥-٨١,٠٪ في عزلات الإيشريكيا القولونية مسؤولة عن نوبات الإسهال، والتي قد تكون لها آثار علاجية في الكلاب المصابة.