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Molecular detection of *rfbO157*, shiga toxins and hemolysin genes for *Escherichia coli* O157: H7 from canine feces in Tikrit and Mosul cities, Iraq

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Abstract

Escherichia coli O157:H7 is considered as an important pathogen of diarrhea in adult dogs and puppies because it contains virulence genes. The study objective was to the molecular detection of the *rfbO157* encoding the O-antigen specific for *E. coli* O157: H7,shiga toxins and hemolysin genes of *E.coli* O157:H7 in feces of dogs that collected from different regions in Tikrit and Mosul cities, Iraq. One hundred fecal swabs were collected from pet and K9 dogs including (72 dogs with diarrhea, and 28 without diarrhea). All the Collected swabs were cultured in the nutrient and MacConkey agars, Then the suspected colonies were cultured in the EMB agar. Metallic sheen colonies were cultured by using the chrome agar. All bacteriological identified isolates were enrolled by using the polymerase chain reaction (PCR) technique. The results of this study showed that 7(9.7%) of 72 dogs suffered from diarrhea were positive for *E. coli* O157:H7 that contained the *rfbO157* gene (n= 6), carry *stx1* gene (n= 3), carry *stx2* gene (n= 3), and *hlyA* gene (n= 1). On the other hand, 2 (7.1%) of 28 dogs without diarrhea were positive for *E. coli* O157:H7 that contained the *rfbO157* gene (n= 1), *stx2* gene (n= 1), and *hlyA* gene (n= 1). In conclusion, dogs can be a significant reservoir for pathogenic *E. coli* O157:H7, particularly dogs with diarrhea.

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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogenic bacterium causes critical gastrointestinal infection in the human range between mild diarrhea and hemorrhagic colitis (1). *E. Coli* O157:H7 serotypes are worldwide zoonotic and significant foodborne pathogens responsible for most extreme cases of human (EHEC) diseases, Shiga toxin (*stx*) a potent cytotoxin, is the main virulence factor associated with hemorrhagic colitis, including *stx1* and *stx2*, While *hlyA* is an important virulence factor in *E. Coli* O157:H7 extraintestinal infections such as those of the upper urinary tract in human and dogs causing hemolytic-uremic syndrome (2,3). Cattle can be considered as reservoir of the serotype O157:H7 and transmit the infection to the human and dogs mainly by oral-fecal route, including contaminated raw milk and meat, dogs are the source of human infection with pathogenic *E. coli* O157: H7. and the infection of dogs is acquired from ruminants that are a storehouse of these bacteria, and the method of transmission is through direct contact between humans and pets animals as well as fecal and urine contamination (4,5). The previous studies have been performed to identify and isolate *E. coli* O157:H7 in dogs. One study conducted over a period of 3 years using 614 fecal samples collected from dogs and cat, only one dog; pet for an infected person, was

positive and possess the *stx1* and *stx2* genes (6). A study of Ojo *et al*. (2014) appeared that the *E. coli* O157:H7 isolates found in 16.1% and 26.9% of the, fecal samples collected from dogs with and without diarrhea (7). In Iraq, Hasan *et al.,* (8) revealed that *E. coli* O157:H7 isolates found in the 32 samples from 350 samples which collected from calves and dogs, (32 samples were positive for the pathogen including 4 isolates from calves with diarrhea and 28 isolates from calves without diarrhea), and included *rfbO157* and *flics H7* genes (8). In additional study, 32 isolates were detected in calves with and without diarrhea in Fallujah city, and contained Stx1, Stx2 and eae genes (9). Furthermore other study of Suleiman *et al*. (9) appeared that the *E. coli* O157:H7 found in the 11 samples from 16 dogs that suffered from diarrhea and 7 samples from dogs without diarrhea in Baghdad (10). In additional study, out of 26 isolates, 24 samples were positive using Congo red dye (11). Another recent study in 2020 by Al-Kubaisi and colleagues recorded 33 (66%) dogs tested positive *E. coli* from samples obtained from Anbar and Salahuddin governorates (12).

The aim of this study was to isolate and identify *E. coli* O157:H7 serotypes in feces of dogs with determining their virulence genes of *rfbO157*, shiga toxins and hemolysin by conventional PCR assay.

Materials and methods

Samples collection

One hundred fecal swabs were collected randomly (age and sex) from pet and K9 dogs in Tikrit and Mosul cities, Iraq, one hundred fecal swabs with transport media were collected from 72 diarrheic dogs and 28 non diarrheic dogs and transported to the college of veterinary medicine, universities of Mosul and Tikrit labs for bacterial isolation and identification.

Isolation and identification of *E. coli* O157:H7

The swabs were cultured on nutrient and MacConkey agars, then suspected isolates were cultured on eosin methylene blue agar EMB agar (LABM™ England), and incubated aerobically at 37°C for 24- 48 hours. Metallic sheen isolates were cultured on Hichrome agar (Himedia™ India), this media used to differentiate *E. coli* O157 from other *E. coli. E. coli* isolates were cultured on Hichrome agar and incubated at 37°C for 24 hours, and appearance of pink to mauve color colonies indicated *E. coli O157:H7*.

Extraction of the DNA

In this study, DNA extraction was performed to all isolates using specific commercial Kit (Presto^{TM} Mini gDNA Bacteria Kit, Geneaid Biotech Ltd, USA). Extraction method included, put 1ml of overnight bacterial growth on nutrient broth media in 1.5 ml of an eppendorf tubes and the trans to centrifuge at (10000) rpm for 1 minute. After centrifuge finished, the supernatant is produced then it removed. The Nanodrop spectrophotometer was used to testing the concentration DNA and hold in the freezer at -20°C.

Detection of *rfbO157, stx1, stx2,* and *hlyA* genes using PCR

Escherichia coli O157:H7 genes (including *rfbO157*, *stx1*, *stx2*, and *hlyA*) was detected using the polymerase chain reaction (PCR) technique as the following:

Primers

Four commercial primers (Bioneer Inc., South Korea) were used for *rfbO157*, *stx1*, *stx2*, and *hlyA* genes (Table 1). Extracted DNA was confirmed in the gel electrophoresis technique using 1% agarose gel. Extracted DNA purity and concentration were measured in nanodrop spectrophotometer.

Table 1: Primers, sequences, and product size used in detection of *E. coli* O157:H7 *rfbO157*, *stx1*, *stx2*, and *hlyA* genes in dogs

Primers		Sequence of the primers (5^{-} to 3^{-})	Size (pb)	Reference
rfb $_{O157}$	F	CGG ACA TCC ATG TGA TAT GG	259	(13)
	R	TTG CCT ATG TAC AGC TAA TCC		
Stx1	F	ACA CTG GAT GAT CTC AGT GG	614	(14)
	R	CTG AAT CCC CCT CCA TTA TG		
Stx2	F	CCA TGA CAA CGG ACA GCA GTT	779	(14)
	R	CCT GTC AAC TGA GCA CTT TG		
hlyA	F	GTC TGC AAA GCA ATC CGC TGC AAA TAA A	561	(15)
	R	CTG TGT CCA CGA GTT GGT TGA TTA G		

Components of PCR mixture for *rfbO157, stx1, stx2* and *hlyA* genes

In this study, the total volume of reaction (20 µL) in 0.5 ml eppendorf tube included template DNA (1µL), PCR master mix (10 µL), the each primer (2 µL), and PCR water (6 µL).

Thermo-cycler programs

The thromocycler program for stx1 and stx2 genes included: (i) one cycle with 3 minutes duration at 94 °C used for denaturation of the template; (ii) 35 cycles, each cycle included 3 processes: denaturation (at 94 °C for 45 seconds), annealing (58°C for 30 seconds), and extension (72°C for 60 seconds); and finally (iii) one cycle with 5 minutes duration

at 72 °C for final extension. The thromocycler program for *rfbO157* gene included (i) one cycle for with 5 minutes duration at 94 °C for initial denaturation; (ii) 35 cycles, each cycle included 3 processes: denaturation (94°C for 60 seconds), annealing (52°C for 30 seconds), and extension (72°C for 60 seconds); and finally (iii) one cycle with 5 minutes duration at 72°C for final extension. The thromocycler program for *hlyA* gene included (i) one cycle with 5 minutes duration at 94°C for initial denaturation; (ii) 35 cycles, each cycle included 3 processes: denaturation (94°C for 60 seconds), annealing (60°C for 45 seconds), and extension (72°C for 60 seconds); and finally (iii) one cycle with 5 minutes duration at 72°C for final extension.

Analysis PCR product using Agarose Gel Electrophoresis

PCR product was analyzed by agarose gel electrophoresis using 2% agarose gel stained with ethidium bromide 0.5 µg/mL, and visualized via UV transilluminator.

Results

The results of the present study showed that 9.7% (7/72) of dogs with diarrhea and 7.1% (2/28) of dogs without diarrhea tested positive for *E. coli* O157:H7 (Table 2). *Escherichia coli* isolates were identified (Figure 1). In addition, the amplified PCR product for *E. coli* O157:H7 In addition, all the genes were detected by using the PCR assay (Figure 2): 259 bp for the *rfbO157* gene (Figure 2, a), 614 bp for the *stx1* gene (Figure 2, b), 779 bp for the gene *stx2* (Figue 2, c), and finally 561 bp for the *hlyA* gene (Figure 2, d). The number of identified genes included the *rfbO157* gene (n= 7), *stx1* gene (n= 3), *stx2* gene (n= 3), and *hlyA* gene (n= 2) (Table 3).

Table 2: Number of *Escherichia coli* O157:H7 isolates in feces of dogs

Dogs	Total	No. (%) positive
With diarrhea	72	7 (9.7%)
Without diarrhea	28	2 (7.1%)
Total	100	9 (9%)

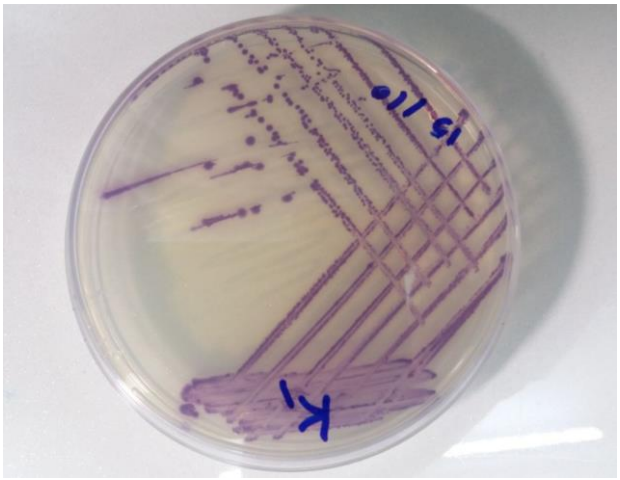

Figure 1: *Escherichia coli* O157:H7 isolate on Hichrom agar.

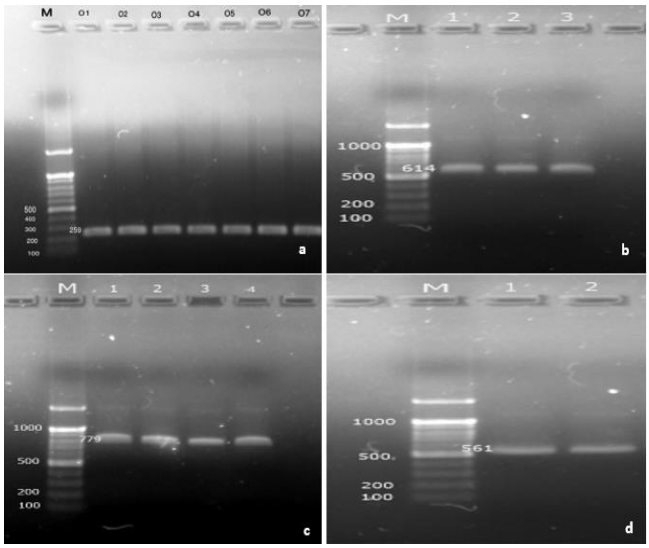

Figure 2: 2% Agarose gel electrophoresis for single gene PCR running of 259 bp fragment for the *rfbO157* gene (a), 614 bp for the *stx1* gene (b),779 bp for the *stx2* gene (c), and 561 bp for the *hlyA* gene (d).

Table 3: Number (percentage) of isolates carried *rfbO157*, *stx1*, *stx2*, and *hlyA* genes in 9 dogs

Animals	No. of positive for *E. coli* O157:H7	No. of *rfbO157* gene	No. of *stx1* gene	No. of *stx2* gene	No. of *hlyA* gene
Diarrheic dogs	7	6 (85.7%)	3 (28.6%)	3(42.9%)	1 (14.3%)
Non diarrheal dogs	2	1 (50%)	0 (0%)	1 (50%)	1 (50%)
Total	9	7 (77.8%)	3 (33.3%)	4 (44.4%)	2 (22.2%)

Discussion

This study indicated that Hichrom agar greatly helped in diagnosis of *E. coli* O157H: 7. Hichrom agar is considered suitable medium for isolation and identification *E. coli* O157H: 7 compared to MacConkey and EMB ager, as Hichrom medium contains sorbitol and chromogenic mixture instead of lactose and indicator dyes respectively.

The chromogenic agent X-glucuronide used in this medium helped in detection of glucuronidase activity of *E. coli* cells that absorb the X-glucuronide. The released chromophore resulted in a light pink to mauve colored colonies (16). The result of present study is in line with Klaif *et al*. (17) who found that the Chrom agar helped in diagnosis of *E. coli* O157:H7 (17).

In this study, *E. coli* O157:H7 was isolated from dogs with diarrhea and without diarrhea. Although only 2 (7%) isolates have been detected from dogs without diarrhea, these dogs are considered the source for the spread of the infection. Our result is in line with a former study in Iraq by Hasan *et al.* (10) that indicated that the pathogenic *E. coli* can be isolated from both diarrhea and non-diarrhea dogs (10). The current study showed that the *E. coli* O157:H7 isolates which carry *stx1* and *stx2* gene were associated with diarrhea in dogs. Our results were agreement with other studies which appeared that *E. coli* carrying virulence factors can be cultured from feces of dogs suffered from diarrhea (14,18,19).

In addition, the *stx1* and *stx2* genes were identified. Most the *E. coli* O157:H7 strains, however, produce *stx2*, although either *stx1*, or *stx2*, or both are produced (20). The results of this study showed that the percentage of the *stx2* gene 44.4% is higher than the ratio of the *stx1*gene 33.3% of all isolates for dogs with and without diarrhea, This results disagree with study in Iran by Torkan *et al.* (21) which appeared the *stx1* gene 64.3% was higher than *stx2* gene 35.7% in *E. coli* O157H:7 isolates from feces of dogs suffering from diarrhea (21).while our results were agreement with study on sheep by Abreham *et al.* (22) showed the percentage of the *stx2* gene 57.1% higher than *stx1* gene 14.2% (22).

Detection of the *stx1* gene mainly occurs in cell lytates as is typically considered cell-associated toxin located in the periplasmic part of the bacteria, unlike the *stx2* that is usually detected in the supernatants of the cultures as it is released outside of the bacterial cell because it is located in the extracellular part of the bacteria (23,24). In addition, hemolysin, which is virulence factor contributed in EHEC *E. coli* pathogenicity, was also detected in this study in both diarrheic and non-diarrheic dogs. This type of the virulence factors for *E. coli* was also identified in calves and cattle affected with diarrhea (25,26).

Identification of the *stx1*, *stx2*, and *hlyA* genes in *E. coli* O157 isolated from diarrheic dogs supports that the presence of these virulence factors are important for EHEC *E. coli* to induce diarrhea and other signs (27). Verotoxin-producing *E. coli* have been convincingly linked to a group of illnesses encompassing watery diarrhea, bloody diarrhea, and hemolyticuremic syndrome in humans (VTl and VT2) (28). In conclusion, the reason for the difference in the prevalence of *E. coli* O157H: 7 in these studies is potentially due to the differences in the level of pollution of the environment where dogs live, water and food in addition to age, immune status, stage of infection and the number of samples analyzed, this is the second study conducted on dogs in Iraq and the first in the cities of Tikrit and Mosul. In conclusion, precaution for human should be taken when handling pet dogs, as a pathogenic EHEC *E. coli O157:H7* is potentially exist in dogs affected diarrhea, and can be isolated from dogs without diarrhea, too.

In conclusion, dogs can be a significant reservoir for pathogenic *E. coli* O157:H7, particularly dogs with diarrhea.

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Conflict of interest

The authors have no conflict of interest.

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التحري الجزيئي عن الجينات *rfbO157* , shigatoxion , hemolysin لجراثيم الاشريشة القولونية نوع O157:H7 والمعزولة من براز الكلاب في مدينتي تكريت والموصل، العراق

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الخلاصة

تعتبر الاشريشية القولونية ذات النمط المصلي O157H:7 من مسببات الاسهال الهامة في الكلاب البالغة والجراء لما تحمله من عوامل ضراوة. هدفت الدراسة الحالية الى التحري الجزيئي عن الجينات الضراوة *rfbO157* , shigatoxin , hemolysin لجراثيم الاشريشية القولونية نوع O157:H7 والمعزولة من براز الكلاب في العراق. مئة ماسحة قطنية اخذت من براز الكلاب الاليفة والكلاب البوليسية في مدينتي تكريت والموصل، تضمنت ٧٢ من الكلاب التي تعاني من الإسهال و ٢٨ من الكلاب التي لا تعاني من الإسهال. زرعت الماسحات القطنية على الوسط المغذي ووسط الماكونكي، ثم حولت العزلات المشكوك فيها الى وسط الايوسين ملين الازرق EMB. زرعت المستعمرات التي اضهرت لمعاناً معدنياً على وسط الكروم التفريقي لجرثومة الاشريشية القولونية نوع O157:H7. أظهرت النتائج أن سبعة (٩,٧%) من الكلاب التي عانت من الاسهال حاملة لجرثومة الاشريشية القولونية من نوع O157:H7، ستة منها حاملة جين *rfbO157*، وثلاثة حاملة جين *stx1*، وثلاثة حاملة جين *stx2*، وواحدة حاملة جين *hlyA*. في الجانب الآخر، اثنان (٧,١ %) من الكلاب التي لم تعان من الإسهال حاملة لجرثومة من نوع O157:H7، واحدة منها حاملة جين *rfbO157*، وواحدة حاملة جين *stx2*، وواحدة حاملة جين *hlyA*. نستنتج من هذه الدراسة أن الكلاب تعد خازناً للايشريشيا القولونية O157:H7، لا سيما الكلاب التي تعاني من الإسهال.