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. 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.

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 eosi 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™ 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 |
|---------|----------------------------------|-----------|-----------|
| rfbO157 | 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 thermocycler 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 \textit{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 \textit{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 \textit{E. coli} O157:H7 (Table 2). \textit{Escherichia coli} isolates were identified (Figure 1). In addition, the amplified PCR product for \textit{E. coli} O157:H7 In addition, all the genes were detected by using the PCR assay (Figure 2): 259 bp for the \textit{rfbO157} gene (Figure 2, a), 614 bp for the \textit{stx1} gene (Figure 2, b), 779 bp for the gene \textit{stx2} (Figure 2, c), and finally 561 bp for the \textit{hlyA} gene (Figure 2, d). The number of identified genes included the \textit{rfbO157} gene (n=7), \textit{stx1} gene (n=3), \textit{stx2} gene (n= 3), and \textit{hlyA} gene (n= 2) (Table 3).

Table 2: Number of \textit{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%)            |

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

| Animals             | No. of positive for \textit{E. coli} O157:H7 | No. of \textit{rfbO157} gene | No. of \textit{stx1} gene | No. of \textit{stx2} gene | No. of \textit{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 \textit{E. coli} O157:H: 7. Hichrom agar is considered suitable medium for isolation and identification \textit{E. coli} O157H: 7 compared to MacConkey and EMB agar, 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* O157:H7 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 lyates 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 (VT1 and VT2) (28). In conclusion, the reason for the difference in the prevalence of *E. coli* O157:H7 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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The summary of the article: The prevalence of Shiga toxin 2 (Stx2) producing Escherichia coli (E. coli) was assessed in the current study. A total of 92 samples were collected from different sources, including tissue samples from camel and human stool samples in Al-Diwaniyah, Iraq. The results showed that Stx2 was detected in 9.7% of the samples. The study concluded that Stx2-producing E. coli is a potential pathogen in the region and might pose a health risk to humans and animals. The authors recommend further research to better understand the prevalence and transmission of Stx2-producing E. coli in the region.