ORIGINAL ARTICLE **Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ**

Clinical and molecular study of *E. coli* O157:H7 isolated from Diarrheic and non-diarrheic dogs

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ARTICLE INFO
Received: 15.03.2016
Revised: 25. 04.2016
Accepted: 29.05.2016
Publish online: 30.05.2016

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Abstract
A clinical study was performed on 104 dogs and puppies with different ages, breeds and sexes. The molecular study was conducted to confirm *E. coli* O157:H7 which was isolated from these animals using a real-time PCR for detection of rfb O157 and flic H7 genes. Totally, eighty seven *E. coli* isolates were isolated from 104 fecal samples by the traditional methods of culturing (on initial enrichment media, gram stain and biochemical tests). From 87 *E. coli* isolates, 26 isolates were belonged to *E. coli* O157:H7, when were cultured on specific media the Chrom agar O157. Only 18 (17.3%) isolates were positive for both O157 and H7 antigens, however, only 8 samples were positive to O157 antigen by latex agglutination test. The results of the real time PCR on 26 isolates showed that 7 (26.92%) were having rfb O157 gene, 18 (69.23%) were possessed rfb O157 and flic H7 genes, while only one (3.85%) was negative for both genes. The 18 animals which were positive for bacterial isolation, showed different clinical signs including: fever, increase respiration and heart rates, diarrhea. Dehydration and congested mucous membrane were seen in 11 animals, while the rested 7 animals didn't showed any clinical signs. Females were more susceptible than male for infection with *E. coli* O157:H7. The results of this study showed also that the global breed were more infected than the local breed. In addition, puppies at age 1-2 months were most susceptible. In conclusion, this study reveals that *E. coli* O157:H7 is important pathogen in dogs and the real time PCR is approved as a best, reliable and faster method for confirmatory diagnosis of these strains.

To cite this article: Afaf Abdulrahman Yousif; Mustafa Salah Hasan and Mohammad J. Alwan. (2016). Clinical and molecular study of E. coli O157:H7 isolated from Diarrheic and non-diarrheic dogs. MRVSA. 5 (2), 1-10.
DOI: 10.22428/mrvsa. 2307-8073.2016. 00521.x

Keywords: *E. coli* O157:H7, real time PCR, dogs.

Introduction

Entero-hemorrhagic *E. coli* (*EHEC*) are major food-borne pathogens responsible for serious infections ranging from mild diarrhea to hemorrhagic colitis and life-threatening

complications. Shiga toxins (*Stx*s) are the main virulence factor of *EHEC* (Pradel *et al*., 2015; Thevenot *et al*., 2015). It is also known as *STEC* (Talaro and Chess, 2013). *Escherichia coli* O157:H7 is now one of the causes of diarrhea worldwide. However, a strain *E. coli* O157:H7 causes bloody diarrhea when it grows in the intestines (Tortora *et al*., 2013). Dogs and puppies regarded as important reservoirs for *E. coli* O157:H7, which is one of the main causes of diarrhea and other diseases in human (Hasan *et al*., 2016). Diarrheic and non-diarrheic dogs of all ages may serve as potential sources of multi-drug resistant *STEC* O157:H7 transmissible to humans (Ojo *et al*., 2014). The RT-PCR assay for *EHEC* O157:H7 and other virulence genes proved to be a rapid test for detection of *EHEC* O157:H7 in complex biological matrices and could also be a potentially to use for the quantification of EHEC O157:H7 in foods or fecal samples (Sharma and Dean-Nystrom, 2003). Rebekka *et al*., (2006) detected sensitivity and accuracy of real-time PCR for *E. coli* O157:H7 and they conclude that the real-time PCR was a quick diagnostics assays in environmental samples for the presence or absence of *E. coli* O157:H7. In Hatay /Turkey, Aslantaş *et al*., (2006) studied the isolation and characterization of verotoxin-producing *E. coli* O157 in rectal swabs from the cattle in a slaughterhouse in each month during a 1-year period by using conventional PCR, the study revealed that the isolates of *E.coli* O157:H7/NM were possessed rbf (O157), EhlyA, *eaeA* genes, vtx2, and for both vtx1 and vtx2. This study was designed to investigate the clinical signs associated with *E. coli* O157:H7 infection from diarrheic and non-diarrheic dogs and puppies and to detect the presence of rfb O157 and flic H7 genes by real time PCR.

Materials and Methods

Clinical study

This study was done on 104 dogs aged from 1 month to 7 years, from different breed and both sexes found in different places from Baghdad Province.

Bacteriological and serological methods

All methods were done according to methods described previously (Hasan *et al*., 2016), by culturing on enrichment media, Gram stain and biochemical test for detection of *E. coli*. All *E. coli* isolates were screened on CHROM O157 agar [The Pioneer of Chromogenic Media/Paris] and Cefixime Tellurite - Sorbitol MacConkey agar (CT-SMAC) [LABM™ (England)] supplemented with potassium tellurite (2.5 mg/L) and cefixime (0.05 mg/L) for differentiation of *E. coli* O157:H7 from other type of *E. coli* (Tahamtan *et al*., 2011). Serotyping was made by using Latex agglutination Test by using commercial kit (Wellcolex *E. coli* O157:H7, Remel) to detect both the somatic antigen O157 and the flagellar antigen H7 according to (Ewing, 1986).

Real time PCR assay

PCR assay was performed in the laboratories of internal and preventive Veterinary Department/ College of Vet. Medicine /University of Baghdad, this assay was done on the 26 isolates of *E. coli* O157:H7 by the following methods:

DNA extraction

All isolates of *E. coli* O157:H7, which confirmed by bacterial and serological techniques, were conducted to the DNA extraction by using genomic DNA extraction Kit from (Presto™ Mini g DNA Bacteria Kit Geneaid. USA).

Measuring the purity and concentration of DNA

The purity and concentration of extracted DNA was measured using Nano-drop spectrophotometer.

Molecular characterization of *E. coli rfb* O157 and *flic* H7 genes by using Real time PCR

Real-time PCR was performed in a 96-well plate using Applied Biosystems (USA) 7500 Fast Real-Time PCR System by using kit from MicroSEQ ® *E. coli* O157:H7 Applied biosystem (USA). The FAM and VIC dyes are used to detect the targets; the NED dye is used to detect the internal positive control (IPC). Two microliters of template DNA was used, and nuclease-free water was added to reach a reaction mixture volume of 30 µl. The real-time PCR conditions were first optimized and were set as follows: activation of TaqMan probe at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 1 s and annealing/extension at 60°C for 20s.

Ethical Approved

This study was approved by the ethical and research committee/ College of Veterinary Medicine/ University of Baghdad, Ministry of High Education and Scientific Research.

Results

Clinical signs in dogs infected with *E. coli* O157:H7

From 104 dogs, 18 animals were at different ages with different types of signs. These were harboring the *E. coli* O157:H7, 11 of these dogs were shown different signs including fever, increase respiratory rate, increasing heart rate, presence of diarrhea, dehydration and congested mucous membrane. The other seven dogs were without any clinical signs, but harbour the *E. coli* O157:H7 (Table 1).

Percentage of *E. coli* O157:H7 with relation of sex

The results demonstrate that 5 (12.20%) dogs were harbor *E. coli* O157:H7 from total 41 fecal samples were taken from all dog. Out of 63 samples from female dogs, thirteen (20.63%) dogs were positive for *E. coli* O157:H7 (Table 2).

Percentage of *E. coli* O157:H7 with relation of age

The results of *E. coli* O157:H7 isolation according to the age revealed different percentage of infection with *E. coli* O157:H7 (Table 3).

Table.1: Shows different signs associated with dogs and puppies infected with *E. coli* O157:H7

Animals with clinical signs (No:11)	Temperature (°C) (Range)	Heart rate (bpm) (Range)	Respiration rate (bpm) (Range)	Diarrhea with blood	Diarrhea Without blood	Dehydration	Congested mucous membrane
Puppies (8)	39.6-40.9	174-209	26-40	3/8	5/8	5/8	3/8
Adults(3)	39.6- 40.9	174-202	26-44	3/3	0/3	2/3	2/3
Rested Animals (7)	No clinical signs						

Table .2: Shows the rate of infection of *E. coli* O157:H7 isolates from both sexes

Sex	Total no. of animals	No. of animals positive to *E.coli* O157:H7	Percentage
Male	41	5	12.20%
Female	63	13	20.63%
Total	104	18	17.3%

Table.3: Shows percentages of *E. coli* O157:H7 with relation of age

Age	No. of animals	No. of animals affected with *E. coli* O157:H7	Percentage
Puppies at 1-2 months	53	12	22.64
Dogs at 3-11 months	12	1	8.33
Dogs at 12-17 months	11	1	9.09
Dogs at 18-24 years	21	3	14.28
Dogs more than 24 months	7	1	14.28
Total	**104**	**18**	**17.3**

Percentage of *E. coli* O157:H7 with relation of breed

The current study showed that 9 (30%) from 30 puppies from the global breed were positive for *E. coli* O157:H7, and 5 (17.86%) from 28 adult dogs. The results of local breed revealed that 3 (13.04%) from 23 puppies and 1(4.34%) from 23 adult dogs were gave positive results for *E. coli* O157:H7 (Table 4).

Bacteriological and serological study

The different methods for detection *E. coli* showed that out of 104 fecal samples, 87 *E. coli* isolates (83.65%) were isolated by the traditional methods of culturing (on initial enrichment media, gram stain and biochemical tests), culturing on specific media Chrom agar O157 and CT-SMAC showed growing of 26 (25%), 23(22.12%) isolates respectively, which were confirmed as *E. coli* O157 and *E. coli* O157:H7 initially (Table 1).

Table. 4: Shows percentages of *E. coli* O157:H7 with relation of breed

Breed	Animals	No. of animals	No. of animals affected with *E. coli* O157:H7	Percentages
Global breed	Puppies	30	9	30%
	Adults	28	5	17.86%
Local breed	puppies	23	3	13.04%
	Adults	23	1	4.34%
Total		**104**	**18**	**17.3**

Table. 5: Shows Number of *E. coli* and *E. coli* O157:H7 by different methods

Animals	No. of fecal samples	No. of *E. coli* isolates by traditional methods	No. of *E. coli* isolates on Chrom agar O157	No. of *E .coli* O157:H7 isolates On CT-SMAC	latex agglutination test	
					No. of *E. coli* O157:H7	No. of *E. coli* O157 -ve H7
Total	104	87	26	23	18	7
Percent		83.65	25	22.12	17.3	6.7

Molecular study

Out of 26 isolates which were tested, 7 (26.92%) isolates were have *rfb* O157 gene, 18 (69.23%) isolates were possessed *rfb* O157 and *flic* H7 genes, while one (3.85%) isolates were negative for both genes (Table 6)(Figure 1).

Table. 6: Shows confirmatory diagnosis of *rfb O*157 and *flic*H7 genes by Real time PCR

No. of gene(s)	Gene name	No. of positive isolates	%
1 gene	*rfb*O157	7	26.92
	*flic*H7	0	0
2 gene	*rfb*O157+*flic*H7	18	69.23
Negative		1	3.85
Total		**26**	**100**

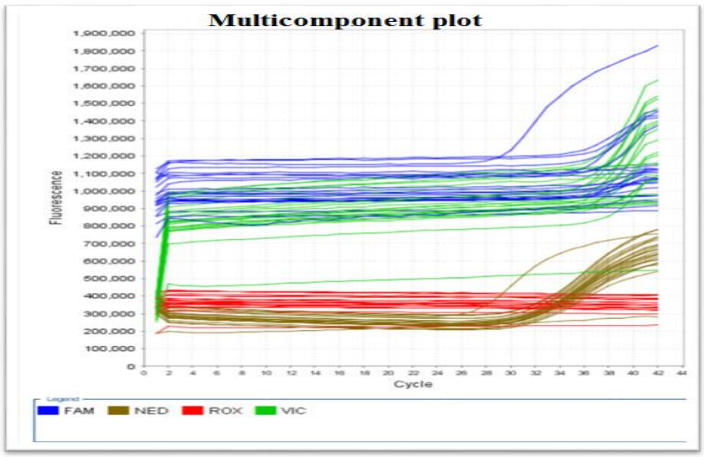

Figure. 1: Real time PCR results, positive samples where CT less than 35

Discussion

Dogs and cats called as companion animals have a closely relationship with humans. For that reason, a companion animal carrying with *EHEC* may become a human health threat (Sancak *et al*., 2004).

Our results showed different clinical signs of *E. coli* O157:H7 in dogs and puppies, these results are in agreement with results of Wang *et al*., (2006), who demonstrated that all dogs developed acute watery or mucoid diarrhoea on the second day after infection with *E. coli* O157:H7. The clinical signs were also accompanied with slightly bloody stool, decreased appetite, nausea and vomiting. The dogs were also exhibit dramatic weight loss and moderate or severe dehydration, fatigue, lethargy. Gouveia *et al*., (2011) was also mentioned that inoculated pups with *EPEC* strain, showed pasty-to-liquid diarrhea, without vomiting for 72 hrs post infection.

The results of present study revealed that puppies were most prevalent for *E. coli* O157:H7 infection. Hancock *et al*., (1998) found that the *EHEC* occurred in younger animals and in animals subjected to transit, feed changes and antimicrobial therapy, possibly due to disturbance in the resident intestinal microflora. Mohawk and O'Brien, (2011) concluded that *E. coli* O157:H7 was infected individuals, particularly the young children. Also, it have been reported that the prevalence of *EHEC* O157:H7 in cattle was peak in post weaned calves and heifers than older animals (Ferens and Carolyn, 2011). Young animals have been shown to be more susceptible to colonization by the *E. coli*

O157:H7 (Hancock *et al*., 1997). The higher prevalence rate of the pathogen is consistent for young beef and dairy cattle and may be attributed to the greater susceptibility of this age group to colonization (Hancock *et al*., 1998). The present study showed that females were susceptible for infection of *E. coli* O157:H7, this may be due to decrease in estrogen levels and increasing progesterone levels during each ovulation, which leading to decline in immune status (Blendinger, 2007) . High prevalence in heifers, but not in young bulls, indicates a role of sex and may involve hormonal shifts associated with pregnancy and/or lactation. Heifers also exhibit high rate of intramammary bacterial infections for as yet unexplained reasons (Nickerson *et al*., 1995; Fox, 2009).

The latex agglutination kit was used for more specific identification of *E. coli* O157:H7. It is used to detect the somatic antigen O157 and flagellar antigen H7 as compared with other methods of diagnosis, this result is incompatible with a result of (Yousif and Al-Taii , 2014 ; Al-Dawmy and Yousif , 2013) . Karmali *et al*., (1999) described latex test as a rapid, reliable, and easy to perform. It was used to reduce the time and effort in isolation, also used to eliminate other stereotypes, which have the same cultural and biochemical characteristics (Al-Dragy and Baqer, 2014; Yousif and Hussein, 2015).

The real-time PCR was used by Jenkins *et al*., (2012) for detection and characterization of verocytotoxigenic *Escherichia coli.* They concluded that real-time PCR is effective, rapid, screening method for the detection of *STEC* from stool specimens. Chui *et al*., (2013) reported that real-time PCR method can use as the "gold standard" for diagnosis of *E. coli* O157:H7. Among bacterial pathogens involved in food-illnesses *STEC* were frequently identified in a study of Mondani *et al*., (2016), who found that using of real time PCR for diagnosis is reduce the time and decrease the limitations which accompanying the other traditional tests. Also, it have been reported that real time PCR is highly recommended for rapid diagnosis of pediatric *STEC* infections (Qin *et al*., 2015). Abbasi *et al*., (2014) showed that, real time PCR can be used as a replacement for conventional PCR assay in the detecting virulence genes of *STEC* and *EPEC* strains. Also the real-time PCR offers the advantage of being a faster, more robust assay, because it does not require post-PCR procedures to detect amplification products. In conclusion, this study reveals that *E. coli* O157:H7 is an important etiological agent in dogs of all ages which can be transmitted to humans and the real-time PCR is an effective and accurate test for diagnosis of *E. coli* O157:H7.

Acknowledgments

This work was supported by College of Veterinary Medicine, Department of Internal and Preventive Veterinary Medicine, University of Baghdad, Iraq.

Author's contribution

All authors contributed equally in all details of this manuscript.

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