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Detection of *Escherichia coli* O157 H7 isolated from infected dogs with urinary tract infections using real time - polymerase chain reaction

Aida H. Ibrahim[1], Ban N.Nadhom[2], Ansam K. Mohammed[3]

College of Veterinary Medicine\ University of Baghdad

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Abstract

This research was approved to isolate, detects, diagnosis and molecular composition of *Escherichia coli* O157 H7 from UTI in Baghdad city. 67 urine samples were collected from the referring dogs into the Adan Teaching Veterinary Hospitals from the period of October 2017 to June 2018. The urine samples were collected by using urethral catheterization method. The samples were cultured, the recovered colonies were diagnosed microscopically (Gram stain), morphorgically and biochemically according to slandered methods. The results showed that the frequency of isolated bacterial spp. were 26 (38.8%) of 67 samples of urine, sex and age disseminations of dogs with bacteriuria .The bacteria were insulated in clean culture from 20 (76.92%) samples and were insulated in diverse culture from 6 (23.07%) samples. A total of 37 strains were isolated from urine samples. Of the strains isolated, 12(32.43%) were as *Escherichia coli*, 7(18.91%) as *Streptococcus species*, 6 (16.21%) as *Staphylococcus species*, 4 (10.81%) as *Klebsiella species*. 3(8.10%) as *Proteus species*. and *Pseudomonas aeruginosa* and 2 (5.40%) as *Corynebacterium species*. Specific forward and reverse primers were designed according to a program from NCBI-Genbank for *Escherichia coli* and *E. coli* O157 H7 To study sequence of these genes after amplification of 16srRNA genes. Genetic identities results showed that *E. coli* and *E. coli* O157 H7 isolates gave 100% matching and resembling the reference strains.

* **Corresponding Author:** Aida H. Ibrahim ✉ aidamedicalmicro@gmail.com

Introduction

Escherichia coli O157:H7 able to persuade wound in humans is a cause of its capability to induce many virulence factors, most remarkably Shiga toxin, which is one of the most potent poisons recognized to man (Griffin Tauxe 1991; Sandvig 2002). Stx has many alternatives (e.g., Stx1, Stx2, Stx2c), and performances like the toxin of plant by preventing synthesis of protein in endothelial and other cells. Endothelial cells line the inner superficial of blood vessels, and are recognized to be very susceptible to *Escherichia coli* O157:H7, which is cytotoxigenic to Endothelial cells (Welinder-Olsson Kaijser 2005).

In adding to Stx, *E. coli* O157:H7 yields many other supposed virulence issues, involving proteins which help in the affection and establishment of the bacteria in the duodenal wall and which can hydrolyse RBCs and release iron to help maintenance *E. coli* absorption (Ball *et al.,* 2008; Johannes 2010).

Medical signs of Urinary Tract Infection involves pollakiuria , dysuria and /or improved resolve of urination. The medical marks are not pathognomonic for infection. Urinalysis is beneficial in distinguishing an unfussy lower Urinary Tract Infection from other illnesses affecting the medical signs, as well as in additional examinations of imaginable essential reasons of Urinary Tract Infection, such as endocrine illnesses. A measure examination is of assessment, as it frequently identifies hematuria and proteinuria in circumstances of Urinary Tract Infection (Hariharan *et al.,* 2016).Current results indicate that the transmission of diarrhoeagenic *Escherichia coli* straining happen amongst humans and dogs. An indicative dogs were recognized as transporters of human pathogenic STEC with *E. coli* 0157:H7 strains and might therefore act a part in eruptions of STEC contaminations in humans (Khakhria *et al.,* 1990) dogs with diarrhea were known as an significant cause of bacterial infection of the surroundings in apartments of dog frames which capacity donate to the feast and spread of pathogenic *Escherichia coli* strains (Trevena *et al.,* 1996; Smee *et al.,* 2013). Therefore, more study should be focused to noticing and typifying diarrhoeagenic *E. coli* kinds in cats and dogs, their host specificity and the likely conversation of pathogenic straining among humans and animals.

Materials and methods

Sample collection

Sixty seven urine tasters were collected from the referring dogs into the Adan Teaching Veterinary Hospitals from the period of October 2017 to June 2018.

The urine samples were collected by using urethral catheterization method which is the passage of a urinary catheter from the external urethral orifice into the urinary bladder.

Biochemical tests

Classical biochemical tests for *E. coli O157:H7* were includes the following tests (Snedecor and Cochran 1980).

The IMViC test consist of four tests:
Indole production test.
Methyl Red test.
Voges Proskauer test.
Citrate utilization test.

Conventional Bacteriological Diagnosis

All urine samples were cultured and the recovered colonies were diagnosed microscopically (Gram stain), morphorgically and biochemically according to slandered methods (Greenwood *et al.,* 2007).

Bacterial isolation and identification

A 10 µL sample of each urine sample was taken by using sterilized and typical immunization loop that brings (0.01 ml or 0.001 ml), and streaked immediately on to blood and Mac Conkey agars plates (Collee *et al.,* 1996). All inoculated agar were raised aerobically at (37°C for 24 hrs). Isolated bacteria were documented conferring to their discoloration capability, form, dye, extent, hemolysis on the blood agar, and lactose fermenter on mac Conkey agar (Tilley and Smith-Jr 2004). All plates were streaked onto eosine methylene blue as a selective media for *E*

coli and for presumptively identify isolates as Gram-negative enteric bacteria incubated for 18 - 24 hr. at 37 °C, only *E coli* give a characteristic features appear as green metallic sheen) (Collee *et al.,* 1996).

Samples were measured –ve for bacterial growing after five days of cultivation. Standards for +ve growth for each assembly technique were founded on typical issued evidence. Ten, eleven growth were measured + ve if they produced ≥10,000 colony forming units /ml in a catheterized taster (Quinn *et al.,* 1994).

Urinalysis

The physical possessions of urine were inspected. Proteinuria, glucosuria, ketonuria, Urine pH, urobilinogen, nitrite, bilirubinuria and urine detailed importance ranks were assessed by lipstick test (Lapstrip®) (Osborne 1995)

Centrifugation of urine samples

Following culturing the samples of urine, were centrifuged (at 3000 rpm for 3 min). Urinary residue inspection was made to calculate epithelial cell casts, WBC, RBC and crystals in centrifuged urine (Osborne 1995; Collee *et al.,* 1996).

Slides preparation and staining

A single colony of the bacterial isolate was transferred and smeared on a clean slide. The prepared smear was discolored with Crystal Violet, preserved with Iodine for the fixation of dye, decolorized with mixture of 70 % alcohol+30% acetone, and then stained with safranine, after that inspected under Microscope (Snedecor and Cochran 1980).

Real-Time PCR (one-step RT-qPCR)

A. Kit description

Some current one-step qPolymerase Chain Reaction assess made well consuming standard cycling state transformed to a rapid, one-step qPolymerase Chain Reaction assess with [KAPA SYBER FAST ONE-STEP qPolymerase Chain Reaction kits]. Usually smallest re-optimization of reaction restrictions is essential: The [KAPA SYBR FAST one-step qRT-Polymerase Chain Reaction kit-Canada] is a sensitive and suitable result for real-time Polymerase Chain Reaction using Ribo Nucleic Acid as template.

The kit includes [KAPA RT mix (50 X) and KAPA SYBR FAST master mix (2 X)]. The KAPA RT mix includes wild-type M-MuLV reverse transcriptase and RNase inhibitor, and is enhanced for quick one-step, one tube Ribo Nucleic Acid quantification.

KAPA SYBR FAST q Polymerase Chain Reaction master mix is intended for high-performance real-time Polymerase Chain Reaction. The kit comprises a original DNA polymerase caused by a procedure of focused development.

The consequence is an exclusive enzyme, definitely planned for qPolymerase Chain Reaction consuming SYBR Green I dye interaction.

The 2 X master mix is a complete to usage mixture comprising all mechanisms for the amplification and recognition of cDeoxyribo Nucleic Acid, excluding primers and template.

B. Procedure

Some current one-step qRT-Polymerase Chain Reaction assess achieved professionally by typical cycling settings can be transformed to a rapid, one-step qReal Time-Polymerase Chain Reaction assess with KAPA SYBR FAST one-step qReal Time-Polymerase Chain Reaction kits.

Step 1: Preparation of qPCR master mix

The KAPA Real Time mix was reserved on ice through usage, and collected responses on ice to evade early cDNA production. Polymerase.

Chain Reaction master mix comprising the suitable capacity of all response mechanisms shared to all or a subset of responses to be done.

Comprised a no template regulator (NTC) and no Real Time regulator (NRT) when essential. The NTC would permit discovery of infection in the response

mechanisms, while the NRT would permit recognition of contaminating genomic DNA. The required volume of each constituent was designed.

Step 2: Setting individual reactions

The suitable capacities of qPolymerase Chain Reaction master mix, template and primers were removed to each well of a Polymerase Chain Reaction tube/plate.

The response tube/plate was caped and centrifuged temporarily.

Step 3: Performing One-Step qRT-PCR

Table Performed conventional qRT-PCR of gene 1 and 2 with cycling protocol

The facts gained after Real Time trials were noticed giving to the Ct standards which intended from cycles and was relative to the initial target copy number [logarithmic scale] using magnification (the point that the fluorescence signal amplified above baseline is the threshold cycle) which are inversely associated with the quantity of initial template that explain the high rate of Ct mentions to the low levels of gene appearance or magnification gene, while low Ct value designate high level of gene appearance or high copy of gene magnification.

Amplification plots appeared when the fluorescent signal from sample is plotted against cycle number; however amplification plots include the accumulation of product through the period of qPCR experiment.

Primers used in the interaction:

As depicted in Table 1 and Table 2.

Statistical analysis

Investigation of consequences was completed by consuming two methods of organization with communication technique and program of SAS (Snedecor and Cochran 1980).

Results

Clinical examination, Bacterial examination

According to the results and characteristic features of bacterial colonies inoculated on blood, mac Conkey, and eosin-methylene blue agars plates, gram's stain and biochemical examination, the frequency of isolated bacterial spp. were 26 (38.8%) of 67 urine tasters.

Table 1. The specific primer STX2of gene.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- CGACCCCTCTTGAACATA- 3'	51.1	50
Reverse	5'- GATAGACATCAAGCCCTCGT- 3'	54	50

The sex and age disseminations of dogs with bacteriuria are shown in table (3). The bacteria were insulated in pure agar growth from 20 (76.92%) samples and were insulated in varied growth from 6 (23.07%) samples.

Table 2. The specific primer SIX1 of gene.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- GTCACAGTAACAAACCGTAACA- 3'	53.0	40.9
Reverse	5'- TCGTTGACTACTTCTTATCTGGA- 3'	52.8	39.1

Thirty seven strains were insulated from urine tasters. Of the strains isolated, 12(32.43%) were as *E. coli*, 7(18.91%) as *Streptococcus spp.*, 6 (16.21%) as *Staphylococcus spp.*, 4 (10.81%) as *Klebsiella spp.* 3(8.10%) as *Proteus spp* and *Pseudomonas aeruginosa*. And 2 (5.40%) as *Corynebacterium spp.* (table 4).

The expression of gene was identified effectively by new molecular procedure which is

RT-PCR (qRT-PCR) with used exact primer. The magnification correctness of gene produce was seen by the value of cycle threshold (Ct) for the triplicate reactions. The statistics gained from RT trials were identified conferring to the Ct standards which designed from cycles and was comparative to the initial target copy number (logarithmic scale) used for magnification. which are associated with the quantity of initial template that mean the great value of Ct mentions to the low levels of gene appearance or magnification gene, while low Ct value designate high level of gene appearance or high copy of gene magnification. Magnification schemes seemed when the fluorescent sign from sample is planned in contradiction of cycle number; though magnification plans comprise the addition of invention through the cycle of qPolymerase Chain Reaction trial as showed in tables (5, 6, 7) fig.(1,2).

Table 3. Age and sex distributions of dogs (years).

Sex					Total	
	Female		Male			
Age	No. of dogs examined	No. of dogs examined with bacteruria	No. of dogs examined	No. of dogs examined with bacteruria	No. of dogs examined	No. of dogs examined with bacteruria
≥2	29	6	22	12	51	18
<2	9	2	7	6	16	8
Total	38	8 (11.94)	29	18 (26.86)	67	26(38.8%)

Table 4. Frequency of bacterial spp. isolated from urine samples and their No.

Bacterial spp.	No. of isolates
E coli	12(32.43%)
Streptococcus spp.	7(18.91%)
Staphylococcus spp	6 (16.21%)
Klebsiella spp	4 (10.81%)
Proteus spp.	3(8.10%)
Pseudomonas aeruginosa	3(8.10%)
Corynebacterium spp.	2(5.40%)
Total	37(100%)

Discussion

In the current revision, bacteria were insulated from 26 (38.8%) of 67 urine samples from dogs with Urinary Tract Infection signs. Hariharan (Hariharan *et al.,* 2016) described that 44 (48.35%) of the urine samples from 91 dogs were bacteriologically + ve and the residue were disinfected. Additional earlier reading informed that bacterial spp. were insulated from 38 (38%) of 100 urine samples from dogs with Urinary Tract Infection signs (Kogika *et al.,* 1995; Trevena *et al.,* 1996). From dogs with Urinary Tract Infections (Etun *et al.*, 2003) described that bacteria were learned from 427 (80.6%) of 530 urine samples from dogs with Urinary Tract Infections or suspected contaminations.

Table 5. Result of nano drop.

sample ID	Nucleic acid conc (ng/ml)	260/280
1	280	1.61
2	180	1.59
3	110	1.86
4	127	1.54
5	140	1.69
6	115	1.63
7	160	1.47
8	196	1.5
9	138	1.51
10	219	1.81

These consequences designate that though medical and urinalysis results are constant with infectious contamination, a identification of bacterial Urinary Tract Infection should not be founded on these results only (Thompson *et al.,* 2011).

Table 6. Qualitative analysis report of PCR analysis result, manual threshold analysis method B, F (Threshold_FAM = 73.9 Threshold_HEX = 5.0).

Result	Ct, Fam	Identificator of the tube	Number of the hole
+	33.0	Sample_1	A1
-		Sample_2	A2
-		Sample_3	A3
-		Sample_4	A4
+	38.9	Sample_5	A5
-		Sample_6	A6
-		Sample_7	A7
-		Sample_8	A8
-		Sample_9	A9
-		Sample_10	A10

Amplification program:

95.0 °C - :10:00 X

95.0 °C - :00:15 X

55.0 °C - :00:10 X

72.0 °C - :00:15 X

Table 7. Qualitative analysis report of PCR analysis result, manual threshold analysis method B, F (Threshold_FAM = 22.0 Threshold_HEX = 0.0)

Result	Ct, Fam	Identificator of the tube	Number of the hole
+	30.1	Sample_1	A1
-		Sample_2	A2
-		Sample_3	A3
-		Sample_4	A4
-		Sample_5	A5
-		Sample_6	A6
-		Sample_7	A7
-		Sample_8	A8
+	30.1	Sample_15	B3

Amplification program:

95.0 °C - :10:00 X

95.0 °C - :00:15 X

55.0 °C - :00:10 X

72.0 °C - :00:15 X.

Agreeing to the earlier available evidence, the greatest regularly insulated bacterium from dogs with Urinary Tract Infection is *E. coli*, followed by *Staphylococcus spp., Proteus spp., Streptococcu spp., Klebsiella spp., Enterobacter spp., and Pseudomonas spp* (Mitra *et al.,* 1994). In the current revision, a whole of 37 strains were insulated from urine samples. Of the strains isolated, 12 (32.43%) were as *E. coli*, 7(18.91%) as *Streptococcus spp.,* 6 (16.21%) as *Staphylococcus spp.,* 4 (10.81%) as *Klebsiella spp.* 3(8.10%) as *Proteus spp.* and *P. aeruginosa*., and 2 (5.40%) as *Corynebacterium spp.* In additional earlier revision, the greatest significant insulates were *E. coli*, *S. aureus*, *Streptococcus spp., Enterobacter*

spp., K. pneumoniae and *P. aeruginosa*. Sharma (Sharma *et al.,* 1992) Isolated *E. coli* (35.3%), *Staphylococcus spp.* (23.5%), *Proteus mirabilis* (15.7%), *Streptococcus spp.* (13.7%), *Klebsiella spp.* (9.8%), *P. aeruginosa* (3.9%), *Enterobacter cloacae* (2.0%), and *Providencia rettgeri* (2.0%), while Ojo (Ojo *et al.,* 2014) reported that *E. coli*, S. aureus, *Pseudomonas spp.* and *P. vulgaris* were insulated from 13 (54.17%), 6 (25%), 3 (12.50%) and 2 (8.33%) cases, correspondingly. Ling (Ling 2000) stated that 10 bacterial types accounted for 96.3% of the urinary insulates, with *E. coli* (44.1%), *Staphylococcus spp.* (11.6%), *Proteus spp.* (9.3%), *Klebsiella spp.* (9.1%), *Enterococcus spp.* (8.0%) and *Streptococcus spp.* (5.4%), and these were the greatest public insulates in both sexes of dogs. It seems from these results that the occurrence of several classes differs significantly from revision to study, *E. coli* is the greatest public causal agent of Urinary Tract Infections in dogs.

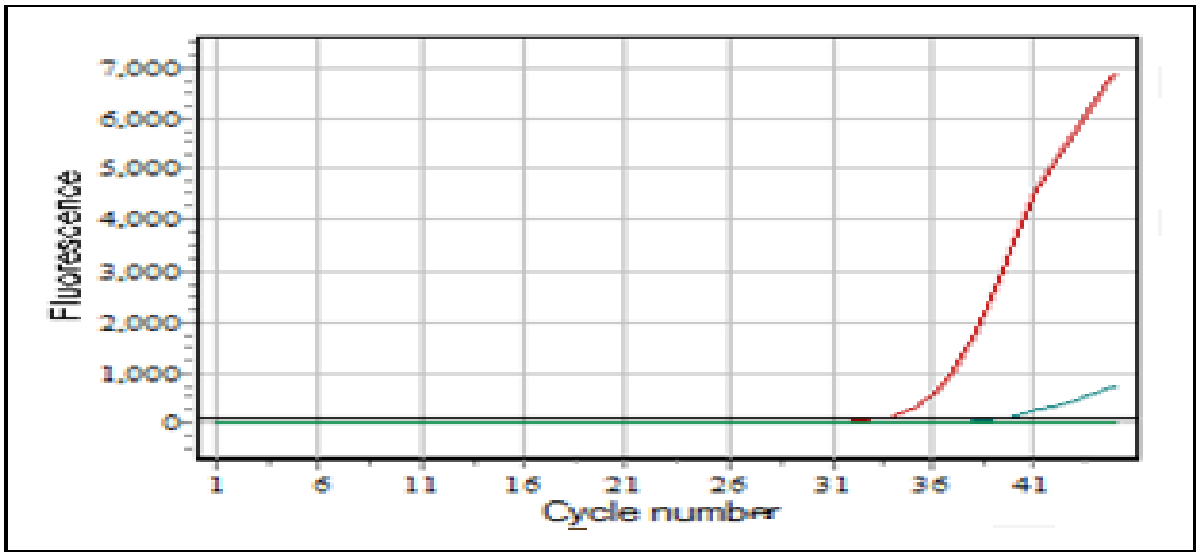

Fig. 1. Dependence of FAM channel fluorescence on cycle number (Threshold_FAM = 73.9 Threshold_HEX = 5.0).

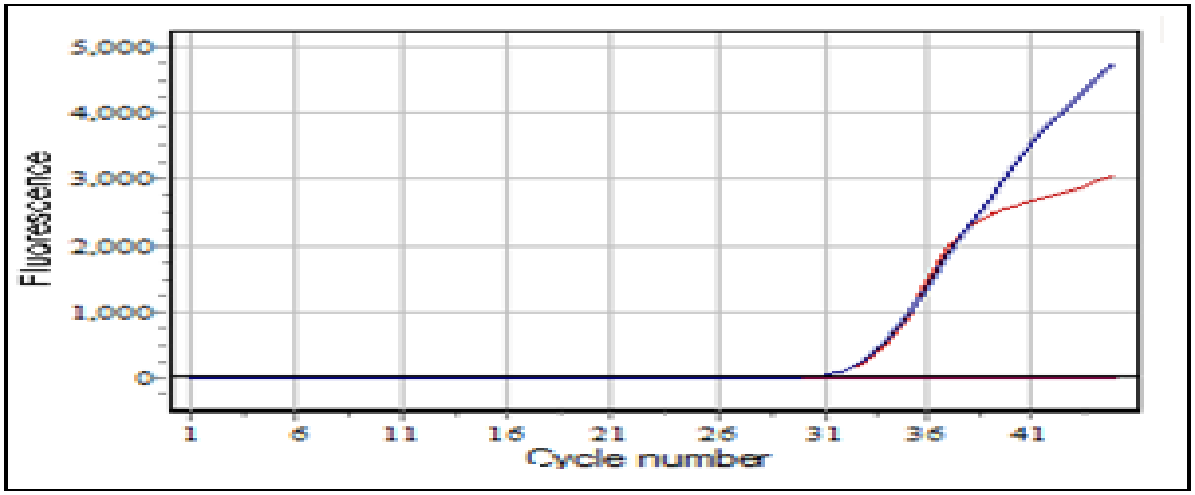

Fig. 2. Dependence of FAM channel fluorescence on cycle number (Threshold_FAM = 22.0 Threshold_HEX = 0.0).

In the present study only 2 (16.6%) cases were give positive result for API 20E system that's used to confirm the presence of *E coli* O157:H7 strains of the infected dogs with UTIs. This reflect important mater that the *E coli* O157:H7 strains must be considered as a bacterial causative agent for developing UTIs in the

dogs since the dogs and puppies observed as significant reservoirs for *E. coli* O157:H7, which is one of the highest reasons of diarrhea and other diseases in human (Ball *et al.,* 2008; Hasan *et al.,* 2016).

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