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Prevalence of Pathogenic Genes of *Escherichia coli* O157:H7 Strains from Patients with Urinary Tract Infections in Iran

Mehdi Kargar[1], Mohammad Zareian Jahromi[2]*, Mohammad Kargar[2] and Reza Robati[3]

[1]Researcher's and Elite Club, Jahrom Branch, Islamic Azad University, Jahrom, Iran.
[2]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran.
[3]Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.

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The aim of this study was to evaluate the presence and frequency of some virulent genes of *E. coli* O157:H7 in patients with urinary tract infections (UTIs). In this research, the presence of *hlyA, eaeA, stx1*, and *stx2* genes in *E. coli* O157:H7 isolates was evaluated using a multiplex PCR method. Two hundred and ninety-eight positive bacterial isolates were obtained from suspected UTI samples, with 206 of the isolates (69.1%) belonging to the *E. coli* category. Of the 206 isolates that were positive for *E. coli*, 16 of them (7.8%) harbored O157 gene, and 13 of them (6.3%) had both O157 and H7 genes. For this group, multiplex PCR was conducted on *stx1, stx2, eae*, and *hly* genes, and the results indicated that the distribution of *stx1, stx2, eaeA* and *hlyA* genes among these isolates was as follows: *eaeA* (15.4%), *stx1* and *eaeA* combined (15.4%), *stx2*(7.7%), and *stx2* and *eaeA* combined (7.7%). No *hlyA* genes were identified in the samples.

Key words: *E. coli O157:H7;* hlyA gene; eaeA gene; stx1 gene; stx2 gene; multiplex PCR**.**

Shiga toxin-producing *E. coli* (STEC) are the fourth most common bacterial foodborne pathogen and responsible for around 1200 illnesses per year in the UK(Tran*et al*., 2014). *E. coli* O157:H7 is a common health concern in most countries around the world (Kiranmayi *et al*., 2010). This was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhea traced to consumption of hamburgers at common chains of fastfood restaurants. *E. coli* O157:H7 was known to be a human pathogen for nearly 32 years (Riley *et al*., 1983). Since then, it has been reported in more than 30 countries on six continents (Brown *et al*., 1997). *E. coli* O157:H7 is highly pathogenic in humans, but it does not induce any clinical disease in cattle and other animals other than diarrhea (Ateba and Mbewe *et al*., 2014). It bears several genes, including the *stx* gene, the Enterohaemolysin-encoding *hly* gene, and the intimin-encoding *eae* gene (Manna *et al*., 2006).

E. coli is an important member of the coliform group. Based on the pathogenecity and variation in biochemical characteristics, *E.coli* has been classified into 6 categories, viz. enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EaggEC) and diffusely adherent (DEAC), of which the EHEC is considered as most important one (Kiranmayi *et al*., 2010). These STEC strains are associated with a variety of disorders in humans including non-bloody diarrhoea, haemorrhagic colitis (HC) and a very

* To whom all correspondence should be addressed.
Tel.: +98(0)9177913007;
E-mail: mohammadzareian@yahoo.com
mzareian@jia.ac.ir

serious complication Haemolytic Uraemic Syndrome (HUS). HUS is the major cause of acute renal failure in children and fatal in up to 2% of cases six. The diarrhoeal-associated or typical HUS is the most common form among all HUS cases and most of them are caused by enterohaemorrhagic *E. coli* (EHEC). EHEC are classified as a subset of STEC carrying the eae gene encoding intimin and being pathogenic to human beings (Khan *et al*., 2011). There are two subunits of shiga toxin that are known as 'A' and 'B.' The B subunit binds to its specific receptor on the host cell, specifically globotriaosylceramide (Gb3). Gb3 is present in greater amounts in renal epithelial tissues, so the shiga toxin may cause renal toxicity(Tran*et al*., 2014). The *E. coli* O157:H7 (EHEC) strain produces two types of toxins namely Shiga toxin 1 (Stx-1) and / or Shiga toxin 2 (Stx-2), which have been also referred as Verotoxin 1 (VT-1) and Verotoxin 2 (VT-2) respectively responsible for food borne illness(Kiranmayi *et al*., 2010).

To the best of the author's knowledge, this study is in Iran that urine samples of patients suspected of having UTIs were collected, bacterial isolation was conducted, and the presence of *hly, eae, stx2,* and *stx1* genes in *E. coli* O157:H7 isolates was tested using the PCR method.

MATERIALS AND METHODS

Sample collection

Urine samples of individuals with suspected urinary tract infections were collected from six hospitals and clinical laboratories during a nine-month period. Two hundred and ninety-eight positive samples were isolated by culturing the samples on blood agar and EMB media and confirming the presence of urinary tract infections in the referred individuals. Subsequently, the bacteria were identified using Gram staining, catalase, oxidase, and biochemical tests. *E. coli* isolates were considered for *E. coli* O157:H7 identification.

Isolation of *E. coli* 0157:H7 and Serological tests

Initially, *E. coli* bacteria were cultivated and incubated on CT-SMAC (Merck - Germany) for 24 h at 37 °C, and sorbitol-negative bacteria were identified (Brandon *et al*., 2009). Then, the sorbitol-negative bacteria were cultured on O157 chrome agar (High Media, India). MUG-negative bacteria were incubated on the Nutrient agar (Merck- Germany) 24 h at 37 °C. Then, using a saline test, bacteria were checked to verify that they were not members of bacteria with spontaneous agglutination. Finally, they were tested with *E. coli* O157 anti-serum. Agglutinated samples were reported as positive samples (Hepburn *et al*., 2002).

DNA extraction

The bacteria that were positive with anti-serum *E. coli* O157:H7 were cultured in medium LB Broth (Sharlua, Spain) at 37 °C for 24 h. Then, using the DNP™ DNA Extraction Kit (Synaclone, Iran) the DNAs of the bacteria were extracted and the DNA concentrations were measured using the OD method.

PCR on gene H7, 0157

Gene O157 was screened by specific primers in the PCR assay. The conditions of the PCR and the thermal cycles were similar to those of virulent genes multiplex PCR (Paton and Paton., 1998). Another PCR amplification analysis was conducted for confirmation of the presence of the flagellar H7 gene. The PCR primers for H7 were previously described by Gannon *et al*. (1997). The oligonucleotide sequence of the primer and expected sizes are listed in Table 1. Both the somatic O157 and the flagellar H7 PCR assays were performed in a final volume of 50µl that contained 2.5 unit ofTaq DNA polymerase (Ferments-Germany), 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, 50 mMKCl, and 20 pmol of one of the flagellar H7 or somatic O157 primers. The PCR reactions were conducted with a GeneAmp PCR thermocycler (Techne, Germany) according to the following protocol: one cycle of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min each, annealing for 2 min at 65 °C for H7 and at 59 °C for O157, extension at 72 °C for 2 min, and the final extension at 72 °C for 5 min. The amplified PCR products were separated on 1.5% agarose gel in TAE buffer, stained with *ethidium bromide*, and photographed under UV illumination (Fig. 2).

Multiplex PCR for *stx1, stx2, eae*A, and *hly*A

Multiplex PCR for the detection of *stx1, stx2, eaeA,* and EHEC *hly*A genes was performed by a GeneAmp PCR thermocycler (Techne, Germany). Oligonucleotide primers for *stx1*, *stx2*,

eaeA, and *hlyA* were synthesized as described previously (Paton and Paton., 1998). The oligonucleotide sequence of the primers and the predicted sizes of the PCR-amplified products are listed in Table 1. Each primer pair had been determined to be specific for *E. coli* and had been shown not to amplify products detectable by agarose gel (Ferments- Germany) electrophoresis using DNA templates derived from a range of Gram-positive and Gram-negative bacterial species from various food and animal sources. Samples (2 ml) of each extract were amplified in 50-ml reaction mixtures containing 200 mM concentrations of deoxynucleoside triphosphates, an approximately 250 nM concentration of each primer, and 1 U of Taq polymerase in 10 mMTris- HCl(pH 8.3), 50 mMKCl, 2 mM $MgCl_2$. (Ferments- Germany) were added to the reaction mixtures. The PCR conditions consisted of an initial 95 °C denaturation step for 3 min followed by 35 cycles of 95 °C for 20 s, 58 °C for 40 s, and 72 °C for 90 s. The final extension cycle was done at 72 °C for 5 min. Amplified DNA fragments were resolved by gel electrophoresis using 1.5% agarose gels in trisacetate-EDTA (TAE) buffer. Gels were stained with 0.5 µl of ethidium bromide per ml and viewed and photographed under UV illumination.

Statistical analysis

The data were statistically analyzed by SPSS version 15 (SPSSInc., Chicago, IL, USA). The chi-square test or the Fisher exact test was provided to compare categorical variables. P-value <0.05was considered statistically significant.

RESULTS AND DISCUSSION

Shiga-toxigenic *E. coli* (STEC), especially *E. coli* O157:H7 are a potential threat to public health due the morbidity and mortality associated with its infection (Pant *et al*., 2014).

In this study, 10,372 urine samples from six hospitals and diagnostic laboratories were analyzed during a nine-month period. Among 298 positive isolates in patients with UTIs, 206 samples containing *E. coli* were detected. After cultivation on the SMAC media, the sorbitol-negative bacteria were cultured on media containing MUG. Then, 63 samples that were MUG-positive for bacteria were selected for serological tests. They were evaluated with specific anti-serum against *E. coli* O157, and 29 samples tested positive for *E. coli* bacteria with the anti-serum. By using PCR on genes O157 and H7, 16 samples were O157 positive which 13 of them were O157: H7 positive and 3 samples were H7 negative. Also, there were 13 anti-serum positive samples out of 29 which did not show the above genes, which indicated which anti-serum test for the detection of *E. coli* O157:H7 is not a valuable method in comparison to PCR. In the present study, *E. coli* O157:H7 was evaluated in suspected samples of patients with urinary tract infections. Statistical analyses of the 298 bacterial isolates from suspected UTI samples showed that 206 (69.1%) of the UTI bacterial isolates belonged to the *E. coli* category. *Klebsiella sp*. was in second place, having been identified in 36 (12.1%) of the isolates. *E. coli*. The data shows that the highest

Table 1. Primers used in multiplex PCR ofstx1, stx2, eae, hly, H7, and O157 genes

Name of gene	Primer Oligonucleotide sequences (5'-3')	Expected size	Reference
stx1	F: ACACTGGATGATCTCAGTGG		
	R: CTGAATCCCCCTCCATTATG	614 bp	Paton and Paton (1998)
stx2	F: CCATGACAACGGACAGCAGTT		
	R: CCTGTCAACTGAGCAGCACTTTG	779 bp	Paton and Paton (1998)
*eae*A	F: GTGGCGAATACTGGCGAGACT		
	R: CCCCATTCTTTTTCACCGTCG	890 bp	Paton and Paton (1998)
*hly*A	F: ACGATGTGGTTTATTCTGGA		
	R: CTTCACGTGACCATACATAT	165 bp	Paton and Paton (1998)
H7	F: GCGCTGTCGAGTTCTATCGAG		
	R: CAACGGTGACTTTATCGCCATTCC	625 bp	Gannon *et al*.(1997)
O157	F: CGGACATCCATGTGATATGG		
	R: TTGCCTATGTACAGCTAATCC	259 bp	Paton and Paton (1998)

Table 2. Frequency of different types of bacteria in patients with UTIs (Total number of positive samples: 298)

Type	Percentage
Total bacteria separated from women	67.4%
Total bacteria separated from men	32.6%
Samples containing blood in urine	52.7%
E. coli percent	69.1%
Klebsiellasp	12.1%
Tested positive womenfor*E. coli*	86.6%
Samples containinganti-serum positive for *E. coli*	14.1%
*E. coli*O157:H7	6.3%

percentage of urinary tract infections was observed in women. Slightly more than seventy-six percent of the women had UTIs, whereas only 32.6% of the men did. After culturing on CT-SMAC medium, transferring of sorbitol-negative *E. coli* to chrome Agar medium, and performing serological tests, a total of 14.1% of the *E. coli* isolates that gave agglutination to *E. coli* O157:H7 antisera were considered as positive isolates. Only 7.8% of the *E. coli* isolates harbored the O157 gene, and 6.3 % of them had both O157 and H7 genes for which multiplex PCR was conducted on *stx1*, *stx2*, *eae*, and *hly* genes (Table 2). The results of the multiplex PCR indicated that the distributions of *stx1*, *stx2*,

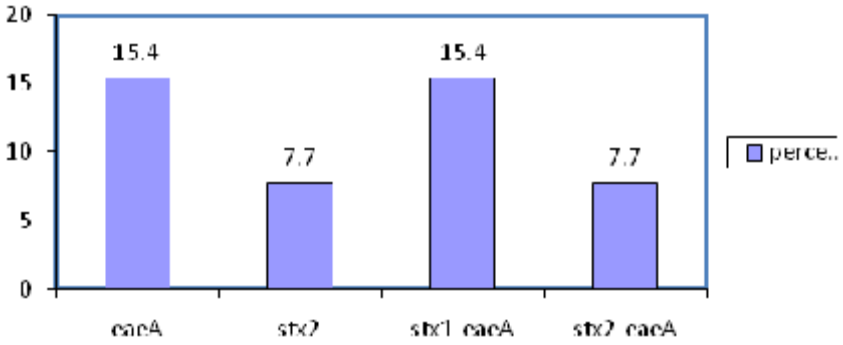
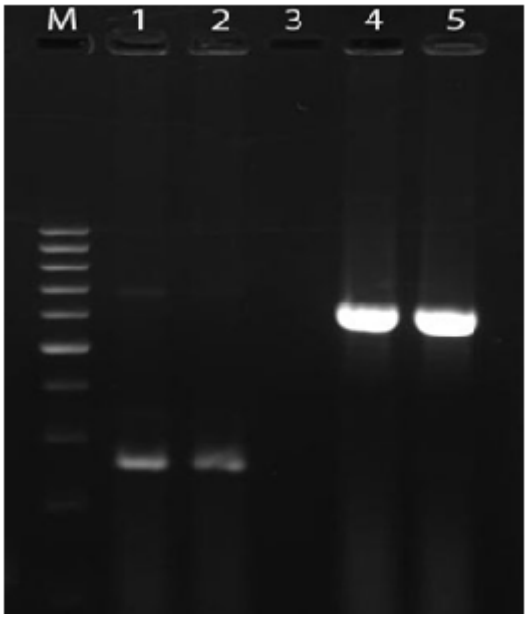


Fig. 1. Percentage of gene frequency in *E. coli*O157:H7



M: marker 100bp, Lanes 1 and2: H7 gene, Lanes 4 and 5: O157 gene, Lane 3: negative Control

Fig. 2. Presence of O157 and H7 genes inbacterial isolates

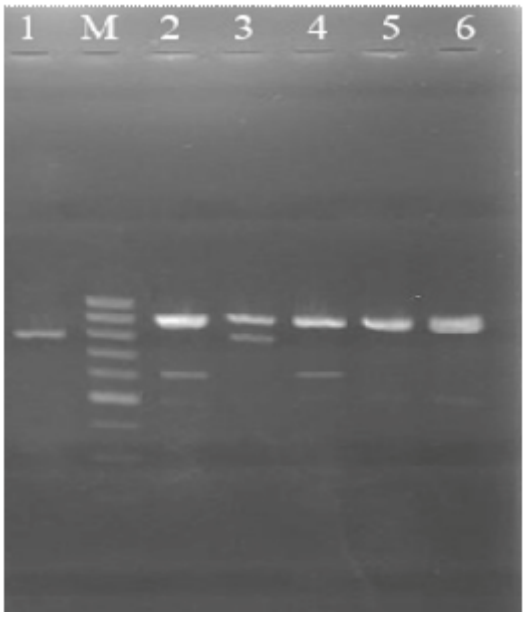


Fig. 3. *E. coli* O157:H7 multiplex PCR productson 1.5% agarosegel

eae, and *hly* genes among these isolates were as follows: eae alone (15.4%), *stx1* and eae together (15.4%), *stx2* alone (7.7%) and *stx2* and *eae* together (7.7%) (Table 2). It is important to note that the *hly* gene was not identified in any of the isolates.

Fig. 3 shows agarose gel electrophoresis of *E. coli* O157:H7 multiplex PCR products.

1) Number 1: *E. coli* O157:H7 isolate with *Stx2* gene of 790 bp.
   M: Marker of 100 bp
3) Number 2: *E. coli* O157:H7 isolate with eae gene of 889bp and *stx1*gene of 614 bp.
4) Number 3: *E. coli* O157:H7 isolate with *eae* gene of 889bp and *stx2*gene of 790 bp.
5) Number 4: *E. coli* O157:H7 isolate with *eae* gene of 889bp and *stx1* gene of 614 bp.
6) Number 5 and 6: *E. coli* O157:H7 isolate with *eae* gene of 889 bp.

Studies have shown that VTEC strain is commonly isolated in hemolyticcolitis and hemolytic uremic syndrome belonging to serogroup O157:H7 (Navidinia *et al*., 2012).

Since most of the urinary tract infections resulted from different strains of *E. coli*, the possibility of illness caused by different strains of O157: H7 was considered. The results of this research are consistent with the findings of some other research Starr *et al*. (2012) and Tarr *et al*. (1996) also showed that O157:H7 can cause urinary tract infections. Christina *et al*. (2011) conducted experiments on non-O157:H7 and O157:H7 that cause disease in children and showed that these bacteria can be the cause of many diseases.

By multiplex PCR performed on the genes *eaeA, stx2, stx1* and *hlyA*, six types of *E. coli* O157:H7 bacteria were isolated from samples of UTI genes that included *stx2, stx1*, and *eaeA* genes, but there were no *hly* genes among them (Schmidt *et al*., 1999). These data were confirmed previously by culturing bacteria on blood agar, which showed no evidence of hemolysis because of the *hlyA* gene.

In his evaluation of the prevalence of *stx*-producing *E. coli* in North America, Johnson examined 597 urine samples and showed that STEC bacteria were not isolated from urine samples (Johnson *et al*., 2002). During a study on urine and stool samples from 6-week-old children, Starr *et al*. (2012) showed that *stx1* and *stx2* genes from *E. coli* were present in the urine of such patients, but the *stx* gene was not detected in their stools.

The results of this research showed that *E. coli* O157:H7 bacteria also can produce disease in the urinary tract, so it is possible that these bacteria are transferred to the urinary tract through fecal contamination. However, studies conducted by Starr *et al*. (2012) and Tarr *et al*. (1996) found no evidence to support this hypothesis. In other research projects, the relationship between urinary tract infections and gastrointestinal infections were reported (Flemming *et al*., 2000). In some studies, the *E. coli* O157:H7 that were isolated contained *stx1* and *stx2* genes; in other studies, the isolates contained *vtx*genes. This may explain the *E. coli* pathogenicity of *E. coli* O157:H7 in the urinary tract (Byrne *et al*., 2003).

Navidinia *et al*. (2012) isolated 378 samples there were positive for *E. coli* bacteria from 12,572 samples in children. Among them, nine samples included EHEC, and five of the EHEC positive samples contained *vtx* genes. Tarr *et al*. (1996) showed that the strains of shigatoxin-producing *E. coli* O103:H2 were isolated from the urine sample of six-year-old girls suffering from urinary tract infections (UTIs), while there were no signs of diarrhea in these children. In 1993 in Denmark, two UTIs caused by *E. coli* were diagnosed. The researchers proposed that *E. coli* strains isolated from UTIs can be verotoxin producers, especially while the HUS prevalence is in the clinical form (Flemming *et al*., 2000). Other studies that have been conducted by scientists on *E. coli* O157:H7 in foods have shown the importance of this microorganism. However, in the previous studies, the frequency and the percentage of *E. coli* O157:H7 in urinary tract infections were low, and this low frequency is very important because the infection can become exacerbated and involve the kidneys.

Bonyadian *et al*. (2010), in a study on patients with diarrhea, isolated 58 samples and found that 16 of the samples contained *stx1* genes, four samples had stx2genes, and eight samples contained both genes. *Stx* Also, 12 samples were found to contain hly genes. None of the *E. coli* bacteria that were isolated contained *eae* genes. They showed that STEC in this study can be the main reason for the occurrence of infections (Bonyadian *et al*., 2010).These bacteria are oral-fecal, so it can be said that there is a relationship

between food poisoning and urinary tract infections caused by *E. coli* O157:H7 (Kiranmayi *et al*., 2010). In research conducted by Tahamtan *et al*. (2010) and Mazhahrei *et al*. (2005), the relationship between *E. coli* O157:H*7* in water drinking and the occurrence of gastrointestinal infections in cattle was evaluated.

Since 67.4% of infected patients are women, the possibility of urinary tract contamination by gastrointestinal tract bacteria should be considered. Our findings supported and reinforced this possibility since we observed that 67.4% of those who had urinary tract infections were women, whereas only 32.6% of patients with this problem are men. The results as well as other studies showed that *E. coli* O157: H7 is a pathogenic factor. This bacterium can be transferred to other people by food, contaminated water, juice, vegetables and fecal (Cooley *et al*., 2007; Kodaka *et al*., 2004), therefore it is necessary to control pollution sources.

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REFERENCES

1. Tran, S .L., Billoud, L., Lewis, S. B., Phillips, A. D., Schüller, S. Shiga toxin production and translocation during microaerobic human colonic infection with Shiga toxin producing *E. coli* O157:H7 and O104:H4. *Cellular Microbiology*., 2014; doi:10.1111/cmi.12281.
2. Kiranmayi, C. B., Krishnaiah, N., Naga, E. M..*Escherichia coli* O157:H7 - An emerging pathogen in foods of animal origin. *Veterinary World*., 2010; **3**(8): 382-389.
3. Riley, L. W., Remis, R. S., Helgerson, S. D., Okott, E. S., Johnson, L. M., Hargratt, N. T., Blake, P. A Cohen, M. L.. Hemorrhagic colitis associated with a rare E.coli serotype. *N .Engl.J .Med.,* 1983; **308**: 681-685.
4. Brown, C .A., Harmon, B .G., Zhao, T., Doyle, M .P. Experimental *Escherichia coli* O157:H7 carriage in calves. *Appl.Environ.Microbiol*., 1997; **63**: 27-32.
5. Ateba, C. N., Mbewe, M. Genotypic Characterization of *Escherichia coli* O157:H7 Isolates from Different Sources in the North-West Province, South Africa, Using Enterobacterial Repetitive Intergenic Consensus PCR Analysis. *International Journal of Molecular Sciences.*, 2014; **15**: 9735-9747; doi:10.3390/ijms15069735.
6. Manna, S. K., Brahmane, M. P., Das, R., Manna, C., Batabyal, S. Detection of *Escherichia coli* O157 in foods of animal origin by culture and multiplex polymerase chain reaction. *J. Food Sci.Technol.,* 2006; **43**(1):77-79.
7. Khan, A. B., Naim, A.Virulence traits of Shiga toxin producing *Escherichia coli*. *The Health*., 2011; **2**(4): 119-127.
8. Brandon, A., Carlson, K. K., Nightingale, G. L., Mason, J. R., Ruby, W., Travis, C., Guy, H.,Loneragan, G. C., Smith, J. N., Keith E. B. *Escherichia coli* O157:H7 strains that persist in feedlot cattle are genetically related and demonstrate an enhanced ability to adhere to intestinal epithelial cells. *Appl. Environ. Microbiol.,* 2009; **75**(18):5927–5937.
9. Hepburn, N. F., Macrae, M., Johnston, M., Mooney, J., Ogden, I. D. Optimizing enrichment conditions for the isolation of *Escherichia coli* O157 in soils by immunomagnetic separation. *Letters in Applied Microbiology.,* 2002; **34**(5): 365-9.
10. Paton, A .W., Paton, J .C. Detection and characterization of shiga toxigenic *Escherichia coli* by using Multiplex PCR assays for *stx1*, *stx2*, *eaeA*, Enterohemorrhagic *hlyA*, *rfb* O111 and *rfb*O157. *J. Clin. Microbiol.*, 1998; **36**(2): 598–602
11. Gannon, P., D'Souza, S., Graham, T., King, R. K., Rahn, K., Read, S. Use of the flagella H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic*Escherichia coli* strains. *J. Clin. Microbiol*., 1997; **35**: 656-662.
12. Pant, S., Rajagunalan. S., Bisht, G., Singh, S .P., Singh, R. Isolation and Characterization of STEC with Particular Eeference to O157 from Human and Animal Sources. *J. Pure. Appl. Micro.,* 2014; **8**(1).
13. Navidinia, M., Karimi, A., Rahbar, M., Fallah, F., Radmanesh, A. R., Malekan1, M. A., HadipourJahromi, M. ,GholineJad, Z.. Study prevalence of verotoxigenic *E.coli* isolated from urinary tract infections (UTIs) in an Iranian children hospital. *The Open Micro. Journal.,* 2012; **6**(2): 1-4.
14. Starr, M., Bennett-Wood, V., Bigham, A. K., Koning-Ward, T. F., Bordun, A. M. D., Lightfoot, K. A., Bettelheim, C. L., Robins-Browne, R. M.. Hemolytic-Uremic Syndrome following

urinary tract infection with Enterohemorrhagic *Escherichia coli*: case report and review. *J. Clin. Infect. Dis.*, 1998; **27**(3): 310-5.

15. Tarr, P. I., Laurie, S. F., Ann, E. S., Richard, A., Wilson, H. H. Hemolytic–Uremic Syndrome in a Six-Year-Old Girl after a Urinary Tract Infection with Shiga-Toxin–Producing *Escherichia coli* O103:H2. *N. Engl. J. Med.*, 1996; **335**(4):635-638.
16. Schmidt, H., Scheef, H. I. J., Huppertz, M., Karch, H. *Escherichia coli* O157:H7 and O157:H2 strains that do not produce Shiga toxin: Phenotypic and genetic characterization of isolates associated with diarrhea and Hemolytic-Uremic Syndrome. *J. Clin. Microbiol.*, 1999; **37**(11): 3491-3496.
17. Johnson, J. R., Catherine, J., Daniel, R., Boster, A., Stapleton, E., Phillip, I. T. Analysis of urinary *Escherichia coli* isolates for ability to produce shiga toxin. *J. Clin. Microbiol.*, 2002; **40**(6): 2247–2248.
18. Flemming, S., Bente, O., Annette, N. Two cases of human urinary tract infection complicated by Hemolytic Uremic Syndrome caused by verotoxin-producing *Escherichia coli*. *Clin. Infect. Dis.*, 2000; **31**(3):815–6.
19. Byrne, C. M., Erol, I. J.E., Call, C. W.,Kaspar, D. R.,Buege, C. J.,Hiemke,P. J.,Fedorka-Cray, A. K., Christina, R.,Hermos, M. J., Linda, L .H., Alexander, J., Adam, M. M. Shiga Toxin-Producing *Escherichia coli* in Children: Diagnosis and Clinical Manife stations of O157:H7 and Non-O157:H7 Infection. *J. Clin. Microbiol*., 2011; 955–959.
20. Bonyadian, M., Momtaz, H., Rahimi, E., Habibian, R., Yazdani, A., Zamani, M. Identification & characterization of Shiga toxin-producing *Escherichia coli* isolates from patients with diarrhoea in Iran. *Indian J. Med. Res*., 2010; **132**(4): 328-331.
21. Tahamtan, Y., Hayati, M., Namavari, M. M.Prevalence and distribution of the stx_1, stx_2 genes in Shiga toxin producing *E. coli* (STEC) isolates from cattle. *Ira. J. Microbiol.*, 2010; **2**(1): 8-13.
22. Mazhaheri NejadFard, R., BehzadianNezhad, G., ZahraeiSalehi, T.,AtashParvar, N . Evaluation of *ehxA*, stx_1, and stx_2 Virulence Gene Prevalence in Cattle *Escherichia Coli* Isolates by Multiplex PCR, *Arch. Razi Ins.*, 2005; 60(2): 55-66.
23. Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M .T., Myers, C., Rose, C., Keys, C., Farrar, J., Mandrell, R. E. Incidence and tracking of Escherichia coli O157:H7 in a major produce production region in California. *Plos One.*, 2007; **2**(11): e1159.
24. Kodaka, H., Uesaka, Y., Kashitani, F. Nissui Glucose fermentative gram-negative rod identification system EB-20 gives a unique profile for typical non-sorbitol-fermenting *Escherichia coli* O157:H7. *J .Clin. Microbiol.*, 2004; **42**(1):354-8.