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Research Article

Evaluation of Shiga toxin 2 genes in Enterobacteriaceae bacteria isolated from stool and urine specimens of the patients referred to the Falavarjan Therapeutic Clinic in Isfahan

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ABSTRACT

Nowadays, bacterial toxins are considering as powerful therapeutic strategies against the pathogenic organisms and other medical application. Shiga toxin is one of the most studied bacterial toxins found in *Shigella dysenteriae* and some *Escherichia coli* serogroups. In this study we screened the presence of Shiga toxin2 **(**Stx2**)** genes between the bacteria isolated from the stool and urine samples of the patients affected by diarrhea. Stool and urine samples were cultivated and microbial and biochemical tests were carried out. Well test was optimized for detection of killer phenotype of the bacteria. Colony-PCR and real-time PCR was performed for confirmation of presence of the Stx2 gene in the extracted genomic DNA of the isolated bacteria, and finally, SDS-PAGE was used for differential analysis of protein profile between killer and non-killer bacteria. We optimized in this study a rapid molecular method for screening of Shiga toxin 2 gene. Our presented condition for assay is referable and confident. Also, we purified, detected and analyzed functionally, Shiga toxin 2 peptide and recorded bacterial strains producing Stx2 toxin. In the future, real-time PCR assays can be developed for detection of *Enterobacteriaceae* virulence genes, including Stx and other its groups. The current study demonstrate that the real-time PCR technique is rapid and simple to use, and it is a promising method for identify *Enterobacteriaceae* toxins in human samples.

1. Introduction

The discovery of bacterial protein toxins, in the late 19th century, opened a path toward extensive biomedical research. The extensive information gained from bacterial toxicology has provided Extensive knowledge about the molecular structure, genetic aspects and interaction of their molecules with various cellular systems, human tissues, and animal organisms (The et al., 2016; Cavaillon, 2018; Henkel et al., 2010).

Shigella dysenteriae and Shiga toxin (Stx), were identified in the nineteenth century by Neisser, Shiga, and Conradi. Around 80 years later, the same toxin was isolated from a group of *E. coli* which is now called Stx1 and is distinct from the toxin (Stx) produced by *S. dysenteriae*. Shiga toxin is known as one of the most powerful

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bacterial toxins found in *S. dysenteriae* and some *E. coli* serogroups. In addition, some strains of *E. coli* produce a second type of Stx (Stx2) with the same function of Stx1. Irrespective of their bacterial origin, this group of biologically toxins are approximately 55%-60% homologous at the protein level and are distinct only in the antigen and the immunology features (The et al., 2016; Melton-Celsa, 2014; Lee et al., 2007).

Uremic hemolytic syndrome (HUS) is the most serious clinical manifestation of Stx-producing *E. coli* (STES)-infected humans. In these cases, most of the strains are the Stx2-producing ones, instead of Stx1, where they are epidemiologically associated with more severe disease. Since their discovery, review studies cover the general and specific aspects of toxins, their structure and function, their interaction with the host cell receptors, and the clinical aspects of the disease. In recent years, significant advances in STEC pathogenesis have been made in order to assist the advancement of the diagnostic methods, as well as the development of treatment and prevention strategies. Recent preventive measures are focused on the functional mechanisms. Lack of appropriate clinical measures in this field requires effective action in the control and prevention of STEC infection in humans (Melton-Celsa, 2014; Vanaja et al., 2013; Paton, & Paton, 1998).

The increase in the number of Shiga toxins and the identification of their structures can provide us with some solutions to deal with pathogens, with diagnostic and therapeutic effects, by eliminating the side effects in a targeted manner which will change world of biomedicine research (both in vivo and in vitro). However, it is obvious that for targeted presentations, more extensive research is needed to demonstrate their performance in future clinical applications.

Objectives

Therefore, in the present study, we aimed to investigate *Stx2* gene in the bacteria isolated from the patients with gastrointestinal disorders (belonging to the *Enterobacteriaceae* family). Genetic analysis of toxins in epidemiology is capable of providing more insights on their extent and limitations. We expect these results to make an efficient, quick, and accessible laboratory diagnosis, and even be used in the treatment of diseases.

2. Materials and Methods

2.1. Sample Collection, Isolation and identification of the Enterobacteriaceae

Stool and urine samples were collected from 60 patients referred to the Falavarjan Therapeutic Clinic Iran, Isfahan. In this study, For *Enterobacteriaceae* detection at the genus level, all samples were routinely cultured on Selective media include MacConkey, GN broth (gram-negative broth) and XLD Agar (Xylose Lysine Deoxycholate Agar) for stool sample and EMB Agar (Eosin-methylene blue Agar) plates and Blood agar media for other Clinical specimens were then placed in an incubator at 37°C for 18-24 hours. The Indole, Methyl Red (MR), Voges-Proskauer (VP), Simmons' Citrate (IMVIC), phenylalanine deaminase production and urease production tests were carried out to identify the coliforms group. Differentiation of the species was done through using standard biochemical and microbiological tests such as Gram-stain, catalase test, carbohydrate fermentation in Kligler Iron agar and cytochrome oxidase production. In addition, in order to assess the movement of bacteria, samples were incubated in SIM (Sulfur-Indole-Motility), as a semisolid medium. To detect *Enterobacter* at the species level, lysine, arginine and ornithine decarboxylase tests were applied (Martinson et al., 2019). One Pure colony from each sample was inoculated in 100ml Luria Bertani Broth medium/glycerol (70/30V/V)and was stored immediately at -20°C

2.2. Well diffusion method for Killer Shiga toxins activity assay

Well test method was carried out for assay of the toxin activity of *Enterobacteriaceae* family (Chen et al., 2000). Similar to the disk diffusion method, the sensitive strain *E. coli*. *BAE77537*, was spread plate cultured with on the surface of Luria-Bertani agar medium. Then, 6 to 8 mm diameter holes were removed from the agar under sterile puncture under sterile conditions and 10 µl of each bacterial sample was inserted into each well. The bacterial produced toxin agent is spread in the agar medium and inhibits the growth of the microbial strain. Then the inhibition zones were measured after 48 h of incubation at 24°C.

2.3. Primers design, genotype identification and Stx gene tracking

Three set of specific primers were developed for detection of *Enterobacteriaceae* organisms related to 16S rDNA and *Stx2* genes. A series of nucleotide sequence of Enterobacteriaceae was collected from NCBI Genbank database returned from Stx2 (Accession number: FR874039.1) genes and 16S rDNA. The conserved motive was identified by ClustalW server and finally, primers was designed and ordered to SinaClon Company (Iran) to synthesize (table1). Genotype identification was done using 16S rRNA genes of *Enterobacteriaceae* isolates were amplified by direct PCR of intact Bacteria (Colony PCR). Also, Colony PCR Assay was performed for Stx Genes screening. The reaction was performed at a defined volume of 25 µl and included a colony of cultured bacteria on LBA plate, 6.95 µl Master Mix (sinaclon, Iran) consisted 2.5 µL of 10X PCR Buffer, 0.5 µL of 10 mM dNTPs, 0.2 µL of Taq polymerase, 18.05 µL of nuclease free water, 0.75 µL of 25mM MgCl2 and 1 µl of 10 pM of forward and reverse primers (SinaClon, Iran). PCR program was optimized in a thermal cycler (TP-Boeco, Germany) including: Initial denaturation at 94°C for 5 min, followed by 35 cycles encompassing a 35sec step at 94°C, a 35sec step at 58°C, and a 35sec step at 72°C and final extension step at 72°C for 5 minutes. PCR products were analyzed by electrophoresis in 1% agarose gel and stained by DNA Green Viewer™ (0.5 µg/ml) for UV light analysis and digitized (UV doc). Finally, three killer phenotype bacteria were subjected to a PCR and sequencing strategy for 16SrRNA gene using 8F and 518R universal primers (Eden, Schmidt, Blakemore, & Pace, 1991, James, 2010). The resulted sequences were submitted to NCBI genomic data bank (accession number: OM065779.1, OM144823.1, and OM144477.1).

2.4. DNA Extraction and Real time PCR

Total bacterial DNA was extracted using DNA extraction kit manufactured by Aron gene Pars Co. (Iran, Cat. No. AGNB102). In order to confirmation of *Stx2* gene presence in the bacterial samples, Real-time PCR reactions for each sample was performed in duplicate in a 10µL reaction volume using forward primer FHSST2C (5'-GGA ATG CAA ATC AGT CGT CAC-3') and reverse primer Rrealstx (5' CGT AAG GCT TCT GCT GTG AC-3') was optimized. A two-step PCR amplification protocol included an initial denaturation step at 94°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 45s. Melting curve was identified between 65°C-95°C with 0.3°C/1min ramping rate.

2.5. Total protein purification and SDS-PAGE analysis

Three bacteria that confirmed by colony PCR and well test method were selected and cultured in LB media. 50CC cultured bacteria was centrifuged in 4000rpm for precipitation of bacteria. Supernatant media was mixed with saturated (NH4)2SO4 at 50% V/V final concentration (2M) and incubated in 4 °C for 24h (Fathi et al., 2017). Total protein was precipitated by centrifugation in 36000g for 30 minutes in 4 °C using a Sigma model: 3-18KHS centrifuge (Sigma co. Germany). Precipitated proteins were dialyzed against PBS 0.1X and resolved in 300µL PBS 1X. Concentration of the precipitated protein was calculated by Bradford method (Bradford, 1976). 30µL of purified protein was mixed with 30µL 2XSDS sample buffer and boiled for 10 minutes and finally was analyzed in SDS-PAGE electrophoresis.

Table 1. List of primers were used in this study

Gene name	primers	Product size (bp)
16S rRNA	**FRRShig:** 5'-CTACGGGAGGCAGCAGTGGG-3' **RRRShig:** 5'-ATCTCTACGCATTTCACCGC-3'	356
Stx2	**FHSST2A:** 5'-ATGAAGTGTATGTTATTTAAATGGG-3' **RHSST2B:** 5'- CTCTGGATGCATCTCTGGTC-3'	535
	FHSST2C: 5'- GGAATGCAAATCAGTCGTCAC -3' **RHSST2D:** 5'- GCAAATAAAACCGCCATAAACATC-3'	559

The electrophoresis was performed at 60–90V in a cold chamber and the protein bands present in gels were stained with 0.025% Coomassie Blue G-250 for 1-2h. The gels were destain by successive washing in acetic acid: methanol: water (7.5:5:87.5 V/V) solution.

3. Results

3.1. Biochemical and physiological tests

In this study, 60 *Enterobacteria* of different genera and species were isolated (Table 2). The results of differential biochemical and microbiological tests are summarized in this table. The typical color of the colonies on media was purple, magenta/mauve, blue-violet, gray, pink with dark pink center, and violet/light purple, for members of Enterobacteriaceae. *E. coli* O157:H7 appears as dark black colonies.

3.2. Well test and killer phenotype screening

We screened all strains of *Enterobacteriaceae* toxins for the killer phenotype with well test, six strains had killer activity (Figure 1). Figure 2 shows a descriptive presentation of the prevalence and type of the isolated bacteria.

3.3. Molecular screening

Figure 3 shows electrophoretic analysis of colony PCR products for 16srRNA. A band equal with 356bp can confirm that the target colony is belong to *Enterobacteriaceae*. Figures 4a shows colony PCR products of Stx2 gene fragments. Target sequence for Stx2 is divided to two amplicon including 535bp and 559bp fragments. Figure 4b shows the extracted DNA from the bacteria with killer phenotype after the cultivation in 10CC LB media. In the next step, the extracted DNA was subjected to a real-time PCR assay as described in the method part. Figure 5 shows the amplification plot and melting curve for *Stx2* gene analysis. This analysis was confirmed the presence of Stx2 gene in some of samples.

3.4. Protein extraction and SDS-PAGE analysis

Mature Stx2 protein contains two subunits included Stx2A and stx2B with 299 and 69 amino acids residues respectively. Figure 6 shows scanned picture of SDS-PAGE for extracted proteins including Stx2A (~33.8kD) and Stx2B (~7.8kD) subunits of Stx2 toxin. Pure recombinant Stx2B protein subunit (69aa) bought from the Sigma-Aldrich company (Cat. No.: SML0655) was used as positive control.

Table 2. Genera of Enterobacteriaceae (n=60) isolated from stool and urine specimens of patients referred to the Falavarjan Therapeutic Clinic Iran, Isfahan.

	Microorganism Isolates (n)	N	Triple Sugar Iron	H_2S	Gas	Motility	PAD	INDOLE	MR	VP	Citrate	Urease	ONPG	MSA
1	*Citrobacter freundii*	2	K/A	+	+	+	-	-	+	-	+	-	+	+
2	**Enterobacter aerogenes*	5	A/A	-	+	+	-	-	-	+	+	-	+	+
3	*E. coli*	21	A/A	-	+	+	-	+	+	-	-	-	+	+
4	*E. coli*	6	K/A	-	+	-	-	+	+	-	-	-	+	+
5	*Klebsiella oxytoca*	1	A/A	-	+	-	-	+	-	+	+	+	+	+
6	*Klebsiella pneumoniae*	11	A/A	-	+	-	-	-	-	+	+	+	+	+
7	*Morganella morganii*	1	K/A	-	+	+	+	+	+	-	-	+	-	-
8	*Proteus mirabilis*	4	K/A	+	+	+	+	-	+	-	+	+	-	-
9	*Proteus vulgaris*	1	A/A	+	+	+	+	+	+	-	-	+	-	-
10	**Serratia marcescens*	1	K/A	-	+	+	-	-	-	+	+	-	+	+
11	*Y. enterocolitica*	1	K/A	-	-	-	-	-	+	-	-	+	+	+
12	**Salmonella Typhi* serogroup (D)	1	K/A	+	-	+	-	-	+	-	-	-	-	+
13	**Shigella flexneri* serogroup (B)	1	K/A	-	-	-	-	+	+	-	-	-	-	+
14	**Shigella sonnei* serogroup (D)	4	K/A	-	-	-	-	-	+	-	-	-	+	+

* Serogrouping: Agglutinins test of antigens *Salmonella* O
* Serogrouping: Agglutinins test of Somatic antigens O for *Shigella*
* DNase test: Differentiation of *Serratia* without pigment from *Entrobacter* & *Klebsiella*

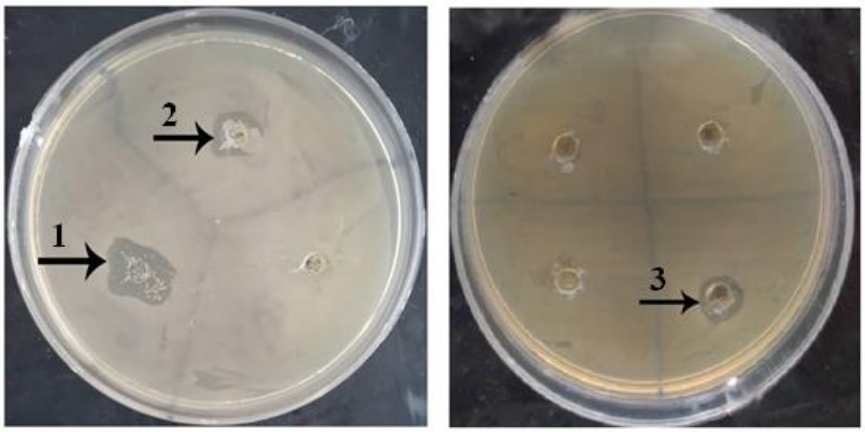

Figure 1. Killer phenotype that was detected using well test method. Bacterial extract was spilled in the wells and control strain was cultivated as spread: 1. *Shigella flexneri* strain SSA(OM065779.1), 2. *Shigella sp.* strain SAF (Accession: OM144823.1) and 3. *Escherichia coli* strain SAF(Accession: OM144477.1).

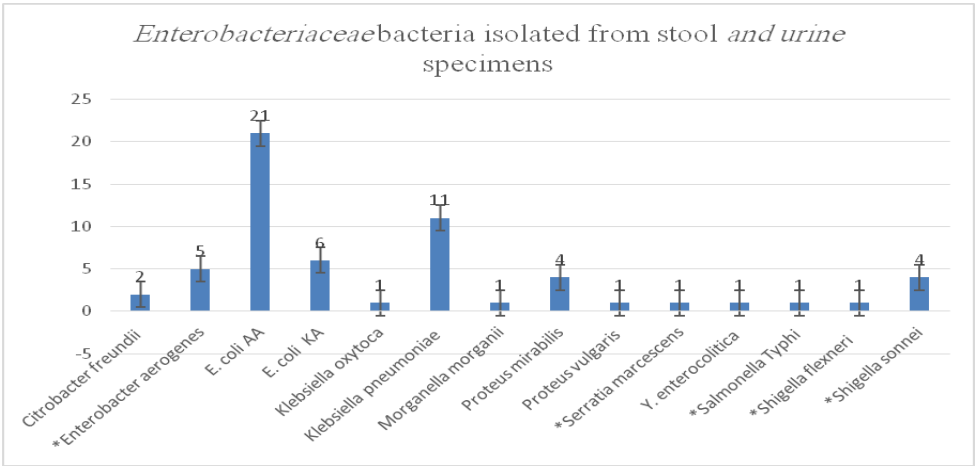

Figure 2. Types and prevalence of the bacteria isolated from stool and urine specimens of the patients referred to the Falavarjan Therapeutic Clinic in Isfahan.

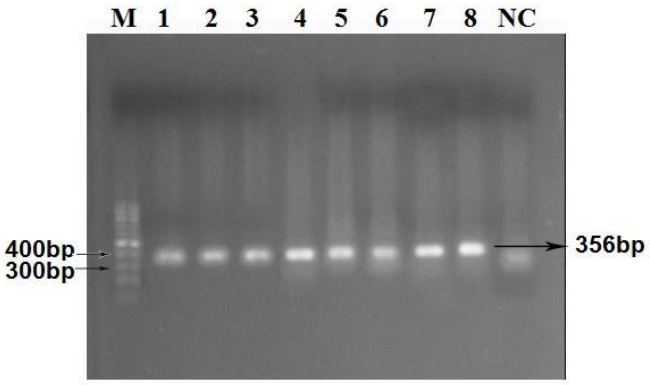

Figure 3. Agarose gel electrophoresis of colony PCR products for 16s rRNA in some of bacterial samples (lane 1-8). M: DNA 100bp size marker. NC: Negative control. A band equal with 356bp can confirm that the target colony is belong to *Enterobacteriaceae*.

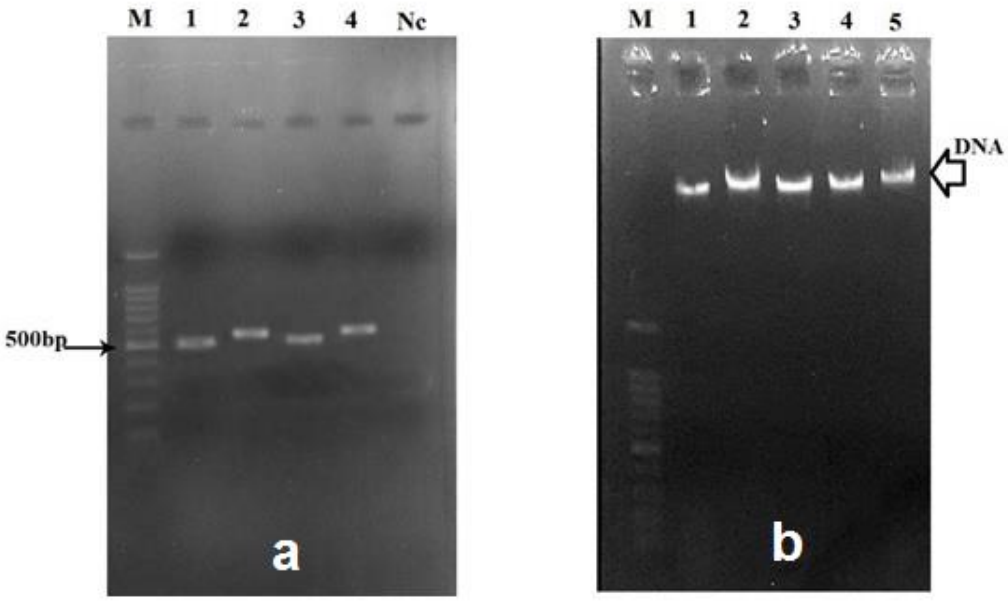

Figure 4. a) PCR products of Stx2 Fragment 1(535bp) and fragment 2(559bp), M: 100bp DNA size marker, 1 and 2 PCR products for the fragments, 3 and 4 are positive Control respectively and Nc is negative control, b) Extracted DNA from some of positive bacteria for Stx2 genes.

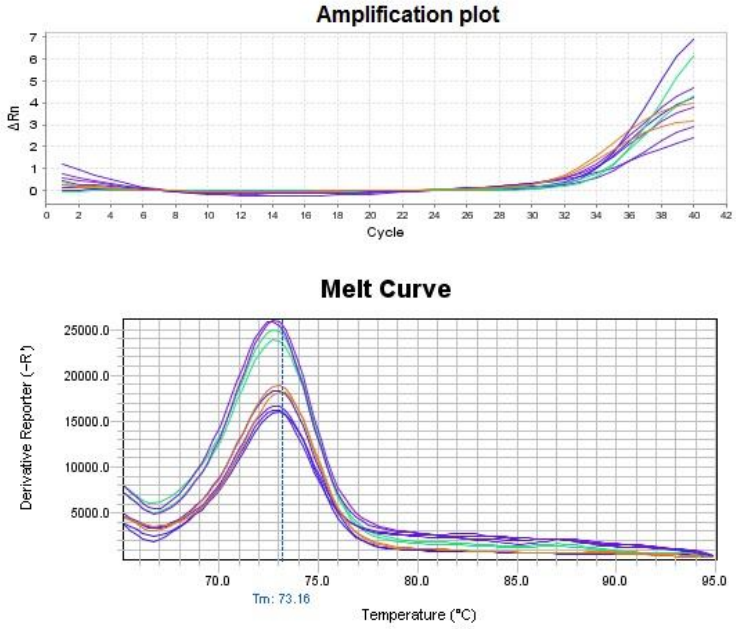

Figure 5. Amplification plot and melting curve of some positive samples for *Stx2* gene.

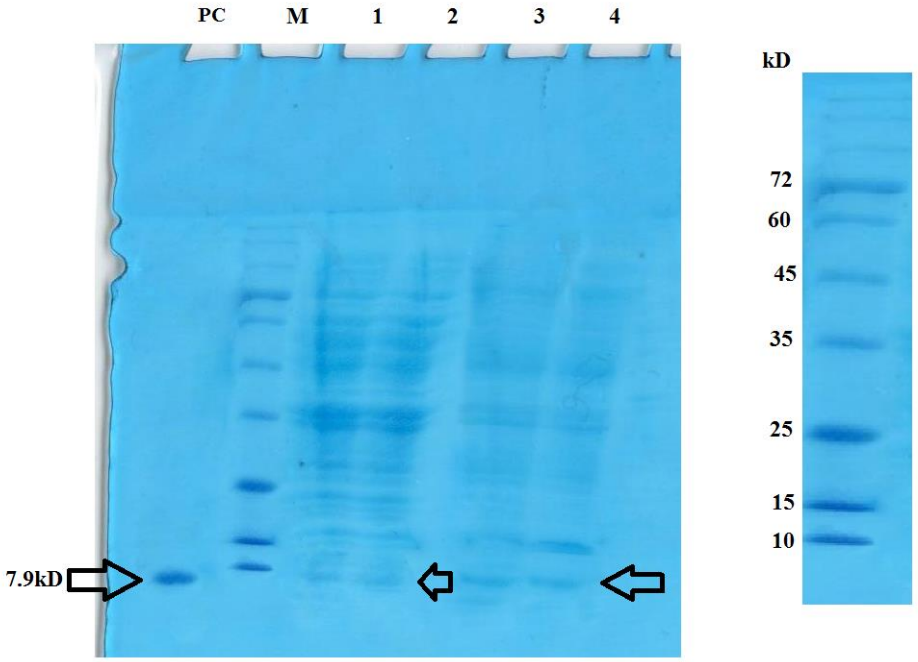

Figure 6. SDS-PAGE for total extracted proteins including stx2A (~33.8kD) and stx2B (~7.8kD) subunits of Stx2 toxin.. M: Protein size marker. PC: positive control: Pure recombinant Stx2B protein subunit (69aa). 1 and 2: total protein of lysate bacteria 3 and 4: $(NH_4)_2SO_4$ purified supernatant total protein.

Some genera of the *Enterobacteriaceae* family include *Salmonella*, *Shigella*, etc. are Shiga toxin–producing bacterial. These are important foodborne zoonotic bacteria. Although these pathogens usually cause mild to moderate self-limiting gastroenteritis, invasive diseases and complications may occur, resulting in more severe cases (hemorrhagic colitis to the potentially fatal hemolytic uremic syndrome (HUS)). Actually *concern over* human health risk associated with non-O157 and O157:H7 STEC serotypes, which might be major responsible for outbreaks serotype O157:H7. This strains produce two major Shiga toxin types, Stx1 and Stx2. Reports indicate that producer strains of Stx2a and Stx2c subtypes are more frequently associated with HUS than other subtypes (Sánchez et al., 2019; Gould, 2012, Campbell et al., 2016).

Since our current surveillance systems have been expressed upon the characterization of bacterial isolates, and the lack of culture confirmation leads to vulnerability in the detection and investigation of outbreaks. Thus important, that the new clinical diagnostic methods combine the clinically operability information of serotype and pathogenicity profile of the infected strain with the serotype information required for public health action, actually can be expected these will be implemented routinely into national surveillance programs in the future (Atikson et al., 2012).

Therefore, clinical microbiology laboratories require a practical, rapid, and sensitive method for diagnostic assays to be able to identify these Shiga toxin subtypes in all serotypes and provide simultaneous detection and qualitative differentiation of Stx1 and Stx2. Actually rapid and short-term isolation of infectious organism helps public health officials that immediate initiate outbreak control measures for infection spread. The diagnostic techniques are already rapidly evolving to reflect the developments in molecular array and sequencing technologies (Sánchez et al., 2019; Atikson et al., 2012; Guerra et al., 2016).

A remarkable feature that is distinction between the Stx1 and Stx2 groups. early and reliable detection of Stx2 subtypes is considered a significant indicator for *Enterobacteriaceae* virulence in diagnostic laboratories, it could detect strains with all combinations of Stx subtypes. Certainly systems differentiating

between the Stx1 and Stx2 subtypes brings added value (Fratamico & Bagi, 2012).

Recently, it has been revolutionized by the availability of information on pure toxins, monoclonal antibodies, and cloned genes with the development of DNA sequencing and amplification technologies, that will be obtained extensive benefits from the complete genome sequences of representative Shiga toxin-carrying strains in the near future. The pathogenesis of *Enterobacter* infections is clearly multifactorial and involves different levels of interaction between the bacterium and the host. The different levels of this pathogenesis provide potential opportunities and goals for the development of treatment and preventative strategies (Paton, 1998).

Due to the availability nucleotide sequences of more Shiga toxin-carrying strains, comparative genomic studies can provide goals to improve diagnosis, virulence profiles, and isolation strategies (Gould, 2012).

The investigation is continuing to determine virulence factors that may predict the severity and long-term prognosis of *Enterobacter* infections. This information is preparing a way for a future paradigm shift in diagnostic methods that offers detailed information to guide patient therapy (Atikson et al., 2012; Paton, 1998).

This study was performed with the objective of screening the Shiga toxin genes (Stx2) from Enterobacteriaceae isolates in different samples. Development of real-time PCR assay for the detection of different serotypes (A and C) of *Enterobacteriaceae* species as an assay that targets additional virulence genes and can be useful for detection of virulence factors. Accordingly, the rapid screening tests available are crucial, this study was followed by real time PCR assays to identify the Shiga toxin gene types of patient specimens. The results revealed that 60 samples, were infected with the targeted Shiga toxin. Method real time, all of the genes of interest in an easy to use format would be done more rapid and less laborious. Many similar studies have been conducted in relation to the screening of Shiga toxin family genes, which differ from our study in some ways. Fluorescent methods and labeled probes were used in some studies (Bélanger et al., 2002). Our results, similar to these types of studies, confirmed the presence of *Stx1* and *Stx2* genes in the bacteria such as *Shigella boydii*, *Shigella sonnei*, and *Shigella flexnerii*. But what sets our study apart is its low cost. Because there is no need for a probe in our research. In another study, Multiplex realtime PCR method and Taqman reagent was used (Li et al., 2017). Such methods have low accuracy due to the possibility of interaction of primers. Perhaps one of the most important advantages of the method used in our research is its simplicity, speed and reasonable cost because of cheaper materials such as SYBER GREEN which is compatible with most qPCR devices. Finally, the number of samples that analyzed in this study can be cited. Because in the previous studies, 27 samples (Bélanger et al., 2002) and even one standard bacterium alone were used (Li et al., 2017). However, the current study confirmed that real-time PCR assays for Shiga toxins detection are rapid, simple and accurate and can be used for screening as a diagnostic kit for these pathogens in human samples.

Conclusion

Results of the current study demonstrate that the real-time PCR technology is rapid and simple to use and it is a promising method for identify and distinction between Enterobacteriaceae toxins in human samples. New types of assays can be designed by incorporating primers and probes for new gene targets. In the future, real-time PCR assays can be developed for detection of *Enterobacteriaceae* virulence genes, including Stx1, Stx2 or others.

Refereces

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