International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-007, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Author(s) retain the copyright of this article.

Full Length Research Paper

Single multiplex PCR assay to identify the shiga toxin

Eisa Tahmasbpour Marzony[1], Mahdi Kamali[2], Mojtaba Saadati[3], Amir Homayoun Keihan[2]*, Abbas Ali Imani Fooladi [4], and Sharareh Sajjadi[5]

[1]Young Research Bashgah, Islamic Azad University, Sari Branch, Tehran, Sari Iran.
[2]Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[3]Department of Biology, Imam Hossein University, Tehran, Iran.
[4]Microbial Products Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
[5]Department of biology, Roudehen Branch, Islamic Azad University, Roudehen, Iran.

Accepted 19 February, 2019

Strains of shiga toxin–producing *Escherichia coli* (STEC) and *Shigella dysenteriae* type1 have been associated with outbreaks of diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. Most clinical signs of disease arise as consequence of the production of shiga toxin/shiga toxin 1 (Stx/Stx1), shiga toxin 2 (Stx2) or combination of these toxins. Here, we designed a Multiplex PCR technique to identify *stx/stx1* and *stx2* genes with the incorporation of *mdh* gene of *E. coli* and *Shigella.* A total of six primers were used: SFI and SRI, which produce a 199bp product that serves as an internal positive control; Ka2F and Ka2R, which yield a 381bp fragment of *stx2* gene, and Ka1F and Ka1R, which amplify a 622bp fragment of *stx/stx1*. The thermal profile, which was preceded by a 5 min incubation at 95°C for 20 to 25 cycles with the following parameters: 95°C at 1 min, 60°C at 1 min, 72°C at 1 min, and 5 min incubation at 72°C as final extension. PCR amplification products identifying the *stx/stx1* and *stx2* gene sequences were observed only in *E. coli* 0157:H7 and *Shigella dysenteriae* type1. Template nucleic acid extracted from other Gram-negative bacteria was found to be negative. The sensitivity of the PCR procedure for detection of shiga toxin genes was determined to be 2.1 pg/µl of total nucleic acid and 320 cfu/µl.

Key words: Shiga toxin, multiplex PCR, diagnosis.

INTRUDUCTION

Shiga toxins (Stx), also known as verotoxins (Vtx), are produced by several enteric pathogens, especially *Shigella dysenteriae* (serotype 1 only) and enterohaemorrhagic *Escherichia coli* (EHEC) (O'Loughlin et al., 2001). Stx and Vtx are important factors in disease pathogenesis and are responsible for spectrum of the severe complications, such as haemorrhagic colitis and the haemolytic uremic syndrome (HUS) (O'Loughlin et al., 2001; Karmali et al., 2010). Stx comprises a family consisting of two major subtypes, shiga toxin 1 (Stx1) and shiga toxin 2 (Stx2) that both of which are composed of one A subunit (StxA) and one B subunit (StxB) (Donohue-Rolfe et al., 1991; Head et al., 1991).

These toxins are attached to the cell surface via a glycolipid receptor known as globotriaosylceramide (Gb3). Following the binding of Stx to the surface receptor glycosphingolipid, (Gb3) via StxB (Lingwood et al., 1987; Hanashima et al., 2008), the toxin is endo-cytosed and transported through the Golgi apparatus (Donta et al., 1995) to the endoplasmic reticulum (Sandvig and van Deurs, 2000). The StxA is then translocated into the cytoplasm and reaches to its target, ribosomal RNA. The N-glycosidase activity of the StxA cleaves an adenine residue from the 28S rRNA, which leads to inhibition of protein synthesis and cell death (Hanashima et al., 2008).

Not only *S. dysenteriae* and *E. coli* can produce these toxins, but also *Citrubacter freundii*, *Aerumoas hydrophila* and *Aerumonas caviae* have been reported to be able to express the toxins (O'Loughlin et al., 2001). At present,

*Corresponding author. E-mail: keyhan@ibb.ut.ac.ir; tahmasb62@yahoo.com. Tel: +98-2188617711. Post code: 1393934453

Table 1. Primer sequences used for polymerase chain reaction and amplicon size.

Gene	Primer	Sequence	Amplicon size (bp)
mdh	SF1	5′-CTAACCCGGTTAACACCACAGT-3′	199bp
	SR1	5′-GGAAGAATGACACCAGAGT-3′	
Stx/stx1	Ka1F	5′-GGGATAGATCCAGAGGAAGG -3′	622bp
	Ka1R	5′-CCGGACACATAGAAGGAAACTC -3′	
Stx2	Ka2F	5′-CTGGCGTTAATGGAGTTCAG -3′	381bp
	Ka2R	5′-CCTGTCGCCAGTTATCTGAC -3′	

different types of stx such as *stx1*, *stx*, *stx2e*, *stx2d*, *stx2c*, *stx2*, *stx1c* and *stx2f* were identified (Gobius et al., 2003; Friedrich et al., 2003; Schmidt et al., 2000).

So far, several methods such as tissue culture cytotoxicity assay, PCR, reverse passive latex agglutination and hybridization are recommended to identify stx (Philpot and Ebel, 2003) . Because of the genetic variations of stx, its detection by the PCR reaction is difficult. Since, in most cases bacteria producing shiga toxin have also other virulence factors, the researchers tried to design a Multiplex PCR reactions to identify other exothermic factors, in addition to stx (Philpot and Ebel, 2003). Therefore, in the present study, a Multiplex PCR (MPCR) protocol was developed using a primer pair specific for identification of stx.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *S. dysenteriae* (biotype 1, 4, 7, 8), *Shigella flexneri* (biotype 2a), *Shigella sonnei*, *Shigella boydii*, *Salmonella paratiphy*, *Vibrio cholera* (strain: ogava and inaba) and *E. coli* (serotype O157:H7) strains were used in this study. *S. dysenteriae* (Biotype 1; RITCC1875) was obtained from the Tarbiat Modares University, but the other strains were provided from the Iran reference Laboratory. For the experiments, the strains were cultured on liquid TSB and incubated at 37°C for 12 h.

DNA template preparation

Template DNA from all bacterial strains was provided by the CTAB (Cetyl trimethyl ammonium bromid) method. For DNA isolation, 1.5 ml broth culture was centrifuged at 5000 rpm for 5 min to pellet the bacteria. Bacterial suspensions were made by solving the pellet into a 1.5 ml Eppendorf tubes containing TE buffer (10 mM Tris HCl, pH 8.0, 10 mM EDTA), 30 µl 10%SDS buffer, 3 µl Proteinase K (20 mg/ml) and were incubated at 37ºC for 3 h. The bacterial suspensions were vortexed by adding the 100 µl 5 M NaCl and 80 µl CTAB solution (4.1 g NaCl, 10 g CTAB and 80 ml DDW) and were then incubated at 65ºC for 15 min. Preparation of DNA required to adding 780 µl chloroform-isoamyl alcohol solution (diluted 24:1) to suspensions and centrifugation at 2500 rpm for 25 min. The supernatant was transferred to a new Eppendorf tube containing a same volume of phenol-chloroform-isoamyl alcohol (diluted 1:24:25). The final supernatant fluid containing genomic DNA was then centrifuged at 2500 rpm for 25 min and was concentrated by adding isopropanol and 70% ethanol. The template DNA was stored in Eppendorf tube containing 100 µl TE buffer (pH 8) and 3 µl RNase enzyme (Sambrook and Russell, 2001) at -20ºC until used. Before storing, the concentration of template DNA (diluted by DDW in 4:50) was determined by UV spectrophotometer (CECIL CE7500) at 260 nm.

PCR primers and amplification conditions

Three primer pairs were used in the multiplex PCR test (Table 1). The multiplex PCR assay was performed as follows. Each 25 µl of reaction mixture contained 0.5 µl of template DNA (210 ng/µl), 1 µl of each primer (5 pmol/µl), 1 µl dNTPs (2.5 mM), 3.8 µl $MgCl_2$ (10 mM), 2.5 µl 10X PCR buffer, 10.9 µl DDW and 0.3 µl Tag DNA polymerase (5 unit/µl). The DNA thermal cycler device (Genamp PCR 9600; Perkin Elmer Cetus, Foster City, CA) was programmed with the following amplification conditions: after initial denaturation for 5 min at 95ºC, 20 cycles, each for 1 min, at 95ºC (denaturation), 60ºC (annealing), 72ºC (extension) were performed. The final cycle was followed by a 72ºC for 5 min. Amplified PCR products were analysed by 2% gel agarose gels stained with ethidium bromide, visualized with UV illumination.

Enzymatic digestion of PCR products

To confirm the PCR products, three restriction enzymes including Hind III, Hae III and EcoR V were used to *stx/stx1*, *stx2* and *mdh* fragments digestion, respectively. After PCR, 6 µl of each amplified fragment was mixed with 1 µl of related restriction enzymes and was incubated at 37ºC for 3 h. The products were then separated on 2% agarose gels by electrophoresis stained with ethidium bromide, visualized with UV illumination.

Specificity of multiplex PCR reaction

Specificity of the multiplex PCR was tested with all bacterial strains. The Multiplex PCR assay was performed on templates DNA from all bacterial strains. Amplified PCR products were separated by 2% gel agarose gels stained with ethidium bromide, visualized with u.v. illumination.

Sensitivity of multiplex PCR reaction

To determine the sensitivity of the multiplex PCR test, both the

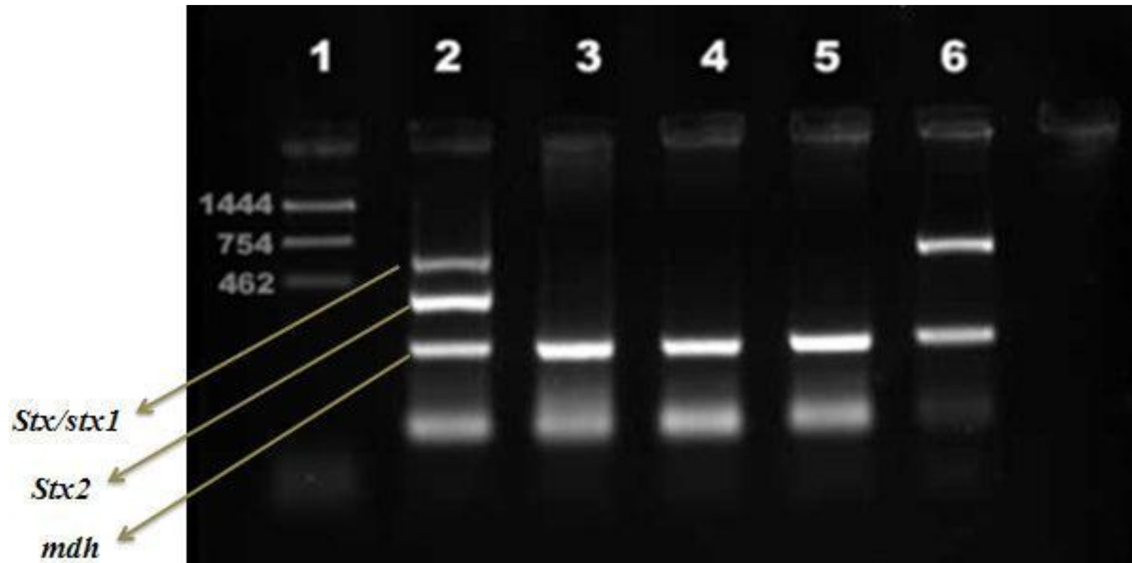

Figure 1. Multiplex PCR assay analysis in different strains. Lane 1. Marker size (pUC18- TagI), lane 2. *E. coli* O157:H7, lane 3. *S. dysenteriae* (biotype 4), lane 4. *S. dysenteriae* (biotype 7), lane 5. *S. dysenteriae* (biotype 8), lane 6. *S. dysenteriae* (biotype 1).

number of viable bacteria and the concentration of extracted DNA were investigated. Serial bacterial dilutions were prepared in LB medium and the number of viable bacteria determined by spectrophotometer at 600 nm. Different dilutions of extracted DNA from *E. coli* (from 10^{-1} to 10^{-10}) was provided in TE buffer. PCR assay was performed for all dilutions and the amplified PCR products were separated by 2% agarose gels stained with ethidium bromide, visualized with u.v. illumination.

RESULTS

DNA concentration

The optical density of extracted DNA from *E. coli* O157:H7 was 0.336 at 260 nm, thus the net concentration was 210 ng/µl.

Multiplex PCR assay analysis of different strains

Multiplex PCR assay provided accurate profiling of either the stx*/stx1*, *stx2* and *mdh* products in *E. coli* O157:H7 (Figure 1). The *stx/stx1* and *mdh* were the only amplified products in *S. dysenteriae* (biotype 1). No amplification products were found in *S. dysenteriae* (biotypes 4, 7 and 8) except *mdh*. The amplification of *mdh* product (as a control) in all strains was the evidence to correct reaction. The exact sizes of each amplified products are shown in Figure 2. DNA template provided from *E. coli* O157:H7.

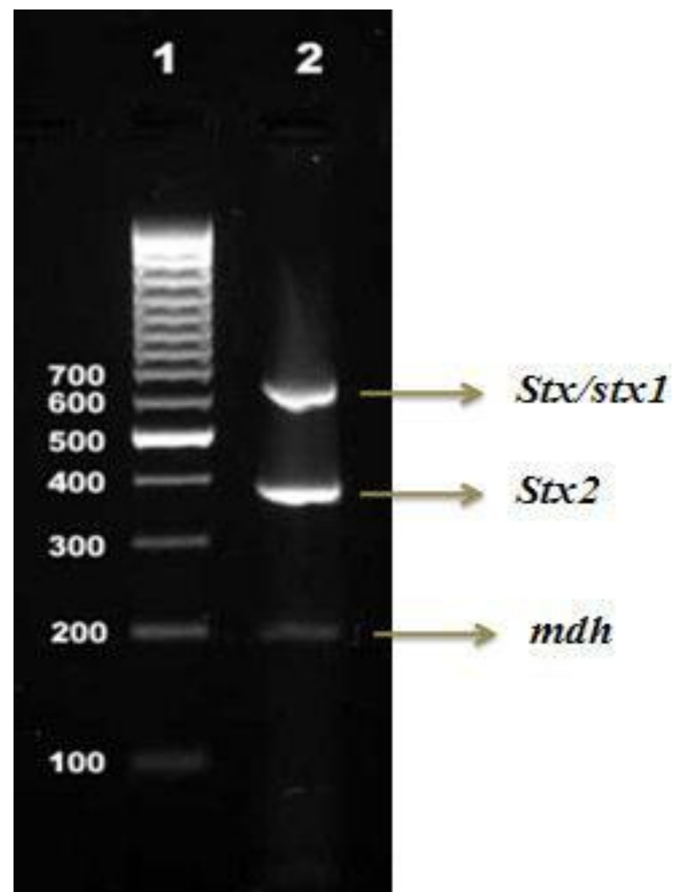

Figure 2. mPCR reaction with DNA template from *E. coli* O157:H7 and amplicon size. Lane 1. Marker size (1000bp), lane 2. The exact size of amplicon products from *E.coli* O157:H7. *mdh* (199bp), *stx2* (381bp) and *stx/stx1* (622bp).

Enzymatic digestion of PCR products

After analysis of amplified products by DNASIS software,

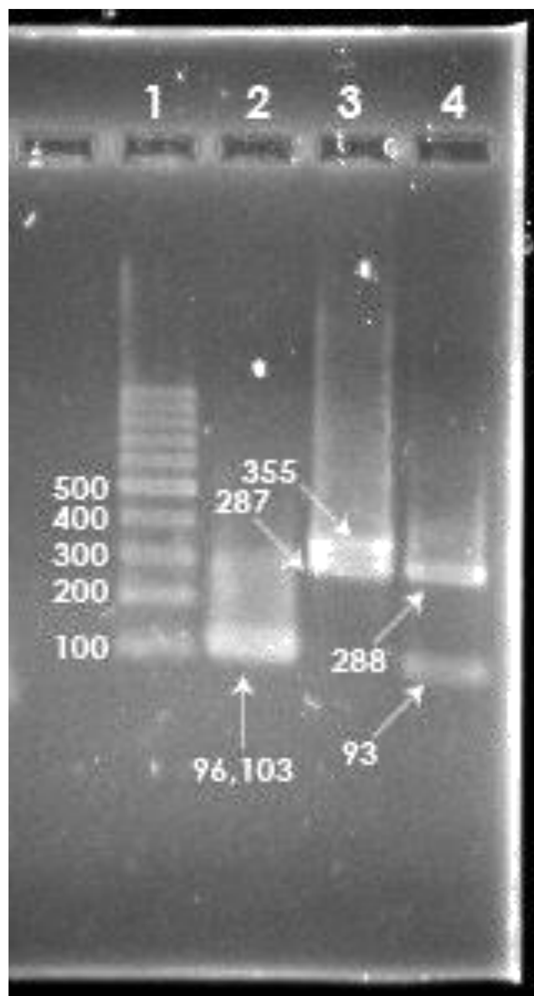

Figure 3. The enzymatic digestion of mPCR products. Lane 1. Marker size, lane 2. Fragments generated by the enzymatic effect of EcoR V on mdh product, lane 3. Fragments generated by the enzymatic effect of Hind III on stx/stx1 product and lane 4. Fragments generated by the enzymatic effect of Hae III on stx2 products.

Hind III, Hae III and EcoR V were used to *stx/stx1*, *stx2* and *mdh* fragments digestion, respectively. These results are shown in Figure 3. Hind III cuts the *stx1* product in 335bp site which generates two fragments of 287bp and 335bp (lane 3) . Hae III cuts the *stx2* product in 288bp site and generates two fragments of 93bp and 288bp (lane 4). EcoR V cuts the *mdh* in 96bp site which generates two fragments of 96bp and 103bp. These fragments are showing in single band, due to the similar size (lane 2).

Specificity of multiplex PCR reaction

The specificity of the mPCR was tested on all strains. As shown in Figure 4, the specific DNA fragments corresponding to the genes defining the appropriate *stx/stx1* and *stx2* only were found in *E. coli* O157:H7 (lane 1). The *mdh* fragment was amplified in 7 bacterial strains (lane 2 to 7), and this fragment was not amplified in the other strains (lane 8 to10).

Sensitivity of multiplex PCR reaction

The agarose gel electrophoresis of different dilutions of extracted DNA from *E. coli* O157:H7 are showing in Figure 5. As shown in Figure 5, the sensitivity of the mPCR assay was estimated to be of 10^{-5} DNA dilution. These results indicate that the mPCR assay is specific and sensitive enough to detect at least 2.1 ng/µl of DNA.

The number of bacteria in all dilutions were determined spectrophotometrically at 600 nm and confirmed by viable counting. As shown in Figure 6, the sensitivity of the mPCR assay was estimated to be of 10^{-3} dilution. These results indicate that the mPCR assay is specific and sensitive enough to detect at least 320 cfu/µl of *E. coli* O157:H7.

DISCUSION

Shiga toxin is one of the exothermic factors produced by *Shigella* and *E. coli*, which initially identified in *S. dysenteriae* (biotype 1). In addition to its destructive effect on gastrointestinal (especially intestine), it can be transferred to central nervous system (CNS) via blood stream and lead to destructive effect on this system (Paton and Paton, 1998). These toxins were divided into two main groups including shiga toxin (Stx) and shiga like toxin (SLT).

Shiga toxins are produced by *S. dysenteriae* (biotype 1), whereas shiga like toxins are produced by certain strains of *E. coli* such as *E. coli* O157:H7 and are divided into two subgroups Stx1 and Stx2 (Philpot and Ebel, 2003). Recently, a number of assays such as cell culture method, ELISA, RPLA, hybridization and PCR methods are available for the detection of shiga toxins. The cell culture method is sensitive but this approach is laborious, time-consuming and requires several days to identify the shiga toxins. The ELISA method can be use for the sensitive and rapid diagnosis of shiga toxins, but it requires to anti-toxin monoclonal antibodies which are expensive (Philpot and Ebel, 2003). RPLA method is required to monoclonal antibodies conjugated to latex particles which working with them is difficult and time consuming (Philpot and Ebel, 2003). Hybridization procedure is a very sensitive and effective molecular technique for specific detection of the biological agents, according to their genetically structure. However, it is effectively used for the identification of shiga toxin genes but it has been made some limitations, including the risk of the using radioactive probes, complexity, time-consuming and the lack of its efficiency for some clinical samples in medical diagnostic laboratories (Philpot and Ebel, 2003).

The PCR assay is an effective method for the detection

Figure 4. Specificity of mPCR reaction. Lane 1. *E. coli* O157:H7 (as Marker and positive control), lane 2. *S. dysenteriae* (biotype 4), lane 3. *S. dysenteriae* (biotype 7), lane 4. *S. dysenteriae* (biotype 8), lane 5. *Shigella flexneri* (biotype 2a), lane 6. *S. sonnei*, lane 7. *Shigella boydii,* lane *8. Vibrio cholera* (strain: ogava), lane 9. *Vibrio cholera* (inaba), lane 10. *Salmonella paratiphy C.*

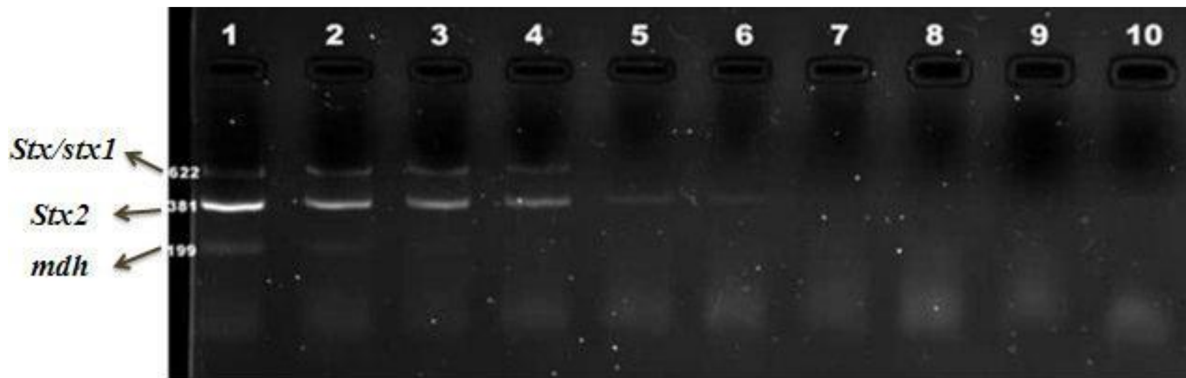

Figure 5. The sensitivity of mPCR reaction in different DNA dilutions. Lane 1. Marker, lane 2. Dilution 10^{-1}, lane 3. Dilution 10^{-2}, lane 4. Dilution 10^{-3}, lane 5. Dilution 10^{-4}, lane 6. Dilution 10^{-5} (the concentration of DNA is 2.1pg/µl), lane 7. Dilution 10^{-6}, lane 8. Dilution 10^{-7}, lane 9. Dilution 10^{-8}, lane 10. Dilution 10^{-9}.

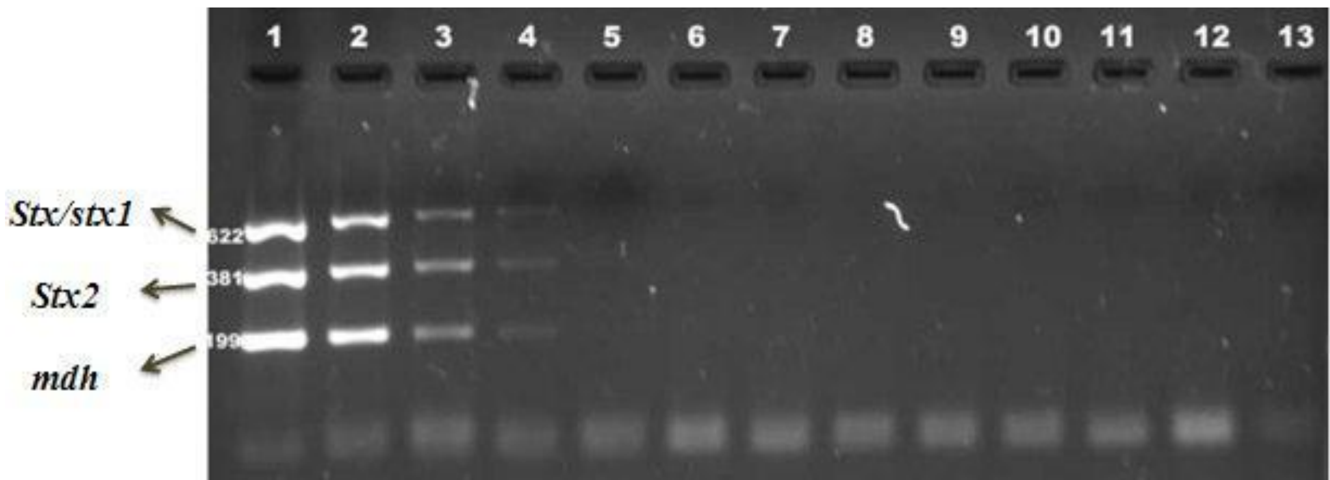

Figure 6. The sensitivity of mPCR reaction in different bacteria counts. Lane 1. Marker, lane 2. Dilution 10^{-1}, lane 3. Dilution 10^{-2}, lane 4. Dilution 10^{-3}, lane 5. Dilution 10^{-4} (bacteria count in this dilution is 320 cfu/µl), lane 6. Dilution 10^{-5}, lane 7. Dilution 10^{-6}, lane 8. Dilution 10^{-7}, lane 9. Dilution 10^{-8}, lane 10. Dilution 10^{-9}, lane 11. Dilution 10^{-10}.

of wide range of genes and biological agents. This technique does not have the described limitations in the other methods. Therefore, these described time-consuming or expensive procedures for identification of shiga toxins can be replaced with a PCR reaction.

In the present study, we show that a rapid and affordable multiplex PCR reaction for the simultaneous detection 2 genetic markers, including both shiga toxins determinants *stx/stx1* and *stx2* of the shiga toxins. Several multiplex PCRs for detection of shiga toxins using different primer sequences had been described (Karch and Meyer, 1989; Cebula et al., 1995; Paton et al., 2003; Pass et al., 2001; Belanger et al., 2002) . The identification of shiga toxin with PCR technique was achieved for the first time by Karch and Meyer (1989). However, this method harbors limitations in terms of the number of targeted genes and specificity to some types of shiga toxins. Therefore, a Multiplex PCR reaction has been suggested to identification of shiga toxins. Primers designed by Cebula et al. (1995) are available to identify the both shiga toxin genes, but they are only able to identify the toxin gene in *E. coli* O157:H7. Primers designed by Paton et al. (2003) are also able to identify the both toxin genes, but the products size is too small and their separation analysis on agarose gel is time-consuming. Primers designed by Pass et al. (2001) are also able to identify both shiga toxins, but the products are not easily separated on agarose gel. Moreover, to the identification of *stx2e*, an additional pair of primer is required. Primers designed by Belanger et al. (2002) can be use to identify shiga toxins, but their analysis on agarose gel is difficult because these amplified products are similar in size. A pair of primer designed by Philpot and Ebel (2003) for the detection of shiga toxins but it was not able to differentiation shiga toxins.

In this study, two pair of primers was used to identify the shiga toxins. Our designed primer does not have the described limitations in the other research. The four primers developed in the present study proved to be specific for the corresponding two genes. Moreover, the amplified products are well resolved by agarose gel electrophoresis. Multiplex PCR with some bacterial strains demonstrated that the MPCR is highly specific and reliable. We conclude that the newly designed primers are specfic for the identification and differentiation of shiga toxins. The MPCR reaction showed the presence of shiga toxin gene in four *Shigella* serotypes, in addition to *E. coli*. Thus, the presence of this amplified product may be a reason for the present of *E. coli* and *Shigella* in a sample.

The cell wall structure in gram negative bacteria, especially in *Enterobacteriaceae*, is very complex and its composed of various lipids, carbohydrates and proteins which causes the inhibition and low sensitivity of PCR reaction. Therefore, the extracted DNA from bacteria must be purified before PCR reaction. Some researcher used SDS-Lysozyme, Tris, glucose and EDTA for DNA purification. The sensitivity of reaction was 100 pg/µl (Pollard et al., 1990; Wang et al., 2002). Franck et al. (1998) used the boiling method for DNA purification but this procedure was unable to remove the involving inhibitors. In the other studies, by using of CTAB and 5 M NaCl, the inhibitors effectively removed from DNA solutions which lead to increasing the sensitivity of reaction to 2.1 pg/µl. This procedure is sensitive enough to detect at least 320 cfu/µl of bacteria that is three time more than that of reaction achieved by Paton et al. (1998). In our study, the initial PCR reaction for extracted template DNA from *S. dysenteriae* (biotype 1) and *E. coli O157:H7* was achieved successfully. In *E. coli*, both *stx1* and *stx2* lanes were found, whereas in *S. dysenteriae* (biotype 1) only 622bp fragment was amplified.

We conclude that the newly designed MPCR primers are specific for the identification and differentiation of shiga toxins and hence can be helpful to diagnosis of shiga toxin in clinical samples.

REFERENCES

Belanger SD, Boissinot M, Menard C, Picard FJ, Bergeron GM (2002). Rapid detection of shiga toxinproducing bacteria in feces by multiplex PCR with molecular beacons on smart cycler, J. Clin. Microb., 40: 1436-1440.

Cebula TA, Payne WL, Feng P (1995). Simultaneous identification of strains of *Escherichia coli* serotype o157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR, J. Clin. Microb., 33: 248-250.

Donohue-Rolfe A, Acheson DW, Keusch GT (1991). Shiga toxin: purification, structure, and function. Rev. Infect. Dis., 13: S293–297.

Donta ST, Tomicic TK, Donohue-Rolfe A (1995). Inhibition of Shiga-like toxins by brefeldin A. J. Infect. Dis., 171: 721–724.

Franck SM, Bosworth BT, Moon HW (1998). Multiplex PCR for enterotoxigenic, attaching and effacing and shiga toxin-producing *Escherichia coli* strains from calves, J. Clin. Microb., 36: 1795-1797.

Friedrich AW, Borell J, Bielaszewska M, Fruth A, Tschape H, Karch H (2003). Shiga toxin 1cproducing *Escherichia coli* strains: Phenotypic and genetic characterization and association with human disease, J. Clin. Microb., 41: 2448-2453.

Gobius KS, Higgs GM, Desmarchelier PM (2003). Presence of activable shiga toxin genotype (stx2d) in shigatoxigenic *Escherichia coli* from livestock sources, J. Clin. Microb., 41: 3777-3783.

Hanashima T, Miyake M, Yahiro K, Iwamaru Y, Ando A, Morinaga N (2008). Masatoshi Noda Effect of Gb3 in lipid rafts in resistance to Shiga-like toxin of mutant Vero cells. Microbial Pathogenesis, 45: 124–133.

Head SC, Karmali MA, Lingwood CA (1991). Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. Evidence for B subunit modulation of a subunit function. J. Biol. Chem., 266: 3617–3621.

Karch H, Meyer T (1989). Single primer pair for amplifying segments of distinct shiga-like toxin genes by polymerase chain reaction. J. Clin. Microb., 27: 2751-2757.

Karmali MA, Gannon V, Sargeant JM (2010). Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol., 140: 360–370.

Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S (1987). Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. J. Biol. Chem., 262: 8834–8839

O' Loughlin EV, Robins-Browne RM (2001). Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. Microbes and Infection., 3: 493−507.

Pass MA, Odedra R, Batt RM (2001). Multiplex PCR for identification of *Escherichia coli* virulence genes, J. Clin. Microb., 38: 2001-2004.

Paton AW, Paton JC (1998). Detection and characterization of shigatoxigenic *Escherichia coli* by multiplex PCR assays for stx1,

stx2, eae A, enterohemorrhagic *Escherichia coli hly A*, *rfb 0111*, and *rfb 0157*. J. Clin. Microb., 36: 598-602.
Philpott D, Ebel F (2003). *E. coli*, Shiga Toxin Methods and Protocols. Humana Press Inc. Totowa, New Jersey, pp. 1-54.
Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR (1990). Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction, J Clin Microb., 28: 540-545.
Sambrook J, Russell DW (2001). Molecular cloning, a laboratory manual. 3 rd ed, New York: Coldspring Harbor laboratory press., 6: 61-62.
Sandvig K, van Deurs B (2000). Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. EMBO J., 19: 5943–5950.
Schmidt H, Scheef J, Morabito S, Caprioli A, Wieler LH, Kawch H (2000). A New shiga toxin 2 variant (*stx2f*) from *Escherichia coli* isolated from pigeons, Appl. Environ. Microb., 66: 1205-1208.
Wang G, Clark GC, Rodgers FG (2002). Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the *O157:H7* serotype, and components of the type 2 shiga toxin family by multiplex PCR, J. Clin. Microb., 40: 3913-3619.