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Identification of *Escherichia coli* O157:H7 Isolated from Cattle Carcasses in Mashhad Abattoir by Multiplex PCR

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Abstract: During the spring of 2008 a number of 120 swab samples were taken from brisket region of male cattle carcasses in the range of 2-4 years of age, which were slaughtered in Mashhad abattoir. The samples were obtained from the post-chilling stage of processing. The samples were initially enriched in modified trypticase soy broth containing novobiocin, followed by plating on sorbitol Mac Cankey agar supplemented with cifixime and potassium tellurite. Consequently, the suspected non sorbitol fermenting (NSF) colonies were confirmed by biochemical tests as *Escherichia coli* and then employed for multiplex-PCR (m-PCR) assay. In the first m-PCR assay, primers specific for O157 and H7 antigens gene were used. A total of 10 NSF *Escherichia coli* colonies were isolated and in the m-PCR assay 8(6.6%) of the samples were confirmed as *Escherichia coli* O157:H7. The second m-PCR assay showed number of two isolates harboring the Shiga toxin (*stx*2) gene. The m-PCR assays employed in this study might be considered as a possible alternative approach for immunological assays which detects somatic and flagellar antigens. Besides this method determines potential of shiga toxin production.

Key words: *Escherichia coli* O157:H7 · Cattle carcasses · m-PCR · *stx*1 · *stx*2

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) strains are the most important recently emerged foodborne pathogens [1]. VTEC may belong to many serotypes, but most severe human infections are caused by strains of *E. coli* O157:H7 [2]. *Escherichia coli* O157:H7 causes hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [3, 4].

Cattle are generally considered as the major reservoir for this organism [5, 6] and frequently excrete these bacteria in their feces [7]. Most of foodborne infections, due to *E. coli* O157:H7 have been associated with foods of bovine origin, particularly ground beef and raw milk [8, 9].

Meat and environment becomes contaminated from intestinal content of the cattle at the time of slaughter and processing may introduce the organism when performs in nonhygienic conditions [2].

E. coli O157:H7 has several virulence factors such as verotoxins (VT 1 and VT 2 or stx1 and stx2), eaeA (intimin) and enterohemolysin [10]. Verotoxins inactivate ribosomal RNA and inhibit protein synthesis and they eventually will cause the death of host cells [10, 11]. *E. coli* O157:H7 can produce VT1, VT2 or both toxins. These toxins and eaeA have been associated with human illnesses [2].

The specific identification of *E. coli* O157:H7 based on culture and biochemical methods is laborious and time consuming and in food products takes 5 or more days to complete [12]. Furthermore, other than the culture methods which are based on biochemical characteristics of the bacteria, many other assays have been developed, including serological techniques, which uses both polyclonal and monoclonal antibodies specific for the O and H antigens [13, 14]. Molecular approaches have also been practiced. In this regard, PCR assays based on the presence or absence of specific genes such as the *stx*, *eae*A and *hly*A have been described [13, 15].

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Several techniques have been described to detect verotoxins [10, 16]. Amongst these, PCR has been widely used and a number of studies have targeted *stx* genes and one or more O157:H7-specific genetic markers [17-20].

The objective of the present study was to isolate *E. coli* O157:H7 from cattle carcasses, slaughtered in Mashhad (northeastern of Iran) abattoir, using conventional culture method and confirming the suspected colonies by a serogroup-specific PCR assay as *E. coli* O157:H7. Afterwards, determination of the presence of *stx*1 and *stx*2 genes by another m-PCR assay was also performed.

MATERIALS AND METHODS

Sampling: During the spring of 2008, numbers of 120 samples were taken from the male cattle carcasses, within the range of 2-4 years of age, using wet and dry swabbing method from 100 cm^2 area of brisket region, which were slaughtered in Mashhad abattoir. The swabs dipped into test tubes containing 2 ml of 0.01% peptone water. The samples were obtained from the post-chilling stage of processing. The samples were brought to the laboratory on ice and held at 4°C until used.

Culture Conditions: 1ml of each sample were aseptically transferred to 9 ml of modified tryticase soy broth (mTSB) containing 20 mg L^{-1} novobiocin, followed by incubation at 37°C overnight. The enriched culture were plated onto sorbitol Mac Conky agar supplemented with cefixime (0.05 mg L^{-1}) and potassium tellurite (2.5 mg L^{-1}) (CT-SMAC). The inoculated CT-SMAC plates were incubated at 37°C for 24 hours. Then non-sorbitol fermenting (NSF) colonies were selected from CT-SMAC plates and streaked onto Eosin Methylene Blue agar (EMB) and incubated overnight at 37°C. These isolates, with typical *E. coli* metallic sheen on EMB, were characterized by biochemical tests, including conventional indol, methyl red, voges-proskauer, citrate and lysine decarboxylase tests.

Multiplex-PCR Assay: NSF colonies on CT-SMAC which were confirmed as *E. coli* by biochemical tests, employed as templates for m-PCR assay. *E. coli* O157:H7 (ATCC-35150) were used as positive control and sterile distilled water as negative control. A whole-cell suspension was prepared by suspending a NSF bacterial colony from CT-SMAC in sterile distilled water. The cell lysate was made by heating the suspension for 10 minutes in a boiling water bath. The lysate were spined for five minutes at 6000 rpm to pellet the cellular debris. Volumes of 2 µl of the supernatant were used as template for amplification by m-PCR. The presence of $fliC_{h7}$ gene encoding the flagellar antigen H7 and rfb_{O157} gene which endcodes the somatic antigen O157 [15, 21] were examined. Confirmed colonies as *E. coli* O157:H7 were subjected to the second m-PCR assay using primers specific for *stx* 1 and *stx* 2 genes [22].

The oligonucleotide primers sequences used in each m-PCR reaction mixture have shown in Table 1. The m-PCR reactions were performed in a 25 µl amplification mixture consisting of 2.5 µl 10x PCR buffer (500mM KCl, 200mM Tris HCl), 0.5 µl dNTPs (10mM), 1 µl Mg Cl_2 (50mM), 1.25 µl of each primer (0.5 µM), 0.2 µl of *Taq DNA* polymerase (5 unit/µl) and 2 µl of template. The thermocycler (Bio Rad) program was started with initial incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, 30 sec of annealing at 52°C and 60 sec of elongation at 72°C and a final extension for 10 min at 72°C was carried out.

The PCR products were separated by electrophoresis in 1.5% agarose gel at 100V for 40 min in tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. 100 bp DNA ladder was used as a size reference for m-PCR assay. The expected size of m-PCR products for *rfb* O157 and $fliC_{h7}$ genes amplification were 259 and 625 bp and for *stx* 1 and *stx* 2 genes were 614 and 779 bp respectively.

Table 1: Target genes, Primers sequence and expected size for *Escherichia coli* O157: H7

Target gene	Primer sequence	Size(bp)	Reference
rfb_{O157}	F: 5'-CGG ACA TCC ATG TGA TAT GG-3'		
	R: 5'-TTG CCT ATG TAC AGC TAA TCC-3'	259	[15, 21]
$fliC_{h7}$	F: 5'-GCG CTG TCG AGT TCT ATC GAG-3'		
	R: 5'-CAA CGG TGA CTT TAT CGC CAT TCC-3'	625	[15, 21]
VT1(Stx_1)	F: 5'-ACA CTG GAT GAT CTC AGT GG-3'		
	R: 5'-CTG AAT CCC CCT CCA TTA TG-3'	614	[22]
VT2(Stx_2)	F: 5'-CCA TGA CAA CGG ACA GCA GTT-3'		
	F: 5'-CCT GTC AAC TGA GCA CTT TG-3'	779	[22]

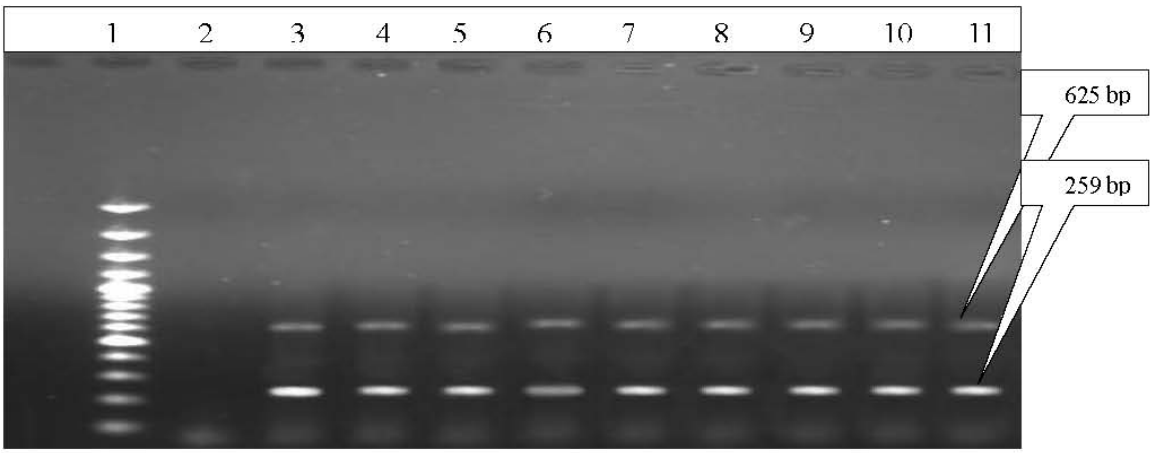

Fig. 1: Results of the m-PCR assay, amplifying 259 base pair segment of rfb_{O157} and 625 base pair of $fliC_{h7}$ genes of *Escherichia coli* O157: H7. 1:100 bp Marker 2: Negative control 3: Positive control 4-11: positive Sample

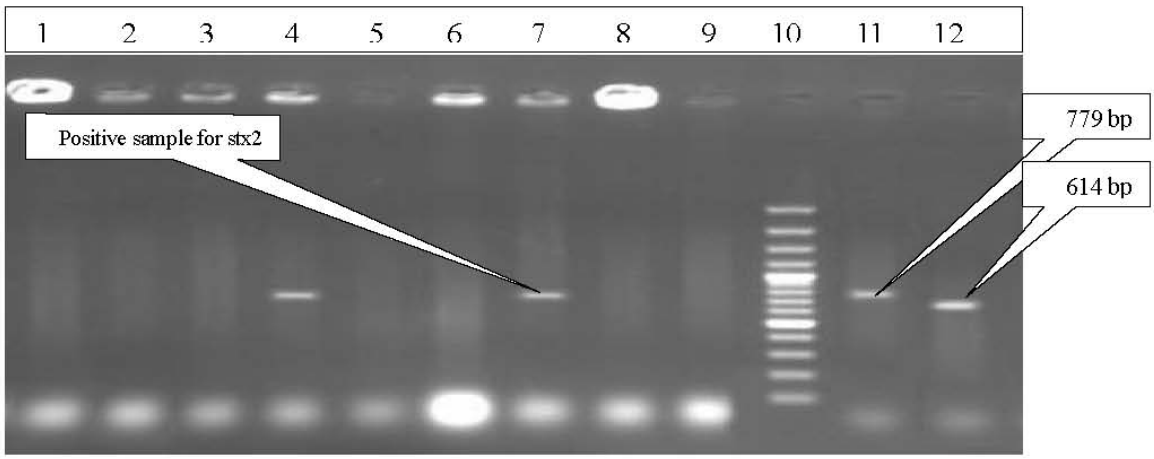

Fig. 2: Results of the m-PCR assay, amplifying 614 base pair segment of *stx1* and 779 base pair of *stx*2 genes of *Escherichia coli* O157: H7. 10:100 bp Marker 9: Negative control 12: Positive control for *stx*1 11: Positive control for *stx*2 4, 7: positive Samples

RESULTS

Conventional Method: From 120 swab samples of cattle carcasses which were slaughtered in Mashhad abattoir, after enrichment and selective plating, number of 10 (8.3%) non-sorbitol fermenting (NSF) colonies were isolated and confirmed as *E. coli* by biochemical tests.

PCR Method: In the first m-PCR assay, using specific primers for rfb_{O157} and $fliC_{h7}$ genes, number of 8(6.6%) samples confirmed as *E. coli* O157: H7 (Fig.1). In the second m-PCR assay, using specific primers for *stx*1 and *stx*2 genes showed that only two isolates were harboring the *stx*2 gene (Fig. 2).

DISCUSSION

In this study, NSF colonies were isolated from 10(8.3%) of cattle carcasses by conventional culture method, but in m-PCR assay from suspected colonies using specific primers for rfb_{O157} and $fliC_{h7}$ genes, number of 8(6.6%) samples confirmed as *E. coli* O157: H7. Our findings do not differ greatly from those reported the isolation of this bacteria from cattle carcasses in other areas of Iran. This has already been reported 6.4% in Isfahan and 9.6% in Shiraz [23, 24]. However the prevalence reported in our study is much higher as compared to the reports from other countries such as 3% from Ireland [25], 2.7% from Mexico [26], 0.4% from France [27], 2.8% from Serbia [28] and 1.4% from England [18].

It should be considered that in the absence of a reliable "gold standard" for determining the presence of EHEC O157 in samples [29], it is difficult to directly compare the reported results. The reported prevalence of *E. coli* O157: H7 depends on the used culture techniques, the age of the animals and the season in which samples are collected [5, 29]. The important finding was the presence of this pathogen in cattle carcasses in this area of Iran. This is the first study which describes the detection and frequency of major virulence genes of *E. coli* O157:H7 isolated from cattle carcasses in Mashhad.

The samples were collected during the spring months. Seasonal distribution of *E. coli* O157:H7 has been reported previously [30], with highest prevalence in summer and lowest in winter, so it is possible that the contamination rate increases more than 6.6% in summer months.

We used modified trypticase soy broth as enrichment stage. It has been proposed that the enrichment before plating on selective agar may increase the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples on selective agar [31, 32].

In this study we did not use IMS (immunomagnetic separation) procedure for enrichment stage, although many researchers have reported that IMS resulted in a greater detection rate for *E. coli* O157 in inoculated or non-inoculated beef and bovine feces [20, 33, 34] but in contrary many other researchers have claimed that using IMS procedure were not significantly different from enrichment plating [26, 32].

After enrichment stage we used CT-SMAC agar for selective plating, because it has been described that the CT-SMAC agar medium yields the best results for selective cultivation of *E. coli* O157:H7 [14, 32]. Although using rainbow agar (a new chromogenic medium for the detection of *E. coli* O157:H7) has been found to be more sensitive than CT-SMAC, but the difference was not significant [20].

We used m-PCR assay for confirming the non sorbitol fermenting colonies as *E. coli* O157:H7. Many researchers have reported multiplex PCR to be a reliable identification method for *E. coli* O157:H7 [35, 36].

We used primers specific for flagellar and somatic antigens genes in the first m-PCR assay. These antigens could be detected by immunological assays as well, but the main advantage of the employed m-PCR method is its ability to detect rough isolates or in another words the isolates having a masked *O* antigen [21]. The method used in this study can also detect O157 H-serotype of *E. coli*, although we did not isolate this serotype in this study.

In the second multiplex-PCR assay we determined the presence of main virulence genes (*stx*1 and *stx*2), which has been widely used by other researchers [16, 37]. According to our results, *stx*2 were detected in 25% of isolates, but *stx*1 was not detected in any of the isolates. It has been reported that *stx*2 gene was more frequent than *stx*1 in most of the studies performed in the USA, Japan and European countries [5, 17, 18, 37, 38]. In a study in the USA, *stx*1 gene was not detected in any strains tested, while most of the isolates were found to carry *stx*2 gene [37]. According to our study 75% of isolates detected as non-toxigenic. In another study in Japan [39] and in a study in France [27], *stx* genes were not detected in *E. Coli* O157:H7 strains.

It seems that using these two multiplex PCR assays are suitable methods for rapid identification of *E. coli* O157:H7 to species level and determining their virulence genes, as it could speed up and simplify the identification procedures that could be completed in 1 working day.

However, the origins and subsequent rate at which carcass contamination with *E. coli* O157:H7 occurs have not been well established, but Hazard Analysis-Critical Control Point (HACCP) system is being used in developed countries to decrease the risk of food-borne illness. It is recommendable to employ this system in developing countries, as well as implementing, evaluating and validating antimicrobial interventions to reduce the presence of potential pathogenic microorganisms.

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