Using multiplex-PCR assay in identification of *Escherichia coli* O157:H7 isolated from hamburger samples in Mashhad, Iran

Jamshidi, A. ¹ *, Bassami, M. R. ², Khanzadi, S. ³, Soltaninejad, V. ⁴

¹. Department of food hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.
². Department of clinical sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.
³. Department of food hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.
⁴. Graduated from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

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Abstract

In this study a number of 100 hamburger samples which were produced industerially from different batches were collected randomly from supermarkerts in Mashhad city, during the autumn months of 2006. For isolation of the bacteria, samples were, firstly 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 assay, using primers specific for O157 and H7 antigens gene. The m-PCR assay employed in this study may be a possible alternative to immunological assays which detects somatic and flagellar antigens. In this study, 9 NSF *Escherichia coli* colonies were isolated, and in multiplex-PCR assay two samples (4%) were confirmed as *Escherichia coli* O157:H7.

Key words: *Escherichia coli* O157:H7, Hamburger, M-PCR.

* Corresponding Author E-Mail address: ajamshid@ferdowsi.um.ac.ir
1- Introduction

*Escherichia coli* O157:H7 serotypes are identified as enterohemorrhagic *E. coli* and categorized in verotoxin producing *E. coli* [1]. Due to the severity of infection which it causes and an infectious dose which may be as low as 10 organisms [2], it has emerged as an important foodborne pathogen of considerable public health concern. It causes haemorrhagic colitis, hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [3-4].

Cattle especially the young ones have been implicated as a principal reservoir of *E. coli* O157:H7 [5,6,7]. Cattle frequently excrete this bacteria in their feces [8-9]. Feces and hides are significant sources of bacterial carcass contamination [10,11,12]. Meat and environment become contaminated from intestinal content of cattle at the time of slaughter, and processing may introduce the organism when performed in non hygienic conditions [13]. Undercooked ground beef is the major vehicle of food-borne outbreaks [14,3].

Different culture methods for the screening of food specimens for *E. coli* O157:H7 are available. Among them Mac Conkey agar containing sorbitol instead of lactose (SMAC), is most commonly used for isolation of *E. coli* O157:H7 [15]. Due to the fact that this bacteria are unable to ferment sorbitol, sorbitol-non fermenting colonies are potentially considered as *E. coli* O157:H7 [15, 16]. Sorbitol-Mac Conkey supplemented with cefixime and tellurite (CT-SMAC) increases the sensitivity of this media. In comparison, broth enrichment methods are superior to direct plating [17]. 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 [18, 19]. Molecular approaches have also been practiced. In this regards, PCR assays based on the presence or absence of specific virulence genes such as the *stx*, *eaeA* and *hlyA* and serogroup-specific genes encoding O-antigen in *E. coli* serogroups have been described [18, 20]. Sequence data for the *fich* gene which encodes the H7 antigen is also available [21].

The aim of the present study was, isolation of *E. coli* O157:H7 from hamburger samples collected from supermarkets in Mashhad city, by conventional culture method and confirmation by a serogroup-specific multiplex-PCR assay.

2- Materials and methods

2-1- Sample collection

During the autumn months of 2006, number of 100 hamburger bag samples from different batches (each bag contained 5 pieces of beef hamburger containing more than 60% meat), from five main suppliers which were produced industrially, were randomly collected aseptically in sterile disposable bags from supermarkets, across Mashhad city, Khorasan province, Iran.

2-2- Culture conditions

The samples were brought to the laboratory on crashed ice. Immediately in the laboratory, 25g of each hamburger bag sample was aseptically transferred to 225ml of modified trypticase soy broth (mTSB) containing 20 mg L⁻¹ novobiocin, followed by incubation at 37°C for 24 hours. 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 innoculated 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 plates containing eosin methylene blue agar (EMB) and were incubated at 37°C for 24 hours. 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.

2-3- Multiplex-PCR assay

Non-sorbitol fermenting (NSF) colonies on CT-SMAC that had been confirmed as *E. coli* were employed as templates for PCR assay. *E. coli* O157:H7 (ATCC-35150) were used as a positive control and sterilized distilled water as negative control. A part of a NSF bacterial colony from CT-SMAC, was suspended in 100µl of sterile DW. The cells were lysed by boiling for 10 minutes in a boiling water bath. The lysate were spined for five minutes at 6000 rpm to pellet the cellular debris. A volume of 2 µl of the supernatant was used as
Table 1 Primers and thermocycling condition for *Escherichia coli* O157: H7

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (name)</th>
<th>Predicted product size(bp)</th>
<th>Thermalcycling program</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rfb</em>O157</td>
<td>F: 5′- CGG ACA TCC ATG TGA TAT GG -3′</td>
<td>259</td>
<td>94ºC for 5min (94ºC for 60s- 52ºC for 30s- 72ºC for 60s)*35 cycles-72 ºC for 10 min</td>
</tr>
<tr>
<td></td>
<td>R: 5′- TTG CCT ATG TAC AGC TAA TCC -3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>flic</em>H7</td>
<td>F: 5′- GCG CTG TCG AGT TCT ATC GAG-3′</td>
<td>625</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5′- CAA CGG TGA CTT TAT CGC CAT TCC-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

template for amplification by m-PCR. The presence or the absence of *flic* H7 gene encoding the flagellar antigen H7 and *rfb* O157 gene which encodes the somatic antigen O157 [20, 22] was examined. Table 1 describes oligonucleotide sequence of primers used in the m-PCR reaction mixture. The m-PCR reaction was 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 Cl2 (50mM), 1.25 µl of each primer (0.5 µM), 0.2 µl of Taq DNA polymerase (5 unit/µl) and 2 microliter 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, annealing at 52˚C for 30 sec and elongation at 72˚C for 60 sec, and final extension at 72˚C for 10 min. The PCR products were seperated 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. The expected size of m-PCR products for *rfb* O157 and *flic*H7 genes amplification were 259 and 625 bp, respectivly.

3-4- Results
From 100 hamburger samples which were collected randomly from different supermarkets in Mashhad, after enrichment and selective plating number of 9 non-sorbitol fermenting (NSF) colonies were isolated and confirmed as *E.coli* by biochemical tests. In m-PCR assay, using specific primers for *rfb* O157 and *flic*H7 genes, only two samples were confirmed to be contaminated with *E.coli* O157: H7 (Figure: 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](image)

3:Positive control  4: Negative control  1 and 2:Sample  5:100 bp Marker

4-5- Discussion
In this study *E.coli* O157: H7 isolated from 2% of hamburger samples by conventional culture method and confirmed by multiplex-PCR assay. Our findings do not differ greatly from those reported abroad from minced beef products and retail meat i.e. 3.8% in Argentina [23]; 2.3% in Switzerland [24];
1.1% in the UK and Netherlands [25,26]. The samples were collected during the autumn months. Seasonal distribution of *E.coli* O157:H7 has been reported previously [25, 27], with highest prevalence in summer and lowest in winter, so it is possible that the contamination rate became more than 2% in summer months.

The samples were chosen from hamburgers which had made industrially, so the products were wrapped and sealed, and thus did not come into contact with the surrounding environment. It is possible that the contamination rate of hand made hamburgers with this bacteria are more higher, because of miss-handlings and cross contamination.

We used modified trypticase soy broth as enrichment medium. It has been proposed that the enrichment befor plating on selective agar may increase the sensitivity of *E.coli* O157: H7 isolation compared to direct plating of test samples on selective agar [28, 17].

In this study, we did not use IMS (immuno magnetic separation) procedure for enrichment stage, because although many researchers have reported that IMS resulted in a greater detection rate for *E. coli* O157 in inoculated or noninoculated beef and bovine feces [29-33] but in contrary many other researchers have claimed that using IMS procedure were not significantly different from enrichment plating [17, 34].

After enrichment stage we used CT-SMAC agar for selective plating, because it has been described that the CT-SMAC agar medium yeilds the best results for selective cultivation of *E.coli* O157:H7 [19,26,17]. Although using rainbow agar (a new chromogenic medium for the detection of *E.coli* O157:H7) has been found to be more senstive than CT-SMAC, but the difference was not significant [33].

We used m-PCR assay for confirming the non sorbitol fermenting colonies as *E.coli* O157:H7. Several groups of researchers have reported multiplex PCR to be a reliable identification method for *E. coli* O157:H7 [24,31,21].

We used primers specific for flagellar and somatic antigens genes in multiplex-PCR assay, these antigens could be detected by immunological assays as well, but the main advantage of the employed m-PCR method is it's ability to detect rough isolates or the isolates having a masked *O* antigen [22]. The method developed in this study can also detect O157 H serotype of *E.coli*, although we did not isolate this serotype in this study.

In this study we did not discriminate between shiga toxin producing strains and non- shiga toxin producing ones, although it is possible to include primers specific for these virulence genes, as well. This multiplex PCR is a suitable method for rapid identification of *E. coli* O157:H7 to species level as it could speed up and simplify the identification procedures that could be completed in 1 working day. However, it is possible that rare sorbitol-positive O157:H7 strains may not be able to be detected using this method.

5- References:


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جداسازی و شناسایی باکتری اشیریشیا کولا O157: H7 از نمونه های همبرگر در شهرستان مشهد با استفاده از روش مولتی پلکس PCR

عبده..جمشیدی 1 *, محمد رضا بسامی 2 *, سعید خانزادی 3, وجهه سلطانی نژاد 4

چکیده

تعداد یکصد نمونه همبرگر از بیش از یک های مختلف که بصورت صمیمی تولید در شرکت‌های مصرفی مواد غذایی در شهرستان مشهد توسط گروه بهداشت غذا، دانشگاه علوم دارویی دامی، دانشگاه ایرانی مشهد جاری شدند. نمونه‌ها در محیط آگوشت ترشح نمونه‌ای از سایه‌گذاری وجود ندارد. سپس در محیط آگوشت ترشح نمونه‌ای از سایه‌گذاری وجود ندارد. سپس توسط واحدهای سایه‌گذاری موجود در ترشح نمونه‌ی اشیریشیا کولا O157: H7 ترکیبی شامل پلوکس PCR (POLYMERASE CHAIN REACTION) با استفاده از دو جفت پرایمر اختصاصی به نام‌های سایه‌گذاری و فلزات در مورد همبرگر اشیریشیا کولا O157: H7 ترکیبی شامل پلوکس PCR (POLYMERASE CHAIN REACTION) با استفاده از دو جفت پرایمر اختصاصی به نام‌های سایه‌گذاری و فلزات در مورد همبرگر اشیریشیا کولا O157: H7 ترکیبی شامل پلوکس PCR (POLYMERASE CHAIN REACTION) با استفاده از دو جفت پرایمر اختصاصی به نام‌های سایه‌گذاری و فلزات در مورد همبرگر اشیریشیا کولا O157: H7 ترکیبی شامل پلوکسPCR (POLYMERASE CHAIN REACTION) با استفاده از دو جفت پرایمر اختصاصی به نام‌های سایه‌گذاری و فلزات در مورد همبرگر اشیریشیا کولا O157: H7 