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

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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 suprermarkets 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, Hamburgr, M-PCR.

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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-ureamic syndrom (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 availabe. 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 potentialy 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 supperior 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 approches have also been practiced. In this regards, PCR assayes 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 *fic*H7 gene which encods 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 transfered 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 methylen 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 steriled 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

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

template for amplification by m-PCR . The presence or the absence of *flic* H7 gene encoding the flagellar antigen H7 and *rfb* 0157 gene which endcodes the somatic antigen O157 [20, 22] was examined. Table 1 describes oligonuclotide 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 Cl_2 (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 extention 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).

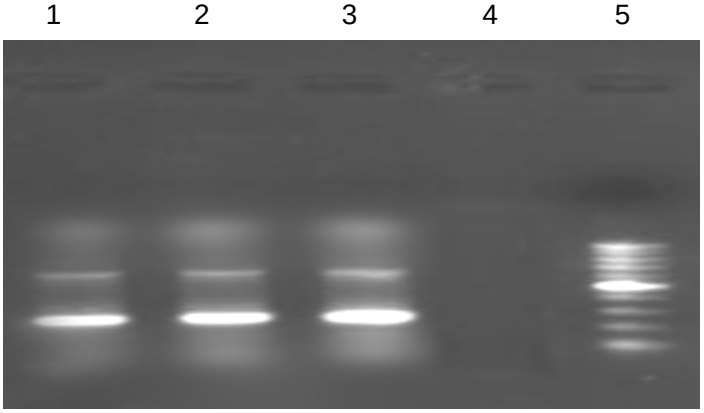

Fig 1 Results of the m-PCR assay, amplifying 259 base pair segment of *rfb O157* and 625 base pair of *flic H7* genes of *Escherichia coli 157: H7*
3:Positive control 4: Negative control
1 and 2:Sample 5:100 bp Marker

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 industerially, 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 tryticase soy broth as enrichment medium. It has been proposed that the enrichment befor plating on selective agar may increase the sensetivity 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 enrichmentplating [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 sensetive 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 detcted 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 virolence 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.

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جداسازی و شناسائی باکتری اشریشیا کولا O157: H7 از نمونه های همبرگر در شهرستان مشهد با استفاده ازروش مولتی پلکس PCR

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چکیده

تعداد یکصد نمونه همبرگر از بچ های مختلف که بصورت صنعتی تولید شده بود در فصل پاییز از سطح فروشگاههای عرضه مواد غذائی درشهرستان مشهد تهیه گردید. جهت جداسازی باکتری **اشریشیا کولا** O157: H7, نمونه ها ابتدا در محیط آبگوشت Trypticase soy حاوی آنتی بیوتیک نوبیوسین غنی سازی گردید. سپس در محیط آگار انتخابی سوربیتول مک کانکی حاوی سیفیکسیم و تلوریت پتاسیم کشت داده شد، از کلنی های غیر تخمیر کننده سوربیتول با آزمایشات بیوشیمیائی باکتری اشریشیا کولای مورد شناسائی قرار گرفت. سپس با روش مولتی پلکس PCR با استفاده از دو جفت پرایمر اختصاصی ژنهای سوماتیک و فلاژلار، سروتیپ O157:H7 مورد شناسائی قرار گرفت. روش مولتی پلکس PCR که در این مطالعه مورد استفاده قرار گرفت می تواند به عنوان جایگزین روشهای ایمیونولوژیک که حضور آنتی ژنهای سوماتیک و فلاژلار را مورد شناسائی قرار می دهد مطرح باشد. در این مطالعه از تعداد ۹ نمونه همبرگر، کلنی های غیر تخمیر کننده سوربیتول جدا سازی گردید که در روش m-PCR در تعداد دو نمونه (۲٪) آلودگی به باکتری اشریشیا کولایO157:H7 مورد تایید قرار گرفت.

کلید واژه گان: اشریشیا کولا O157: H7، مولتی پلکس PCR، همبرگر.

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