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Quantification of *Escherichia coli* O157:H7 in milk by MPN-PCR method

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Abstract

Due to the severity of infection which E. coli O157:H7 causes and an infectious dose which may be as low as 10 organisms, it has emerged as an important foodborne pathogen of considerable public health concern, so it's enumeration in foods is critical. Number of 101 to 105 ml -1 of bacteria were inoculated in sterilized milk. Modified MPN dilutions from inoculated milk sample (10, 1, 0.1, 0.01, 0.001 ml) with three replicates per dilution were prepared, using modified m-TSB (containing 20 mg L-1 novobiocin) Different background microorganisms including gram positive and gram negative bacteria were also inoculated. Enumeration was performed by DNA extraction from tubes showing turbidity and performing multiplex-PCR using primers specific for O157 and H7 antigens gene.This MPN-PCR proved to be a rapid and reliable method for enumerating Escherichia coli O157:H7 in milk, including low contaminated samples and may facilitate the enumeration of Escherichia coli O157:H7 for routine analyses in milk without excessive work.

Key words: Escherichia coli O157:H7, milk, MPN-PCR, enumeration.

Introduction

Verocytotoxin-producing Escherichia coli (VTEC) strains specially O157:H7 is the most important recently emerged food-borne pathogens (1). Escherichia coli O157:H7 causes haemorrhagic colitis, hemolytic-ureamic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (11,8). Other than the culture methods and serological techniques, Molecular approaches have also been practiced. Criteria for tolerable levels of Escherichia coli O157:H7 in foods have not been described in several countries, including in our country (Iran), but the international commission on microbiological specification for foods (ICMSF) has proposed this criteria as ≤ 1cfu 250g -1.

In the present study, we enumerated the inoculated Escherichia coli O157:H7 in milk samples with the combination of the MPN method and the species-specific PCR targeting the somatic and flagellar antigen gene, to evaluate the sensitivity and specificity of the method.

Materials and Methods

Bacterial reference strain:

In this study E.coli O157:H7 (ATCC-35150) purchased from Mast International Inc, was used for the experiment.

Inoculation:

Stock cultures of the E.coli O157:H7 were prepared in Tryptone Soya Agar (Himedia) slants, stored at 4 °C and subcultured every 4 weeks. Pure cultures of E.coli O157:H7 were prepared by subculturing the test strain into 10 mL of Brain Heart Infusion Broth (Merck), following incubation at 37 °C for 24 h. The concentration of the resulting culture of E.coli O157:H7 was determined by preparing serial dilutions and viable counts by surface plating on MacConkey agar (Himedia). The absorbance of the cultured media were also determined in 600 nm wave length, using a spectrophotometer apparatus in order to inoculate the same dose of bacteria in repeating the experiment. To combine the principles of most-probable-number (MPN) statistics and the conventional PCR technique to enumerate Escherichia coli O157:H7, sterilized milk were used as matrix, and 10^1 to 10^5 ml -1 of the reference strain were inoculated.

MPN-PCR procedure:

Modified MPN dilutions from inoculated milk sample (10, 1, 0.1, 0.01, 0.001 ml) with three replicates per dilution were prepared, using modified trypticase soy broth (m-TSB) containing 20 mg L-1 novobiocin, followed by incubation at 37°C for 24 h, but only three dilutions were used in computing the MPN. In order to performing multiplex-PCR, the DNA extraction were performed from tubes showing visible turbidity, using phenol-chloroform DNA extraction method. For the multiplex PCR, the sequence of the two pairs of primers are shown in Table:1, The flic H7 primers are specific for the flagellar antigen (H7), and rfb O157 primers are specific for somatic antigen (O157) (10,3). The PCR products were subjected to electrophoresis and stained with ethidium bromide and viewed under UV light. The expected size of m-PCR products for rfb O157 and flic H7 genes amplification were 259 and 625 bp, respectively. The turbid tubes which showed the expected size in m-PCR were considered as positive in computing the MPN.

Results and discussion

Considering the detection limit of the modified MPN set whit five dilution and three replicates per each dilution (< 3×10-2 /100 ml to >2.4×105/100 ml), and then performing m-PCR assay from turbid tubes, using specific primers for somatic and flagellar antigens gene, the method could accurately enumerate the inoculated Escherichia coli O157:H7 in milk samples (Fig:1).

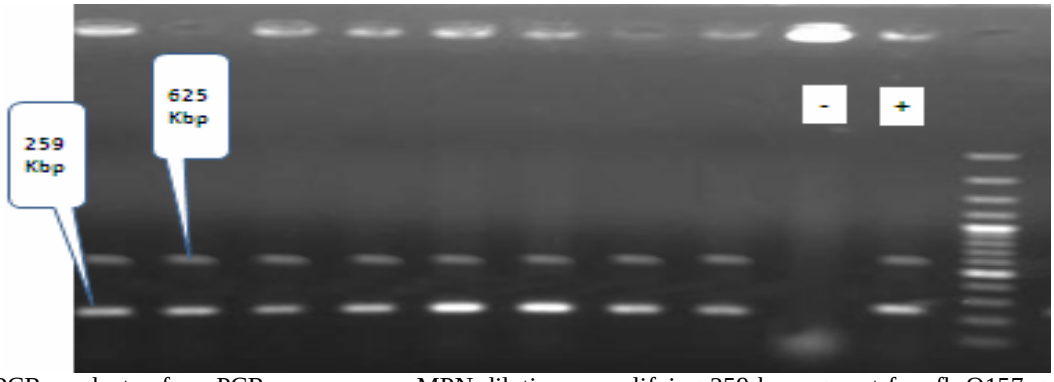

Fig 1: PCR products of m-PCR assay on our MPN dilutions, amplifying 259 bp segment for rfb O157 gene, and 625 bp segment for flic H7 gene. 100 plus bp markers is used.

Quantification of different bacteria has traditionally been carried out by the most-probable-number (MPN) method. The MPN technique consists of an estimation of the density of viable organisms in a sample and is particularly useful for low levels of micro-organisms (≤10–100 MPN g−1). Numerous modern methods have been developed for the quantification of Escherichia coli O157:H7 such as real-time PCR (4). However, these methods are, in general, too expensive and not suitable for routine analysis in many laboratories. PCR detection methods have been extensively used in diagnostic microbiology. Besides it has been reported that the MPN-PCR method may be more convenient and reliable than the MPN-culture method, especially for samples that show many colonies other than the target bacterial colonies. Furthermore, utilization of the PCR technique reduces the time and labor required for the biochemical identification tests used in the MPN-culture method. (7). The combination of MPN method with a species-specific PCR method enables the completion of enumeration within 2 days. The MPN-PCR method could facilitate the enumeration of Escherichia coli O157:H7 in milk samples without the interference of background micro-organisms, because of using specific primers to somatic (O157) and flagellar (H7) antigens gene. The use of m-TSB broth as the culture medium avoids the most undesirable micro-organisms and permits the growth of Escherichia coli O157:H7. Although PCR appears to be the most sensitive and rapid option, but it should be noticed that some food or enrichment medium components can inhibit the reaction. Inhibition of PCR may be overcome by dilution, centrifugation, filtration of the sample, aqueous two-phase systems, adsorption methods, DNA extraction (5), Chelex or EGTA treatment of the sample (2).
In this study for detection and enumeration of Escherichia coli O157:H7 serotype, we used specific primers. Considering the presence of different background microorganisms the method showed the specificity of 100% and it's sensitivity determined as 10 cfu-ml -1. It seems that the MPN-PCR method, using the specified primers, is a convenient and reliable method for enumeration of Escherichia coli O157:H7 in milk samples, and could be considered as an alternative to MPN- culture techniques.

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شمارش باکتری اشریشیا کولای سویه O157:H7 در شیر با استفاده از روش MPN-PCR

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چکیده

باکتری اشریشیا کولای سویهO157:H7 یک باکتری شدیدا بیماریزا با دوز عفونی پایین (در حدود ۱۰ سلول باکتری) می باشد، که از طریق مصرف مواد غذائی به انسان منتقل می گردد و شمارش باکتری در مواد غذائی از اهمیت ویژه برخوردار می باشد. در این بررسی جهت کاربرد توام روش MPN و روش PCR برای شمارش باکتری اشریشیا کولای O157:H7 در شیر تعداد 10^{1}-10^{5} سلول باکتری در محیط شیر پاستوریزه تلقیح گردید. میکرو ارگانیسم های دیگر به عنوان زمینه شامل انواع باکتریهای گرم مثبت و منفی نیز به شیر تلقیح گردید. شمارش باکتری با استفاده از روش سه لوله ای و رقتهای ۱۰،۱،۰,۱، ۰,۰۱، ۰,۰۰۱ در لوله های حاوی محیط m-TSB (دارای نوبیوسین) صورت گرفت. سپس از لوله های کدر استخراج DNA به عمل آمد و با استفاده از پرایمر های اختصاصی ژن های تولید کننده آنتی ژن های O157 و H7 تست PCR صورت گرفت. نتایج بدست آمده نشان داد، روش مورد استفاده دارای حساسیت و ویژگی بالا می باشد و می تواند به عنوان روش جایگزین روشهای مرسوم بکار رود.

واژه‌های کلیدی: اشریشا کولای O157:H7، شیر, MPN-PCR ، شمارش .