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Full Length Research Paper

Quantification of *Escherichia coli* O157:H7 in milk by most probable number –polymerase chain reaction (MPN-PCR) method

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***Escherichia coli* O157:H7 has been recognized as one of the most important causes of potentially life-threatening human diseases such as haemorrhagic colitis, haemolytic ureamic syndrome and thrombotic thrombocytopenic purpura. To combine the principles of most-probable-number (MPN) statistics and the conventional polymerase chain reaction (PCR) technique to enumerate *E. coli* O157:H7 in milk, from 10^1 to 10^5 ml^{-1} of bacterial cells were inoculated in sterilized milk. Different background microorganisms including Gram positive and Gram negative bacteria were also inoculated. Modified MPN dilutions from inoculated milk sample with three replicates per dilution were prepared and enumeration was performed by DNA extraction from tubes showing turbidity and performing multiplex-PCR (m-PCR) using primers specific for O157 and H7 antigens gene. This MPN-PCR proved to be a rapid and reliable method for enumerating *E. coli* O157:H7 in milk at the lowest level (10^1 $cfu.ml^{-1}$), even in the presence of different Gram positive and Gram negative background microorganisms. It may facilitate the enumeration of *E. coli* O157:H7 for routine analyses in milk without excessive work and could be considered as an alternative to MPN- culture techniques.**

Key words: *E. coli* O157:H7, milk, most probable number-polymerase chain reaction (MPN-PCR), most probable number (MPN) - culture, multiplex- polymerase chain reaction (m-PCR).

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) strains are the most important recently emerged food-borne pathogens (Armstrong et al., 1996). VTEC may belong to many serotypes, but most severe human infections are caused by strains of *E. coli* O157:H7 (Mead and Griffin, 1998). *E. coli* O157:H7 causes haemorrhagic colitis, hemolytic-ureamic syndrome and thrombotic thrombocytopenic purpura (Zhao et al., 1998).

Cattle frequently excrete these bacteria in their feces (Molina et al., 2003; Van Donkersgoed et al., 1999). Since dairy cattle are asymptomatic carriers (Heuvelink et al., 1998) of *E. coli* O157:H7, milk products are thought to be risky foods. Therefore insufficient heat-treatment of raw milk forms a potential infection risk (Betts, 2000). Cheese made with unpasteurised milk is potential vehicle for transmission of *E. coli* O157:H7 to the consumers (Oksuz et al., 2004).

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 (Oberst et al., 1998). 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 (Kimura et al., 2000). Molecular approaches have also been practiced. In this regards, polymerase chain reaction (PCR) assays based on the presence or absence of specific genes such as the *stx*, *eaeA* and *hlyA* have been described (Kimura et al., 2000; Pilpott and Ebel, 2003).

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Table 1. Primers and predicted product size for *Escherichia coli* O157: H7.

Target gene	Primer sequence(name)	Predicted product size(bp)
rfbO157	F: 5′- CGG ACA TCC ATG TGA TAT GG -3′ R: 5′- TTG CCT ATG TAC AGC TAA TCC -3′	259
flicH7	F: 5′- GCG CTG TCG AGT TCT ATC GAG-3′ R: 5′- CAA CGG TGA CTT TAT CGC CAT TCC-3′	625

However, most of them require a selective enrichment step and consequently, absence or presence can be determined, such results, are meaningless in terms of quantification (Hussein et al., 2002).

Most probable number- polymerase chain reaction (MPN-PCR) method have been described previously for enumeration of micro-organisms in soil (Fredslund et al., 2001), water (Savill et al., 2001) and foods (Mantynen et al., 1997; Miwa et al., 1999; Jeyaletchumi et al., 2010). Overall, this method was found to be a rapid and reliable method that could facilitate the enumeration of different micro-organisms.

In the present study, we enumerated the inoculated *E. 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

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 (Jenway 6105, Essex, England), 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 *E. coli* O157:H7, sterilized milk were used as matrix, and 10^1 to 10^5 cells-ml $^{-1}$ of the reference strain were inoculated.

A variety of Gram positive and Gram negative background microorganisms including: *Staphylococcus aureus* (ATCC-25923), *Bacillus cereus* (ATCC- 10876), *Clostridium perfringenes* (ATCC-13124), *Listeria monocytogenes* (ATCC- 7644), *Salmonella typhimurium* (ATCC- 14028) and *Campylobacter jejuni* (ATCC-33291) were grown in appropriate broth media and number of 10^3 bacterial cells were inoculated. Enumeration of background microorganisms were performed the same way as for reference strain.

MPN-PCR procedure

Amount of 10, 1, 0.1, 0.01 and 0.001 ml of each milk sample containing 10^1 to 10^5 cells-ml $^{-1}$ of *E. coli O157:H7* and 10^3 cells-ml $^{-1}$ of background microorganisms with three replicates per dilution were inoculated in tubes containing 10 ml of modified trypticase soy broth (m-TSB) with 20 mgL^{-1} novobiocin, double strength medium was prepared for testing 10 ml quantities of samples. Inoculated tubes were incubated at 37°C for 24 h. In order to obtain the most reliable results, three consecutive dilutions which were positive at lower dilutions and negative at higher dilutions were selected for computing the MPN.

In order to perform multiplex-PCR, the DNA extraction were performed from tubes showing visible turbidity, using phenol-chloroform DNA extraction method (Simon et al., 1996). 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* 0157 primers are specific for somatic antigen (O157) (Pilpott and Ebel, 2003; Desmarchier et al., 1998).

The m-PCR reaction were performed in a 25 µl amplification mixture consisting of 2.5 µl of 10× PCR buffer (500 mM KCl, 200 mM Tris HCl), 0.5 µl dNTPs (10 mM), 1 µl Mg Cl_2 (50 mM), 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 s, annealing at 52°C for 30 s and elongation at 72°C for 60 s, and final extension at 72°C for 10 min. 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, respectively. The turbid tubes which showed the expected size in m-PCR were considered as positive in computing the MPN.

RESULTS

In order to enumerate the inoculated *E. coli* O157:H7 in sterilized milk samples, the modified MPN method whit five dilution and three replicates per each dilution with the detection limit of < 3×10^{-2} /100 ml to >2.4×10^5/100 ml were used, then the DNA extraction and m-PCR assay from turbid tubes were performed using specific primers for somatic and flagellar antigens gene. The method

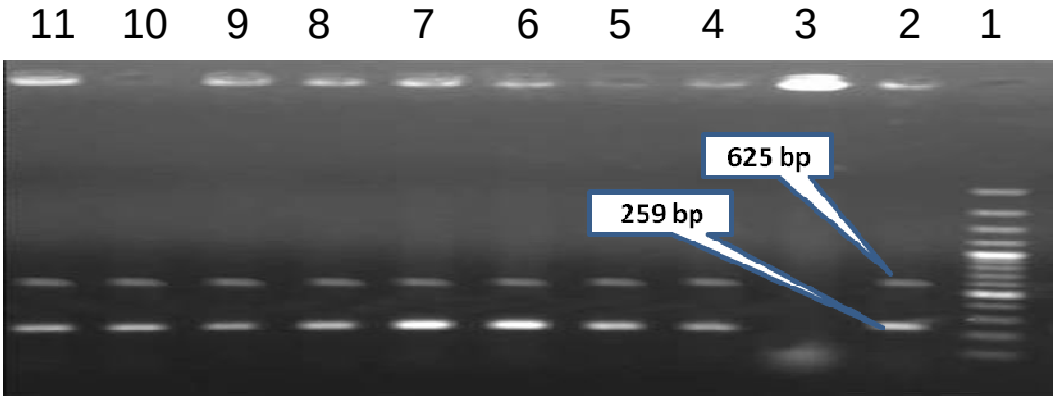

Figure 1. Results of the PCR assay, amplifying 259 base pair segment of *rfbO157* and 625 base pair of *flicH7* gene of *E. coli* O157: H7. Lane 1: 100bp marker. Lane2: positive control. Lane 3: negative control. Lanes 4-11: positive samples.

could accurately enumerate the inoculated *E. coli O157:H7* in sterilized milk samples at the lowest level (10^1 cfu.ml^1), even in the presence of different Gram positive and Gram negative background microorganisms (Figure 1). The sensitivity of the method determined as enumeration of 10 cfu.ml $^{-1}$ and it's specificity as 100%.

DISCUSSION

Numerous modern methods have been developed for the quantification of *E. coli* O157:H7 such as real-time PCR (Ibekwe and Grieve, 2003) and flow cytometry (Hussein et al., 2002). 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. However, a lack of a simple and reliable method for quantification of the PCR products has partly hindered the use of PCR in routine food laboratories. The quantification of PCR products can be done by combining the principles of MPN statistics and PCR technique (Mantynen et al., 1997). It seems that direct plating of tubes with turbidity on CT-SMAC agar (MPN-culture method) is not an appropriate method for the enumeration of *E. coli* O157:H7 in milk samples, since the plates may be covered by a slime of other micro-organisms.

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 (Miwa et al., 2003).

The major inconveniences of the MPN method coupled with traditional confirmation techniques are the amount of material, the workload, and above all, the time needed to complete identification (4 to 5 days). However using chromagar could be cost-effective and reduces the number of some biochemical and serological tests (Church et al., 2007). 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 *E. 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 *E. 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, substances which have been proved to be PCR inhibitory such as calcium ions in milk (Bickley et al., 1996), and proteinases (Powell et al., 1994), haem compounds (Akane et al., 1994), chelating agents, and proteins may be present in other foods (Rossen et al., 1992; Aymerich et al., 2003). Inhibition of PCR may be overcome by sample preparation using dilution, centrifugation, filtration, aqueous two-phase systems, adsorption methods, DNA extraction (Lantz et al., 1994), and Chelex or EGTA treatment of the sample (Bickley et al., 1996).

Picozzi et al. (2004) reported that MPN-PCR showed values of relative accuracy, sensitivity and specificity of, respectively, 85.8, 81.6 and 93.5% in enumeration of *E. coli* in minced meat, using primers specific for the *uidA* gene. In this study for detection and enumeration of *E. 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 enumeration of 10 cfu.ml $^{-1}$ (the lowest level of inoculation). It seems that the MPN-PCR method, using the specified primers, is a convenient and reliable method for enumeration of *E. coli* O157:H7 in milk samples, and could be considered as an

alternative to MPN- culture techniques.

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