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QUANTIFICATION OF *LISTERIA MONOCYTOGENES* IN MILK BY MPN-PCR AND MPN-CULTURE METHODS

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ABSTRACT

The aim of this study was to compare the MPN-PCR (Most Probable Number- Polymerase Chain Reaction) and MPN-Culture methods in enumerating of Listeria monocytogenes in milk. In order to compare the accuracy of these methods, 10^3 cell/ml Listeria monocytogenes and different background bacteria which may be present in raw milk, were inoculated in sterilized milk. After preparing serial dilutions, three replicates per dilution were inoculated in tubes containing listeria enrichment broth. After 48 hours of incubation, for MPN-Culture three inoculated replicates were subcultured on Oxford agar and suspected colonies were confirmed by performing by biochemical tests.
For MPN-PCR assay, the DNA extraction was performed from the three inoculated replicates which were already used for MPN-Culture and PCR assay was performed using primers specific for *Listeria monocytogenes*.
The experiment was repeated three times and the average of enumerated bacteria was calculated by each method separately.
Statistical analysis using one sample Wilcoxon signed rank test showed that enumeration by MPN-PCR method was more accurate than enumeration by MPN-Culture method.
The result of this study showed that MPN-PCR method in comparision with MPN-Culture even in the presence of different background microorganisms is more rapid and reliable. It is concluded that MPN-PCR method facilitates the enumeration of *Listeria monocytogenes* without excessive work and could be considered as an alternative to MPN-Culture technique.

Keywords: MPN-PCR, MPN-Culture, *Listeria monocytogenes*, milk

INTRODUCTION

Among the genus of *Listeria*, which causes the infection of listeriosis in both animals and humans, *Listeria monocytogenes* is a major pathogenic microorganism **(Jami *et al.*, 2010)**. This organism is an important opportunistic food-borne pathogen and it can cause severe problems, especially in pregnant women, neonates, the elderly and immunocompromised individuals **(Jeyaletchumi *et al.*, 2010)**.
Food is the most important source of infection, although other ways of transmission exist **(Martin *et al.*, 2004)**. The reported incidence of human cases is low compared with that for other foodborne bacterial pathogens, such as *campylobacter* and *salmonella*, but the mortality rate in human listeriosis is the highest among human food-borne bacterial pathogens **(Gyles *et al.*, 2010)**.
Several outbreaks of listeriosis were proven to be associated with consumption of contaminated milk and are causing great concern in the dairy industry due to the number of cases and the nearly 30% overall mortality rate of these outbreaks **(Jami *et al.*, 2010)**. There is a hypothesize that cells of *L. monocytogenes* enter a dormant, long-term-survival (LTS) phase and become more barotolerant and thermotolerant due to cytoplasmic condensation during their transition from rods to cocci **(Wen *et al.*, 2009)** and can rapidly resume metabolic activities and transit back to log phase with decreased baro- and thermotolerance **(Wen *et al.*, 2011)**. *L. monocytogenes* may be present in food processing plants in a resistant long-term-survival state, especially inside hard-to-clean harborage sites or biofilms and can contaminate a wide variety of raw and ready-to-eat foods (**Tompkin, 2002; Gandhi and Chikindas, 2007**). Some environmental factors may affect the transition of *L. monocytogenes* to the LTS phase, including cell density and pH **(Wen *et al.*, 2013)**. The growing importance of *L.monocytogenes* on a global scale has prompted improves analytical procedures for the detection and enumeration of this pathogen in foods **(Jeyaletchumi *et al.*, 2010)**.
The specific identification of *L.monocytogenes* based on culture and biochemical methods is laborious and time consuming and in food products requires up to a week for species identification according to the international organization for standardization. Therefore variety of faster techniques have been applied including nucleic acid amplification, immunoassay based methods, immune-latex agglutination based methods, isothermal nucleic acid amplification and PCR **(Martin *et al.*, 2004)**. The quantification of PCR products can be done by combining the principles of MPN statistics and PCR techniques **(Jamshidi *et al.*, 2011)**. The MPN technique consists of an estimation of the density of viable organisms in a sample and is particularly useful for low levels of microorganisms **(Martin *et al.*, 2004)**.
The aim of this study was to compare MPN-PCR and MPN-Culture methods in enumerating *L.monocytogenes* in milk.

MATERIAL AND METHODS

Inoculation

Pure cultures of *Listeria monocytogenes* (ATCC-7644) was prepared by subculturing the test strain on Oxford agar (Merck, Darmstadt, Germany), following incubation at 30°C for 48 h and background microorganisms including *Lactobacillus*, *Streptococcusa galactiae*, *Salmonella typhimurium*, *Micrococcus loteus*, *Eschrchia coli*, *Bacillus cereous*, *Staphylococcus aureous* were prepared by subculturing on Brain heart agar (Merck, Darmstadt, Germany), following incubation at 37°C for 24 h.
In order to inoculate the same dose of bacteria for each bacterium a suspension with concentration of 0.5 Mcfarland was prepared and its concentration of these suspensions was determined in 600 nm wave length using a spectrophotometer apparatus (Jenway 6105, Essex, England).
To combine the principles of most probable number (MPN) statistics and the conventional cultural technique and also PCR method to enumerate *L. monocytogenes*, sterilized milk was used as matrix, and 10^3 cells ml^{-1} of the reference strain and background microorganisms were inoculated.
Serial dilutions of 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared from inoculated milk. From each dilution amount of 1 ml was inoculated in listeria enrichment broth (LEB) in 3 replicates. Inoculated tubes were incubated at 30°C for 48 h. in order to obtain the most reliable results, three consecutive dilutions which were positive (turbid) at lower dilutions and negative at higher dilutions were selected for computing the MPN.

MPN-Culture procedure

In order to perform MPN-Culture, 0.1 ml from each dilutions (10^{-3}, 10^{-4}, 10^{-5}) was surface plated on Oxford agar and incubated at 30°C for 48 h. For confirmation of suspected colonies as L. monocytogenes on agar plates, morphological and biochemical tests, including gram staining, catalase, oxidase and motility test were performed.

MPN-PCR procedure

For MPN-PCR assay, the DNA extraction was performed from the three replicates which were already used for MPN-Culture. The tubes were incubated at 100°C for 10 min, and then centrifuged at 15000 rpm for 15 minutes. The supernatants were transferred to sterile nuclease free microtubes and freezed at -18 °C until use **(Bansal *et al.*, 1996)**.
The sequence of primers was as follow: (F) GAT ACA GAA ACA TCG GTT GGC and (R) GTG TAA TCT TGA TGC CAT CAG. These primers are specific for the *prf* A gene of *monocytogenes* which amplifies a 274 bp product.
The PCR reaction was performed in an amplification mixture consisting of 2.5 µl of 10x PCR buffer (500 mMKCl, 200 mMTrisHCl), 0.5 µl dNTPs (10 mM), 1 µl Mg Cl_2 (50 mM), 1.25 µl od each primer, 0.3 µl of Taq DNA polymerase (5 unit/µl) and 3 µl of extracted DNA and deionized water to make a final volume of 25 µl.The reaction mixture was amplified in a thermocycler (Bio-radiCycler) using the following PCR conditions: initial incubation at 94ºC for 5 min, denaturation at 94ºC for 45 sec, annealing at 54ºC for 30 sec and extension at 72ºC for 1 min, and final extension at 72ºC for 5 min. The PCR product was seprated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in Tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transllluminator and documented by a gel documentation apparatus. 100 bp DNA ladder was used as a size reference for PCR assay. The tubes with expected size in PCR were considered as positive in computing the MPN.

Statistical analysis

One sample Wilcoxon signed rank test was used to compare medians of inoculated bacteria estimated by each method, with reference value. Statistical analysis was performed using SPSS software (version 21).

RESULTS

In this study, enumerating of inoculated *L. monocytogenes* in milk was performed using two methods.The experiments were repeated three times and the average of enumerated bacteria were calculated by each method separately.The conventional MPN-Culture procedure was performed using culturing in tubes containing listeria enrichment broth (LEB) and then plating on supplemented Oxford agar and finally morphological and biochemical tests. To perform the MPN-PCR assay the DNA was extracted from the same turbid tubes, and the PCR assay was performed using *LM lip1* primers that amplify a 274 bp fragment of the *prf* A gene of *L. monocytogenes*. The average number of enumerated by MPN-Culture method was equal to 3.4×10^2 cell ml^{-1} and the average number of enumerated by MPN-PCR method was equal to 1.3×10^3 cell ml^{-1}, whereas the amount of inoculation was 1×10^3 cell ml^{-1} (Fig 1).

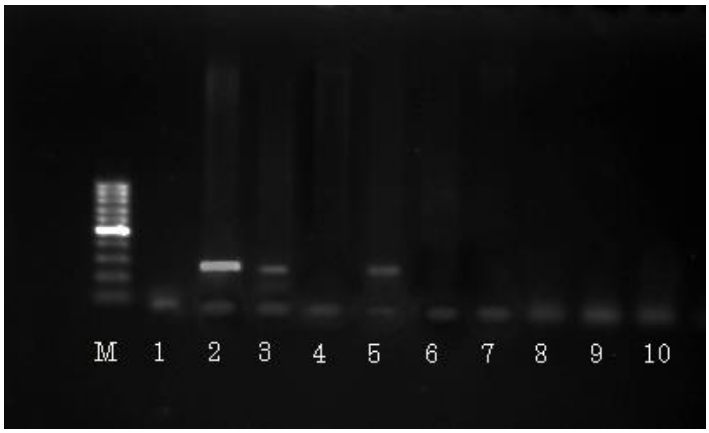

Figure 1 Results of the PCR assay, amplifying 274 base pair segment of *prf* A gene of *L. monocytogenes*.
Lane M: 100bp marker. Lane 1: negative control. Lane 2, 3 and 4from10^{-3} dilutions. Lane 5, 6 and 7 from 10^{-4} dilutions. Lane 8, 9 and 10 from 10^{-5} dilutions.

Statistical analysis at p=0.1 level, showed significant difference between the number of inoculated bacteria and estimated medians obtained by MPN-culture method, whereas there was no significant difference between the number of inoculated bacteria and medians obtained by MPN-PCR method.

DISCUSSION

L. monocytogenes is the cause of the many sporadic and epidemic foodborne diseases in humans **(Aguado *et al.*, 2004).** To investigate the source or route of infection, it is important to know which types of foods are contaminated with *L. monocytogenes* and also its frequency and dose **(Okutani *et al.*, 2004)**. Since the number of required bacteria to cause infection is unknown, the ability to detect the organism in low numbers in food is essential **(Churchill *et al.*, 2006)**.
Rapid detection and enumeration of pathogenic Listeria spp. in foods using conventional culture method is laborious and time-consuming. The recovery of low numbers of *L. monocytogenes* from foods and environmentalsamples requires the use of enrichment cultures followed by selective plating and, where injured organisms are likely to be present, a pre-enrichment step is also necessary **(Reissbrodt, 2004)**. In contrast, PCR-based techniques allow for the identification of microorganisms regardless of their culturability **(Jeyaletchumi *et al.*, 2010)**. In this study some dilutions that were unable to form colonies on the selective media, were recognized as positive in the PCR assay. This indicates that molecular methods have more sensitivity in detecting the *L. monocytogenes* in foods. Viable but non-culturable (VBNC) cells present a major public health problem since they are unable to be detected by conventional culture methods and remain potentially pathogenic under favorable conditions **(Marian *et al.*, 2012)**.
Luan *et al.* (2008) reported that the sensitivity of direct PCR was about 100 times lower than that of MPN-PCR, They showed that during subculture of enriched cultures of *Vibrio parahaemolyticus* on TCBS agar, the target cells did not produce isolated colonies because of the background of other bacteria, including other *Vibrio* species. It has been demonstrated that PCR can amplify DNA from dead cells, but MPN-PCR overcame this limitation and realized a rapid quantitative detection of live target bacteria. The MPN-PCR is a useful enumeration method because of its rapidity and sensitivity, and both of which are critical to any assay for the detection of any bacterium **(Luan *et al.*, 2008)**.
In this study the duration time for detection and enumeration of target organism by MPN-PCR method was two days, while it was 5 days in MPN-Culture method.
Martin *et al.* (2004) has been reported that initial counts could only be determined by MPN-PCR, as interfering bacteria were highly abundant in the plating technique, making a reliable enumeration impossible. The MPN-PCR method facilitated the enumeration of *L. monocytogenes* in chorizo without the interference of background micro-organisms and the MPN-PCR method may be a more reliable method, when interfering bacteria are highly abundant in the samples **(Martin *et al.*, 2004)**. 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)**.
Jamshidi *et al.* (2011) used the MPN-PCR method in enumerating of *escherchia coli* in milk. This MPN-PCR proved to be a rapid and reliable method for enumerating this microorganism in milk at the lowest level, even in the presence of different Gram positive and Gram negative background microorganisms **(Jamshidi *et al.*, 2011)**.
Picozzi *et al.* (2004) also reported that MPN-PCR is a reliable method for low concentrations of microorganisms **(Picozzi *et al.*, 2004)**.
The MPN-PCR applications which has been described for soil microorganisms have revealed that soil contains components and materials which inhibit PCR, and careful purification steps are needed **(Mantynen *et al.*, 1997)**. Also it should be noticed that some food or enrichment medium components can inhibit the PCR reaction **(Amagliani *et al.*, 2007; Jamshidi *et al.*, 2011)**. Substances such as calcium ions in milk and proteinases, haem compounds, chelating agents, and proteins have been proved to be PCR inhibitor **(Jamshidi *et al.*, 2011)**. Therefore, quality and purity of extracted nucleic acids are primary requirements for a PCR-based detection assay and the selection of a proper extraction method is essential for a successful and valid PCR analysis **(Amagliani *et al.*, 2007)**. Inhibition of PCR may be overcome by sample preparation using dilution, centrifugation, filtration, aqueous two-phase systems, adsorption methods **(Lantz *et al.*, 1994)** and chelex or EGTA treatment **(Bickley *et al.*, 1996)**.

CONCLUSION

In the present study bacterial quantification, using MPN-PCR method was more accurate than MPN-Culture method, even in the presence of different background microorganisms.
Overall it seems that the MPN-PCR method, using the specific primers is a convenient and reliable method for enumeration of *L. monocytogenes* in milk and could be considered as an alternative to MPN-Culture techniques.

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