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Full Length Research Paper

Monitoring paneer for *Listeria monocytogenes*- A high risk food pathogen by multiplex PCR

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A multiplex polymerase chain reaction (PCR) assay was developed and applied to spiked and natural paneer samples to detect *Listeria monocytogenes*, a high risk food pathogen. The sensitivity of the assay on *L. monocytogenes* spiked paneer samples was 10^4 cells prior to enrichment, was improved to 10^3 cells after 4 h enrichment and one to 10 cells after 6 h enrichment as indicated by the amplification of 1.2 kb and 713 bp PCR products. The multiplex PCR assay when applied to 10 randomly sampled natural paneer products did not reveal any amplification products, indicating the absence of this organism from these samples.

Key words: *Listeria monocytogenes*, multiplex PCR, 16S rRNA, hemolysin, paneer products.

INTRODUCTION

Paneer is an extremely popular and delicious, indigenous acid coagulated dairy product, extensively used in a variety of preparations in almost every household in India and other parts of the subcontinent. It is also called as vegetarian's meat and hence could be an attractive alternative to non-vegetarian diet because of its high nutritive value. Paneer is highly nutritious due to high protein content, fats, vitamins and soluble minerals like calcium and phosphorus. Though paneer is rich in food value, it is extremely prone to contamination by different microorganisms due to adequate moisture and relatively high humid and temperature conditions prevailing in India. The unhygienic conditions under which product is manufactured and marketed by halwais in India is far from satisfactory. Hence, the product is susceptible to contamination by various microorganisms including potential pathogens like *Salmonella* sp., *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Among these pathogens, *L. monocytogenes* is considered as a high risk food pathogen as it may cause serious health problems associated with listeriosis in human beings. *L. monocytogenes* can gain access into highly perishable and vulnerable products from various sources.

In this regard, there are scanty of reports available in India. The one such report available in India regarding the incidence of *Listeria* sp. (25%) and *L. monocytogenes (*10%) in paneer samples analysed by Lister test kit came from a small study carried out at Molecular Biology Unit, National Dairy Research Institute (NDRI), Karnal by Grover et al. (1993; Personal communication). The outcome of the study does not undermine the seriousness of the problem and possible implication of paneer with this pathogen. This is particularly important because the occurrence of *L. monocytogenes* in dairy foods in India has been underreported chiefly due to lack of systematic studies and cumbersome isolation protocols currrently in use. However, polymerase chain reaction (PCR) technique is a very popular tool for diagnosis of serious foodborne illnesses and can promptly detect the aetiological agents of these illnesses. PCR has also been explored for detection of *L. monocytogenes*.

In this context, different PCR formats have been developed by different investigators (Cooray et al., 1994; Bubert et al., 1999; O'connor et al., 2000) for detection of *L. monocytogenes* by targeting different genes and have been applied in dairy foods including soft cheeses (Fitter et al., 1992; Makino et al., 1995). In this study, we explored the possibilty of applying a multiplex PCR technique developed in our laboratory for rapid detection

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of *L. monocytogenes* in paneer samples to assess their safety for human consumption.

MATERIALS AND METHODS

Bacterial cultures and their maintenance

L. monocytogenes strains used in this study included *L. monocytogenes* ATCC 7644 which was purchased from Oxoid Culture Media (HIMEDIA) and *L. monocytogenes* Scott A procured from DM Division, NDRI, Karnal. These strains were propagated in brain heart infusion (BHI) broth at 37°C for 18 h, preserved on BHI agar slants and stored in refrigerator or as glycerol stocks stored at –70°C until further use. The cultures were activated in BHI broth prior to use by sub-culturing at biweekly intervals.

Preparation of template DNA

Broth cultures

The template/genomic DNA was prepared from broth cultures of *L. monocytogenes* by following boiled lysate method (Witham et al., 1996). The boiled lysate was prepared by harvesting the overnight grown culture of the test organism followed by heating the bacterial suspension in 50 µL milliQ water for 5 min in a boiling water bath and then centrifuging for 5 min at 10,000 rpm to separate the supernatant containing DNA.

Spiked paneer samples

Fresh paneer samples obtained from Experimental Dairy, NDRI, Karnal, were used in this study. An aliquot of 11 g of paneer sample was homogenized using 99 ml of 2% sodium citrate solution as diluent in a flame sterilised pestle and mortar and dispensed in 1 ml aliquots each in sterilized Eppendorf tubes. The aliquots of paneer samples were separately inoculated with *L. monocytogenes* at different levels ranging from 10^7 to 10 cfu/ml. The DNA was then extracted from the spiked samples before and after enrichment in Listeria enrichment broth (LEB), Oxoid (peptone, 23.4 g/L; yeast extract, 5.0 g/L; lithium chloride, 10.0 g/L; ferrric ammonium citrate, 0.1 g/L; sodium chloride, 5.0 g/L; pH, 7.4) for 4 and 6 h at 37°C by following the NDRI method developed previously in our laboratory (Suresh, 1999). In brief, the steps used in the method included solvent treatment in order to remove the fat layer from the paneer samples. One milliliter aliquots from the samples were then centrifuged and the pellet dissolved in 400 µL of guanidine thiocyanate (GTC) buffer and 400 µL of phenol saturated with Tris EDTA (TE) pH 8.0. Chloroform extraction was then carried out. The samples were centrifuged and aqueous phase transferred to a new tube and the DNA was precipitated by adding one volume of isopropanol or two volumes of ethanol. The DNA was pelleted, dried and dissolved in TER buffer containing 10 µg / ml of RNaseA.

Natural paneer samples

The paneer samples were collected from the local market. The samples were first pre-enriched for 6 h in Listeria enrichment broth. Template DNA was extracted from 10 ml aliquots of paneer samples before and after enrichment in LEB using the above described 'NDRI' method. The samples were also analysed microbiologically using LEB agar for typical *L. monocytogenes* colonies.

Multiplex PCR assay

The PCR amplification for detection of *L. monocytogenes* was performed using Eppendorf Master Cycler Gradient, 5331, Germany. The selected oligonucleotide primers for detection of *L. monocytogenes* were gotten custom synthesised (Bangalore Genei, India). The description of the primer pairs namely Lm3 and Lm5 targeting 16S rRNA gene (Wiedmann et al., 1993) and ELMHLYF and ELMHLYR targeting hemolysin gene (Klein and Juneja,1997) used in this study is given in Table 1. The PCR assay was performed in 25 µL reaction mixture comprising of 100 ng of template DNA, 1X PCR buffer, 0.2 mM (each of primers), 0.2 mM (each) dNTPs and 1 unit of Taq DNA polymerase (Boehringer Mannheim). Appropriate positive and negative controls with each reaction were also set up. The PCR parametres included initial denaturation at 95°C for 4 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min.

Analysis of PCR products

The PCR amplified products were electrophoresed on 2% agarose gel containig 0.5 µg/ml of ethidium bromide. The gel was visualised under UV transilluminator and photographed using Polaroid 667 packfilm with MP4 system polaroid camera (Photodyne, USA). The molecular size marker (100 bp DNA ladder) was also run on the gel to monitor the size of the amplified PCR products.

RESULTS AND DISCUSSION

Due to the process of globalization recently being witnessed, the demand for indigenous dairy products like paneer is growing tremendously for local consumption and possible export avenues. In the light of this changing scenario, the microbiological quality and safety of this highly perishable dairy product have assumed added significance. In view the scope and potential market of paneer in our country, it is imperative that this soft cheese like indigenous dairy product should confirm to high microbiological standards particularly in relation to high risk food pathogens like *E. coli* O157:H7 and *L. monocytogenes*. This requires rigorous monitoring of paneer for the potential food pathogens to completely rule out any risk to the health of the consumers.

Since *L. monocytogenes* was previously detected from paneer, in this study we targeted this organism for developing a DNA based method for its rapid detection in paneer. In this context, a multiplex PCR assay was established in our laboratory by targeting 16S rRNA (Listeria specific) and *'hlyA'* (*L. monocytogenes* specific) genes for rapid detection of *L. monocytogenes* in dairy foods. The authenticity of multiplex PCR assay was established by nested PCR using an internal set of primers located within the amplified product of *'hlyA'* gene. The assay was also found to be reliable in terms of its analytical specificity and sensitivity. Hence, initially we tested the efficacy of multiplex PCR assay in terms of sensitivity in paneer samples after spiking with different levels of *L. monocytogenes* cells. The results are shown in Figure 1. The PCR amplified products of

Table 1. Description of the primers used in the present investigation.

Target gene	Primer	Primer sequence	Size of amplified product (bp)	Reference
16S rRNA	Lm 3 Lm 5	5′-ggA CCg ggg CTA ATA CCg AAT gAT AA-3′ 5′-TTC ATg TAg gCg AgT TgC AgC CTA – 3′	1200	Wiedmann et al., 1993
Hemolysin	ELMHLYF ELMHLR	5′ – TCC gCC TgC AAg TCC TAA gA – 3′ 5′ – gCg CTT gCA ACT gCT CTT TA– 3′	713	Klein and Juneja, 1997

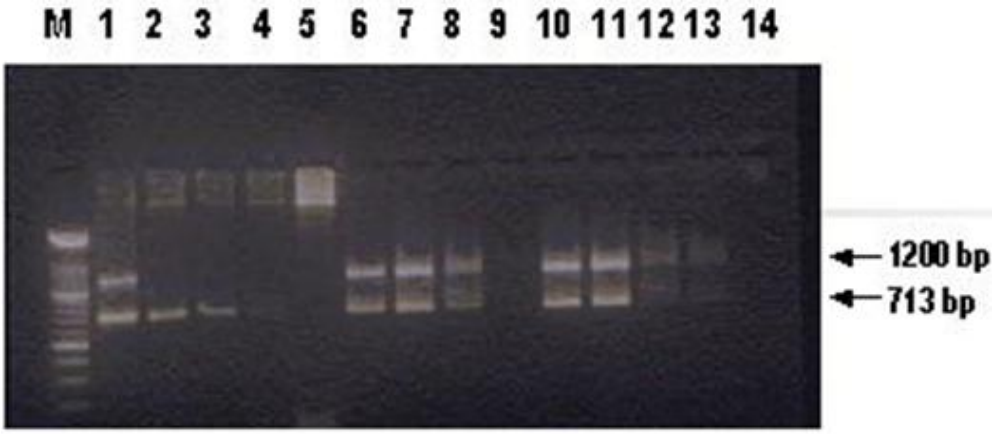

Figure 1. Sensitivity of multiplex PCR assay for detection of *L. monocytogenes* in paneer inoculated with different levels of target cells. Lanes: M, 100 bp marker; 1 to 5 (cfu/ml before enrichment): 1, 10^7; 2, 10^6; 3, 10^5; 4, 10^4; 5, 10^3; 6 to 9: (cfu/ml, 4 h enrichment): 6, 10^5; 7, 10^4; 8, 1000; 9, 100; 10 to 12 (cfu/ml, 6 h enrichment): 10, 10^3; 11, 10^2; 12, 10; 13, 1; 14, negative control. PCR, Polymerase chain reaction.

1.2 kb (16S rRNA) and 713 bp (*'hlyA'*) could be detected when template was used from samples spiked with 10^7, 10^6, 10^5 and 10^4 cells before enrichment indicating the sensitivity of multiplex PCR assay in paneer was limited to 10^4 cells (Figure 1, Lanes 1 to 4).

However, the intensity of 1.2 kb band is low in the gel picture at 10^6, 10^5, 10^4 cells (Figure 1, Lanes 2 to 4) although both the bands were clearly discernible on the agarose gel. After a 4-h enrichment in listeria enrichment broth, the sensitivity of the assay improved to 10^3 cells (Figure 1, Lane 8) and after a 6-h enrichment as low as 10 to 100 cells (Figure 1, Lanes 12 and 13) could be detected. Since there is not enough report available on application of PCR based assay on paneer samples, we cannot substantiate our findings on sensitivity of multiplex PCR in paneer samples. However, we can corroborate our findings by comparing with similar findings of other investigators carried out on soft cheese because of close proximity between the two products. Previously, Fitter et al. (1992) detected a minimum level of 10 to 100 cfu/g from spiked soft cheese samples after overnight enrichment by their PCR assay. Our findings are further substantiated by Makino et al. (1995) who detected a minimum level of 10^3 cfu per 0.5 g of soft cheese by targeting Listeriolysin 'O' gene in their PCR assay. Based on these results, the high level of sensitivity of our multiplex PCR assay in paneer may be attributed to more homogenous nature of the product compared to soft cheese, which may interfere in the recovery of template DNA. The efficient extraction protocol used in our study for isolation of template DNA from paneer samples as has been reported previously (Suresh,1999).

Application of multiplex PCR in naturally contaminated paneer samples

Since paneer could be a potential source of *L. monocytogenes* contamination as indicated from a previous study from our laboratory, attempts were also made in this study to monitor some paneer samples collected from local market for *L. monocytogenes* with our multiplex PCR assay. A total of 10 paneer samples were analysed and the results pertaining to the amplification of template extracted from these samples

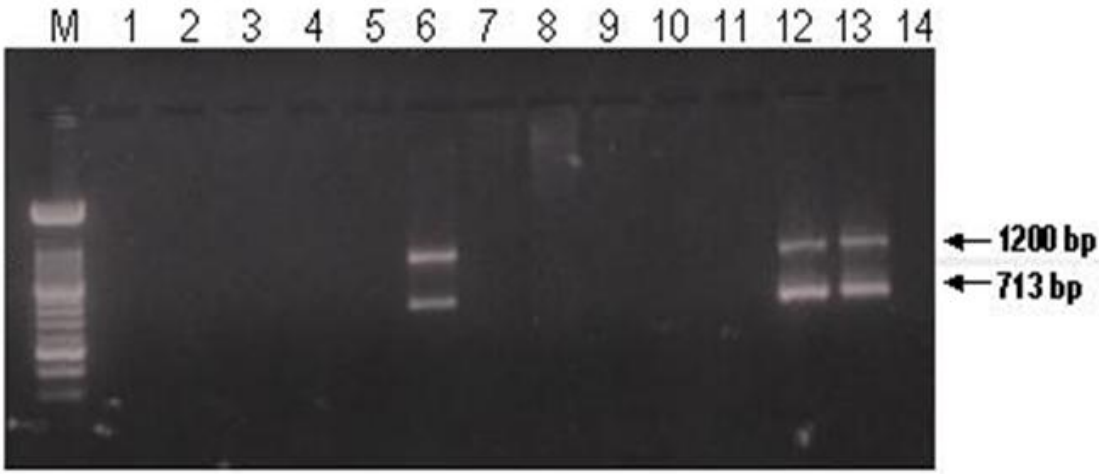

Figure 2. Application of multiplex PCR assay for monitoring paneer samples for *L. monocytogenes*. Lanes: M, 100 bp marker; 1, PN-1; 2, PN-2; 3, PN-3; 4, PN-4; 5, PN-5; 6, PN-5 (spiked); 7, PN-6; 8, PN-7; 9, PN-8; 10, PN-9; 11, PN-10; 12, PN-10 (spiked); 13, LmScottA; 14, negative control. PCR, Polymerase chain reaction.

by 'NDRI' method in multiplex PCR are shown in Figure 2. None of the samples showed amplification of the 1.2 kb and 713 bp PCR products, thus indicating possible absence of *L. monocytogenes* in these samples. To check the functioning of the assay and to rule out any false negative results, parallel studies were carried by spiking the same samples with the test organism at 10^6 cfu/g which showed positive signals (Figure 2, Lanes 6 and 12). The negative PCR results obtained with PCR samples are in agree-ment with the microbiological analysis of these samples which did not show any typical *L. monocytogenes* counts on LEB agar.

Singh et al. (2009) applied the molecular beacon based duplex real time PCR assay on 60 market samples, including 20 samples of two popular indian indigenous products (10 each of kulfi and paneer) and they revealed that only one sample of raw milk was positive for *L. monocytogenes*. Whereas, Ramirez et al. (2010) reporterd that out of 30 cheese samples, only six samples showed confirmatory test for genus Listeria. However, PCR could amplify only two samples for *L. monocytogenes*. Meanwhile, there is no other direct reference by which we can substantiate our PCR results on paneer. Nevertheless, our findings can be supported by almost similar observations obtained with soft cheese made by Fitter et al. (1992) and Makino et al. (1995) who could detect *L. monocytogenes* from spiked soft cheese samples only with their respective PCR assays. However, more extensive studies are required to be carried out on these lines since paneer and soft cheeses unlike raw milk have a more complex matrix and hence may require special treatment during preparation of template DNA from the samples. The problem of PCR inhibitors also need to be addressed before drawing any meaningful conclusion.

A critical appraisal of the results based on this limited study may not represent the true picture as far as occurrence of *L. monocytogenes* in milk and milk products is concerned. Therefore, to draw more authentic conclusions from this type of study, a large number of representative samples of these foods from various sources need to be analysed.

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REFERENCES

Bubert A, Hein I, Rauch M, Lehner A, Yoon B, Goebel W, Wagner M (1999). Detection and Differentiation of *Listeria* sp. by a single reaction based on multiplex PCR. Appl. Environ. Microbiol. 65: 4688-4692.

Cooray KJ, Nishibori T, Xiong H, Matsuyama TC, Fujita M, Mitsuyama M (1994). Detection of multiple virulence-associated genes of *Listeria monocytogenes* by PCR in artificially contaminated milk samples. Appl. Environ. Microbiol. 60: 3023-3026.

Fitter S, Heuzenroeder M, Thomas CJ (1992). A combined PCR and selective enrichment method for rapid detection of *Listeria monocytogenes*. J. Appl. Bacteriol. 73: 53-59.

Grover S, Batish VK, Tatini SR (1993). Emerging pathogens of concern to dairy industry and their rapid detection. Indian Dairyman, 45: 343-348

Klein PG, Juneja VK (1997). Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol.

63: 4441-4448.
Makino SJ, Okada Y, Maruyama T (1995). A new method for direct detection of *Listeria monocytogenes* from foods by PCR. Appl. Environ. Microbiol. 61: 3745 -3747.
O'Connor L, Joy J, Kane M, Maher M (2000). Rapid polymerase chain reaction / DNA probe membrane based asay for the detection of *Listeria* and *L. monocytogenes* in food. J. Food Prot. 63: 337-342.
Ramírez LG, Morón de Salim A, Alfieri Graterol AY, Gamboa O (2010). Detection of *Listeria monocytogenes* in white cheese by polymerase chain reaction (PCR). Arch. Latinoam Nutr. 60(3): 254-260.
Singh J, Batish VK, Grover S (2009). A molecular beacon-based duplex real time polymerase chain reaction assay for simultaneous detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in milk and milk products. Foodborne Pathol. Dis. 10: 1195-1201.
Suresh Kumar S (1999). Detection of *E.coli* O157:H7 in milk by PCR. Thesis submitted to National Dairy Research Institute (Deemed University), NDRI, Karnal.
Wiedmann M, Barany F, Batt CA (1993). Detection of *Listeria monocytogenes* with a nonisotopic polymerase chain rection assay. Appl. Environ. Microbiol. 59: 2743-2745.
Witham PK, Yamashiro CT, Livak KJ, Batt CA (1996). A PCR based assay for detection of *Escherichia coli* shiga Like toxin genes in ground beef. Appl. Environ. Microbiol. 62: 1347-1353.