# PCR-based detection of *Listeria monocytogenes* in dairy foods

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**Consumption of dairy foods has been recognized as an important transmission route for human listeriosis, causing health hazards among public. This demands a successful detection assay to declare dairy foods free of *Listeria monocytogenes*, the causative pathogen. Although PCR-based detection of *L. monocytogenes* in dairy foods is associated with problems of PCR inhibition and carry-over contamination, PCR have been applied widely for successful detection of *L. monocytogenes* in dairy foods and found useful for suggesting, monitoring and control measures. Therefore, in the present report, PCR-based detection protocols with special reference to overcome PCR inhibition by dairy foods and culture media constituents over the last 5 years are discussed. Also, critical tips to be kept in mind while performing PCR-based *L. monocytogenes* detection assay are enumerated.**

CONSUMPTION of safe food has always been the desire of man. It has now acquired commercial importance with more and more people consuming prepacked food and dairy products. Consequently, the detection of pathogens became cardinal in any centralized food preparation. Although *Listeria monocytogenes* has been recognized as a cause of disease in humans and animals for over 50 years, recognition of listeriosis as an important public health problem dates from the documentation of common source food-borne outbreaks in the last decade[1–11] with special risk groups of pregnant women, newborns, elderly and immuno-compromised patients (Figure 1).

*Listeria monocytogenes* (Table 1) is a human and animal pathogen which is widespread in nature. It is a transient constituent of the intestinal flora excreted by 1–10% of healthy humans. It is extremely hardy and can survive for many years in the cold in naturally infected sources. *L. monocytogenes* has been isolated from a wide variety of foods, including dairy, meat and fish. Most of the food-borne listeriosis outbreaks have been linked to the consumption of dairy foods. Also the evidence that gastrointestinal tract is an important route of infection and that the epithelial cells of the intestine may be primary site of entry for these bacteria has been provided by electron microscopic studies of tissues of infected guinea pigs[12]. It is now recognized that listeriolysin O, a 60-kDa protein, is one of the major virulence factors of the organism. All strains of *L. monocytogenes* are pathogenic by definition although some appear to be more virulent than others.

Besides the above epidemics, *L. monocytogenes* have also been found in dairy foods spanning other parts of the world, viz. Egypt[13], Singapore[14], United Arab Emirates[15], Scotland[16], Spain[17], Ethiopia[18], Jordan[19], Taiwan[20], Italy[21], Tokyo[22], France[23], Poland[24,25], Brazil[26], New Zealand[27], China[28], Denmark[11], and England and Wales[29]. The presence of *L. monocytogenes* in dairy foods is mainly due to contamination of raw milk, survival following improper pasteurization, post-pasteurization contamination, resistance to production technologies, contamination during cheese ripening, and survival and growth during product storage[30]. Therefore, whether the dairy foods are manufactured from raw milk or pasteurized milk, strict detection for *L. monocytogenes* is required to be undertaken.

There is no study available on the incidence of *L. monocytogenes* in India. Since the dairy processing industry in India is growing rapidly as was in the developed world before epidemics, the possibility of such epidemics in India, therefore, cannot be ruled out completely. Consequently, methods for detection of dairy foods-borne *L. monocytogenes* are required for protecting the health of public. Further, questions regarding the epidemiology of the disease, the extent of dairy foods contamination and the importance of the foodborne route of transmission remain unanswered partly due to lack of successful detection assay. A successful assay must be simple, sensitive, specific, reliable, accurate and above all rapid, for detection and identification of *L. monocytogenes* so as to warn against the use of contaminated dairy foods (which have limited shelf life) well in advance. Since an oral infective dose of 105 virulent *L. monocytogenes* is required to cross the intestinal barrier in healthy immunocompetent humans, future studies aiming to determine the potential hazard of *L. monocytogenes* in foods should focus on quantitative rather than qualitative bacteriological aspects[31].

Various approaches, viz. classical culture, ELISA, DNA probing, mihiaturized biochemical assays, etc.

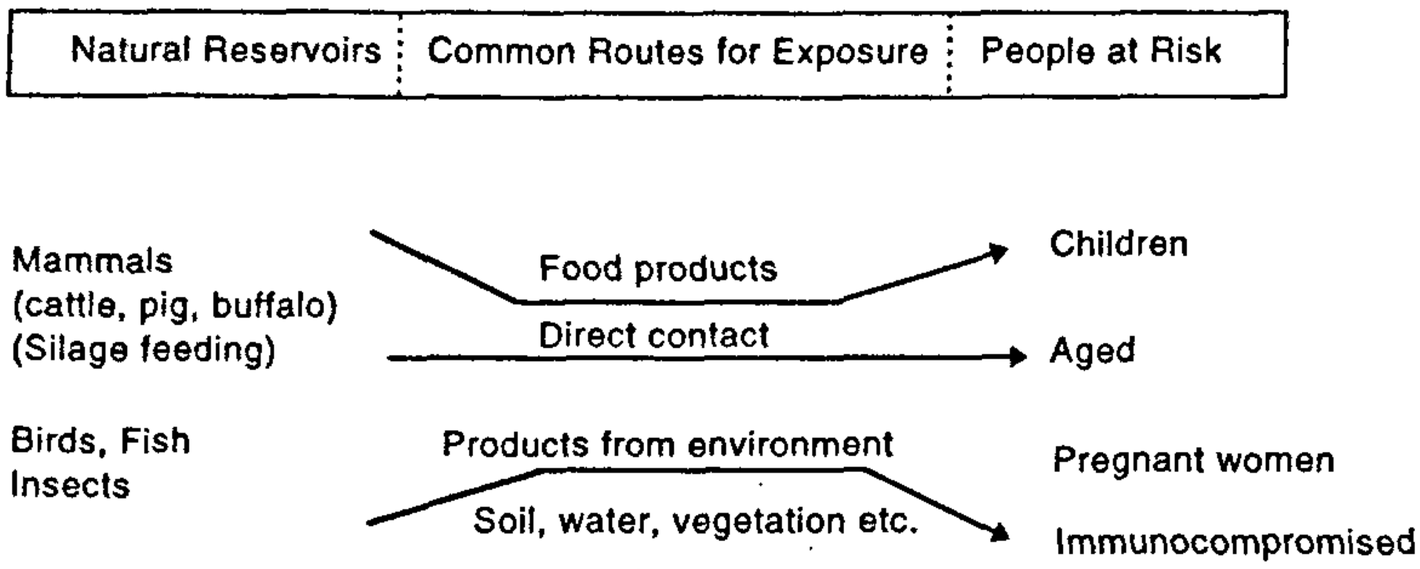


**Figure 1.** Natural reservoirs, common routes for exposure and people at risk of *L. monocytogenes*.

**Table 1.** General description of *L. monocytogenes*

| Character | Description |
|---|---|
| General | Rod shaped, 2*0.5 μM, Non spore former, Gram +ve |
| Motility | Tumbling |
| Habitat | Soil, vegetation, sewage, silage, water and food |
| Route of infection | Food and water |
| Virulence factors | 60 kDa, 49 kDa, 40 kDa and 34 kDa |
| Antibiotic sensitivity | Ampicillin, chloramphenicol, erythromycin, neomycin, tetracycline, cephalosporin |

have been used to detect *L. monocytogenes*. The ELISA assay was able to detect *Listeria innocua, L. monocytogenes, L. murrayi* and *L. welshimeri* after 48 h in artificially contaminated cheese with high sensitivity but without specificity[32]. A hydrophobic grid-membrane filter (HGMF) colony hybridization method using a digoxigenin-labelled DNA probe is potentially useful for automated detection of the organisms in foods[33]. No interference with the present background flora was noticed in a DNA probe assay with the colonies, grown on the selective agar plate, swabbed and washed prior to analysis[34].

Approaches used in the past lack one or other criteria of successful detection assay. Standard methods which rely on cultivation of presumptive *Listeria* colonies is time consuming taking 3 to 4 weeks before a species identification is possible[35]. Therefore, by the time results are available, the product is already consumed. Identification of *L. monocytogenes* by colony hybridization with a specific DNA probe has been reported[36,37] but here also prior cultivation of the organisms was necessary. A sensitive, quantitative hybridization assay using riboprobes against *Listeria* 16S rRNA has been described[38], although the specificity for *L. monocytogenes* was not reported.

The development of primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, i.e. polymerase chain reaction (PCR)[39] and its application to amplify specific fragments of bacterial DNA precede a detection assay which meets all the criteria for successful detection, identification and confirmation of *L. monocytogenes*. Sensitivity of the PCR method for cheese wash-water was higher than classical and the DNA colony-hybridization method[40]. PCR applied to a 2-step enrichment was the most powerful assay for detecting *L. monocytogenes* among selective agar plating, by 'Gen-Probe' DNA hybridization and by the polymerase chain reaction (PCR)[41]. With the widespread studies on *L. monocytogenes* in dairy foods, a number of research reports have accumulated over the past 5 years describing various protocols and experimental modifications for its detection.

## PCR-based detection assay

PCR in an *in vitro* method for amplification of specific DNA fragments (Figure 2). Two oligonucleotide primers, each complementary to the extremes of opposite strands of DNA separated by a region to be amplified, direct the synthesis of a complementary strand towards each other to produce an exact copy of DNA flanked by primers. Repeating the cycle of 3 independent steps carried out at defined temperatures doubles the numbers, thus increasing the copy number of target DNA in a geometrical fashion (approximately $m2^n$). Here $m$ is the initial copy number of target DNA and $n$ is the number of PCR cycles. The steps involved are denaturation (94–97°C), primer annealing (55–72°C) and extension of annealed primers (at about 72°C) by a thermostable DNA polymerase.

Detection of foodborne microbial pathogens using PCR has been applied widely and reviewed[42,43]. Denner and Boychuk[44] developed a species-specific

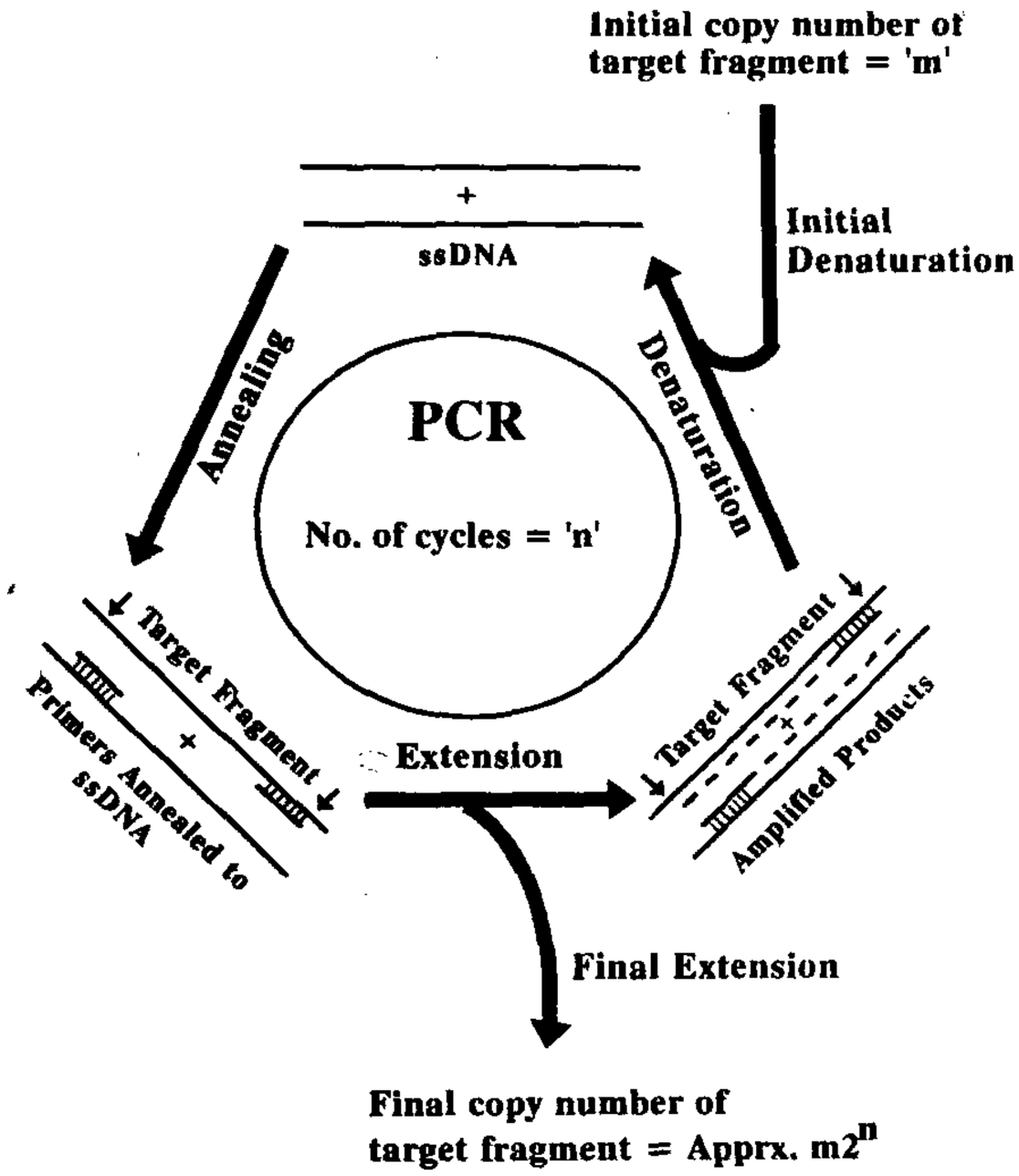


**Figure 2.** Digrammatic representation of PCR.

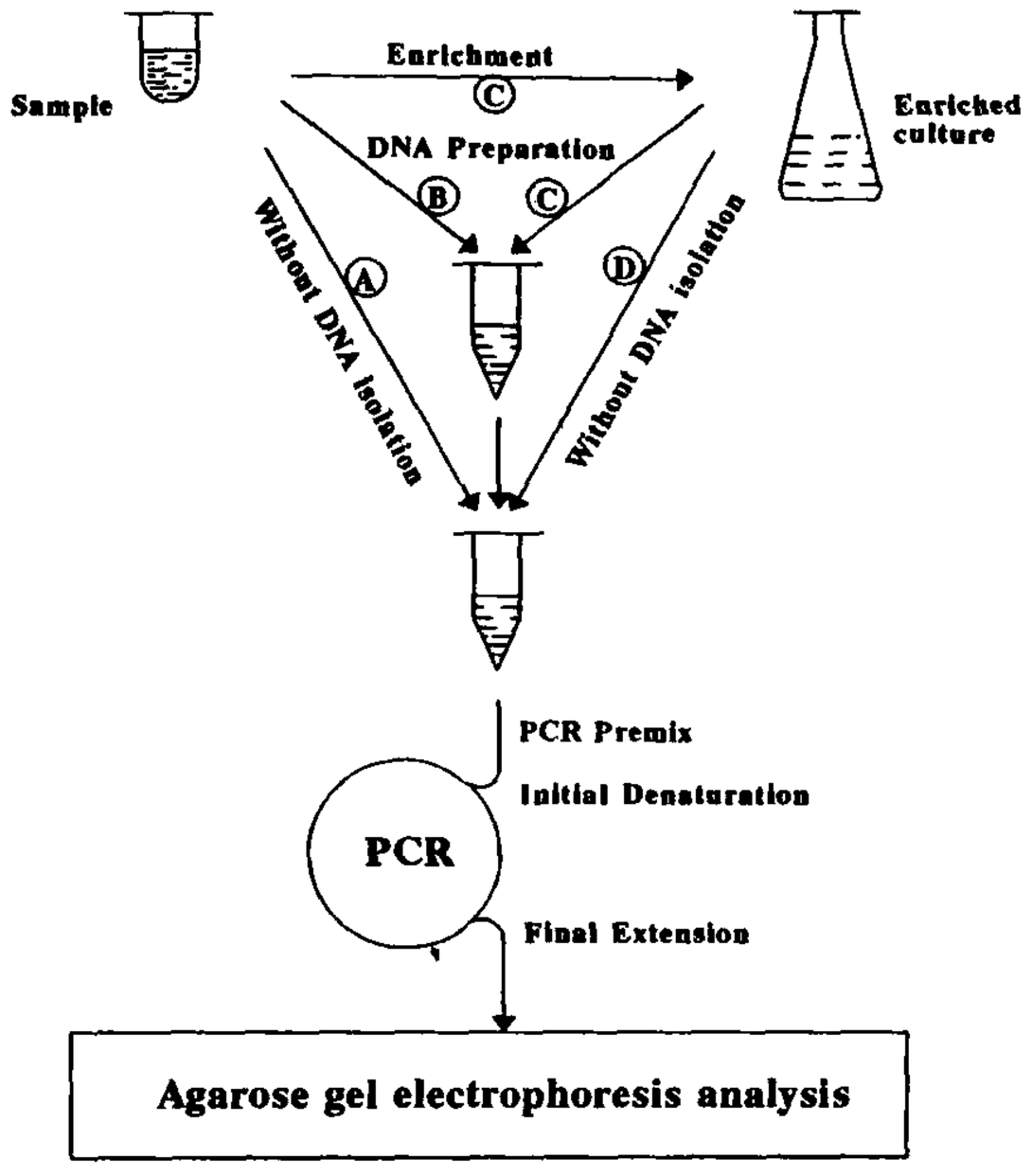


**Figure 3.** Flowchart for PCR-based detection of *L. monocytogenes* during path: ***A***, without enrichment and without DNA isolation; ***B***, without enrichment and with DNA isolation; ***C***, with enrichment and with DNA isolation and ***D***, with enrichment and without DNA isolation.

detection assay of *L. monocytogenes* by DNA amplification. Niederhauser and his associates[45] detected *L. monocytogenes* in food using a PCR-based assay. Since then, a number of research reports have accumulated describing various protocols and experimental modifications for detection of *L. monocytogenes* dairy foods.

In comparison to other methods, PCR offers the following advantages as summarized[46]: (i) a short time requirement improves public health security and minimizes personnel costs; (ii) the method is able to identify microorganisms that are difficult to culture; (iii) the culture and enrichment of pathogens are not necessary for quality control; (iv) PCR reagents are more readily available and easier to store than those required for serological procedures; (v) animal models are not needed; (vi) the choice of primers determines specificity, which contrasts with the fragment cross-reactivities of antisera utilized in immunoassays; (vi) elaborate diagnostic equipment and media are not required, thereby increasing the flexibility as to locations where PCR may be performed and (vii) automated thermocyclers are available.

## Principle of the detection assay

Small portion from the sample dairy food is taken for investigation (Figure 3). PCR with *L. monocytogenes* specific pair of primers (Table 2) is applied to either (i) DNA isolated with or without enrichment, or (ii) directly to sample or enriched broth to amplify DNA sequences present in *L. monocytogenes* only. The amplified products are resolved on an agarose gel and presence of a specific band signals the sample to be *L. monocytogenes* contaminated. Both positive (isolated template DNA) (PC) and negative controls (NC) (reagents without template DNA) are included to ascertain success of PCR and detect contamination of exogenous DNA respectively.

## Reagents

A complete kit as well as individual reagents are available with commercial suppliers and should be used in accordance with manufacturers' instructions. Oligonucleotide primers are specific to each assay employed and can be synthesized in the laboratory or from custom oligonucleotide synthesis facility of commercial suppliers. All components may be premixed, aliquoted and stored in a separate section of the freezer dedicated for PCR reagents located near a laminar flow hood.

**Table 2.** Primer pairs used in *L. monocytogenes* detection assay

| Target gene | Nucleotide sequence from 5' to 3' | Amplified product (bp) | Reference |
|---|---|---|---|
| Listeriolysin O (*hlyA*) | GCATCTGCATTCAATAAAGA<br>TGTCACTGCATCTCCGTGGT | 174 | 44 |
| | CGGAGGTTCCGCAAAAGATG<br>CCTCCAGAGTGATCGATGTT | 234 | 62 |
| | ATTGCGAAATTTGGTACAGC<br>ACTTGAGATATATGCAGGAG | 234 | 63 |
| | ATTGCGAAATTTGGTAC<br>CGCCACACTTGAGATAT | 240 | 50 |
| | AACCTATCCAGGTGCTC<br>CTGTAAGCCATTTCGTC | 267 | 50 |
| | GACATTCAAGTTGTGAA<br>CTGTAAGCCATTTCGTC | 299 | 50 |
| | GAATGTAAACTTCGGCGCAATCAG<br>GCCGTCGATGATTTGAACTTCATC | 388 | 64 |
| | CATAGACGGCAACCTCGGAGA<br>ATCAATTACCGTTCTCCACCATTC | 417 | 51 |
| | AACCTATCCAGGTGCTC<br>CGCCACACTTGAGATAT | 520 | 50 |
| | GACATTCAAGTTGTGAA<br>CGCCACACTTGAGATAT | 560 | 50 |
| Dth 18 gene | GAAGCACCTTTTGACGAAGC<br>GCTGGTGCTACAGGTGTTTC | 122 | 53 |
| | CCGGGAGCTGCTAAAGCGGT<br>GCCAAACCACCGAAAAGACC | 326 | 53 |
| β-Hemolysin (*iap A*) | ACAAGCTGCACCTGTTGCAG<br>TGACAGCGTGTGTAGTAGCA | 131 | 62 |

*Primers.* Success of detection assay basically depends upon the sequence of primers. The mistakes here will either result in no amplification or non-specific amplification. Availability of nucleotide sequence of target fragment is a must. There are no set rules, but oligonucleotide primers are generally in the range of 18–30 bases in reverse orientation to each other, have similar GC content (>50%), similar $T_m$, minimal secondary structure (i.e. self-complementarity) particularly in the 3′ region (to reduce primer dimer formation), low complementarity to each other, and are specific to target DNA with no cross hybridization to non-target DNA sequences from the same or related species[47]. These are generally designed with the help of a computer program and a number of such programs are available from commercial software suppliers[48]. Some of the primer pairs used in detection assay are given in Table 2.

The optimal concentration of primers to be used in the reaction mixture ranges between 0.1 and 0.5 μM. Higher primer concentration should be avoided as this may promote misspriming, resulting in nonspecific amplification. Annealing temperatures should be 5–10°C below $T_m$. However, annealing at higher temperatures for slightly extended times, especially in first few cycles reduces misspriming and helps in increasing the specificity of the primer pair used in the detection assay. Hot start reduces primer dimer formation[49].

*PCR buffer.* Composition of PCR buffer particularly the concentration of $Mg^{++}$ has profound effects on the specificity and yield of amplification. Apparently low $Mg^{++}$ may result due to the presence of EDTA or other chelators in primer or template DNA stocks. Excess of $Mg^{++}$ may result in accumulation of nonspecific products. Therefore, titration of $Mg^{++}$ is highly desirable. Nowadays, PCR buffer without $MgCl_2$ along with a stock solution of $MgCl_2$ is supplied for this purpose (Boehringer Mannheim). Inclusion of Triton-X-100 and/or gelatin has stabilizing effect on enzymes used in PCR and result in better yield. Some recent protocols have recommended the use of 10% DMSO to reduce secondary structures of target DNA.

*Deoxynucleotide triphosphates.* The dNTPs bind $Mg^{++}$ quantitatively, therefore, dNTPs' concentration in a reaction mixture will determine free $Mg^{++}$ available for enzyme activity. dNTPs are used at 200 μM final concentration and approximately 50% of dNTPs are left unused after PCR amplification cycles. The pH of the dNTPs stock solution should be neutral. A number of biotechnology companies have come up with stock solution of 100 mM ready-to-use solutions.

**Table 3.** PCR protocols for detection of *L. monocytogenes* in dairy foods

| Sample preparation | Dairy food | Sensitivity | Target gene with amplified product (bp) | PCR parameters | Reference |
|---|---|---|---|---|---|
| Direct detection | Inoculated milk | 10 bacteria/10 ml | *hly A* 234 bp, *iap* 131 bp | D = 30″, 95°C; A = 1′, 55°; E = 1′, 72°C; FE = 5′, 72°C; C = 40 | 62 |
| Direct DNA extraction | Inoculated soft cheeses | $10^3$–$10^8$ cfu/0.5 g | *Dth* 112 bp, *dth* 326 bp | ID = 3′, 94°C; D = 1′, 94°C; A = 2′, 54°C; E = 3′, 72°C; C = 30 | 53 |
| 18 h enrichment before DNA extraction | Inoculated yoghurt, skim milk, mature cheese, soft cheese cream | 10–100 cfu/g food inoculated with $10^4$ cfu/g | *hly A* 417 bp | ID = 7′, 94°C, D = 1′, 15″, 95°C; A = 30″, 62°C; E = 30″, 72°C; FE = 1′, 72°C; C = 30 | 51 |
| Overnight enrichment before DNA extraction | – | 5–50 cells | *hly A* 175 bp | ID = 4′, 95°C; D = 45″, 95°C; A = 45″, 60°C; E = 1′, 72°C; FE = 5′, 72°C, two rounds of 35 cycles | 44 |
| Overnight enrichment before DNA extraction | Inoculated pasteurized 2% milk | 0.1 cfu/ml of milk | *hly A* 240 bp, *hly A* 267 bp; *hly A* 299 bp; *hly A* 520 bp; *hly A* 560 bp | D = 1′, 94°C; A = 1′, 55°C; E = 2′, 72°C; FE = 5′, 72°C; C = 36 | 50 |
| 48 h enrichment before DNA extraction | Milk, ice-cream | 10 cfu/25 g food before enrichment and 100 cfu/ml of enrichment broth | *hly A* 388 bp | D = 5′, 94°C; D = 1′, 94°C; A = 1′, 65°C; F = 2′, 70°C; C = 30 | 64 |

ID = Initial denaturation; D = Denaturation; A = Annealing; E = Extension; FE = Final extension; C = Cycles.

*Enzymes.* Thermostable DNA polymerase is the enzyme required for amplification and is available with a number of manufacturers. Taq DNA polymerase, the commonly used enzyme in PCR, has been isolated from *Thermus aquaticus*, which has 5′ to 3′ polymerase activity.

*Template DNA.* Template DNA used in PCR varies from pure genomic DNA to crude preparation of cells as in dairy foods. Whatever the method employed, the sample should always be taken in either pure sterile triple glass distilled water or 1X PCR buffer.

## *Protocols*

PCR is carried out for amplification of *L. monocytogenes* specific DNA fragment according to the standard procedure as described by Saiki *et al.*[39] with modifications to favour optimum amplifications (Table 3). A sensitive and specific method for detection of *L. monocytogenes* in dairy foods consists of culturing samples in listeria enrichment broth (LEB) and subculturing them from LEB to listeria plating media, followed by DNA extraction and species-specific detection of the organism using the PCR[50]. A short enrichment period before PCR amplification allowed detection of the organisms in a range of complex foods contaminated with 104 c.f.u./g, within 24 h or 2 d in soft cheese[51]. A novel method employing PCR-coupled ligase chain reaction (LCR) has also been developed for specific detection of *L. monocytogenes*[52].

The natural samples of dairy foods contain components that inhibit the action of the polymerase enzyme and necessitate either their specific removal or DNA purification. A considerable decrease of PCR inhibition was obtained by phenol extraction, but, it simultaneously reduced the total amount of DNA which is available for amplification. However, Qiagen columns (prepacked with an ion exchange resin) specifically bind DNA and release this DNA upon elution with a buffer of high ionic strength. Purification of cheese extracts on these columns, in contrast to phenolization, did not lead to excessive loss of DNA in the final preparation[53]. The direct detection, using PCR of *L. monocytogenes* added to cow milk was also inhibited at some milk concentrations. This inhibitor was moderately heat stable. Inhibition could be prevented by the addition of bovine serum albumin or proteinase inhibitors to the PCR and the evidence suggests that the inhibitor was plasmin[54].

The polymerase chain reaction detection method, in which the enrichment culture was directly tested, rendered false negative results for 3 soft cheese samples due to the presence of components in the enrichment culture of this cheese that inhibit the PCR[40]. A new detection system – magnetic immuno polymerase chain reaction assay (MIPA) – separates listeria cells from PCR inhibitory factors present in enrichment broths containing food samples by using magnetic beads coated with specific monoclonal antibodies (MAbs)[55]. But in a sensitivity test, PCR was strongly inhibited by cheese components and not by the ingredients of the enrichment broth. Use of DNA purification matrices (DNA Capture Reagent and Geneclean II) may increase the detection limit approximately hundredfold[56]. DNA extracted

directly from mozzarella cheese artificially contaminated with *L. monocytogenes* samples was amplified by PCR. Amplification was obtained for all samples even at a concentration of 3 cells/g cheese and no interference from natural microflora cheese was observed[57].

PCR detects both viable and dead *L. monocytogenes*. Therefore, during initial screening with PCR, samples detected positive for *L. monocytogenes* must be tested subsequently using culture methods to confirm for viability of *L. monocytogenes*. Alternatively, PCR protocol may be modified to detect mRNA which represents active and viable cells. Since an oral dose of 105 virulent *L. monocytogenes* is required to cross the intestinal barrier in healthy immunocompetent humans, for a PCR based method to be completely successful, quantitation may also be undertaken in addition to qualitative detection. Perhaps in future, the development of kinetic PCR[58] and its application to real-time monitoring of DNA amplification reaction run against a concentration standard may provide solution to quantitation problems using PCR.

## Analysis of amplified products

The amplified products are resolved on 2–3% agarose gel. The ethidium bromide stain is included in the gel for it increases the clarity of the results as compared to staining the gel after run. The gel is run at constant volts (5–10 V/cm) for 30–70 minutes in 1X TAE buffer and visualized under UV lights. Although the basic setup is same in all cases, the length of amplified fragment is different for each pair of primers. If an expected pattern for PC and NC is obtained, then the assay is valid and there is no contamination. Results can be interpreted accurately. If NC shows PC pattern (due to contamination), then no interpretation is possible.

## Avoiding false positives

Since the PCR is a powerful technique to detect even a single copy of target DNA, a false positive may occur if exogenous DNA finds its way to PCR tube. PCR amplifies the target fragment to a large copy number and each fragment is capable of serving as template for the next PCR. Therefore, even if a single copy of amplified product finds its way to the next PCR tube, the detection assay will give a wrong signal. This is called carry over contamination to distinguish it from contamination from naturally-arising DNA. Separate working areas should be allocated to pre-PCR, PCR and post-PCR work. Use of positive displacement pipettes is recommended to avoid pipette barrel contamination. Also, separate sets of pipettes should be allocated for PCR and post-PCR works. The pipette set used for PCR work should be labelled with red tape and in no circumstances should be used for post-PCR work. Disposable gloves are advised at all the times during PCR set up and frequent change of gloves. All the tubes are closed to avoid air-borne contamination. Given below are the tips which should be kept in mind while performing *L. monocytogenes* detection using PCR[59–61].

*Pre-PCR work.* Prepare reagents in a separate area, premix to 2X stock solutions, aliquot and store these in a freezer section dedicated to store PCR reagents. Record the number of each lot so that if contamination is suspected, it can be traced out easily. Use dedicated chemicals for PCR including water. Autoclave PCR tubes, tips, triple distilled water and mineral oil. Use screw-capped tubes for stocks or tubes that do not require much force to open. This is to avoid splashes and associated contamination. Use siliconized tubes.

*PCR-work.* Assemble PCR in a clean laminar flow hood having UV lamp. Switch the UV lamp on when the hood is not in use. Expose PCR premix for 5 minutes under UV (254 nm) before assembling PCR in case contamination is suspected. This destroys the template DNA through cross linking whereas primers are safe. Set up positive controls at the beginning and negative control at the end. This represents that the same premix is being added to all tubes and reflects that there was no contamination of premix during reaction set up.

*Post-PCR work.* This is the area where carry over contamination can arise and maximum care should be taken to prevent spread of carry over contamination. Amplified product containing tubes, gels, tips used for gel loading should be sealed in plastic bags along with a little bleach and disposed off safely. Under no cirumstances should these disposables find their way to the pre-PCR area or the PCR area. To prevent 'carry over' contamination from equipment used in post-PCR area, gel apparatus should be soaked in 1 M HCl to depurinate any residual DNA. Surface of the UV transilluminator should be covered with a fresh sheet of plastic wrap for each gel.

## Conclusion

Consumption of dairy foods has been recognized as an important transmission route for human listeriosis causing health hazards among public. This demands a successful detection assay to declare dairy foods free of *L. monocytogenes*, the causative pathogen. Various approaches have been used to detect *L. monocytogenes*. PCR offers a successful assay for detection of *L. monocytogenes* in dairy foods. A single copy of specific target DNA is sufficient, enhancing its sensitivity. The assay is rapid enough to provide results on the same day. In addition, it is simple, reliable and accurate. Also, per-

sons without formal education in molecular biology can perform the assay after training. Although the assay is quite easy to perform, it is vulnerable to carry over contamination giving ambiguous results. Therefore, we caution against the spread of contamination. As the methods to distinguish between genuine sample and contamination-related amplification become available, detection assay will be feasible on a single bench without the fear of contamination. In addition, there is need to modify PCR protocols to target mRNA for active and viable *L. monocytogenes* detection sparing dead ones. Also, efforts should be directed towards evolving a quantitative assay.


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RESEARCH ARTICLE

# Comparative sequence analysis and expression in *E. coli* of the subgroup I-specific antigen VP6 from a G2 serotype human rotavirus IS2

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**VP6, the intermediate capsid protein of the virion, specifies subgroup specificity of rotavirus. It is also the most conserved, both at nucleotide and amino acid levels, among group A rotaviruses and is the target of choice for rotavirus detection. In this study we report the sequence of the subgroup I (SGI)-specific VP6 from the serotype G2 strain IS2 isolated from a child suffering from acute diarrhoea in Bangalore and its comparison with the published VP6 sequences. Interestingly, IS2 gene 6 shared highest homology with that from bovine UK strain and the protein contained substitutions by lysine at amino acid positions 97 and 134. In contrast, the amino acids Met and Glu/Asp at these respective positions are highly conserved in all the other group A rotaviruses sequenced so far. These observations have obvious implications for the evolution of serotype G2 and G2-like strains circulating in India. The SGI VP6, of a human rotavirus, possessing epitopes that are conformationally similar to those found in the native protein in the virion, was successfully expressed in *E. coli* and purified for the first time by single-step affinity chromatography.**

DIARRHOEAL diseases are the major cause of morbidity and mortality among infants and young children, especially in developing countries. Of the many diarrhoeal agents, rotavirus is the single most important cause of severe, acute infantile gastroenteritis in humans and a variety of domestic animals[1]. Rotavirus diarrhoea occurs throughout the year and is estimated to account for about a million deaths annually among young children, thus representing an important public health problem[1,2]. In spite of the staggering economic burden, an effective vaccine against rotavirus diarrhoea is yet to be developed.

Rotavirus belongs to the family Reoviridae and consists of a triple-layered capsid[3]. The outer shell consists of two proteins, VP4 and VP7, the intermediate shell comprises of VP6 and the inner shell is composed of VP2 that encloses a genome of 11 segments of double-stranded (ds) RNA[3,4]. The genome encodes 6 structural