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Short communication

Multiplex PCR assay for the routine detection of *Listeria* in food

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Abstract

The development and validation of a multiplex PCR assay for the detection of *Listeria* that can be employed in routine investigation of food samples are described. The assay, which employs a short culture enrichment step followed by isolation of bacterial cells and detection by multiplex PCR reaction, is highly sensitive and specific for the detection of *Listeria monocytogenes* and all other *Listeria* species. Over 350 food samples were tested in parallel by standard cultural procedures and the PCR assay, with no false-positive or false-negative results obtained with the PCR assay. Compared to the standard cultural methods the PCR assay is highly sensitive, cost effective and extremely rapid with results obtained within 48 h from sample receipt.

Keywords: *Listeria* detection; PCR; Rapid methods

1. Introduction

A number of immunoenzymatic and nucleic acid probe based rapid methods have been described for the detection of *Listeria* (Butman et al., 1988; Chenevert et

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al., 1989; Datta et al., 1988; Farber and Speirs, 1987). The detection threshold of most commercially available nucleic acid probe tests is at least 10^4–10^5 bacterial cells (Bobbitt and Betts, 1992). Significantly, methods based on immunoassays are hampered by varying levels of cross-reactivity to related non-*Listeria* organisms and the sensitivity of detection for most immunoassays is in the range of 10^5–10^6 bacterial cells (Mattingley et al., 1988). By contrast, polymerase chain reaction (PCR)-based methods have the sensitivity and specificity to detect a limited number of organisms, or even a single organism. This may be of great importance in the analysis of food as *Listeria* populations in contaminated foods are usually very low ($< 1\%$ of total bacterial count).

Recently, the use of PCR technology for the detection of *L. monocytogenes* has been reported (Bessesen et al., 1990; Border et al., 1990; Deneer and Boychuk, 1991; Rossen et al., 1991; Wernars et al., 1991; Jaton et al., 1992; Ericsson et al., 1995; Gray and Kroll, 1995; Makino et al., 1995; Simon et al., 1996). However, none of the applications published thus far employed PCR internal quality control measures in the PCR procedure and they do not appear to be suited for routine screening of a large number of food samples.

In this study we have investigated the reliablity and applicability of using a PCR-based assay with built in quality assurance measures which can be used after culture enrichment for the routine examination of foods for *Listeria* spp. and *L.monocytogenes*.

2. Material and methods

2.1. Test samples

Food samples were collected at local food outlets by the NSW Health Department's food inspectors and comprised a wide variety of products including seafood, vegetables, dairy products, processed meats and raw poultry. Artificially contaminated foods were prepared by spiking food homogenates with *L.monocytogenes* at a level of approximately 1 cfu/g.

2.2. Cultural procedures for analysis of food samples

Apart from dairy products, all food samples were tested by the double selective enrichment method published by the USDA-FSIS (McClain and Lee, 1989). Dairy products were tested by an Australian Standard Method (Standards Australia, 1991).

2.3. Nucleic acid isolation

Quantities of 10 ml of overnight primary enrichment broths (McClain and Lee, 1989) were subjected to low speed centrifugation, at $500 \times g$ for 10 min, at 4°C to remove food debris. The supernatant was decanted and bacterial cells were pelleted

by centrifugation at $6000 \times g$ for 20 min at 4°C. When testing secondary enrichment broth, only high speed centrifugation was used, due to the very low level of sample debris. The pelleted cells were washed in cold phosphate-buffered saline (PBS) before nucleic acid extraction.

Two procedures were used to recover nucleic acids for the PCR assay. In the first procedure, high molecular weight genomic DNA was purified by the guanidium thiocynate method described by Pitcher and Saunders (1989). In the second procedure, the washed cells were resuspended in 1 ml of cold sterile distilled water and stored at 4°C. Prior to PCR assay, the intact cells were heat denatured at 96°C for 12–15 min on a hot block. After centrifugation at $12\,000 \times g$ for 10 min and the clear supernatant containing nucleic acids was used for subsequent PCR reaction.

A control culture of *L. monocytogenes* was always included as a positive control in every experiment. The cell pellet from 10 ml of control culture (rice-grain size) was used as a reference point for appropriate dilution of the food sample pellet, thereby ensuring that the level of target DNA present in every PCR assay was approximately the same.

2.4. PCR assay

DNA samples (~ 10 ng) were amplified in an optimized 50 μl reaction mixture as described by Bansal (1996). A multiplex of synthetic oligonucleotide primers was used in the PCR assay, which comprised: (a) genus-specific primers (U1 and LII), complementary to ribosomal DNA sequence for detecting all *Listeria* species (Border et al., 1990); (b) species-specific primers (LR and LF) complementary to the nucleotide sequence of the virulence gene listeriolysin O, for the detection of *L. monocytogenes*, (Bansal, 1996) and (c) *Listeria* internal postive control (LIPC) fragment, 2 fg of which were included in every PCR assay. The species-specific primers co-amplify the LIPC. The primers used yielded 938, 750 and 525 bp PCR products, respectively. The amplification products of the multiplex assay were analyzed by agarose gel electrophoresis as previously described by Bansal (1996).

3. Results and discussion

The two procedures utilized in this study for the isolation of nucleic acids prior to the PCR assay yielded nucleic acids which were readily amplifiable by *Taq* polymerase.

Consequently, we aborted the use of the guanidium thiocyanate method of Pitcher and Saunders (1989), thus avoiding the lengthy extraction, purification and precipitation steps involved. Moreover, the much simpler extraction method used in this investigation reduces the need for manipulations which may result in cross contamination and, as a result, erroneous results.

In order to evaluate the specificity, sensitivity and reliability of the PCR

procedure, a wide range of food samples were tested in a series of parallel tests, over a period of 2 years by the cultural methods and the PCR procedure for the presence of *Listeria* spp. and *L. monocytogenes*. The food samples investigated included raw meats, poultry, cooked meat products, seafoods, vegetables, and dairy products. Full agreement between results of the cultural methods and the PCR procedure was obtained for the 350 samples analysed. The PCR procedure detected *Listeria* in four additional samples which were negative by the cultural methods. Two of the four samples were shown by the PCR procedure to be *L. monocytogenes*. In each of these four cases, the bands on the ethidium bromide stained gels were faint, indicating low levels of *Listeria*. These PCR positive and culture negative may be false positives or PCR artefacts, but this is highly unlikely, as in two of the *L. monocytogenes* positive samples both the Listeria species-specific (938 bp) and L. monocytogenes-specific (750 bp) bands were observed. The primers used in the multiplex PCR assay are very reliable, specific and have a high affinity for the correct target sequence. This is indicative by the facts that there is no spurious priming evident using these primers. In addition to this, there is no cross-reactivity (amplification products) with 'target' DNA from other bacteria commonly associated with food (Bansal, 1996).

Of the 350 food samples tested by the PCR procedure, 97 (28%) contained *Listeria* spp. and 54 (16%) were positive for *L. monocytogenes* (Table 1). The incidence of *Listeria* was the highest in raw poultry (47%), followed by smallgoods (38%), and seafoods (32%). The remaining categories of foods had *Listeria* incidence ranging from 0–25%. The results of multiplex PCR amplification of some samples are represented in Fig. 1.

Some food types such as pate, salami, ham and cheese which are often contaminated with *Listeria* were found to be negative by both the culture methods and by the PCR procedure. Samples of these foods were artificially spiked with *L. monocytogenes* at a level of less than 1 cfu/g. Eighteen artifically contaminated foods samples were tested by both methods. Identical results were obtained by both methods, *L. monocytogenes* was detected in fifteen samples. The fact that no *Listeria* spp. were detected in three spiked samples by both methods may be attributed to the extremely low level of spiking used. In some food samples as few as 10 organisms were used to spike 25 g of food sample. After the enrichment procedure it was possible to detect *L. monocytogenes* in these samples. Therefore, indicating that PCR could detect very low levels of contamination in food samples. Under ideal conditions, PCR can amplify from a single target cell. However, ideal conditions are not likely to be encountered when food matrices are involved.

On two occasions, the PCR assay was employed on samples received for inter-laboratory trials, totalling 13 samples distributed to laboratories in Australia and New Zealand. On both occasions the PCR results were in full agreement with the correctly reported culture method results, as indicated by the convenors of the trials.

Moreover, the PCR procedure substantially reduced the total analysis time as results could be obtained within 48 h from sample receipt-as opposed to the 14 day period needed to accomplish analysis using the cultural methods. The PCR procedure very well suited for rapidly screening out negative samples and to give presumptive positive results. The fact that the PCR assay uses a small portion of the enrichment broth, the remainder can be used for confirmation and isolation of

Table 1
Results of foods tested by the cultural methods and the PCR assay

Food	No. of samples	No. of samples positive for	
		Listeria	*L. monocytogenes*
Dairy Products			
Soft cheese	10	0	0
Ice cream	5	3	3
Milk powder	2	0	0
Subtotal	17	3 (18)[a]	3 (18)
Seafoods			
Smoked salmon	63	14	10
Smoked trout	11	3	1
Crab highlighter	12	3	2
Salmon products	33	17	12
Other seafoods	11	4	1
Subtotal	130	41 (32)	26 (20)
Vegetables			
Tofu	25	0	0
Ready to eat salads	16	0	0
Cooked vegetables	4	2	0
Subtotal	45	2 (4)	0 (0)
Cooked meats			
Chicken	8	1	0
Roast beef	8	3	2
Subtotal	16	4 (25)	2 (13)
Smallgoods			
Salami	49	21	8
Ham, mortedella, turkey, pastrami, silverside	15	3	0
Subtotal	64	24 (38)	8 (13)
Pate	15	0 (0)	0 (0)
Raw poultry	19	9 (47)	8 (42)
Inter-lab trials	13	12 (92)	7 (54)
Miscellaneous	31	2 (6)	0 (0)
Total	350	97	54

[a]Values in parenthesis are percentages.

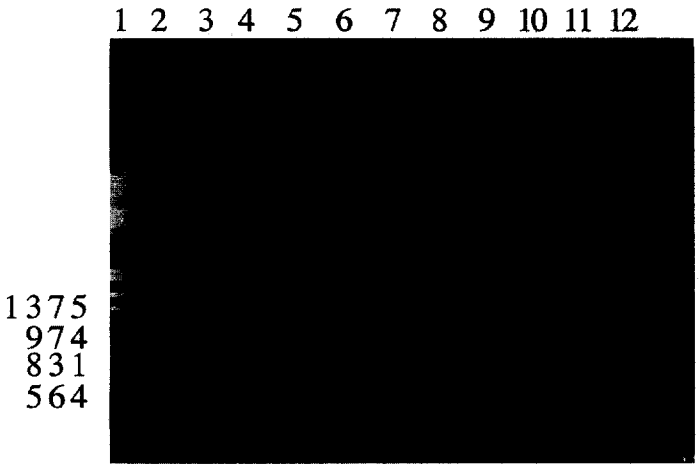

Fig. 1. Agarose gel electrophoresis of PCR-amplified products from different food samples. Target DNA in lanes 2–10, was prepared from 10 ml of Fraser broth. Lanes 2–9 represent different food samples, where lanes 2 and 3 are *Listeria* spp. postive food samples; lanes 4, 5, 7 and 8 are *Listeria* negative food samples; lane 9 is a *L. monocytogenes* positive food sample; lane 6 shows a PCR amplification failure; lane 10 is a positive control *L. monocytogenes* (FDA/V7); lane 11 is a positive control *L. innocua* (FDA/C1-94); lane 12 is a negative control (water blank) and lane 1 is a molecular size marker (Lambda DNA cut with *Eco*RI plus *Hin*dIII).

bacterial colonies by the standard culture procedure. Hence, the PCR procedure can be used in conjuction with the standard culture procedure.

The application of PCR assay after cultural enrichment offers distinct advantages: (1) it increases the amount of target sequence, as the levels of target organism may be initially low and not be uniformly distributed; (2) it dilutes out non-*Listeria* DNA and other components (inhibitors) that might interfere in the PCR assay; and (3) it ensures target DNA is obtained from viable (dividing) cells.

In every PCR assay, *Listeria* internal PCR control (LIPC) was included. The LIPC proved to be a sensitive indicator of PCR failure, which may be caused by inhibitors of PCR present in food samples or as a result of a human error in setting up PCR. The PCR amplification was inhibited completely in less than 1% of the food samples tested in this study (see Fig. 1, lane 6). However, the food samples that failed the PCR assay were all negative for *Listeria* by cultural methods. The intensity of ethidium bromide-stained band of LIPC was determined to be equivalent to 100 organisms/ml. Therefore, these bands can also be used to estimate semi-quantitatively the number of *Listeria* cells present in contaminated food samples.

In conclusion, on the basis of the results obtained with 350 food samples, our results demonstrate that our PCR-based method is superior in reliability and performance as compared with the standard culture methods. The PCR procedure proved to be highly sensitive, substantially faster, and more cost effective than standard culture procedures. Our results also indicate that the PCR procedure can

be employed as a routine diagnostic procedure for the definitive identification of *Listeria* and *L. monocytogenes* in a diverse range of food types. Therefore, this rapid method should be of great benefit to the food industry as well as to regulatory or public health laboratories engaged in establishing the safety of food products and the management of listeriosis.

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