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Evaluation of Diagnostic PCR for the Detection of *Listeria monocytogenes* in Food Products**

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Summary

Conventional methods for the detection of *Listeria* in foodstuffs are generally cumbersome and time consuming. The use of primary enrichment in ½ strength Fraser broth and the use of Oxford and RAPID'L. mono agars were assessed in comparison with polymerase chain reaction (PCR) for their ability to accurately detect and confirm the presence of *L. monocytogenes* in food products. Of the 27 food samples tested, 74 % were presumptively positive for *Listeria* on Oxford agar, while 44 % were presumptively positive for *L. monocytogenes* on RAPID'L. mono. Only 37 % of samples were confirmed to be positive for *L. monocytogenes* by PCR amplification of the *hly* gene (732 bp). PCR was able to eliminate the false positives and detect all *L. monocytogenes* in the food products, unlike the conventional methods used in the industry. In addition to the fact that the incidence of *Listeria* species was higher than *L. monocytogenes* on selective media, there was also the presence of *Listeria*-like organisms. These organisms had the typical appearance of *Listeria* on selective media, but were non-*Listeria* species, as confirmed by the PCR and API *Listeria* (bioMérieux). PCR proves to be a sensitive and rapid technique to be included in the procedure of detection of *L. monocytogenes* in food products.

Key words: *Listeria monocytogenes*, PCR, diagnostic, food products

Introduction

Listeria monocytogenes, a ubiquitous, Gram-positive, intracellular pathogen is the causative agent of listeriosis, which is characterised by septicaemia, meningitis and abortion in animals and man. Epidemiological studies have indicated that both epidemic and sporadic cases of human listeriosis are foodborne (*1*). Unlike infection with other common foodborne pathogens, such as *Salmonella*, which rarely results in fatalities, listeriosis is associated with the highest case fatality rate of approximately 30 % (*2*,*3*).

In its most severe form, listeriosis is an invasive disease that infects the immunocompromised and results in a high rate of foetal loss (including stillbirths) and serious neonatal disease due to perinatal infection. In immunocompromised persons, it can also cause severe disease, as well as benign febrile gastroenteritis (*3*). Data indicate that the infective dose of *L. monocytogenes* in contaminated food is usually more than 100 CFU/g (*4*), but the food vehicle responsible for the listeriosis outbreak in the United States in 1998, however, contained less than 0.3 CFU/g of the bacterium (*5*).

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Many different enrichment broths and selective media have been adapted in the past fifty years for selective cultivation of *Listeria* species. Detection and isolation remains complicated due to the inability of researchers to identify a single procedure sensitive enough to detect *L. monocytogenes* in all types of foods within a reasonable amount of time (*6*). Despite these challenges, classical cultivation techniques still remain the official method used although many rapid molecular methods exist.

PCR is one of the most promising techniques for rapid detection of microorganisms in food. This process has provided increased sensitivity for detection and therefore enhanced the likelihood of detecting bacterial pathogens (*7*).

The aim of this study was to evaluate the use of PCR in the detection of *Listeria monocytogenes* in food products.

Materials and Methods

Products tested

Twenty-one sample of various food products (Table 1), including 7 samples of cheese, 3 raw meat and fish products, 5 ready-to-eat meat and chicken products, one spice and one dried fruit mixture, obtained from retail outlets, as well as wholesalers, were tested. All products had been properly stored or refrigerated and tested before their respective 'sell-by' dates. One evaluated meat sample was artificially inoculated.

Five presumptive positive *Listeria monocytogenes* colonies from previously tested products similar to products in Table 1 were also evaluated. These colonies were taken from RAPID'L. mono (Biorad, France) plates. When performing these tests, the colony served as a sample and no food product was added.

Growth media

Listeria monocytogenes strains were detected using a two-step enrichment procedure followed by subculturing on Oxford agar (Oxoid CM856), which is based on the principle of esculin hydrylosis, and a new chromogenic agar, RAPID'L. mono agar (Biorad), which is based on phospholipase C detection and the inability of *L. monocytogenes* to metabolise xylose. The protocol for the identification of *L. monocytogenes* from foods was a modification of the EN ISO 11290-1 (*8*) and Sanofi protocols (Biorad). A food sample of 25 g was added to 225 mL of ½ strength Fraser broth (Oxoid CM895) with supplement SR156 (Oxoid). Samples were homogenized with a stomacher (Seward stomacher 400) for 30–60 s and then incubated at 30 °C for 24 h. This served as the primary enrichment phase. From this primary enrichment, 0.1 mL was then inoculated into 10 mL of Fraser broth and incubated at 37 °C for 24–42 h in a shaking incubator. This served as the secondary enrichment. The primary and secondary enrichments were subcultured on both Oxford and RAPID'L. mono (Biorad, France) agar plates after their respective incubation periods. Presumptive *L. monocytogenes* colonies were streaked on nutrient agar (Oxoid CM1) and incubated at 37 °C for 18–24 h. The colonies on RAPID'L. mono and nutrient agar were then used as templates in PCR reactions. The same protocol was used when selected colonies from previously tested products were used as a substitute for the food sample. All food samples were evaluated in duplicate.

API Listeria

Confirmation of *Listeria* species, *i.e. L. monocytogenes* and *L. ivanovii,* by means of API *Listeria* (bioMérieux) was performed according to manufacturer's instructions.

Inoculum preparation

Inocula of the controls used in the PCR reaction, *i.e. L. monocytogenes* UWC1 (positive control), *L. ivanovii* UWC1 and *Bacillus cereus* UWC1 (negative controls), were prepared by inoculation of a loopful of glycerol stock culture into 5 mL of brain-heart infusion (BHI) broth (Oxoid CM025) and incubated in a shaking incubator at 37 °C for 18–24 h.

Listeria monocytogenes presumptively positive colonies on RAPID'L. mono agar, *i.e.* blue colonies, were subcultured on nutrient agar (Oxoid CM1) and incubated at 37 °C for 18–24 h. A loopful of this growth was used to inoculate BHI broth.

DNA extraction

The extraction procedure was based on a protocol previously described for the detection of *L. monocytogenes* in food products (*9*). A few colonies were resuspended in 50 μL of 1×PCR buffer in a 2-mL microcentrifuge tube with an interlocking cap. A solution of 2 % Triton X (50 μL) was then added to this cell suspension and thoroughly mixed. This mixture was heated at 100

Table 1. Products tested by means of conventional methods and the PCR

Cheeses	Meat and chicken products	Fish products	Other
1) Italian-style blue cheese	1) RTE chicken (smoked)	1) Smoked salmon slices*	1) Dried fruit
2) Blue cheese	2) Vienna sausages	2) Trout paté*	2) Black ground pepper
3) Camembert**	3) Lean mince (beef and ostrich)*	3) Fish paté*	3) Salad*
4) Feta with black pepper**	4) Hamburger patty*	4) Fish patty*	4) Brown mushroom paté**
5) Brie**	5) Lean mince (artificially inoculated)*	5) Salmon paté	5) Cutting board*
6) Prince Albert Royal cheese	6) Polony		6) Three cheese salad*
7) Mozzarella**	7) Traditional smoked beef frankfurters		7) Rosa tomatoes**

*tested presumptive and PCR positive for *Listeria monocytogenes*
**tested presumptive positive but PCR negative for *Listeria monocytogenes*

°C for 10 min and then allowed to cool to room temperature. For PCR amplification, 5 μL of this crude cell lysate were used.

PCR

PCR assays were performed in 50-μL reaction volumes. The primer pair consisting of primer A [5′-CAT TAG TGG AAA GAT GGA ATG -3′] and primer B [5′--GTA TCC TCC AGA GTG ATC GA -3′] was used for the amplification of a 730 bp region of the *hly* gene (*9*). PCR was performed in the Perkin Elmer GenAmp PCR system 2400 thermal cycler. Amplification conditions were optimised to the thermal cycler and were as follows: 80 °C for 10 min, an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, then a final extension at 72 °C for 2 min. The amplified DNA was analysed by gel electrophoresis on a 1.2 % agarose gel stained with ethidium bromide (3 μL/100 mL). A 100 bp ladder (Promega) was used as a reference marker. Tris-borate EDTA (0.5×) was used as the running buffer and the gel was viewed using UV transillumination at a wavelength of 254 nm.

Isolation of yeasts

All presumptive yeast colonies were inoculated on malt extract agar (Oxoid CM 59) and the pH value of the media was adjusted to 3.5, with lactic acid, to suppress the growth of bacteria, and then incubated at 28 °C for 4 days. Colonies were then examined microscopically for typical yeast morphology. The yeasts were not identified.

Results and Discussion

Of the total number of samples tested, 74 % were presumptively positive for *Listeria* on Oxford agar while 44 % were presumptively positive for *L. monocytogenes* on RAPID'L. mono agar. Samples confirmed to be positive for *L. monocytogenes* by means of PCR and API *Listeria* totalled 37 %. The difference in presumptive and confirmed presence of *L. monocytogenes* was therefore 37 and 7 % for Oxford and RAPID'L mono agars, respectively. Neither Oxford nor RAPID'L. mono agars detected all of the positive samples, with Oxford agar detecting 9/10 and RAPID'L. mono detecting 8/10 of the samples positive for *L. monocytogenes*.

The regulatory protocols for isolating listeriae from food samples, *e.g.* the Food and Drug Administration and the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service methods, usually use one or two enrichment broths, followed by plating on *Listeria*-selective media. Although used as the standard method in many countries, these procedures are not totally effective, allowing approximately 30 % of *Listeria*-positive food samples to escape detection (*10*).

A high number (74 %) of samples tested were identified as presumptively positive for *Listeria* species using Oxford agar. Oxford agar does not differentiate between *Listeria* species. Non-pathogenic species of *Listeria* therefore cannot be excluded when selecting suspect colonies for confirmation. The coexistence of several *Listeria* species on the same food is not unusual, and often the incidence of *Listeria* species other than *L. monocytogenes* is higher than that of *L. monocytogenes* itself (*11*). It has been shown that the non-pathogenic species *L. innocua* outgrows the pathogenic species *L. monocytogenes* by a wide margin during enrichment in both UVM and Fraser broths (*12,13*). This makes selection of suspect colonies for confirmation from Oxford agar all the more difficult, since the incidence of non-pathogenic strains is higher than that of *L. monocytogenes* and all species demonstrates the same phenotype. Some *Listeria*-positive samples go undetected due to overgrowth by other *Listeria* species and/or natural background flora during enrichment and differing abilities of *Listeria* strains to grow competitively (*10*). This may explain why negative results were obtained from PCR when using presumptive positive colonies from Oxford agar, while PCR from RAPID'L. mono plates, which are selective for *L. monocytogenes*, proved positive for *L. monocytogenes* (results not shown). Presumptive positive results for *L. monocytogenes* on RAPID'L. mono were more modest than presumptive results for *Listeria* species on Oxford agar. *Listeria monocytogenes* colonies are distinctively different in phenotype from other *Listeria* species on RAPID'L. mono, making selection and identification of *L. monocytogenes* far easier, and thereby improving the accuracy of confirmation tests.

Confirmation (by PCR) of these presumptively positive colonies from both Oxford and RAPID'L. mono agars showed that results from RAPID'L. mono plates were more indicative of the true prevalence of contamination by *L. monocytogenes* than those from Oxford. Food

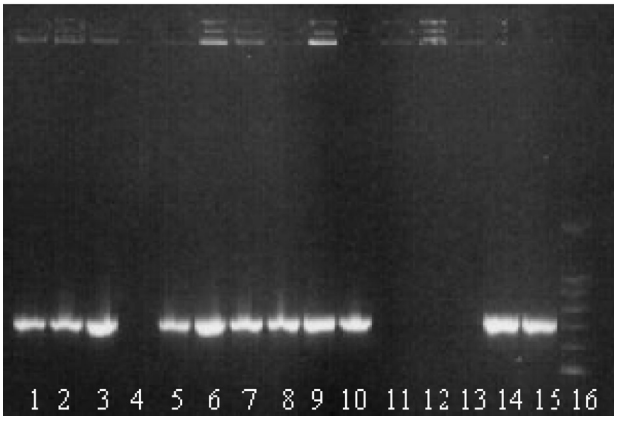

Fig. 1. Agarose gel electrophoresis of the PCR amplification products of various food samples presumptively positive for *L. monocytogenes* on either Oxford or RAPID'L. mono agars. Colonies were subcultured onto a non-selective agar before DNA extraction.

Lane 1: salad (Oxford), lane 2: salad (RAPID'L. mono), lane 3: salad*, lane 4: brown mushroom pate*, lane 5: cutting board (Oxford), lane 6: cutting board (RAPID'L. mono), lane 7: cutting board*, lane 8: 3-cheese salad (Oxford), lane 9: 3-cheese salad (RAPID'L. mono), lane 10: 3-cheese salad*, lane 11: Rosa tomatoes*, lane 12: *Listeria* species (negative control), lane 13: *Bacillus cereus* (negative control), lane 14: *L. monocytogenes* (positive control), lane 15: *L. monocytogenes* (positive control)*, lane 16: 100 bp DNA ladder

Samples marked by * were taken directly from the chromogenic media plate (RAPID'L. mono)

samples and enrichment media may be inhibitory to PCR and thereby can lower its detection capacity. Therefore subculturing on a non-selective medium often precedes the PCR (*14*), as it did in this study. DNA extraction performed directly from suspect colonies from RAPID'L. mono, however, showed no inhibition of PCR (Fig. 1). When comparing PCR results obtained from either subcultured suspect colonies, or colonies taken directly from RAPID'L. mono, no significant differences were observed. There was no decrease of intensity of detected DNA when comparing subcultured colonies to those taken directly from the differential medium, RAPID'L. mono. This could significantly decrease the time taken for confirmation of suspect colonies as it eliminates the need for subculturing onto a non-selective agar.

The use of selective media highlighted significant failings. Competitive bacteria, which should be inhibited by primary and secondary enrichments, as well as the chromogenic media, grew on RAPID'L. mono. Although other bacterial species, *e.g. Bacillus cereus* and *Staphylococcus aureus*, also produce phospholipase C and therefore their growth on this media results in a colorimetric reaction, they can still be distinguished by their appearance. In our study, yeasts which do not produce phospholipase C were also detected on this media, and were phenotypically no different from *L. monocytogenes* colonies. Colorimetric reaction with the yeast took longer to occur than with *L. monocytogenes*, but it occurred within the specified time for detection (<48 h). There is no explanation for their reaction with this medium. It is suspected that there was a lack of inhibition by the antifungal agent in this media due to either insufficient concentrations of the agent, or the adverse effects of shipping and storage.

Selection of suspect colonies from Oxford agar was also problematic. In addition to the fact that the incidence of non-pathogenic species of *Listeria* was higher than the incidence of *L. monocytogenes*, there was also the occurrence of *Listeria*-like organisms. These organisms had the typical appearance of *Listeria* on the selective medium, but were not *Listeria* species (as confirmed with API *Listeria* and PCR) (results not shown). Phenotypic properties by which the bacteria are identified when using culture methods may not always be expressed and may be difficult to interpret or classify (*15*).

PCR is deemed to be more reliable than conventional identification since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable (*16*). With bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and selective media, the transition from conventional methods of detection to genetic methods should be carried out. This method proved to be reliable, cost effective and time saving.

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Otkrivanje *Listeria monocytogenes* u prehrambenim proizvodima PCR-om

Sažetak

Konvencionalni postupci za otkrivanje bakterija roda *Listeria* u hrani su dugotrajni i vrlo zahtjevni. Prisutnost *L. monocytogenes* u hrani određivana je u napola razrijeđenoj Fraserovoj podlozi te na Oxford i RAPID'L. mono agaru te uspoređena i provjerena PCR metodom. Od 27 ispitanih uzoraka hrane 74 % bilo je vjerojatno pozitivno na rod *Listeria* u pokusima na Oxford agaru, a 44 % na RAPID'L. mono agaru. PCR amplifikacijom *hly* gena (732 parova baza) bilo je pozitivno samo 37 % uzoraka. PCR postupak omogućio je eliminaciju prividno pozitivnih vrsta i pronalaženje *L. monocytogenes* u prehrambenim proizvodima, za razliku od konvencionalnih postupaka koji se primjenjuju u industriji. U odabranim uzorcima hrane nađeno je čak više vrsta bakterija roda *Listeria*, a prisutne su bile i bakterije slične rodu *Listeria*. Ti organizmi izgledaju kao tipične vrste roda *Listeria* na selektivnim podlogama, ali ne pripadaju tom rodu, što je dokazano PCR-om i primjenom API *Listeria* (bioMérieux). Dokazano je da je PCR osjetljiva i brza metoda koju treba uvrstiti pri otkrivanju *L. monocytogenes* u prehrambenim proizvodima.