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DNA Extraction and PCR Methods for the Detection of *Listeria monocytogenes* in Cold-Smoked Salmon

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Protocols for the specific detection of *Listeria monocytogenes* in cold-smoked salmon were developed. PCR was used as the method of detection. Inhibitors of PCR present in the food samples were removed by ether extraction or column purification, or their effect was overcome by the use of Tween 20 as an enhancer. These protocols are many times more rapid than conventional detection methodologies and also have the potential for automation.

Cold-smoked salmon is a common commercially prepared, ready-to-eat foodstuff. Smoking is used to add flavor to the fish and also to preserve the meat by inhibiting microbial action; however, since it is generally performed at less than 28°C, the process can often be insufficient to kill many of the microorganisms which may have been present on the fresh fish or which may have infected it during the earlier steps of brining or salting and rinsing.

Several surveys have reported the presence of the pathogen *Listeria monocytogenes* in cold-smoked salmon (1, 4, 5), and although there are no reports of listeriosis caused by the consumption of this foodstuff, there have been cases in which other seafoods have been implicated (1). Conventionally, detection of *L. monocytogenes* involves preenrichment and selective growth and then confirmatory identification tests, and this can take several days to complete (3, 5, 6). It would be useful to have a rapid detection protocol to screen for the presence of *L. monocytogenes* in cold-smoked salmon. PCR is a technique which possesses rapidity, sensitivity, and specificity and could therefore be employed to facilitate rapid diagnosis of *L. monocytogenes* contamination. However, smoked salmon contains phenolics, cresols, and aldehydes, which are potential inhibitors of the PCR (10), and their presence must therefore be overcome before PCR can be used successfully. Here, we describe protocols which allow the detection of *L. monocytogenes* in cold-smoked salmon in less than 36 h by DNA extraction followed by PCR. Inhibition of the PCR is surmounted either by removing the inhibiting substances or by the addition of a compound which enhances the reaction.

MATERIALS AND METHODS

***Listeria* strains.** *L. monocytogenes* NCTC11994 (serotype 4b), *Listeria innocua* NCTC11288, and *Listeria ivanovii* NCTC11007 were obtained from the National Collection of Typed Cultures, London, United Kingdom. *Listeria seeligeri*, an isolate from salmon, was obtained from the Scottish Agricultural College, Aberdeen, United Kingdom.

Inoculum preparation. Inocula of *Listeria* spp. were prepared by inoculating 10 ml of TSY broth (30 g of tryptose soya broth mix and 6 g of yeast extract liter^{-1}) and by incubation at 37°C until the mid-log phase of growth. Cells were enumerated by plating serial dilutions of the inoculum onto PALCAM selective medium (Oxoid, Unipath Ltd., Basingstoke, Hampshire, United Kingdom). Culture dilutions were prepared in tryptose soya extract broth.

DNA extraction. Samples (5 g) of locally purchased smoked salmon were seeded with 5 ml of bacterial culture, or dilutions thereof, and were homogenized for 2 min in 45 ml of homogenizing buffer (10 mmol of Tris-HCl and 10 mmol of EDTA liter^{-1} and 0.5% sodium dodecyl sulfate [SDS; wt/vol]; pH 7.8). Total DNA was then extracted by following one of four protocols, each of which was based on the hexadecyl trimethylammonium bromide (CTAB) method described by Murray and Thompson (8).

Protocol 1. CTAB extraction. Homogenate was centrifuged (7,000 × *g* for 10 min), and the supernatant was discarded. The pellet was resuspended in 9.5 ml of TE buffer (10 mmol of Tris-HCl and 1 mmol of EDTA liter^{-1}; pH 7.8) containing 1% (wt/vol) SDS and 100 μg of proteinase K ml^{-1}, and the suspension was incubated at 37°C for 1 h. Then, 1.8 ml of 5 mol of NaCl liter^{-1} and 1.5 ml of CTAB-NaCl solution (10% [wt/vol] CTAB in 0.7 mol of NaCl liter^{-1}) were added, and the sample was incubated at 65°C for 20 min. Proteins were removed by a single chloroform-isoamyl alcohol (24:1) extraction, and DNA was precipitated with 0.6 volumes of cold isopropanol. The DNA was pelleted by centrifugation (10,000 × *g* for 10 min), washed once with 70% (vol/vol) ethanol, and air dried for 15 to 30 min. The final pellet was resuspended in 150 to 200 μl of sterile distilled water.

Protocol 2. CTAB extraction with ether separation. Protocol 1 was followed up to and including the chloroform-isoamyl alcohol extraction step. The aqueous phase was then transferred to a separating funnel, mixed with an equal volume of diethyl ether, and left to separate for 20 min. The aqueous phase was drained into a clean sterile tube, and the DNA was precipitated with 0.6 volumes of cold isopropanol. The DNA was pelleted (10,000 × *g* for 10 min), washed with 70% (vol/vol) ethanol, air dried, and resuspended as before.

Protocol 3. CTAB extraction with column purification. Protocol 1 was followed up to and including chloroform-isoamyl alcohol extraction of the DNA. The aqueous phase was then passed through a 100-mg silica column (NP silica 60 Angstrom 15 μm; Amicon, Beverly, Mass.) equilibrated with 55% (vol/vol) ethanol, and the column was washed with 15 ml of 55% (vol/vol) ethanol. DNA was eluted with 0.5 ml of sterile distilled water, precipitated with ethanol, and pelleted at 10,000 × *g* for 5 min. Finally, the pellet was washed with 70% (vol/vol) ethanol, air dried, and resuspended as described above.

Protocol 4. CTAB extraction with particle separation and column purification. Following the 65°C incubation step of protocol 1, the sample was loaded onto a Centriprep Particle Separator (Amicon), which is a centrifugal filtration device which removes particulate matter of greater than 0.2 μm from the extract. The separator was centrifuged at 2,500 × *g* three times for 30 min each, and the filtrate was collected and passed through a silica column as described for protocol 3.

Unless used immediately, all DNA samples obtained by these protocols were stored at −20°C until required for PCR detection.

Heat extraction of DNA. For each *Listeria* sp., a 10-ml culture was grown to mid-log phase in TSY broth, and 1 ml of cells was pelleted by centrifugation (7,000 × *g* for 5 min). The cells were washed with 1 ml of sterile distilled water, resuspended in distilled water to give approximately 10^5 cells ml^{-1}, and heat lysed at 95°C for 5 min. Cell debris was pelleted by centrifugation (7,000 × *g* for 5 min), and the supernatant containing DNA was transferred to a clean sterile tube.

PCR. A nested PCR detection protocol employing the *prfA* gene, which is involved in the regulation of listeriolysin synthesis (7), as the target was designed. The first round used primers PRFA and PRFB, which were directed against nucleotides 181 to 207 and 1462 to 1482 of the sequence (7 [GenBank accession number M55160]); they amplify a product of 1,060 bp (14). Each 50-μl reaction mixture contained 50 mmol of KCl and 10 mmol of Tris-HCl (pH 9.0) liter^{-1}; 0.1% (vol/vol) Triton X-100; 3 mmol of $MgCl_2$ liter^{-1}; 200 μmol each of dATP, dTTP, dCTP, and dGTP liter^{-1}; 0.5 μmol of primer liter^{-1}; and 1 U of *Taq* DNA polymerase (Promega, Southampton, Dorset, United Kingdom). The reaction conditions were 94°C for 2 min and then 35 cycles of 94°C for 30 s, 60°C for 30 s, and 74°C for 1 min, with a final extension period of 5 min at 74°C. A 5-μl

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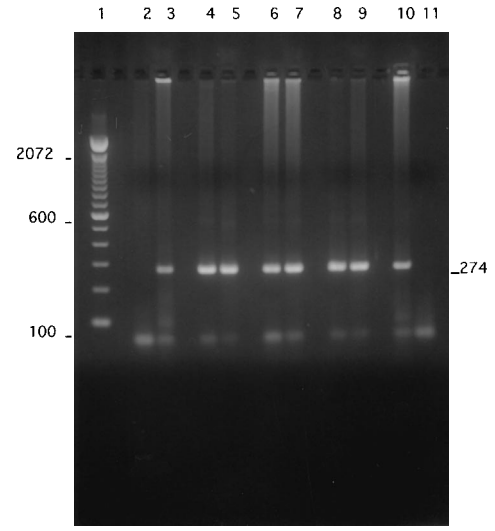

FIG. 1. PCR detection (after secondary amplification) of *L. monocytogenes* in cold-smoked salmon samples with target DNA prepared by standard CTAB extraction (lanes 2 and 3), ether separation (lanes 4 and 5), column purification (lanes 6 and 7), and particle separation with column purification (lanes 8 and 9). PCR enhancer (Tween 20) was added to samples run in lanes 3, 5, 7, and 9. Lanes: 1, 100-bp size markers; 10, positive control (100 ng of amplified *L. monocytogenes* DNA); 11, negative control. The small (<100-bp) bands are unbound primers.

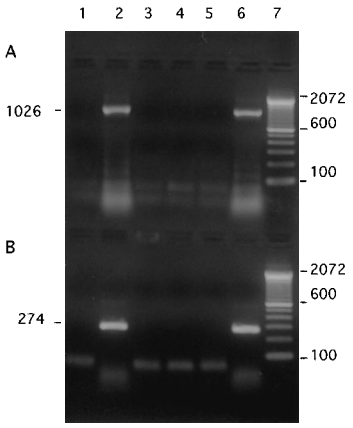

FIG. 2. Specificity of PCR detection of *L. monocytogenes*. (A) Primary amplification; (B) secondary amplification. Lanes: 1, negative control; 2, positive control (100 ng of amplified *L. monocytogenes* DNA); 3, *L. seeligeri*; 4, *L. ivanovii*; 5, *L. innocua*; 6, *L. monocytogenes*; 7, 100-bp size markers. The small (<100-bp) bands are unbound primers.

volume of target DNA was added to each reaction mixture. A reaction mixture containing 5 μl of sterile distilled water was included as a negative control. A reaction mixture containing 5 μl of a 20-ng μl^{-1} solution of *L. monocytogenes* DNA (prepared from pure culture) was included as a positive control.

The second round employed primers LIP1 and LIP2, which were directed against nucleotides 634 to 654 and 886 to 907 of the sequence and which were internal to the product amplified by PRFA1 and PRFA2; they amplify a product of 274 bp. The reaction mixture contained 50 mmol of KCl and 10 mmol of Tris-HCl (pH 9.0) $liter^{-1}$; 0.1% (vol/vol) Triton X-100; 3 mmol of $MgCl_2$, 150 μmol of each deoxynucleoside triphosphate, and 0.25 μmol of primer $liter^{-1}$; and 1 U of *Taq* DNA polymerase, giving a final volume of 50 μl. Reaction conditions were 94°C for 2 min and then 45 cycles of 94°C for 30 s, 60°C for 30 s, 74°C for 1 min, with a final extension phase of 74°C for 5 min. A 2-μl volume of completed first-round reaction mixture was added to each reaction mixture as target DNA. A reaction mixture containing 2 μl of sterile distilled water was included as a negative control, and a reaction mixture containing 2 μl of a 20-ng μl^{-1} solution of *L. monocytogenes* DNA (prepared from pure culture) was included as a positive control.

For each sample in both primary and secondary rounds of PCR, two separate sets of reactions were run, the second set having 2.5% (vol/vol) Tween 20 included in the reaction mixture as an enhancer.

For detection, 25 μl of PCR mixture was electrophoresed on a 2% (wt/vol) agarose gel, stained with ethidium bromide (0.1 μg ml^{-1}), and viewed under UV light.

RESULTS

L. monocytogenes present in cold-smoked salmon could be detected by PCR with target DNA prepared by all four protocols described above. However, the sensitivity of detection varied according to the method by which target DNA was prepared. Sensitivity was adjudged from the results of the second rounds of the nested PCRs.

When PCR was performed with target DNA prepared by protocol 1, *L. monocytogenes* could not be detected (Fig. 1, lane 2). The PCR was inhibited, probably by the presence of phenolic compounds carried over into the final DNA sample. However, when Tween 20 was incorporated in the reaction mixture, a signal could be obtained (Fig. 1, lane 3). The limit of detection was 6×10^4 CFU of *L. monocytogenes* g of cold-smoked $salmon^{-1}$ (data not shown). A range of concentrations of Tween 20 was tested, and 2.5% (vol/vol) was found to be optimal for the reaction. By adapting the CTAB extraction protocol to include ether separation to remove phenolic contaminants from target DNA (protocol 2), a PCR product was detected regardless of whether enhancer was included in the reaction mixture (Fig. 1, lanes 4 and 5). The sensitivity of the procedure was also substantially increased, and 94 CFU g^{-1} could be detected (data not shown).

For the third DNA extraction protocol, a silica column rather than ether separation was used to purify DNA. Again, inhibitors were removed, since PCR generated a product without the need for addition of enhancer (Fig. 1, lanes 6 and 7). With this extraction procedure, 216 CFU g of $sample^{-1}$ could be detected (data not shown). This protocol was modified, replacing organic solvent extraction by particle separation (protocol 4). The particle separator removed debris from the extract, which could then be run through the silica column without clogging it. With this procedure, there was no requirement for PCR enhancer (Fig. 1, lanes 8 and 9), and 8×10^3 CFU g^{-1} could be detected (data not shown).

A combination of protocols 2 and 3 in which the sample underwent ether extraction and was then passed through the silica column was tried; however, the detection limit was not increased beyond that obtained by either method alone (data not shown).

To confirm the specificity of the detection, DNA was extracted from pure cultures of *L. innocua*, *L. ivanovii*, and *L. seeligeri* by heat lysis, and PCR was carried out in the presence of Tween 20; no signal could be obtained from either round of amplification (Fig. 2). In addition, smoked salmon samples were inoculated with 3×10^5 CFU g of each of these $cultures^{-1}$, and DNA was extracted by protocol 2. No signal was obtained from any of these samples (not shown).

DISCUSSION

The experiments described above show that *L. monocytogenes* contamination can be detected in cold-smoked salmon by protocols to extract total DNA and then selectively amplify sequences from the pathogen. This technique has been used to detect *L. monocytogenes* in a variety of other foodstuffs including milk (12) and soft cheese (13). The protocols described here differ from these others in that they incorporate steps to

combat the inhibition of the PCR caused by substances within the food samples under examination.

Many substances, such as dimethyl sulfoxide, Tween 20, and polyethylene glycol 6000, have been reported to enhance the efficiency of the PCR, but their modes of action are uncertain and they may not work under all reaction conditions (9). It was found that the addition of Tween 20 could allow detection of the *L. monocytogenes* sequences when inhibitors endogenous to the samples were obviously present in the reaction mixtures described here; however, other potential enhancers, such as dimethyl sulfoxide, did not have this effect. It may be beneficial to try PCR enhancers to rescue reactions which may be inhibited by other food components, e.g., sucrose or ovalbumin (10).

Nested PCR, in which primers complementary to sequences amplified by the primary reaction were used in a secondary amplification reaction, was employed in these experiments. Secondary amplification can result in a sensitivity of detection that is several orders of magnitude greater than that with primary amplification alone and has the added benefit of conferring greater specificity, since it should be unlikely that any nonspecific product which may be produced during the primary reaction will contain sequences complementary to the primers of the secondary reaction. In the experiments described here, it was found that detection achieved by primary PCR could be increased by at least 3 orders of magnitude by performing secondary amplification.

Phenolic compounds were deemed to be the likeliest inhibiting substances present in the smoked fish samples. Diethyl ether is often used in DNA extraction methods to remove residual phenol from the extract (11) and, therefore, it was used in protocol 2. Binding of nucleic acids to silica or other matrices within a column and then washing away of other materials which do not bind are also widely used as a purification step, for example in the method described by Boom et al. (2).

When testing for bacterial contamination of food, it is essential that the method used is sufficiently sensitive to detect low levels of contamination. By using whole cells derived from pure cultures, the nested-PCR protocol used in these experiments is capable of detecting approximately 1 *L. monocytogenes* CFU per reaction mixture (11a). Applying extraction procedures necessary to obtain total DNA from foodstuffs prior to PCR inevitably leads to some loss of target, and this will account for the lower detection limits achieved in the cold-smoked salmon experiments. Of the DNA extraction methods detailed within the protocols, the third protocol, which used ether separation, gave the best level of detection at approximately 100 CFU g of food^{-1}. However, in view of what can be achieved under optimal conditions, it is possible that further development of these protocols will lead to greater sensitivity of detection. Ether is extremely flammable, and it may not be desirable to use it routinely in the workplace; however, a reasonably similar level of detection could be achieved by substituting silica column purification, as in protocol 3. Modification of the column matrix to increase the efficiency of DNA binding and/or elution may enhance the overall detection level.

It is also desirable to use the most rapid detection method possible, especially if testing is to be performed routinely. All of the protocols described here can be completed within 2 working days, yielding results well within the time taken by conventional techniques. A further advantage of these protocols is that, by incorporating PCR detection, they provide both an indication of the pathogen's presence and confirmation of its identity in one step, thus obviating the requirement for the time-consuming selective-plating procedures normally used for confirmation (6). Thus, it is considered that DNA extraction and PCR detection could become a viable alternative to the conventional methods for detection of *L. monocytogenes* in cold-smoked salmon and other seafood products. In looking toward future possibilities of routine monitoring, the ease of use of particle separation and column purification, as in protocol 4, may facilitate automation of the process, allowing large numbers of food samples to be processed by minimum numbers of skilled personnel.

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