Enhanced Sensitivity in PCR Detection of *Listeria monocytogenes* in Soft Cheese through Use of an Aqueous Two-Phase System as a Sample Preparation Method

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PCR is one of the most promising techniques for rapid detection of microorganisms in food. However, the usefulness of PCR for detection of microbes in soft cheeses and other complex samples is limited by the presence of factors that inhibit PCR (7, 10, 14).

The aim of the present study was to investigate whether an aqueous two-phase system (1, 12) can provide the basis for a sample preparation method which separates PCR-inhibitory factors from the bacteria in a food sample rather than vice versa, which is the common approach (2, 5, 13). The approach taken was to employ PCR for direct detection of *Listeria monocytogenes* cells after their extraction in the aqueous two-phase system.

**PCR conditions.** The PCR assay comprised two DNA amplification steps (Fig. 1). Primers LM2 (5'-CTTTGACCT CTGGAGCACAGAC-3') and LM1 (5'-GGAGCTAATCCC ATAAAATCTTA-3') were designed on the basis of published nucleotide sequences of 16S rRNA genes (4). The first step involved amplification with oligonucleotides LM2 and ru8 (5'-AAGGAGGTCTACCA[G/A][C/CG][C/CG]TTC-3' [8]) for 30 cycles, and the second amplification step was performed with oligonucleotides LM1 and ru8 for 30 cycles, the latter after 1:10 dilution of the product obtained from the first PCR incubation. The 553- and 275-bp PCR products were visualized by ethidium bromide-stained 1.5% agarose gel electrophoresis (11).

The PCR mixture (50 µl) contained 1.5 U of Taq DNA polymerase (Boehringer GmbH, Mannheim, Germany), 1× PCR buffer (Boehringer), each primer at 0.5 µM, and each of the deoxynucleoside triphosphates at 0.2 mM. The sample volume was 10 µl. The reaction tubes were placed in a thermal cycler (Perkin Elmer Cetus). Each amplification cycle consisted of heat denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 7 min.

**Preparation of food samples.** A sample (25 g) of Danish Blue Castello soft cheese was mixed with physiological saline solution (225 ml), homogenized in a stomacher for 2 min, inoculated with *L. monocytogenes* 167vet (obtained from the Swedish Meat Research Institute, Kävlinge, Sweden), and homogenized again for 30 s.

**PCR detection of *L. monocytogenes* in an aqueous two-phase system.** Aqueous two-phase systems were prepared from stock solutions of the following polymers in water: 20% (wt/wt) dextran 500 (Pharmacia Biotech Norden AB, Sollentuna, Sweden), 20% (wt/wt) dextran 40 (Pharmacia), 40% (wt/wt) polyethylene glycol (PEG) 8000 (Merck, Darmstadt, Germany), and 40% (wt/wt) PEG 4000 (Merck). In preparing the aqueous two-phase system, the polymers were weighed and mixed with water, 0.25 ml of phosphate buffer (100 mM; pH 7), 0.25 ml of sodium chloride (1 M), 0.1 ml of the sample, and water to a total weight of 2.5 g. The sample, in turn, was either a bacterial culture or an inoculated cheese homogenate. The sample and the other constituents of the aqueous two-phase system were mixed by inversion approximately 20 times and then left for 30 min at room temperature to separate.

When the cheese homogenate, inoculated with increasing concentrations of *L. monocytogenes*, was subjected to PCR amplification, a visible PCR product was first obtained at a concentration of 10³ CFU/ml. However, 5 of 10 amplifications were PCR negative at this concentration. The inconsistency was probably due to the variability of the cheese homogenate. In contrast, the results were less variable after partition of the inoculated cheese homogenate in aqueous two-phase systems. The same result was then obtained from experiments repeated 10 times.

When bacterial cultures were partitioned in four aqueous two-phase systems, the *Listeria* cells were detected by PCR in

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**FIG. 1.** Locations of primers LM2, LM1, and ru8 in the 16S rRNA gene. Primers LM2 and LM1 were designed on the basis of published nucleotide sequences of 16S rRNA genes (4), and primer ru8 was obtained from Rådström et al. (8). Universal (U) and variable (V) regions are marked with black and white squares, respectively (the nomenclature used is that of Gray et al. [6]). The dashed regions between the universal and variable regions are semivariable.
both the dextran and PEG phases (Table 1). On the other hand, when an inoculated cheese homogenate was extracted in the same aqueous two-phase systems, PCR products were obtained from the dextran phases only. Samples from the PEG phases were PCR negative. When the PEG phase of these systems was removed and extracted with a new dextran phase, positive PCR results were again obtained from the bottom phase (data not shown). Thus, the PCR templates (Listeria cells or DNA) present in the PEG phase were distributed to the new dextran phase in the second extraction and the PCR-inhibitory factors remained in the PEG phase.

None of the single-polymer solutions in the aqueous two-phase systems inhibited the PCR, since the intensities of the PCR products from inoculated polymer solutions were the same as those of the positive controls, as visualized by ethidium bromide-stained agarose gel electrophoresis.

**Partition of L. monocytogenes in an aqueous two-phase system.** A pure bacterial culture and an inoculated cheese homogenate were added to an aqueous two-phase system (Table 2), and samples were withdrawn from the top and bottom phases for viable-count determination on supplemented Listeria Selective Agar Base (Oxoid CM 856 and SR 140; Unipath Ltd., Basingstoke, England) after incubation at 37°C for 48 h. Most of the L. monocytogenes detected was partitioned to the dextran phase. However, the numbers of L. monocytogenes organisms recovered in the aqueous two-phase system were 1.3 to 2 and 0.2 to 0.4 orders of magnitude below the added amount of L. monocytogenes when a pure bacterial culture and an inoculated cheese homogenate, respectively, were extracted in the system.

One reason for the loss of L. monocytogenes is that bacteria and other large particles are, to some extent, partitioned to the interphase (3). Furthermore, microscopic investigations of samples from the aqueous two-phase systems revealed aggregates of four to five bacteria, as has been observed earlier (9).

**Sensitivity tests with inoculated cheese homogenate.** When an inoculated cheese homogenate (100 μl) was added to water, polymer solutions, and an aqueous two-phase system for a final weight of 2.5 g, L. monocytogenes was detected only in the aqueous two-phase system (Table 3). For the aqueous two-phase system, the detection level was 10^5 CFU/ml of cheese homogenate (Fig. 2).

In conclusion, the aqueous two-phase systems described can, when optimized, be useful at least for partitioning of the PCR inhibitors and bacteria to the PEG and dextran phases, respectively. Extraction in an aqueous two-phase system prior to carrying out PCR was found in the present study to be a fast and simple technique for preparation of samples containing PCR-inhibitory substances originating from soft cheese.

**TABLE 3. Sensitivity of the PCR-based detection assay after sample treatment in water, polymer solutions, and an aqueous two-phase system composed of 8% (wt/wt) PEG 4000 and 11% (wt/wt) dextran 40.**

<table>
<thead>
<tr>
<th>CFU/ml of cheese homogenate</th>
<th>PCR result* obtained with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water 8% (wt/wt) PEG 4000</td>
<td>PEG 4000 Dextran 40</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>Dextran 40</td>
</tr>
<tr>
<td>10^7</td>
<td>–  – –</td>
</tr>
<tr>
<td>10^6</td>
<td>–  – –</td>
</tr>
<tr>
<td>10^5</td>
<td>–  – –</td>
</tr>
<tr>
<td>10^4</td>
<td>–  – –</td>
</tr>
<tr>
<td>10^3</td>
<td>–  – –</td>
</tr>
</tbody>
</table>

\* +, L. monocytogenes detected by PCR; –, negative PCR test result.

FIG. 2. PCR products from the top and bottom phases after extraction of inoculated cheese homogenate in an aqueous two-phase system composed of 8% (wt/wt) PEG 4000 and 11% (wt/wt) dextran 40. Lanes: M, molecular size markers (lambda DNA cleaved with PstI); 1, PEG phase after extraction of a cheese homogenate containing 10^5 CFU/ml; 2, dextran phase after extraction of a cheese homogenate containing 10^5 CFU/ml; 3, PEG phase after extraction of a cheese homogenate containing 10^4 CFU/ml; 4, dextran phase after extraction of a cheese homogenate containing 10^4 CFU/ml; 5, PEG phase after extraction of a cheese homogenate containing 10^3 CFU/ml; 6, dextran phase after extraction of a cheese homogenate containing 10^3 CFU/ml; 7, positive control; 8, negative control.
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REFERENCES