Direct Detection of Viable Bacteria, Molds, and Yeasts by Reverse Transcriptase PCR in Contaminated Milk Samples after Heat Treatment

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A fast, sensitive, and target contaminant-modulable method was developed to detect viable bacteria, molds, and yeasts after heat treatment. By reverse transcriptase PCR with elongation factor gene (EF-Tu or EF-1α)-specific primers, the detection level was 10 cells ml of milk⁻¹. The simplicity and rapidity (4 h) of the procedure suggests that this method may be easily transposable to other foods and other contaminants.

Methods to estimate the total number of living contaminants in food samples accurately and reliably have not yet been encountered and therefore remain a public health concern. The definition of viability of microorganisms being neither simple nor straightforward (14), in this study viable cells are considered to be those capable of mRNA production of a protein synthesis elongation factor. The translation elongation factor EF-Tu (for eubacteria) (reviewed in reference 21) or EF-1α (for archaeabacteria and eukaryotes) (reviewed in reference 11) plays a basic role in protein synthesis, guiding the aminoacylated tRNAs to the acceptor site in ribosomes under conditions of GTP consumption (19). Currently employed standard methods such as plate counting, microscopic enumeration, and indirect activity measurements are usually time-consuming and inadequate to protect human health (1). New methods using fluorescent nucleic acid stains seem efficient for measuring bacterial viability but are nonspecific (15). A technique that offers the greatest potential for the detection and/or measurement of bacterial viability but are nonspecific is DNA-DNA binding. By reverse transcriptase PCR with elongation factor gene (EF-Tu or EF-1α)-specific primers, the detection level was 10 cells ml⁻¹. The simplicity and rapidity (4 h) of the procedure suggests that this method may be easily transposable to other foods and other contaminants.

In order to detect contaminants in milk, serial concentrations ranging from 10⁴ to 1 cell ml⁻¹ were performed in commercially available cow milk pasteurized at an ultrahigh temperature. PCR interference by the milk was eliminated by four cycles of washing with phosphate-buffered saline (PBS) as mentioned by Cooray et al. (3). A filtration step was added. The final pellet of washed milk was resuspended with PBS to the original volume of milk and filtered through a PBS-pre-soaked 25-mm-diameter polycarbonate membrane (0.8-μm pore size; Millipore). The filter was transferred to a 15-ml Falcon tube (Becton Dickinson). One milliliter of PBS was added and vortexed vigorously for 30 s. After recovery, 0.5 ml more of PBS was used and the resulting suspensions were collected and then centrifuged for 5 min at 5,000 × g. To extract RNA, 40 U of RNase inhibitor (Boehringer) and H₂O adjusted with 0.05% diethylpyrocarbonate to inactivate nucleases (14) were added to a final volume of 50 μl. Cells were then disrupted by adding 50 mg of glass beads (0.5-mm diameter; Biorep Prod, Bartlesville, Okla.). The tubes were shaken twice for 20 s at 900 × g, at 10-s intervals, and left on ice between periods of shaking. After decanting, 10 μl of the lysate was transferred to a new tube. A solution containing 1× EZ buffer (Perkin-Elmer), 2.5 mM manganese acetate, and 10 U of DNase I (RNase free; Boehringer) was added to a final volume of 20 μl. This DNase step was included in order to prevent amplification of contaminating genomic sequences (20). After incubation for 10 min at 37°C, DNase was inactivated by raising the temperature to 95°C for 5 min. Both steps, reverse transcription and PCR, were performed successively in the same tube. For the reverse transcription step, 15 μl of the lysate digested with DNase I, 1× EZ buffer, 1.4 mM manganese acetate, 0.3 mM deoxyribonucleoside triphosphate (Perkin-Elmer), 0.4 μM each primer (upper and lower strands), and 5 U of rTth DNA polymerase (Perkin-Elmer) were added to a final volume of 50 μl. The mixture was incubated for 2.5 min at 95°C, 20 min at 60°C, and 1 min at 95°C. For each step, 40 cycles were directly performed, each consisting of 15 s at 95°C and 30 s at 60°C. A final extension was performed for 10 min at 60°C. False-positive detection due to DNA contamination was verified by adding 5 μl of the lysate digested with DNase I, 1× Taq buffer (Boehringer), 0.2 mM deoxyribonucleoside triphosphate, 0.4 μM each primer, and 2.5 U of Taq

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TABLE 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer pair sequences</th>
<th>Specificity</th>
<th>GenBank or EMBL accession no.</th>
<th>Length (bp)</th>
<th>Microorganism(s) tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGCTGGAAGGCGACGMRRAG3</td>
<td>M. leprae, tuberculosis, E. coli, Brevibacterium linens Streptomyces, ramocissimus</td>
<td>X57091, X76863, X67057</td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td>CGGAAGTAGAACTGCGGACGGTAG3</td>
<td>M. leprae, tuberculosis, E. coli, Brevibacterium linens Streptomyces, ramocissimus</td>
<td>X57091, X76863, X67057</td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td>TCCATGGTACAAGGGTTGGGAA3</td>
<td>C. albicans, Saccharomyces cerevisiae</td>
<td>X01638, M29934, M29935</td>
<td>95</td>
<td>59</td>
</tr>
<tr>
<td>GCGAATCTACCTAATGGTGGGT3</td>
<td>Neurospora crassa, Mucor racemosus, Aspergillus nidulans, Humicola grisea, Trichoderma reesei, Absidia glauca, Trichoderma reesei</td>
<td>J02605, M16352, X17475, X17476, D45837, Z23012, X54730, U19723, U14100, X7352</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>GCCAAGCCTGCAAAGAAGAAAGAA3</td>
<td>D13147</td>
<td>Triticum aestivum</td>
<td>431</td>
<td>431</td>
</tr>
<tr>
<td>GCCAAGCCTGCAAAGAAGAAAGAA3</td>
<td>T. aestivum</td>
<td>D13147</td>
<td>431</td>
<td>431</td>
</tr>
</tbody>
</table>

DNA polymerase (Boehringer) to a final volume of 50 μl. The amplification products (10 μl) were separated by horizontal gel electrophoresis (1 to 2% agarose in Tris-acetate-EDTA) and visualized by ethidium bromide staining and UV illumination. As a positive RT-PCR control, 0.1 μg of total RNA was analyzed and extracted by using the RNAgent Total RNA Isolation kit (Promega). The amount of RT-PCR products was normalized with respect to a reference template corresponding to the reverse transcription and amplification of EF-1α from 0.1 μg of total RNA of Triticum aestivum added in samples. To confirm the identity of amplified products, the bands of expected size were gel purified (Gel Extract kit; Qiagen), and then 1 μl of the 50 μl of DNA recovery solution was used as a template in a second PCR using internal primers (data not shown).

Using the above-mentioned RT-PCR setting, several controls were routinely carried out: negative control without cells (Fig. 1, lane 1), RT-PCR control with pure extracted total RNA (Fig. 1, lane 2), and DNase I control with single PCR on lysate digested with DNase I (Fig. 1, lane 3). No detectable PCR products occurred; thus, inclusion of the DNase I step appears to prevent amplification of contaminating genomic sequences. Due to the fast RNA extraction procedure we used, we systematically cocultured DNA that gave false positives (data not shown). Other authors previously mentioned this problem (9, 20), even though some others did not observe the same phenomenon (18). The importance of the RT step was highlighted by performing RT-PCR on lysate without DNase I and RT-PCR on lysate without DNase I but with RNase (Fig. 1, lanes 4 and 5, respectively). In the second case, RNA only is responsible for the PCR product, giving a weaker signal.

We then tried to detect E. coli, S. cerevisiae, and M. racemosus suspended at serial concentrations ranging from 10^5 to 1 cell ml^-1 in milk. With all the strains, it was possible to detect the elongation factor at contaminant concentrations as low as 10 cells ml^-1, corresponding to 5 to 10 cells per reaction tube in our procedure (Fig. 2A to C, lanes 5). No transcripts were detected at lower contaminant concentrations and in noncontaminated milk (Fig. 2A to C, lanes 6 and 7, respectively). The same intensity of specific band from T. aestivum total RNA suggests the level of RT-PCR is roughly the same in each dilution (Fig. 2D). We used the same method on contaminated beer and yogurt, and microorganisms were detected with the same level of sensitivity.

The correlation between appearance of RT-PCR products and cell viability after heat treatment was verified by performing a time course experiment during heat treatment at 60°C. One half of the cells was used for our RT-PCR procedure and the other half was used for plating. For E. coli, no amplified products of EF-Tu mRNA were detected 6 min after heat
treatment (Fig. 3A, lane 7), and no colonies were detected on plates after 4 min. For *S. cerevisiae*, no amplified products of EF-1α mRNA were detected 8 min after heat treatment (Fig. 3B, lane 9) and no colonies were detected on plates after 2 min. We thus conclude that detection of mRNA for EF-Tu or EF-1α is appropriate for measuring cell viability and that the half-lives of these mRNAs are less than 6 min for EF-Tu and less than 8 min for EF-1α after heat treatment.

The RT-PCR procedure was then applied to a mix of living and dead cells after heat treatment of *E. coli*, *S. cerevisiae*, and *M. racemosus* in contaminated milk. When all cells had died (verified by plating), RT-PCR detection was negative (Fig. 4, lanes 1), indicating that the RT-PCR method developed in this study appears to detect only living cells. Although production of quantitative results is quite difficult by conventional RT-PCR, since the yield of both amplification (7) and reverse transcription (6) steps can be grossly variable in different reactions, the gel electrophoresis analysis showed a quantitative increase in the intensity of the specific band as the number of viable cells increased (Fig. 4, lanes 2 to 5), indicating the apparent increase in RT-PCR target DNA as the number of viable cells increased.

In some cases, foods are contaminated by several microorganisms of different families and different kingdoms. Therefore, it seemed helpful to develop a procedure in which various contaminants could be detected simultaneously in a simple step. In this way, we performed a simultaneous RT-PCR detection of the EF-Tu gene of *E. coli* and the EF-1α gene of *S. cerevisiae* and *M. racemosus* in one reaction. Using milk contaminated with *E. coli*, *S. cerevisiae*, and *M. racemosus*, the RT-PCR-amplified products of expected size (Table 1) were simultaneously detected when two (Fig. 5, lanes 4 to 6) or three (Fig. 5, lane 7) pairs of primers were placed in the same reaction mixture without modification of RT-PCR conditions.

RT-PCR is a powerful method in the detection of viable contaminants due to its high potential to increase detection sensitivities and its speed. The detection level was 10 cells ml⁻¹ of milk⁻¹, and the described RT-PCR setting, including washing and filtering of the milk sample, could be carried out in 4 h without need of a preenrichment step. The choice of the target gene is of importance. Unlike other genes (9), the use of primers specific for the coding region of the elongation factor gene seems to be reasonable for several reasons. First, the elongation factor gene may be considered as an appropriate viability marker since inactivation of both the prokaryotic and
FIG. 5. Gel electrophoresis of RT-PCR products of the EF-Tu gene of E. coli and of the EF-1α gene of S. cerevisiae and M. racemosus obtained in a single RT-PCR. All cells were suspended in milk at 10^2 cells ml^{-1}. Lane 1, yeast primers; lane 2, bacterial primers; lane 3, mold primers; lane 4, bacterial and yeast primers; lane 5, yeast and mold primers; lane 6, bacterial and mold primers; lane 7, bacterial, mold, and yeast primers; lanes M, 1-kb ladder (Gibco BRL).

eukaryotic corresponding genes is a lethal event (2, 4). Second, the elongation factor gene encodes one of the most abundant prokaryotic and eukaryotic proteins (8, 17), allowing a considerable increase in the sensitivity level. Third, the function conserved throughout prokaryotes and eukaryotes as well as the similarity of primary structures (10) of the elongation factor allows the modulation of the specificity of detection. As far as we know this study is the first description of simultaneous detection of viable organisms belonging to two kingdoms. Fourth, due to the short mRNA half-lives, detecting elongation factor mRNA would indicate the presence of living cells that had been present within about the last 6 to 8 min after heat treatment, depending on the microorganism studied. We successfully transposed the procedure described in this study to use with yogurt and beer. This method can be used throughout prokaryotes and eukaryotes as well as the similarity of primary structures (10) of the elongation factor gene encodes one of the most abundant prokaryotic and eukaryotic proteins (8, 17), allowing a considerable increase in the sensitivity level. Third, the function conserved throughout prokaryotes and eukaryotes as well as the similarity of primary structures (10) of the elongation factor allows the modulation of the specificity of detection. As far as we know this study is the first description of simultaneous detection of viable organisms belonging to two kingdoms. Fourth, due to the short mRNA half-lives, detecting elongation factor mRNA would indicate the presence of living cells that had been present within about the last 6 to 8 min after heat treatment, depending on the microorganism studied. We successfully transposed the procedure described in this study to use with yogurt and beer. This method can be used for the detection of viability after heat treatment of other contaminants in other foods, provided appropriate primers and food pretreatment are available. We are currently investigating more precise quantitative analysis by real-time quantitative RT-PCR.

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REFERENCES