A Research Note

Polymerase Chain Reaction Identification of Enteroinvasive Escherichia coli Seeded into Raw Milk

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(Received for publication July 24, 1991)

ABSTRACT

Molecular biological techniques for identifying pathogenic foodborne bacteria use small amounts of bacterial nucleic acids that must be purified and concentrated from complex food matrices. The polymerase chain reaction (PCR) amplifies specific segments of DNA and speeds the identification of bacterial strains. Methods for preparing DNA for PCR analysis were tested by seeding enteroinvasive Escherichia coli (EIEC) cells into raw milk. Test samples of milk were heated at 35°C and were treated with proteinase and detergent. As few as 10^2 cells of EIEC per ml of seeded milk were identified after amplification of a plasmid-borne gene required for invasion.

Milk is a major component of the American diet; however, it can present some microbiological risks if not collected, processed, distributed, and stored with appropriate care. Although more than 12 million microbiological tests are conducted on milk and milk products each year (Richard Saunders, Food and Drug Administration, personal communication), dairy products accounted for 6% of the outbreaks and 30% of the cases of foodborne disease in the United States from 1983 to 1987 (1).

Identification of the pathogenic microorganisms in milk and milk products can present problems. Cultural enrichment methods often are not specific enough to select low numbers of a particular bacterial strain from among a high background of indigenous microflora. Furthermore, additional work and time are required to isolate and characterize pure cultures. Physiological stress during enrichment may cause the cells to lose plasmids; thus, pathogenic isolates may not be identified (3). Rapid and accurate methods, therefore, are needed to screen milk for the presence of pathogenic microorganisms.

The polymerase chain reaction (PCR) is a rapid and sensitive method that is used to identify specific strains of pathogenic bacteria in some foods (4); however, preparing the test samples for this method can be labor intensive. A reasonably rapid and simple technique, therefore, was sought for preparing milk for PCR analysis. The PCR-based test for invasive Shigella species developed by Lampel et al. (5) was applied to the identification of enteroinvasive Escherichia coli (EIEC) in raw, whole milk. Several protocols for preparing bacterial DNA from seeded milk were compared for use in extracting PCR templates.

MATERIALS AND METHODS

Milk seeding and preparation

Raw, unhomogenized milk was seeded with EIEC strain M4163 (2,5) and bacterial DNA was extracted (Fig. 1). Bacteria for seeding experiments were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C. Seeded milk was incubated at 37°C for 0, 2, 4, or 24 h, and then held at room temperature for 4 h or refrigerated overnight. After centrifugation at 1,500 rpm for 1 min, the supernatant was incubated at 35°C for 30 min or treated with proteinase K (0.1 mg/ml; Sigma Chemical Co., St. Louis, MO) for 60 min, or subjected to the next procedure without further manipulation. This supernatant was centrifuged at 8,000 rpm for 5 min, and the pellet was treated in one of the following ways: i) resuspended in 100 ul Tris-buffered saline (TBS) and boiled at 98°C for 8-10 min; or ii) resuspended in 100 ul TBS, prewarmed to 37°C, and a) treated with proteinase K as above, or b) incubated at 60°C, for 1 h after addition of a 0.1-volume of lysis solution, containing proteinase K (50 mg/ml), Brij 35 [nonionic detergent, 3% w/v (Sigma)], 0.1 M EDTA, and 0.2 M Tris HCl (pH 7.5), or c) not manipulated, and then boiled as above; or iii) resuspended in 100 ul TBS, prewarmed to 37°C, and a) treated with proteinase K as above, or b) incubated at 60°C, for 1 h after addition of a 0.1-volume of lysis solution, containing proteinase K (50 mg/ml), Brij 35 [nonionic detergent, 3% w/v (Sigma)], 0.1 M EDTA, and 0.2 M Tris HCl (pH 7.5), or c) not manipulated, and then boiled as above; or iii) resuspended in 100 ul TBS, and then incubated at 60°C for 1 h after addition of a 0.1-volume of 5.88 M guanidine isothiocyanate (GITC) stock solution. DNA was then precipitated by adding a 0.1-volume of 3 M sodium acetate and 2 volumes of -20°C ethanol (100%) and placing the mixture on ice for 30 min. After centrifugation at 10,000 rpm for 15 min, the ethanol was removed by aspiration; the pellet was then washed in cold 70% ethanol and centrifuged again at 10,000 rpm for 5 min. The ethanol was removed by aspiration, and the pellet was allowed to dry before being resuspended in 20 ul of sterile deionized water.

PCR reagents (Perkin Elmer/Cetus, Norwalk, CT; Amersham International Inc., Arlington Heights, IL; and United States Biochemical Corp., Cleveland, OH) were used for the reactions. A

JOURNAL OF FOOD PROTECTION, VOL. 55, MAY 1992

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100-pmol amount of each primer, KL1 and KL8 (5), and 2 µl of a DNA preparation were added to a standard 100-µl reaction. After inactivation of nucleases for 10 min at 94°C, 30 to 45 cycles were carried out as follows: denaturation, 1 min at 94°C; annealing, 2 min at 62°C; extension, 2 min at 72°C in a Perkin Elmer/Cetus DNA thermal cycler. Purified EIEC plasmid was used as a template for positive-control reactions.

Agarose gel electrophoresis

Twenty µl from each completed PCR reaction was run for 1 h at 150 V on a 1% agarose gel. A 123-bp ladder (BRL, Gaithersburg, MD) was used as a molecular weight (mol wt) standard.

RESULTS AND DISCUSSION

Bacterial DNA was prepared by several combinations of treatments for use as PCR templates (Fig. 1). The ability of these DNAs to serve as templates was determined by the relative intensities of bands on agarose gels (Figs. 2-4). Many treatment combinations yielded negative results (data not shown).

Several methods for preparing DNA templates for the PCR were compared. Fig. 2 shows the analysis of the PCR products by gel electrophoresis. Possibly by liquefying milk fats, incubation for 30 min at 35°C allowed more efficient extraction and recovery of template DNA, or resulted in fewer PCR inhibitors than did treatment with proteinase K and detergent (Fig. 2).

The sensitivity was determined by seeding raw milk with various amounts of EIEC (Fig. 3). Incubation times were also varied and test portions were refrigerated overnight (except for the test portion incubated for 24 h). Samples seeded with 10^4 cells/ml yielded positive results even with no incubation at 37°C. At 10^2 cells/ml, EIEC cells were identified only after 4 h of incubation at 37°C. In initial experiments, test portions incubated overnight were not refrigerated before DNA extraction. Insoluble materials were much more difficult to remove during low speed centrifugation and were subsequently precipitated with the DNA and may have interfered with the PCR, causing negative results even at high seeding levels. When these samples were refrigerated overnight, the insoluble material was removed and results were positive; however, analysis time was increased.

To determine the effects of incubation and enzyme treatment, raw milk was seeded with various numbers of EIEC cells and held at room temperature for 4 h. These samples were incubated at 35°C for 30 min, pelletted, treated with proteinase K, boiled, and added as template to amplification reactions. Raw milk seeded with as few as 10^2 EIEC cells/ml yielded positive results (Fig. 4). A comparison of these results with those shown in Fig. 3 shows that incubation at 35°C for 30 min gave greater assay sensitivity than did refrigerating the samples overnight.

Two approaches for designing protocols for test sample preparation were explored: i) extracting bacterial DNA directly from the seeded food matrix, and ii) eliminating as much of the food matrix as possible before proceeding with bacterial DNA extraction. Sensitivity was improved with the latter approach. Because milk fat made samples difficult to handle, it was removed by either of two methods. Fats were allowed to set during extended (up to 24 h) refrigeration at 4°C and bacterial cells were then removed by pelleting while milk fat remained at the top of the tube. Alternatively, fats were liquefied and milk proteins solubilized by incubation at 37°C and bacterial cells were removed by centrifugation.

SUMMARY AND CONCLUSIONS

PCR was used to identify as few as 10^2 cells/ml of EIEC seeded into raw milk. The greatest sensitivity was obtained by incubating seeded milk at 37°C for 30 min to liquefy milk fats, followed by treatment with proteinase K, boiling, and ethanol precipitation. Because only 10% of the final DNA preparation was used in the PCR, a sensitivity as low as 10 cells/ml may be possible.
Figure 2. Raw milk seeded with $10^6$ cells/ml of EIEC was incubated overnight at 37°C and centrifuged at low speed to separate liquid from solids and fat. The resulting liquid was treated in various ways before it was boiled, precipitated with ethanol and used as a template for PCR. Lanes: 1, incubated at 35°C for 30 min; 2, proteinase K, washed, proteinase K plus Brij 35; 3, washed, proteinase K plus Brij 35; 4, pH decreased to 3.8 with acetic acid; 5, plasmid control; M, 123-bp mol wt standard.

Figure 3. Raw milk was seeded with different amounts of EIEC and incubated for various times at 37°C. Samples were refrigerated overnight and centrifuged at low speed; supernatants were treated with proteinase K and boiled. DNA was then precipitated with ethanol and used as a template for PCR. Lanes: 1, 0 cells/ml, 0 h; 2, $10^2$ cells/ml, 0 h; 3, $10^2$ cells/ml, 2 h; 4, $10^4$ cells/ml, 2 h; 5, $10^6$ cells/ml, 4 h; 6, $10^6$ cells/ml, 4 h; 7, 0 cells/ml, overnight; 8, $10^2$ cells/ml, overnight; 9, positive plasmid control; M, 123-bp mol wt standard.

Figure 4. Various amounts of EIEC were seeded into raw milk, held for 4 h at room temperature, incubated at 35°C for 30 min, pelleted, treated with proteinase K, boiled, and added to PCR tubes. Lanes: 1, 0 cells/ml; 2, $10^2$ cells/ml; 3, $10^2$ cells/ml; 4, $10^4$ cells/ml; 5, plasmid; M, DNA positive control.

ACKNOWLEDGMENTS

We thank Peter Feng, Al Sheldon, Darcy Hanes, and Keith Lampel, Food and Drug Administration, Washington, DC, and Eddie Pahuski, Promega Corp., Madison, WI, for assistance.

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