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Bacteriological Analytical Manual

Chapter 28: Detection of Enterotoxigenic *Vibrio cholerae*

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Introduction

Recent epidemics of cholera in various parts of the world have emphasized the urgent need for rapid and reliable detection methods for *Vibrio cholerae*, especially in food and water. Classical microbiological methods are sensitive and specific; however, they require several days to complete (**see** Chapter 9) and may result in considerable loss of perishable foods. Since cholera toxin production (encoded by the *ctxAB* genes) is the major factor in the pathogenesis of cholera, a polymerase chain reaction (PCR) method that selectively amplifies a DNA fragment within the *ctxAB* operon of *V. cholerae* has been developed and applied to various foods (19).

The PCR was first described by Mullis et al. in 1985, and since then has revolutionized most of the biological sciences (25). In this technique, double-stranded target DNA is denatured to provide single-stranded templates to which specific oligonucleotide primers are hybridized, followed by primer extension with a thermostable DNA polymerase (26). Primer pairs complementary to opposite strands of a DNA region are chosen so that 5' to 3' directional extensions are toward one another. Thus repetitive denaturation, annealing, and primer extension cycles exponentially amplify a unique DNA fragment bordered by the primers. The process is extremely rapid (as little as 30 min for 25 cycles with certain thermocyclers) and sensitive (amplification of gene sequences from a single cell is possible). Further, the process can be designed to be specific for genus, species, or allele.

Use of the PCR as a detection method for microbial pathogens in foods has been documented in approximately two dozen PCR-based, detection procedures published by early 1994 (Table 1). PCR-based methods have been developed to detect a wide variety of foodborne pathogens, including *Listeria monocytogenes*, enterotoxigenic *Escherichia coli* (**see** Chapter 4), *V. vulnificus*, *V. cholerae*, *Shigella flexneri*, *Yersinia enterocolitica*, various *Salmonella* and *Campylobacter* species, and the Hepatitis A (15) (**see** Chapter 26) and Norwalk viruses.

Table 1. PCR methods developed for the detection of pathogenic microbes in foods

Organism	Target gene/gene product	Reference
Campylobacter spp.	16S rRNA	14
C. jejuni	*flaA-flaB* intergenic sequences	32
C. coli	*flaA-flaB* intergenic sequences	32
Escherichia coli	*malB*, LT1, ST1	5
Escherichia coli	LT	34
Escherichia coli	SLTI, SLTII	13
Escherichia coli	VT1, VT2 and VTE	23
Escherichia coli	plasmid invasion gene (*ial*)	1,20
Listeria monocytogenes	listeriolysin O (*hly*)	3,4,9,10,12,21,22,24,29,34
Listeria monocytogenes	16S rRNA	30
Listeria monocytogenes	Listeria cell surface protein	31
Salmonella spp.	*oriC*, chromosomal origin of replication	11
Shigella flexneri	plasmid invasion gene	20
Vibrio cholerae	*ctxAB*	19
V. vulnificus	cytotoxin-hemolysin	16
Yersinia enterocolitica	virulence plasmid *virF* gene	35
Yersinia enterocolitica	*yadA*	17
Hepatitis A virus[(a)]	polymerase gene	2,15
Norwalk virus[(a)]	polymerase gene	2

[a] Reverse transcription PCR (RT-PCR).

Many of these PCR methods have relied on extraction of DNA from contaminated foods, an additional step which adds several hours to the procedure and often requires modification of each diverse food matrix tested. An advantage of PCR is that the amplification reaction often proceeds well with crude lysates of cells, in some cases requiring only brief boiling of a bacterial suspension.

Although the PCR method, in principle, can detect a single bacterial cell with extended cycle regimens (50-60), the detection limit of direct PCR is effectively confined to about 10^4 bacteria per gram of food. This limitation is due to reaction volume constraints (25-100 µl), the increased propensity to amplify nonspecific products at high cycle numbers, and the inhibitory effect of many food components on *Taq* polymerase. Thus, the coupling of enrichment procedures with the PCR has effectively served two purposes: 1) It increases the sensitivity of detection to as few as 0.1 organism per gram of food; 2) it demonstrates by comparison of pre- and post-enrichment inocula that the food contains **viable** organisms. The added sensitivity and information regarding cell viability warrants the additional 4-24 hours (depending on the growth characteristics of the organism) required for such a procedure, and overcomes an early criticism that PCR would give false-positive results because it amplifies any DNA, including that of dead or nonviable organisms.

Results of amplification reactions are usually obtained by the resolution of products based on size via agarose gel electrophoresis and visualization by UV-induced fluorescence after staining with ethidium bromide. The complete PCR amplification and agarose gel analysis generally requires only about 2-4 hours after enrichment. In terms of sensitivity, specificity, and analysis time, selective enrichment followed by PCR is clearly a powerful, rapid, and robust methodology for detecting foodborne bacterial pathogens.

V. cholerae of the Inaba and Ogawa serotypes which lack the cholera toxin genes have been isolated; however, such strains are generally nonpathogenic. Since the presence of the cholera toxin operon is a prerequisite for pathogenicity, various PCR methods for the detection of pathogenic *V. cholerae* have all used the *ctxAB* genes as a target for amplification (8,18,28); these and the PCR method described here, will not detect nontoxigenic *V. cholerae*. As a practical matter, this PCR detection method allows one to define food samples as negative for the presence of toxigenic *V. cholerae* much more quickly than by following the complete microbiological identification scheme. However, it is recommended that alkaline peptone water (APW) enrichment broths used for PCR analysis also be plated onto selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar (**see** Chapter 9) for isolation and direct confirmation of the presence of *V. cholerae* in samples that give positive PCR results.

A. Equipment and materials

1. For APW enrichment of *V. cholerae* (**see** Chapter 9).
2. Programmable automatic thermocyler
3. Horizontal gel electrophoresis apparatus
4. Electrophoresis constant-voltage power supply
5. Heating plate
6. Microcentrifuge tubes, 1.5 and 0.6 ml
7. Variable digital micropipettors (e.g., 0.5-20 µl, 20-200 µl)
8. Aerosol-resistant pipet tips
9. Microcentrifuge
10. UV transilluminator
11. Polaroid camera
12. Polaroid film

B. Media and Reagents

1. Alkaline peptone water (APW) (**see** Chapter 9)
2. Cholera toxin PCR primers, 10 pmol/µl stock solutions (5'-TGAAATAAAGCAGTCAGGTG-3', 5'-GGTATTCTGCACACAAATCAG-3'; **see** ref. 19)
3. *Taq* DNA polymerase (native available from various vendors) or Amplitaq® (Perkin-Elmer)
4. 2'-Deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP, dTTP); stock solution 1.25 mM of each dNTP
5. 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$)
6. Light mineral oil
7. Sterile deionized water
8. 10× TBE (0.9 M Tris-borate, 0.02 M EDTA, pH 8.3)
9. Agarose (nucleic acid electrophoresis grade)
10. Ethidium bromide solution, 10 mg/ml
11. 6× sample loading buffer (**see** ref. 27)
12. DNA molecular weight markers (e.g., 123 bp ladder, Bethesda Research Laboratories, Gaithersburg, MD)

C. Procedure for amplification of cholera toxin gene sequences from *V. cholerae* using APW enrichment broth

Food sample preparation and APW enrichment (**see** Chapter 9).

APW enrichment lysate preparation. Prepare APW washes or blends (**see** Chapter 9). Sample and freeze **immediately** (about 1 ml). After enrichment (6-24 h), prepare crude APW lysates for PCR by boiling 1 ml samples in 1.5 ml microcentrifuge tubes for approximately 5 min. Lysates may be used for PCR immediately or stored in a -20°C freezer until use. **NOTE**: Due to the enormous amplification possible with the PCR, minute levels of contamination can result in false positives. It is recommended that sample preparation, PCR reaction set-up, and PCR product analysis be physically separated from one another to minimize contamination. For an excellent discussion of considerations in setting up a PCR laboratory, see *PCR Methods and Applications* **3**(2):S1-S14, (1993) A Manual Supplement, Section 1: Establishing PCR in the Laboratory (6,7). Minimally, use of aseptic technique in handling all PCR reagents and solutions is absolutely necessary. Use aerosol-resistant pipet tips for preparing samples and reagents for PCR reactions, and, if possible, a separate set of pipettors for analysis of PCR reaction products.

PCR reaction preparation. To minimize cross contamination of PCR reagents, it is recommended that master mix solutions be prepared, aliquoted, and stored frozen. Master mixes contain all necessary reagents except *Taq* polymerase and the lysates being amplified. The final reaction contains 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, and dTTP; 2 to 5% (v/v) APW lysate; 0.5 µM of each primer and 2.5 U *Taq* polymerase per 100 µl; reaction volumes of 25-100 µl may be used. Add *Taq* polymerase to the master mix and add APW lysate upon distribution to 0.6 ml microcentrifuge tube reaction vessels. Cover with approximately 50-70 µl of mineral oil.

Temperature cycling. While there is some variability in the heating and cooling dynamics of thermocylers from different manufacturers, use of the following temperature cycling regimen should yield efficient amplification of the *ctx* gene fragment: Denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension 72°C for 1 min, repeated for no more than 35 cycles. Increasing the cycle number beyond 35 cycles often leads to the formation of nonspecific amplification products, including primer dimers.

Agarose gel analysis of PCR products. Mix 10-20 µl portions of PCR reactions with 6× gel loading buffer (choose one of four common buffers from *Molecular Cloning: A Laboratory Manual* by Sambrook et al. (27) and load into sample wells of 1.5-1.8% agarose gel submerged in 1× TBE containing 1 µg/ml ethidium bromide. After appropriate migration with a constant voltage of 5-10 V/cm, illuminate the agarose gel with a UV transilluminator and visualize bands relative to molecular weight marker migration. The primers listed above give rise to a 777 bp

fragment (19). Take Polaroid photographs of gels to document results. Further details regarding gel electrophoresis analyses may be found in the above-mentioned *Molecular Cloning Laboratory Manual* (27).

Proper controls. The need for a number of control reactions to ensure accurate interpretation of PCR results cannot be overemphasized. Minimally, for PCR analysis of food types previously optimized for this method (e.g., vegetable washes, oyster, crab and shrimp blends; **see** ref. 19), include a master mix contamination control containing no lysate and a toxigenic *V. cholerae* APW positive control in **every** analysis. For every **new** food blend to be analyzed by this PCR method, determine the potential inhibitory effects of that food. Minimally, this entails spiking 1 ml of a 1:10 and 1:100 APW food blend **post-enrichment** with about 5×10^6 organisms per ml (or an equivalent amount of positive control lysate). A direct comparison of these spiked samples with the APW (no food) lysate containing identical numbers of *ctx*$^+$ cells, allows one to determine if any inhibition occurs at either of the two food concentrations and prevents the occurrence of false negatives. It is unlikely that food washes (e.g., fruits and vegetables) will inhibit the PCR reaction unless the fruits are bruised and washing releases excessive acidity to the APW wash.

References

Andersen, M.R., and C.J. Omiecinski. 1992. Direct extraction of bacterial plasmids from food for polymerase chain reaction amplification. Appl. Environ. Microbiol. 58:4080-4082.

Atmar, R.L., T.G. Metcalf, F.H. Neill, and M.K. Estes. 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. Appl. Environ. Microbiol. 59:631-635.

Blais, B.W., and L.M. Phillippe. 1993. A simple RNA probe system for analysis of Listeria monocytogenes polymerase chain reaction products. Appl. Environ. Microbiol. 59:2795-2800.

Bohnert, M., F. Dilasser, C. Dalet, J. Mengaud, and P. Cossart. 1992. Use of specific oligonucleotides for direct enumeration of Listeria monocytogenes in food samples by colony hybridization and rapid detection by PCR. Res. Microbiol. 143:271-280.

Candrian, U., B. Furrer, C. Hofelein, R. Meyer, M. Jermini, and J. Luthy. 1991. Detection of Escherichia coli and identification of enterotoxigenic strains by primer-directed enzymatic amplification of specific DNA sequences. Int. J. Food Microbiol. 12:339-351.

Dieffenbach, C.W., and G.S. Dveksler. 1993. Setting up a PCR Laboratory. PCR Methods Appl. 3(2):S1-S7.

Dragon, E.A. 1993. Handling reagents in the PCR laboratory. PCR Methods Appl. 3(2):S8-S9.

Fields, P.I., T. Popovic, K. Wachsmuth, and O. Olsik. 1992. Use of the polymerase chain reaction for detection of toxigenic Vibrio cholerae O1 strains from the Latin American cholera epidemic. J. Clin. Microbiol. 30:2118-2121.

Fitter, S., M. Heuzenroeder, and C.J. Thomas. 1992. A combined PCR and selective enrichment method for rapid detection of Listeria monocytogenes. J. Appl. Bacteriol. 73:53-59.

Fluit, A.C., R. Torensma, M.J. Visser, C.J. Aarsman, M.J. Poppelier, B.H. Keller, P. Klapwijk, and J. Verhoef. 1993. Detection of Listeria monocytogenes in cheese with the magnetic immuno-polymerase chain reaction assay. Appl. Environ. Microbiol. 59:1289-1293.

Fluit, A.C., M.N. Widjojoatmodjo, A.T. Box, R. Torensma, and J. Verhoef. 1993. Rapid detection of salmonellae in poultry with the magnetic immuno-polymerase chain reaction assay. Appl. Environ. Microbiol. 59:1342-1346.

Furrer, B., U. Candrian, C. Hoefelein, and J. Luethy. 1991. Detection and identification of Listeria monocytogenes in cooked sausage products and in milk by in vitro amplification of haemolysin gene fragments. J. Appl. Bacteriol. 70:372-379.

Gannon, V.P., R.K. King, J.Y. Kim, and E.J. Thomas. 1992. Rapid and sensitive method for detection of Shiga-like toxin-producing Escherichia coli in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol. 58:3809-3815.

Giesendorf, B.A., W.G. Quint, M.H. Henkens, H. Stegeman, F.A. Huf, and H.G. Niesters. 1992. Rapid and sensitive detection of Campylobacter spp. in chicken products by using the polymerase chain reaction. Appl. Environ. Microbiol. 58:3804-3808.

Goswami, B.B., W.H. Koch, and T.A. Cebula. 1993. Detection of hepatitis A virus in Mercenaria mercenaria by coupled reverse transcription and polymerase chain reaction. Appl. Environ. Microbiol. 59:2765-2770.

Hill, W.E., S.P. Keasler, M.W. Trucksess, P. Feng, C.A. Kaysner, and K.A. Lampel. 1991. Polymerase chain reaction identification of Vibrio vulnificus in artificially contaminated oysters. Appl. Environ. Microbiol. 57:707-711.

Kapperud, G., T. Vardund, E. Skjerve, E. Hornes, and T.E. Michaelsen. 1993. Detection of pathogenic Yersinia enterocolitica in foods and water by immunomagnetic separation, nested polymerase chain reactions, and colorimetric detection of amplified DNA. Appl. Environ. Microbiol. 59:2938-2944.

Kobayashi, K., K. Set, S. Akasaka, and M. Makino. 1990. Detection of toxigenic Vibrio cholerae O1 using polymerase chain reaction for amplifying the cholera enterotoxin gene. J. Jpn. Assoc. Infect. Dis. 64:1323-1329 (In Japanese with English summary).

Koch, W.H., W.L. Payne, B.A. Wentz, and T.A. Cebula. 1993. Rapid polymerase chain reaction method for detection of Vibrio cholerae in foods. Appl. Environ. Microbiol. 59:556-560.

Lampel, K.A., J.A. Jagow, M. Trucksess, and W.E. Hill. 1990. Polymerase chain reaction for detection of invasive Shigella flexneri in food. Appl. Environ. Microbiol. 56:1536-1540.

Niederhauser, C., U. Candrian, C. Hofelein, M. Jermini, H.P. Buhler, and J. Luthy. 1992. Use of polymerase chain reaction for detection of Listeria monocytogenes in food. Appl. Environ. Microbiol. 58:1564-1568.

Niederhauser, C., C. Hofelein, J. Luthy, U. Kaufmann, H.P. Buhler, and U. Candrian. 1993. Comparison of "Gen-Probe" DNA probe and PCR for detection of Listeria monocytogenes in naturally contaminated soft cheese and semi-soft cheese. Res. Microbiol. 144:47-54.

Read, S.C., R.C. Clarke, A. Martin, S.A. De Grandis, J. Hii, S. McEwen, and C.L. Gyles. 1992. Polymerase chain reaction for detection of verocytotoxigenic Escherichia coli isolated from animal and food sources. Mol. Cell. Probes 6:153-161.

Rossen, L., K. Holmstrom, J.E. Olsen, and O.F. Rasmussen. 1991. A rapid polymerase chain reaction (PCR)-based assay for the identification of Listeria monocytogenes in food samples. Int. J. Food Microbiol. 14:145-151.

Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of Beta-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shirai, H., M. Nishibuchi, T. Rammurthy, S.K. Bhattacharya, S.C. Pal, and Y. Takeda. 1991. Polymerase chain reaction for detection of the cholera entertoxin operon of Vibrio cholerae. J. Clin. Microbiol. 29:2517-2521.

Thomas, E.J., R.K. King, J. Burchak, and V.P. Gannon. 1991. Sensitive and specific detection of Listeria monocytogenes in milk and ground beef with the polymerase chain reaction. Appl. Environ. Microbiol. 57:2576-2580.

Wang, R.F., W.W. Cao, and M.G. Johnson. 1992. 16S rRNA-based probes and polymerase chain reaction method to detect Listeria monocytogenes cells added to foods. Appl. Environ. Microbiol. 58:2827-2831.

Wang, R.F., W.W. Cao, and M.G. Johnson. 1992. Development of cell surface protein associated gene probe specific for Listeria monocytogenes and detection of the bacteria in food by PCR. Mol. Cell. Probes 6:119-129.

Wegmuller, B., J. Luthy, and U. Candrian. 1993. Direct polymerase chain reaction detection of Campylobacter jejuni and Campylobacter coli in raw milk and dairy products. Appl. Environ. Microbiol. 59:2161-2165.

Wernars, K., E. Delfgou, P.S. Soentoro, and S. Notermans. 1991. Successful approach for detection of low numbers of enterotoxigenic Escherichia coli in minced meat by using the polymerase chain reaction. Appl. Environ. Microbiol. 57:1914-1919.

Wernars, K., C.J. Heuvelman, T. Chakraborty, and S.H. Notermans. 1991. Use of the polymerase chain reaction for direct detection of Listeria monocytogenes in soft cheese. J. Appl. Bacteriol. 70:121-126.

Wren, B.W., and S. Tabaqchali. 1990. Detection of pathogenic Yersinia enterocolitica by the polymerase chain reaction [letter; see comments]. Lancet 336(8716):693.

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