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ORIGINAL ARTICLE

Simultaneous detection of Escherichia coli O157:H7, toxigenic Vibrio cholerae, and Salmonella typhimurium by multiplex PCR

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ABSTRACT

Background: Escherichia coli O157:H7, Vibrio cholerae, and Salmonella typhimurium are pathogenic bacteria found in contaminated water and food. No assay method is currently available on simultaneous detection or identification of all the three pathogens. Our aim was to develop a rapid and reliable method for this purpose.

Materials and methods: A protocol for sample collection, and a PCR procedure was designed specifically for the assay. Selected fragments of 239 bp, 432 bp, and 360 bp for E. coli O157 lipopolysaccharide (LPS) gene (rfbE), V. cholerae toxin gene (ctx), and Salmonella typhimurium putative cytoplasmic protein gene (STM4497), respectively, were amplified from the extracted bacterial DNA samples in a single tube by multiplex PCR. The multiplex PCR products were analyzed by gel electrophoresis.

Results: All unknown samples were verifiably identified. The assay was sensitive enough to detect and identify as few as 100 cells of E. coli O157:H7, V. cholerae and Salmonella typhimurium. The presence of other bacteria did not interfere with the analysis.

Conclusion: This assay is a specific and reliable tool which allows cost-effective detection of all three bacterial pathogens in one reaction tube.

Keywords: *Escherichia coli O157:H7, Vibrio cholerae, Salmonella typhimurium, Multiplex polymerase chain reaction.*
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INTRODUCTION

Escherichia coli O157:H7, Vibrio cholerae, and Salmonella typhimurium are mainly isolated from contaminated water and various food products (1-3). E. coli O157:H7 is one of the most harmful food borne pathogenic bacteria and is alone responsible for many cases of infection and deaths worldwide (4,5). Although infection is usually self-limiting, the bacterium can cause life-threatening complications, including hemorrhagic colitis and hemolytic uremic syndrome in children and the immunocompromised patients (6).

Members of the bacterial genus Salmonella are among the major pathogens that cause infections in humans and animals. Most human Salmonella infections are thought to be associated with food borne transmission from contaminated animal–derived meat and dairy products (7). Salmonella enterica subspecies enterica serotype typhimurium is the commonly isolated serotype in industrialized

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countries (8). S. typhimurium is the most common cause of food borne infections. In immunocompromized people, that is the elderly, young, or people with depressed immune systems, Salmonella infections are often fatal if remained untreated (9,10). Since the traditional microbiological method for the detection of Salmonella requires up to 5–7 days, and involves several subcultivation steps followed by biochemical and serological tests, quicker approaches have been searched for, mainly at the DNA level.

V. cholerae is a waterborne pathogen with a major virulence factor identified as cholera enterotoxin (1). Infection is characterized by vomiting and rice-like diarrhea (11). The ubiquitous and virulent nature of these bacterial pathogens creates a need for specific, sensitive, and rapid detection techniques. Traditional bacterial detection methods have relied upon selective media, biochemical reactions and other parameters for bacterial identification, and are of course time-consuming and tedious (12,13). The results obtained therein do not always provide the information rapidly enough to allow the appropriate actions needed to protect the consumer. The simultaneous detection and identification of several pathogens by use of one differential medium plate is not possible. Molecular techniques such as PCR have been invaluable tools for the detection of pathogens (14,15). When multiple target genes need to be amplified, multiplex PCR (mPCR) can be performed and may provide a simple and sensitive tool for the simultaneous detection of multiple pathogenic bacteria (16). By recognizing conserved genomic DNA sequences unique to a particular organism and amplifying that region of the genome to increase sensitivity, contamination by that organism can be ascertained (17).

We report here the simultaneous detection of E.coli O157:H7, toxigenic V. cholerae, and S. typhimurium. mPCR methods for detecting several genes of these bacteria that have been reported previously for E. coli O157:H7 (2,3,18), V. cholerae (19-21) and S. typhimurium (22,23). However, none of the reported mPCR methods simultaneously detected all three pathogens present in the same sample. This multiplex PCR assay was also tested for cross-reactions against a number of other food borne bacteria from a variety of food products and water to determine specificity of the assay.

MATERIALS and METHODS

Bacterial strains and culture media: All V. cholerae (ATCC 14035, ATCC 14735 ATCC 51394) were grown onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) for 24 hours at 35°C. Salmonella enterica serotype typhimurium (ATCC 43971, ATCC 13076) and E. coli O157:H7 (ATCC 43889, ATCC 43890) were grown on brain heart infusion media (Difco) for 24 hours at 37°C. Meats, dairy products (milk, and cheese) and drinking water were purchased from the retailers. Samples (25g) of each food product and 50 ml of sterile mineral water were immediately inoculated with 1 ml of a bacterial suspension containing S. typhimurium, E. coli O157:H7, and V. cholerae at concentrations of 3×106 CFU/ml. Following a 10 minutes hold at room temperature, the food and watery samples were homogenized in 100 ml of buffered peptone water and were incubated at 37°C and 35°C for 18 hours prior to testing.

DNA isolation: DNA was extracted from pure bacterial cultures and from food samples. One ml of enriched medium was collected, centrifuged at 9000g for 5 minutes, and the supernatant was discarded. The cell pellets were washed in 1 ml phosphate buffered saline (PBS) and mixed with 1% triton X100. Bacterial DNA was released by heating the sample for 10 minutes at 100°C. After vortexing and centrifugation (12,000g, 4°C/5 min), an aliquot of the supernatant was used for the PCR

reactions. Standard microbiological assays were performed to confirm the presence and concentration of each isolate in the PCR reaction.

PCR primers: Forward and reverse primer pairs were designed for V. cholerae ctx gene, and the S. typhimurium STM4497 gene, as shown in table 1. The primers were analyzed for melting temperature (Tm), hairpin structures, and dimers by Oligo Analyzer 2.5 software. The primers were synthesized by MWG Biotech (Ebersberg, Germany).

Multiplex-PCR conditions: Each PCR reaction mixture contained 10mM Tris–HCl (pH=8.3), 50mM KCl, 3mM MgCl2, 0.2mM of each deoxynucleoside triphosphate, 1U of Taq DNA polymerase (Fermentas), 1µl of DNA template, and 0.2mM of each primer. Temperature conditions were as follows: initial denaturation at 94°C/2min, denaturation at 94°C/45s, hybridization at 57°C/45s, and polymerization at 72°C/45s. Thirty PCR cycles were run. PCR amplicons (10µl) were electrophoresed at 70 volts for approximately 0.5 hour through 1.5% agarose (Fermentas). The gel was then stained with ethidium bromide (10mg/ml) and visualized under U.V light. When a negative amplification was obtained a new PCR was performed and 20µl of the resultant solution was run on an agarose gel to confirm the result.

Sensitivity and specificity of primers: The primers were tested for their sensitivity of simultaneous detection of S. typhimurium, V. cholerae and E. coli O157 various concentrations (10-105) of bacteria homogenate. Aliquots (10µl) of selected dilutions were made up to 1ml with buffered peptone water and spread on plate count agar. Having incubated at 37°C, the colonies were counted. Possible cross-reactions with other potential food and water contaminants were also investigated. Shigella dysenteriae (ATCC 13313), Pseudomonas aeruginosa (ATCC 35554), Listeria monocytogenes (ATCC 15313), Salmonella typhi (ATCC 49469), Campylobacter jejuni (ATCC 35921), Klebsiella pneumoniae (ATCC 13883), and Yersinia enterocolitica (ATCC 51871) were substituted independently for the original target bacteria in the multiplex PCR assay and any cross-reactivity was determined. Each experiment was carried out in triplicate.

RESULTS

In designing primers, we evaluated the sequence similarities of potential primers used in this assay against all known sequences found in Gene Bank. To achieve specificity, we designed PCR primers that were unique to a single region of each target gene from toxigenic V. cholerae and S. typhimurium. The sequences and Tm of the forward and reverse primers are summarized in table 1. These primers enabled the generation of different-sized PCR products, allowing easy differentiation of these products by subsequent agarose gel electrophoresis. Consequently, primer pairs with different Tm were used. Each primer pair was initially evaluated separately for specificity and functionality. As shown in figure 1, all three target genes were successfully amplified.

Table 1. Sequences primer pairs for Escherichia coli O157:H7, Vibrio cholerae, and Salmonella typhimurium

Reference	Target gene	Amplicon(bp)	sequence, 5´_3´	TM	
E. coli O157:H7	rfbE	239 bp	F: GTGCTTTTGATATTTTTCCGAGTAC R: TTTATATCACGAAAACGTGAAATTG	63 63	Morin, et al.,2004
V. cholerae	ctx	432 bp	F: ATTTGTTAGG CACGATGATG R: ATCGATGATCTTGGAGCATTC	57 60	This work
S. typhimurium	STM4497	360 bp	F: TTAACGAGGATTCAATGTCG R: TTATTTGACCGCGTCTGTCA	57 60	This work

They include a 432-bp region of the V. cholerae ctx gene, a 360-bp region of the S. typhimurium STM4497 gene, a 239-bp region of the E.coli O157:H7 rfbE gene.

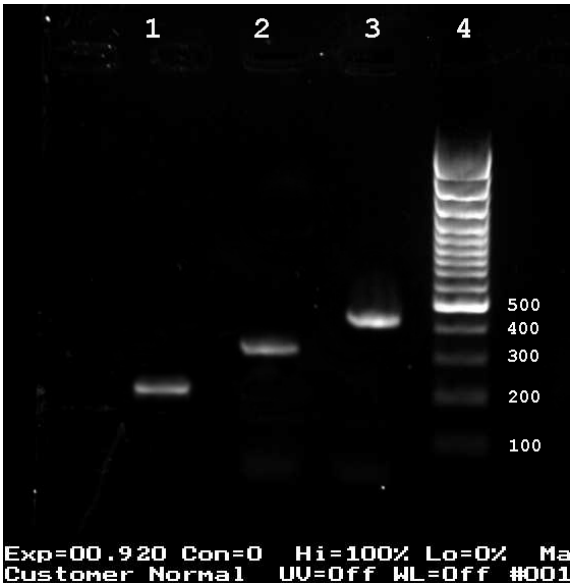

Figure 1. Agarose gel electrophoresis of PCR products of toxigenic V. cholerae, S. Typhimurium, and E. coli O157:H7. Lane 1: 236-bp PCR product of the STM4497 gene of S. typhimurium; lane 2: 360-bp PCR product of the ctx gene of V. cholerae; lane 3: 432-bp PCR product of the rfbE gene of E. coli O157:H7; lane 4: 100-bp DNA ladder.

Standard microbiological assays verified the presence and ascertained the concentration of each target bacterium. When all three pathogens were present, the multiplex PCR assay generated three amplicon products. In three consecutive trials using artificially contaminated foods and water (table 2), the multiplex PCR assay detected all three bacteria in all samples tested (Fig.2). Identical food and water samples from the same product batches, but not pre-seeded with the target bacteria prior to PCR detection, gave negative results in both microbiological and multiplex PCR assay. Multiplex PCR assay with seven different food and water contaminating bacteria revealed no cross-reactions with positive or negative controls (Fig.2).

Table 2. Compatibility of PCR with various food samples

Food	E.coli O157	Salmonella typhimurium	Vibrio cholerae
Chicken	+	+	ND
Minced beef	+	+	ND
Sausage	+	+	ND
Milk	+	+	ND
Yogurt	+	+	ND
Cottage cheese	+	+	ND
Buttermilk	+	+	+
Tap water	+	+	+
Mineral water	+	+	+

ND: not done

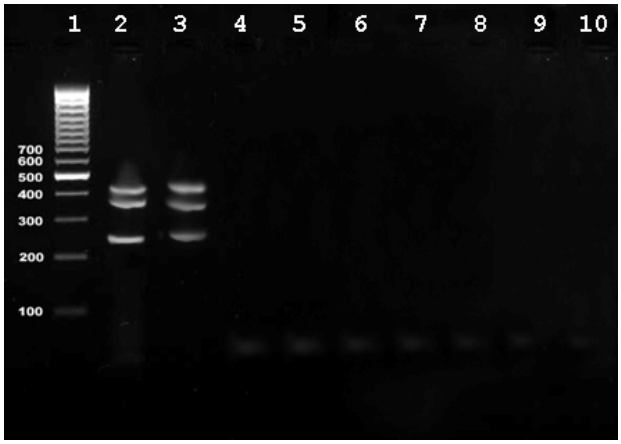

Figure 2. Specificity of multiplex PCR. Lane 1: 100 DNA ladder; lane2: positive control; lane 3: food sample contaminated with S. typhimurium, toxigenic V. cholerae and E. coli O157:H7; lan4-10: Shigella dysenteriae, Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella typhi, Campylobacter jejuni, Klebsiella pneumoniae, Yersinia enterocolitica.

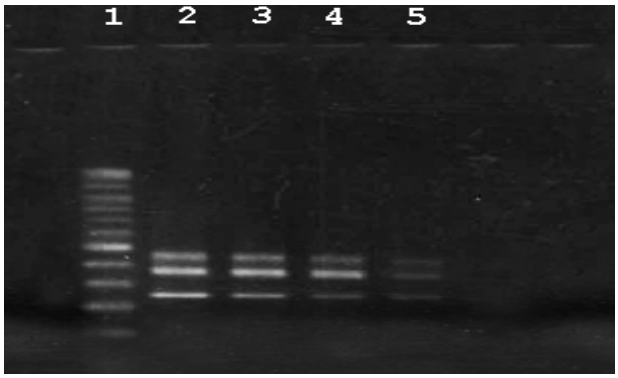

Figure 3. Sensitivity for simultaneous detection of S. typhimurium, toxigenic V. cholerae and E. coli O157:H7. Lane 1: 100 DNA ladder; lane 2-5: PCR products amplified from 10^5, 10^4, 10^3 and 10^2 cells of each strain per assay.

When serial dilutions of the three bacterial suspensions were tested in multiplex PCR assay, a positive result was obtained at concentrations of 10^2-10^5 cells (Fig.3).

The sensitivity of the multiplex PCR assay was also determined with pure cultures. The sensitivity of this method was as few as 50 cells for each bacterium.

DISCUSSION

E. coli O157:H7, V. cholerae, and S. typhimurium are extremely virulent and can cause severe gastrointestinal illness. We aimed to detect a panel of food and water borne pathogens by multiplex PCR in order to replace the current tedious, less sensitive and specific detection technologies in clinical and food microbiologic analysis. This method should also be considered to be a more parsimonious use of PCR reagents than the individual locus PCR testing protocols described by others (24).

Three specific genes were selected from the three target bacterial pathogens: the rfbE gene of E. coli O157, the ctx gene of V. cholerae, and the STM4497 gene of S. typhimurium that transcribed putative cytoplasmic protein. The rfbE gene of E. coli O157 encodes the O157 LPS and is therefore unique to the E. coli O157 serogroup (25). This gene has been identified as a good marker because it is transcribed in all growth phases from early exponential to late stationary phase (26).

Only strains of V. cholerae that produce cholera toxin have been associated with epidemics and pandemics; therefore, production of cholera toxin has become an important marker for identifying isolates with the potential to cause epidemics. By incorporating primers into a single detection assay, we were able to develop a multiplex PCR procedure to concurrently detect these bacteria.

An important factor in evaluating any DNA-based test is the specificity of the DNA sequence chosen for the genes and strains of interest. The advantage of our method is the ability of PCR to amplify specifically a gene or a segment of gene directly from a sample containing various bacterial strains. One of the problems often encountered with multiplex PCR is a reduction in sensitivity. This may be because of the competition between individual reactions for dNTPs and Taq polymerase when multiple primer sets are combined in a single reaction (27). However, we were able to demonstrate that products could be amplified from all three targets in the same reaction. Attempts at improving the detection sensitivity by increasing the number of amplification cycles or amount of polymerase by 2-4 folds (data not shown) were unsuccessful. Our approach does not rely on DNA extraction (24); boiling of cultures provides adequate nucleic acid to detect sequences of interest. Boiling the bacterial cells in the presence of 1% triton X-100 in this study enhanced the efficiency of DNA extraction being an additional advantage of the present method. To overcome PCR inhibition problems and to increase the sensitivity of the assay, enrichment method was employed. The higher sensitivity of the assay may be due to the dilution of inhibitory substances in the enrichment broth and the increased number of organisms. Media enrichment also helps hike in viable bacterial population that are always looked for in the routine microbiological detection procedures. Hence the probability of hitting DNA from dead cells is significantly reduced to its minimum possible level. Our sensitivity ranges were within the range of previous reports (28,29).

Our approach also provides simultaneous specific detection of three different microorganisms in a single attempt. This assay has been shown to detect the target pathogens without interference by other commonly associated food borne pathogens.

In conclusion, we have developed a rapid, simple and convenient m-PCR-based assay for the specific and simultaneous detection of three major food and water borne pathogens. This method

rendered final results in hours rather than lengthy and equally expensive biochemical methods. Application of this method in food industries and municipal water supply departments is an additional benefit attributed to this technique.

REFERENCES

1. Bilge SS, Vary J, James C, Dowell SF, Tarr PI. Role of the Escherichia coli O157:H7 side chain in adherence and analysis of an rfb locus. Infect Immun 1996;64: 4795–801.

2. Call DR, Brockman FJ, Chandler DP. Detecting and genotyping Escherichia coli O157:H7 using multiplexed PCR and nucleic acid micro arrays. Int J Food Microbiol 2001;67:71–80.

3. Fratamico PM, Sackitey SK, Wiedmann M, Deng MY. Detection of Escherichia coli O157:H7 by multiplex PCR. J Clin Microbiol 1995;33:2188–91.

4. Gannon VPJ, D'souka S, Graham T, King RK, Rahn K, Read S. Use of flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic Escherichia coli strains. J Clin Microbiol 1997;35:656–62.

5. Gilbert C, Winters D, O'Leary A, Slavik M. Development of a triplex PCR assay for the specific detection of Campylobacter jejuni, Salmonella spp. Mol Cell Probes 2003;17:135–38.

6. Gryko R, Sobieszczanska BM, Stopa PJ, Bartoszcze MA. Comparison of multiplex PCR, and an immunochromatographic method sensitivity for the detection of Escherichia coli O157:H7 in minced beef. Pol J Microbiol 2006;51;121–9.

7. Gubala AJ, Proll DF. Molecular-beacon multiplex real-time PCR assay for detection of Vibrio cholerae. Appl Environ Microbiol 2002;72:6424-28.

8. Herikstad H, Motarjemi Y, Tauxe RV. Salmonella surveillance: a global survey of public health serotyping. Appl Environ Microbiol 2002;129:1–8.

9. Parry CM, Hien TT, Dougan G, White NJ, Farrar J. Typhoid fever. N Engl J Med 2002;347:1770–82.

10. Houng HS, Sethabutr O, Echeverria P. A simple polymerase chain reaction technique to detect and differentiate Shigella and enteroinvasive Escherichia coli in human feces. Diagn Microbiol BMC infectious diseases 1997;28:19–25.

11. Ibekwe AM, Watt PM, Grieve CM, Sharma VK, Lyons SR. Multiplex fluorogenic real-time PCR for detection and quantification of Escherichia coli O157:H7 in dairy wastewater wetlands. Appl Environ Microbiol 2002;68:4853–62.

12. Koch WH, Payne WL, Wentz BA, Cebula TA. Rapid polymerase chain reaction method for detection of Vibrio cholerae in foods. Appl Environ Microbiol 1993;59:556–60.

13. Kumar A, Arora V, Bashamboo A, Ali S. Detection of Salmonella typhi by polymerase chain reaction: implications in diagnosis of typhoid fever. Infect Genet Evol 2002;2:107–10.

14. Liu Y, Lee MA, Ooi EE, Mavis Y, Tan AL, Quek HH. Molecular typing of Salmonella enterica serovar typhi isolates from various countries in Asia by a multiplex PCR assay on variable-number tandem repeats. J Clin Microbiol 2003;41:4388–94.

15. Li Y, Mustapha A. Simultaneous detection of Escherichia coli O157:H7, Salmonella, and Shigella in apple cider and produce by multiplex PCR. J Food Prot. 2004;67:27–33.

16. Mahon J, Murphey CK, Jones PW, Barrow PA. Comparison of multiplex PCR and standard bacteriological methods of detecting Salmonella on chicken skin. Lett Appl Microbiol 1994;19:169–72.

17. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Diseases 1999;5:607-25.

18. Morin NJ, Gong Z, Li XF. Reverse transcription-multiplex PCR assay for simultaneous detection of Escherichia coli O157:H7, Vibrio cholerae O1, and Salmonella typhi. Clin Chem 2004;50:2037–44.

19. Hoshino K, Yamasaki S, Mukhopadhyay AK, Chakraborty S, Basu A, Bhattacharya SK, et al. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic Vibrio cholerae O1 and O139. FEMS Immunol Med Microbiol 1998;20:201-7.

20. Singh DV, Isac SR, Colwell RR. Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in Vibrio cholerae and Vibrio mimicus. J Clin Microbiol 2002;40:4321–24.

21. Panicker G, Call DR, Krug, MJ, Bej AK. Detection of pathogenic Vibrio spp. in shellfish by using multiplex PCR and DNA microarrays. Appl Environ Microbiol 2004;70:7436-44.

22. Jofre´A, Martin B, Garriga M, Hugas M, Pla M, Rodriguez Liazaro D, et al. Simultaneous detection of Listeria monocytogenes and Salmonella by multiplex PCR in cooked ham. Food Microbiol 2005;22:109–15.

23. Soumet C, Ermel G, Rose V, Rose N, Drouin P, Salvat G, et al. Identification by a multiplex PCR-based assay of Salmonella typhimurium and Salmonella

enteritidis strains from environmental swabs of poultry houses. Lett Appl Microbiol 1999;29:1–6.

24. Van der Vossen JMBM, Hofstra H. DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementation. Int J Food Microbiol 1996;33:35–49.

25. Wang RF, Cao WW, Cernaglia CE. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J Appl Microbiol 1997;83:727–36.

26. Wang G, Clark CG, Rodgers FG. Detection of Escherichia coli of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and the components of the type 2 Shiga toxin family by multiplex PCR. J Clin Microbiol 2002;40: 3613–9.

27. Winters DK, O'Leary AE, Slavik MF. Polymerase chain reaction for rapid detection of Campylobacter jejuni in artificially contaminated foods. Lett Appl Microbiol 1998;7:163–7.

28. Winters DK, Slavik MF. Evaluation of a PCR based assay for specific detection of Campylobacter jejuni in chicken washes. Mol Cell Probes 1995;9:307–10.

29. Winokur PL. Molecular epidemiological techniques for Salmonella strain discrimination. Mol Cell Probes 2003;8:14–24.