Triplex PCR for Rapid Detection of *Escherichia coli* O157:H7 Directly from Ground Pork

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Summary and Implications

A polymerase chain reaction (PCR) procedure is being developed to detect *Escherichia coli* O157:H7 and associated Shiga-like toxins (STX_1 and STX_2) directly from pork, bypassing the time-consuming microbial culture steps for identification. A triplex PCR procedure was adapted and conditions experimentally determined to efficiently amplify three target genes: *uidA* with unique sequence in *E. coli* O157:H7, and stx_1 and stx_2. A meat sample preparation technique developed in previous research was modified to allow detection of 0.2-2.0 colony forming units per gram (CFU./g.) of meat. Time required to complete the procedure after overnight incubation of samples is seven hours.

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) continues to draw attention as an important foodborne pathogen. This is because of the rising number of the EHEC cases and foodborne outbreaks associated with *E. coli* O157:H7 and the potentially life-threatening diseases it produces, i.e., hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Acheson and Keusch, 1996; Padhey and Doyle, 1992). Outbreaks of foodborne infection with *E. coli* O157:H7 have been most often associated with the consumption of undercooked beef products. It has been shown that cattle are a major reservoir for *E. coli* O157:H7 and, in most cases, the ultimate source of the contamination has been traced to cattle (Acheson and Keusch, 1996).

Pork may be a source of *E. coli* O157:H7 infection. A survey of retail meats disclosed that 1.5% of pork is contaminated with *E. coli* O157:H7 (Doyle, 1991). The percent of pork harboring other STX-producing *E. coli* is even higher - 18% (Samadpour et al., 1994). Another concern is swine edema disease caused by *E. coli* producing variant of STX, called STX_e, which is closely related to STX_1 and STX_2. Recently a case of HUS has been reported caused by an STX_e-like producing E. coli strain isolated from healthy pigs (Franke et al., 1995).

PCR is currently being widely explored for detection of STX-producing *E. coli* from different sources: foods, meat, water, feces. To our knowledge there are no reports of sensitive, rapid, and specific PCR procedures which could specifically detect *E. coli* O157:H7 and other STX-producing bacteria directly from meat. Previously described PCR methods applied directly to meat involve labor intensive DNA extraction procedures or laborious sample preparation steps necessary for removal of PCR inhibitors from meat (Begum and Jackson, 1995; Gannon et al., 1992).

The purpose of our research is to develop a rapid and sensitive PCR-based procedure that allows detection of *E. coli* O157:H7 and strains with STX types directly from ground pork.

Materials and Methods

Development of a PCR technique

A multiplex PCR was developed to simultaneously identify *E. coli* O157:H7 and the two STXs it ordinarily encodes. Sequences for the three pairs of primers employed in multiplex PCR were obtained from published references (Gannon et al., 1992; Cebula et al., 1995). The target genes were conserved regions of each *stx* and *uidA* gene with a unique sequence for *E. coli* O157:H7.

Conditions of multiplex PCR were adjusted to optimize synthesis of the target sequences by using boiled colonies from *E. coli* strains with a previously known STX type (information kindly provided by Dr. T. Casey; National Animal Disease Center).

Positive (*E. coli* O157:H7 with both STX types) and negative colonies (non-STX *E. coli*) were prepared by boiling colonies for 10 min. in 500 Fl. water containing 4mM NaOH. After boiling, samples were cooled on ice for 10 min. and centrifuged at 14,000 RPM. for two minutes. Fifty Fl. PCR reactions contained 10 mM. Tris-HCl (pH 8.3); 50 mM. KCl; 0.2 mM. (each) dATP, dGTP, dCTP, and dTTP (Perkin and Elmer); 1 :M. each primer; and 5.0 U. of Taq DNA polymerase (Perkin and Elmer).

Amplifications were performed in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin and Elmer) for 40 cycles with 4 min. initiation at 94EC followed by cycles for 1 min. at 94EC, 1 min. at 58EC, 1.5 min. at 72EC, and a final extension at 72EC for 7 min.

After PCR amplification, DNA samples were analyzed by agarose gel electrophoresis (60 min. at 80V.), visualized by UV light, and photographed. Molecular size marker - 1 Kb. DNA Ladder (Gibco, BRL) was included in each gel.

Meat sample preparation

Ground pork was purchased from a local retail store and checked bacteriologically for presence of *E. coli* O157:H7 as described in the literature (Okrend et al., 1990). Meat negative on culture for *E. coli* O157:H7 was used in experiments.

Shiga-like toxin-producing *E. coli* strains from the National Animal Disease Center collection were kindly provided by Dr. T. Casey together with the STX gene probe data (Table 1).

For each meat experiment 10-fold or 100-fold dilutions of the inoculum were made to seed 10 g. meat samples to yield approximately 0.02 to 2000 CFU's per gram. In addition, a control meat sample was included to which 100 Fl. of phosphate buffer solution (PBS) was added. To adjust inoculum, STX-producing *E. coli* strains were grown overnight in Trypticase Soy Broth (TSB, Fisher Scientific) and centrifuged at 7,000 RPM. for 15 min. in an IEC Clinical centrifuge. Supernatant was removed and the resultant pellet was resuspended in PBS to give 50% light transmittance at 400 nm. in a Bausch and Lomb Spectronic 20. Decimal dilutions to 10^{-9} were made in PBS. One hundred Fl. of dilutions 10^{-3} - 10^{-9} were used to inoculate meat samples to determine the lowest detectable amount CFU/g . After inoculation, meat samples were left on the counter for one hour to allow bacteria to adhere to meat. Thirty-five ml. of TSB were added to each sample afterwards and blended for two minutes in Stomacher 80. Prepared meat samples were incubated at 37EC overnight.

Sample processing

The next day the EHEC-contaminated samples were processed by repeated centrifugation and washing. Meat samples were centrifuged at 1000 RPM. for 15 min. in an IEC UV centrifuge. Supernatant was separated from meat particles and subjected to centrifugation at 3000 RPM. for 15 min. Supernatant was removed and the pelleted cells resuspended in 30 ml. PBS. High speed centrifugation (3000 RPM. for 15 min.) was repeated and the supernatant discarded. The resulting pellet was resuspended in 1 ml. water. The DNA was released from this crude cell pellet by boiling in 20 Fl. 1N NaOH (4 mM) for 10 min. After immediate cooling and high speed centrifugation 10 Fl. of supernatant was added to the PCR mixture and subjected to amplification according to the conditions already described.

Results and Discussion

In this study we continue to explore the suitability of PCR as a rapid method for *E. coli* O157:H7 detection directly from ground pork. Our specific goals were to:

1) develop a PCR for specific detection of *E. coli* O157:H7 along with STX types;

2) modify meat sample preparation techniques to improve sensitivity (threshold);

3) determine the lowest detectable inoculum CFU/g.

A multiplex PCR procedure was established by choosing three target genes. Two sets of primers were directed to the conserved regions within the genes encoding for stx_1 and stx_2 (Gannon et al., 1992). The third set of primers was directed at the *uidA* gene, which encodes $-glucuronidase in *E. coli*. Although O157:H7 isolates do not exhibit glucuronidase activity, they carry the *uidA* gene which has a single base difference from wild *E. coli* strains. This unique conserved base is used to specifically identify O157:H7 isolates (Cebula et al., 1995).

Multiplex PCR conditions, i.e., the number of cycles and annealing temperature, were varied to optimize synthesis of target sequences in *E. coli* strains with known STX profiles. Total agreement was achieved between *stx* gene probe data and the established PCR procedure when colonies from 15 STX-producing *E. coli* strains (Table 1) were subjected to amplification for 35-40 cycles. All primers generated PCR products of the predicted size as analyzed by agarose gel electrophoresis (Table 2). Thus, a PCR technique was developed which could identify the types of STX encoded by *E. coli* strains and at the same time discriminate other STX producing *E. coli* from O157:H7, the major serotype implicated in disease.

The established PCR procedure was applied to ground beef samples inoculated with 6 selected STX-producing *E. coli* and one wild-type *E. coli*. The inoculum varied from 0.5 to 15 CFU/g. All samples resulted in generating PCR products of expected profile. Again, there was 100% agreement with STX gene probe data. As anticipated, no products were amplified from wild-type *E. coli*.

The experiment was carried out further to determine the lowest limit (CFU/g.) which can be detected by current procedures. Ground pork samples were inoculated with 10-fold (experiment #2 and #3) or 100-fold dilutions (experiment #1) of *E. coli* O157:H7 producing both STX_1 and STX_2 (ATCC strain 35150). The experiment was repeated three times. The results are summarized in Table 3.

It was expected that meat samples inoculated with the smallest inoculum 0.01-0.001 CFU/g. might not yield PCR products, as theoretically they may not have received a single cell. The lowest inoculum detected by PCR from seeded ground pork averaged 1.0-2.0 CFU/g.

Experiments showed promising results as far as sensitivity of the PCR procedure and ability to determine STX type and to discriminate O157:H7 serotype from other *E. coli*. Our research produced a rapid PCR-based procedure that allowed detection of 1.0-2.0 CFU. of *E. coli* O157:H7 per gram of ground pork. The time necessary to process samples after overnight incubation in our laboratory was seven hours.

Larger scale experiments are currently underway. They utilize ground beef and ground pork. The possible influence of STX-producing *E. coli* strain differences in this PCR is being explored. The PCR results will be compared with bacteriologic test results, and test specificity and sensitivity will be determined.

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Publications

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2. Matise, I., Shelton, M., Phillips, G., and Will, L. A. 1995. PCR detection of *Escherichia coli* O157:H7 directly from pork. In: *Swine research report*, pp. 201-203. Iowa State University

Table 1. NADC *E. coli* collection: serotypes and *stx* gene probe data.

Strain ID	Serotype	stx_1	stx_2	uidA
2162	O139:H(+)	-	+	-
2228	O139 NM	-	+	-
2482	unknown	-	+	-
2597	O139:H1	-	+	-
2602	unknown	-	+	-
3081	O157:H7	+	+	+
3094	O111	+	-	-
3108	O26	+	-	-
3128	O113	+	+	-
3239	O165	-	+	-
3244	O119:H16	-	+	-
4718	O157:H7	+	+	+
4719	O157:H7	+	+	+
4720	O157:H7	-	+	+
4721	O157:H7	+	-	+

Table 2. Oligonucleotide primers used in the study.

Target gene	Protein encoded	Predicted size (base pairs)
stx_1	Shiga-like toxin 1	614
stx_2	Shiga-like toxin 2	779
uidA	β-glucuronidase	252

Table 3. PCR detection limits (CFU/g.) when applied directly to inoculated ground pork samples.

Experiment #1		Experiment #2		Experiment #3	
Inoculum CFU/g.	Result	Inoculum CFU/g.	Result	Inoculum CFU/g.	Result
1300	+	2000	ND	1000	+
130	ND	200	ND	100	+
13	+	20	+	10	+
1.3	ND	2	+	1	+
0.13	-	0.2	-	0.1	ND
0.013	ND	0.02	-	0.01	ND
0.0013	ND	0.002	ND	0.001	ND
Control[2]	-	Control	-	Control	-

1 ND - not determined
2 Control meat sample to which 100 μl of PBS was added instead of *E. coli* 0157 H7