Sensitive PCR Method for Detection of *Escherichia coli* 0157:H7 and Other Shiga toxin-producing Bacteria in Ground Meat

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Summary and Implications

Sensitivity of a polymerase chain reaction (PCR) procedure was evaluated for *Escherichia coli* O157:H7 and Shiga toxin gene (stx) detection in ground beef and ground pork at contamination levels of 0.14, 1.4, and 14 colony forming units per gram (CFU/g) of meat. The PCR procedure, developed during our previous research, amplifies three target genes simultaneously: uidA that is specific for *E. coli* O157:H7, and stx1 and stx2, the genes for Shiga toxins 1 and 2, respectively. Detection of the uidA gene by the PCR was 91% sensitive in beef and 55% sensitive in pork, and 90% and 71% sensitive for stx gene detection in beef and pork, respectively. In comparison, detection of *E. coli* O157:H7 by culture, done simultaneously with PCR, was 53% sensitive in beef and 11% sensitive in pork. Results indicate that this PCR procedure is a rapid and sensitive method for STEC and *E. coli* O157:H7 detection in meat at contamination levels less than 1 CFU/g.

Introduction

Shiga toxin-producing *Escherichia coli* O157:H7 is a zoonotic foodborne pathogen of major importance. The diseases associated with *E. coli* O157:H7 infection are hemorrhagic colitis (HC) and hemolytic uremic syndrome (8, 13), both life-threatening conditions. Infection is contracted through consumption of contaminated food products. Most of the outbreaks associated with *E. coli* O157:H7 infection have been traced to the consumption of undercooked beef products. Epidemiologic analyses indicate that cattle are a reservoir for *E. coli* O157:H7 (3, 15, 16). Information from outbreaks suggests that the infectious dose for *E. coli* O157:H7 is low (7, 10).

The pathogenicity of *E. coli* O157:H7 is attributed to the production of Shiga toxins 1 and 2 (Stx1 and Stx2), previously know as Verocytotoxins because of their toxicity on Vero cells (7, 9). However, knowledge of pathogenesis is incomplete. It is unclear why *E. coli* O157:H7 is the predominant serotype associated with HC and HUS when more than 100 STEC strains are known. Importantly, O157:H7 is not the only STEC serotype known to be involved in HC and HUS (11). In recent years, the incidence of HC and HUS associated with STEC strains other than O157:H7 has been increasing (1, 14).

Routinely, laboratory diagnosis of *E. coli* O157:H7 HC and HUS cases is accomplished by isolation of *E. coli* O157:H7 from specimens by bacteriologic culture followed by O157 antigen detection, toxin detection, or biochemical characterization. These methods, although reliable and easily performed, are not rapid enough and cannot detect Shiga toxins simultaneously with O157:H7 serotype. The PCR technique has enabled the detection of genetic markers rather than the use of biochemical properties to identify bacteria in cultures and clinical specimens. Multiple target sequences can be amplified at the same time, therefore, specimens can be rapidly tested for several genes of interest.

In our earlier research we developed a PCR-based procedure that detects specifically *E. coli* O157:H7 and other Stx-producing bacteria in ground meat by avoiding time-consuming DNA extraction procedures. The purpose of current research was twofold: (i) to determine sensitivity and specificity of the PCR procedure in comparison to bacteriologic culturing methods by testing seeded meat samples, and (ii) to determine whether the fat content of ground beef influences PCR test results.

Materials and Methods

Experimental design. The experiment was designed to determine the sensitivity and specificity of PCR for uidA and stx gene detection at four contamination levels in ground beef of varying fat content and in ground pork. To compare PCR sensitivity with *E. coli* O157:H7 detection sensitivity by culture, the same seeded samples were cultured on selective media as described below. The experiment was conducted in four blocks, each block employing one kind of meat: 80% lean ground pork, 70% lean ground beef, 80% lean ground beef, and 93% lean ground beef (% lean according to retail store label). Meat negative for *E. coli* O157:H7 as detected by culturing was used. Ten grams of meat samples was inoculated with randomly selected STEC strains from a pool of 30 strains. The 30 STEC strains included 22 *E. coli* O157:H7 and eight STEC other than O157:H7. For each block, 100 (l of decimal dilutions 10-5, 10-6, 10-7, and 10-8 of eight randomly chosen STEC strains was used to inoculate 32 meat samples. The dilutions delivered approximately 14, 1.4, 1.4 (10-1, and 1.4 (10-2 CFU STEC per gram of meat, respectively. Eight control meat samples, each receiving 100 (l of PBS, were included in the block. The experimental design provided use of 40 samples in a block and a total of 160 samples in the experiment.

Bacterial strains and meat. Twelve *E. coli* strains with known stx gene data were provided by Dr. T. Casey (National Animal Disease Center). These and another 18 STEC strains, all *E. coli* O157:H7 with unknown stx genotypes, provided by Dr. J. Dickson (Iowa State

University), were analyzed by PCR in our laboratory prior to their inoculation onto meat.

Ground beef (single batches of 70, 80, and 93% lean) and ground pork (two batches of 80% lean) were purchased from local retail stores. For each meat batch, total aerobic and coliform plate counts were determined twice and results averaged. Twenty-five grams of meat was blended with 225 ml phosphate buffer solution (PBS) for 2 min in a Stomacher 80 to yield a 1:10 dilution. One hundred (l of 10-fold dilutions made in PBS was plated on plate count agar (PCA, Difco) and violet red bile agar (VRBA, Difco). Plates were incubated at 37°C for 24 (VRBA) or 48 hours (PCA), after which colony counts were determined (Table 1).

Table 1. Ground pork and ground beef aerobic and coliform plate counts.

Meat	Plate counts CFU/g	
	Aerobic	Coliforms
Ground pork[a]	3.1×10^5 1.5×10^6	1.8×10^4 5.0×10^2
Ground beef[b] 70% lean	6.7×10^5	6.0×10^2
Ground beef 80% lean	2.3×10^5	1.8×10^2
Ground beef 93% lean	5.2×10^4	1.0×10^3

[a] Two separately purchased ground pork batches were used.
[b] All ground beef samples were purchased from single lots.

Inoculation of STEC onto meat. To adjust inoculum, STEC strains were grown overnight in trypticase soy broth (TSB; Difco) and centrifuged (15 min; 5125 (g) in an IEC clinical centrifuge. The supernatant fluid was removed and the pellet was resuspended in PBS to give 50% light transmittance at 400 nm in a Bausch and Lomb – Spectronic 20. Decimal dilutions up to 10-8 were made in PBS. To determine the inoculum for seeding meat samples, 100 (l of the 10-5 and 10-6 dilutions was plated in duplicate on SMAC and incubated overnight at 37(C.

After inoculation, all contaminated and control meat samples were left on the counter for 1 hour. Thirty-five milliliters of TSB was then added to each sample and blended in a Stomacher 80. Prepared meat samples were incubated at 37°C overnight.

Meat processing prior to PCR. Meat samples were centrifuged for 15 min at 110 (g in an IEC, model UV, centrifuge. The supernatant was separated from meat particles and centrifugation repeated. Supernatant fluids were collected and centrifuged for 15 min at 1,000 (g. Centrifugation was done twice with removal of supernatants and resuspension of bacterial pellets in 30 ml of PBS between centrifugations. After the second centrifugation the supernatant fluids were removed and the resulting pellets were resuspended in 1 ml of water. Each sample was divided in half: 500 (l of each suspension was frozen for later analysis, and the other 500 (l were used immediately in a PCR reaction, as described below.

PCR procedure. A PCR for simultaneous detection of *E. coli* O157:H7 specific sequence and the two Shiga toxin genes (stx1 and stx2) encoded on bacteriophages was developed in our earlier research. Sequences for the three pairs of primers employed in the PCR were specific to conserved regions of each stx gene, and the uidA gene as reported previously (2, 6). Oligonucleotides were synthesized at the Iowa State University Nucleic Acid Facility.

The 50 (l PCR reaction solutions contained 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl; 0.2 mM (each) of dATP, dGTP, dCTP, and dTTP (Perkin and Elmer); 1 mM of each primer; 2.5 units of Taq DNA polymerase (Perkin and Elmer); and 10 (l of template. To target the DNA liberation from the meat samples, 10 (l of 1 N NaOH solution was added to 500 (l cell suspensions. All samples were boiled for 10 min, cooled on ice for 10 min, centrifuged (2 min; 16,000 (g) in an Eppendorf centrifuge 5415 C, and the supernatant subjected to PCR. Amplifications were performed in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin and Elmer) for 40 cycles with 4 min initiation at 94°C followed by cycles of 1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C, and a final extension at 72°C for 7 min. After PCR amplification, DNA samples were analyzed by agarose gel electrophoresis, visualized by UV light, and photographed. A molecular size marker, 1 kb DNA ladder (Life technologies, Inc.), was included in each gel.

E. coli O157:H7 cultural detection. The protocol for cultural detection of E. coli O157:H7 was adapted from the National Animal Disease Center Bacteriology Laboratory procedure (4). Ten grams of control and seeded ground meat samples were blended with 35 ml of TSB for 2 min in a Stomacher 80. Samples were incubated overnight at 37°C. The next day, 10-fold dilutions in PBS were made from sample homogenates. From each sample, 100 (l of dilutions 10-4, 10-5, and 10-6 was plated on sorbitol-MacConkey agar (SMAC, Difco) and incubated for 24 hours. A plate with a count between 30(300 clear colonies was chosen from each sample following incubation and the total number of sorbitol negative colonies determined. Eight sorbitol-negative (clear) *E. coli* O157:H7 characteristic colonies were inoculated to media containing 0.08% (-D-glucuronide (Sigma) and streaked on MacConkey agar (MAC, Difco). After overnight incubation, lactose-positive (red on MAC) and (-D-glucuronidase-negative (no fluorescence) colonies were tested for indole production by liquid indole reagent (Bactidrop Spot Indole, Remel). Indole-positive (blue) *E. coli* O157:H7 suspect colonies were tested with the latex O157 antigen (ECOLEXTM, Orion Diagnostica) test.

Statistical analyses. Statistical analyses were applied to the pooled results of the experiment. General linear model (SAS program) was used to determine the influence of meat type and to compare PCR with culturing for 10-5,

10-6, 10-7, and 10-8 inocula levels separately. Values 1 and 0, respectively, were assigned to positive and negative samples. Test sensitivity and specificity were calculated for both PCR and bacteriology by pooling results from all samples inoculated with doses varying from 1.4 (10-1 to 14 CFU/g. The 1.4 (10-2 CFU/g inoculum was considered too low to deliver a single cell per 10 g of meat sample and, therefore, was not used in calculation of the sensitivity of the technique.

Results and Discussion

We report the results of series of experiments that were conducted to determine the sensitivity of PCR for detection of low STEC contamination levels in meats. The PCR procedure amplifying three target genes, uidA, stx1 and stx2, was previously developed in our laboratory. The particular set of genes was chosen for several reasons. The uidA gene encodes (-glucuronidase in *E. coli* and although *E. coli* O157:H7 strains do not exhibit glucuronidase activity, the uidA gene is present on the chromosome. This gene has some conserved sequences that are unique to the *E. coli* O157:H7 serotype (2, 5) and can be used to specify this serotype. However, it is not the serotype that determines the pathogenicity of the bacteria but the production of Stx. Because HUS cases associated with STEC other than O157:H7 have been reported at an increasing rate (11), the simultaneous targeting of stx1 and stx2 sequences was important in detection of foods contaminated with a virulent STEC.

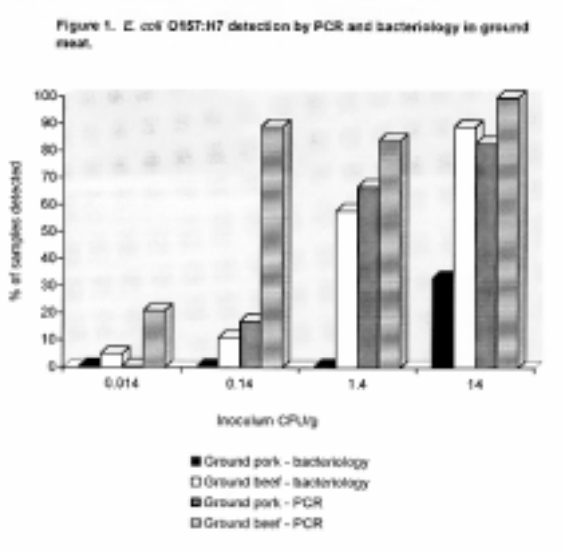
The sensitivity of the PCR procedure was determined by analysis of 160 STEC-contaminated samples. Meat was inoculated with a fairly low inoculum (0.14 to 14 CFU/g) to simulate a natural low infectious dose that is believed to be as low as a few cells (7, 10). The analysis of meat samples seeded with a dose of 14 CFU of *E. coli* O157:H7 per g of meat showed that PCR was 100% and 83% effective in detection of the uidA sequence in ground beef and ground pork samples, respectively (Figure 1). Positive sample detection at an inoculum level of 1.4 CFU/g diminished to 84% and 67% for ground beef and pork, respectively. The difference due to meat type at these inocula levels was not significant (P=0.07 and P=0.4 for inocula of 14 and 1.4 CFU/g, respectively). The PCR detected 89% of the positive beef samples and 17% of the pork samples contaminated with a dose of 0.14 CFU/g. Furthermore, the triplex PCR procedure detected 21% of ground beef samples seeded with 10-fold fewer cells, 1.4 (10-2 CFU/g, but none of the seeded pork samples. When the E. *coli* O157:H7 detection levels were compared in beef samples of differing fat content, no statistically significant difference was found at any of the inocula levels (P>0.6).

The presence of the stx sequences was correctly identified in 88% of ground pork samples and 100% of ground beef samples seeded with 14 CFU/g. When meat samples were seeded with 10-fold fewer cells (1.4 CFU/g), stx genes were identified in 88% of contaminated beef and pork samples. The identification rate dropped to 38% in ground pork samples and to 83% in ground beef samples inoculated with 0.14 CFU/g

The specificity of PCR procedure in detection of uidA sequences was 100%; that is, it correctly identified the uidA sequence in all *E. coli* O157:H7 strains. Moreover, it correctly distinguished between *E. coli* O157:H7 and other STEC-contaminated meat samples, emphasizing the specificity of the uidA primers used in this procedure for *E. coli* O157:H7 detection. The triplex PCR procedure did not detect the uidA sequence in any of the noncontaminated samples, but found stx genes in two of them. Two possible explanations are that (a) these were true findings, meat samples contained bacteria producing Stx, or (b) these were false positive samples.

Results of *E. coli* O157:H7 detection by PCR were compared with the *E. coli* O157:H7 detection by culturing Like most of the standard cultural methods for detection of *E. coli* O157:H7 (12), our culturing protocol was based on the inability of *E. coli* O157:H7 to ferment sorbitol in SMAC media.

As shown in Figure 1, the bacteriologic culturing procedure identified 89% of the ground beef samples seeded with 14 CFU/g of meat and 33% of ground pork



samples inoculated with the same dose. This trend also was observed at an inoculum level of 1.4 CFU/g; however, at a considerably lower detection level for both beef and pork with only 58% of seeded ground beef samples and none of the seeded pork samples being identified by cultural isolation procedures. The ability to detect *E. coli* O157:H7-contaminated samples at these two inoculation levels differed significantly depending on meat type, i.e., beef versus pork (P=0.005). With the decrease of inoculum to 0.14 CFU/g, bacteriologic culture techniques identified only 11% of the inoculated beef samples and none of the pork samples. This difference was not dependent on meat type (P=0.43). The specificity of bacteriology was 100% as none of the control meat samples were positive for *E. coli* O157:H7. The fat content of beef, however, was not a significant factor influencing *E. coli* O157:H7 recovery (P>0.4).

In summary, PCR analysis determined that the overall sensitivity of this method was high for both uidA and stx gene detection in beef (91 and 90%, respectively) but not as high in pork (55 and 71%, respectively). In comparison, the overall sensitivity of culturing was only

about 53% in ground beef and 11% in ground pork, and the difference between sensitivities of the two methods was statistically significant (Table 2).

Statistical analysis of separate inocula levels revealed that the sensitivity at each inoculum level was greater in beef than in pork for both methods. We explored possible explanations. As regards PCR, fat content as a PCR inhibitor was one of our considerations. We excluded this possibility for two reasons: (i) ground pork was on average 80% lean, which was the same as one of ground beef batches, yet PCR sensitivity for uidA gene detection in pork was 33% compared with 100% in ground beef 80% lean, and (ii) PCR analysis of beef samples differing in fat content (70, 80, and 93% lean) determined that fat content was not a significant factor influencing PCR outcome.

As regards bacteriology, several factors were investigated. Inconsistency in technique was excluded because bacteriology on pork and beef samples was done by the same person. Further, we compared total aerobic plate counts (APC) and coliform counts of ground beef and ground pork to determine whether the number of microorganisms in meat interfered with the cultural recovery of *E. coli* O157:H7. We found that one of two ground pork batches had a 10- to 100-fold higher APC and the other had 10- to 100-fold higher coliform count compared with the rest of the meat batches (Table 1). The comparison of bacteriology results of seeded ground pork samples revealed consistently low O157:H7 detection (one positive of nine seeded) in both ground pork batches, indicating that factors other than the total number of aerobic bacteria or coliforms account for the sensitivity differences in beef and pork by culture.

Table 2. Comparison of the sensitivity[a] of culturing anand triplex PCR methods for *E. coli* O157:H7 and *stx* gene detection.

Meat type[b]	Detection method[c]	Detection target	No. of samples pos/total	% of samples detected
Ground pork	Bacteriology	*E. coli* O157:H7	2/18	11
	PCR	*uidA*	10/18	55
	PCR	*stx* genes	17/24	71
Ground beef (average)	Bacteriology	*E. coli* O157:H7	30/57	53
	PCR	*uidA*	52/57	91
	PCR	*stx* genes	65/72	90
Ground beef 70% lean	Bacteriology	*E. coli* O157:H7	11/24	46
	PCR	*uidA*	21/24	88
	PCR	*stx* genes	21/24	88
Ground beef 80% lean	Bacteriology	*E. coli* O157:H7	8/12	67
	PCR	*uidA*	12/12	100
	PCR	*stx* genes	23/24	96
Ground beef 93% lean	Bacteriology	*E. coli* O157:H7	11/21	52
	PCR	*uidA*	19/21	90
	PCR	*stx* genes	21/24	88

[a] Sensitivity calculated for pool of contaminated meat samples consisting of an equal number of samples inoculated with 14 , 1.4 and 1.4 x 10^{-1} CFU/g.
[b] Significant differences between pork and beef samples within each detection method.
[c] Significant difference between PCR and bacteriology for *E. coli* O157:H7 detection regardless of meat type.

We analyzed the number of sorbitol-negative colonies (data not shown) in non-inoculated meat samples and found that the total number was higher in ground pork compared with ground beef. As we were testing eight sorbitol-negative colonies according to our culturing protocol, the larger total number of sorbitol-negative colonies in ground pork samples could have contributed to a greater chance to miss the true *E. coli* O157:H7 colonies. To determine whether there was a difference in *E. coli* O157:H7 growth rates in beef and pork, we compared the number of sorbitol-negative colonies in seeded and corresponding control samples.

The results revealed that after overnight enrichment there was a larger increase in the number of sorbitol-negative colonies in ground beef samples than in ground pork (35% increase in beef versus 25% increase in pork). Although we don't know the actual increase in *E. coli* O157:H7 numbers, the last observation supports the hypothesis that ground pork is not as favorable a medium for *E. coli* O157:H7 growth as ground beef. It also

validates the sensitivity differences between pork and beef detected by PCR because the overnight enrichment determined the number of bacteria subjected to PCR analysis. More extensive research is needed on this subject, especially because there are no other studies comparing *E. coli* O157:H7 growth in ground beef and ground pork.

In summary, we have described a new PCR procedure that is superior to bacterial culture in *E. coli* O157:H7 detection in meat. Because PCR cannot determine whether the detected genes derive from a single STEC strain, the primary use of the reported PCR technique is to identify meat samples containing STEC. Use of overnight sample enrichment prior to PCR allowed detection of low levels of bacterial contamination after a simple preparation procedure, while eliminating detection of DNA derived from nonviable bacterial cells. This technique can be applied to PCR detection of other meatborne organisms. Several improvements of the PCR protocol are under consideration for enhanced sensitivity. These include use of higher quality *Taq* polymerase and development of an automated PCR product detection system such as an ELISA that would exceed electrophoretic product analysis in speed and precision.

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