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Restriction-Site-Specific PCR as a Rapid Test To Detect Enterohemorrhagic *Escherichia coli* O157:H7 Strains in Environmental Samples

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is an important food-borne pathogen in industrialized countries. We developed a rapid and simple test for detecting *E. coli* O157:H7 using a method based on restriction site polymorphisms. Restriction-site-specific PCR (RSS-PCR) involves the amplification of DNA fragments using primers based on specific restriction enzyme recognition sequences, without the use of endonucleases, to generate a set of amplicons that yield "fingerprint" patterns when resolved electrophoretically on an agarose gel. The method was evaluated in a blinded study of *E. coli* isolates obtained from environmental samples collected at beef cattle feedyards. The 54 isolates were all initially identified by a commonly used polyclonal antibody test as belonging to O157:H7 serotype. They were retested by anti-O157 and anti-H7 monoclonal antibody enzyme-linked immunosorbent assay (ELISA). The RSS-PCR method identified all 28 isolates that were shown to be *E. coli* O157:H7 by the monoclonal antibody ELISA as belonging to the O157:H7 serotype. Of the remaining 26 ELISA-confirmed non-O157:H7 strains, the method classified 25 strains as non-O157:H7. The specificity of the RSS-PCR results correlated better with the monoclonal antibody ELISA than with the polyclonal antibody latex agglutination tests. The RSS-PCR method may be a useful test to distinguish *E. coli* O157:H7 from a large number of *E. coli* isolates from environmental samples.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has received much attention in recent years as the cause of numerous food-borne diarrhea outbreaks in developed countries. First identified in 1982 as an etiologic agent of hemorrhagic colitis (22), *E. coli* O157:H7 is now a major public health problem, causing an estimated 20,000 infections and 250 deaths per year in the United States (2). While most outbreaks have been associated with the consumption of beef and dairy products, outbreaks related to contaminated apple juice (16), alfalfa sprouts (4), and a water park (13) have been documented. The pathogenesis of *E. coli* O157:H7 is not clearly understood, but it is believed to involve a number of specialized virulence factors, including Shiga-like toxins (SLTs), adherence factors, and a plasmid-encoded hemolysin (17). Although other bacterial pathogens such as *Shigella* and *Campylobacter* spp. also are associated with bloody diarrhea in the United States, *E. coli* O157:H7 is now the agent most commonly isolated from fecal specimens containing blood (23).

Due to the rising incidence of *E. coli* O157:H7 infections in the United States and, thus, the need for improved epidemiologic surveillance, the development of simple and rapid *E. coli* O157:H7 detection methods is of utmost importance. Many assays have been developed for isolating and identifying the organism in food and clinical specimens. Culture methods based on biochemical characteristics, such as the inability of *E. coli* O157:H7 to ferment sorbitol on sorbitol MacConkey agar, are frequently used in clinical laboratories (17). Serological techniques such as enzyme-linked immunosorbent assay (ELISA) (6, 9, 21), dipstick immunoassays (14), and other antibody-based methods for detection of *E. coli* O157:H7 have also been developed (1, 5). More recently, the development of molecular approaches and PCR-based methods, which detect *E. coli* O157:H7 based on the presence or absence of specific virulence genes such as the *stx* and *eaeA* genes, have been described (3, 8–10, 33). Oberst et al. developed a PCR-based method that incorporates fluorogenic probes in a 5′ nuclease assay and have shown it to be rapid and specific in the detection of *E. coli* O157:H7 from environmental samples (18). While these methods have allowed for improved detection, many such techniques are labor-intensive, time-consuming, expensive, or often not specific enough for accurate identification. For example, antibodies often cross-react with various antigens, and many *E. coli* serotypes other than O157:H7 are known to produce verotoxins (19).

This study reports a simple method called restriction-site-specific-PCR (RSS-PCR) for the detection of *E. coli* O157:H7 strains. RSS-PCR is a technique that is based on the principle of restriction fragment length polymorphism (RFLP) but which is unique in that it does not require the use of restriction endonucleases. The RSS-PCR method is based on the use of primers that are homologous to specific restriction enzyme recognition sequences that are 10 to 18 bp long. The primers are designed in such a way that they will amplify genomic DNA segments that lie between the restriction site sequences on which the primers are based. The rationale for this procedure is that genetically different bacteria exhibit variations in the numbers and locations of different restriction site sequences throughout the genome. Harris et al. (11) applied the tech-

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nique to dengue virus for differentiating strains belonging to serotypes 2 and 3 and have shown that RSS-PCR has a level of discriminatory power comparable to a more labor-intensive subtyping method, which involves nucleotide sequencing of the dengue virus envelope (E) gene. The application of this method allows amplification of fragments of various lengths, yielding a unique collection of DNA fragments or "fingerprint" pattern for each different serotype. Thus, the RSS-PCR method can be used as a rapid and specific screening assay for *E. coli* O157:H7 isolates from food and clinical samples.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* O157:H7 strains F4637, F4761, G5244, H2294, H2493, and H2548 from six distinct domestic outbreaks were provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. The 54 *E. coli* feedlot isolates are environmental sample isolates from beef cattle feedyards in Kansas, obtained from Kansas State University. Representative *E. coli* strains from each of the other major enteric pathogenic *E. coli* groups (enteropathogenic *E. coli* [EPEC] [O55:H7, O111:NM], ETEC [H10407], enteroinvasive *E. coli* [strain 1], and enteroaggregative *E. coli* [strain 25-2]) and *Salmonella enterica* serovar Enteritidis strain phage types 4 and 8 were obtained from the CDC. To grow the bacteria, we picked a loopful of a bacterial colony from a tryptic soy agar slant or plate and inoculated it into Luria broth (LB). All bacterial strains were grown in 2 ml of LB broth overnight at 37°C on a shaker until the bacteria reached stationary phase (optical density at 600 nm [OD_{600}] of 3.0 to 4.0; CFU of $\sim 10^8$/ml). The overnight culture was pelleted and resuspended in 1 ml of distilled water. The reconstituted bacterial pellet was diluted 10-fold and boiled for 10 to 15 min and immediately frozen for at least 20 min at −70°C. The boiled and frozen bacterial preparation was thawed at room temperature prior to use for PCR.

Primer design. The primers used in this study were based on the restriction enzyme recognition sequences of *Bbv*I and *Taq*II found in the *chuA* gene of *E. coli* O157:H7 strain EDL933 (GenBank accession U67920). *chuA* encodes a 69-kDa outer membrane heme receptor that has been shown to be responsible for iron transport and is specific to *E. coli* (27). We identified four *Bbv*I recognition sites and three *Taq*II recognition sites within the *chuA* gene. Primer 1 (EC-1), an 18-mer, was designed to hybridize to the recognition sequence of *Bbv*I. Primer 2 (EC-2), an 18-mer, was designed to hybridize to the recognition sequence of *Taq*II. The sequences of the two primers are as follows: EC-1, 5′-GGC-AGC-CAG-CAT-TTT-TTA; EC-2, 5′-CAC-CCA-ACA-GAG-AAG-CCA.

PCR amplification. All PCR assays were performed in 50-μl volumes containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 10% dimethyl sulfoxide, 0.8 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 4 μM concentrations of each primer, 5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer/Roche, Foster City, Calif.) and 5 μl of bacterial template DNA. The reactions were performed in an automated DNA Thermal Cycler, Model 480 (PE Biosystems, Foster City, Calif.). Each reaction was carried out with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, 2 min of annealing at 42°C, and 5 min of primer extension at 72°C. A final extension step of 10 min at 72°C was carried out at the end to ensure complete amplification. Each reaction included a negative control, which was a reaction mixture that did not include a template DNA, and positive controls that included purified extracted DNA from a known strain of *E. coli* O157:H7, as well as *E. coli* O157:H7 bacterial cells subjected to the same treatment as the test *E. coli* samples prior to the PCR test. All mixtures were prepared in a UV-irradiated, PCR-dedicated biosafety cabinet, using filtered, aerosol-resistant pipettor tips (ART; Molecular BioProducts) to prevent carryover contaminations.

PCR amplification products were resolved on a 1.5% agarose gel and stained with ethidium bromide for 30 min to 1 h. Each gel was electrophoresed for 10 min at 40 V and then for 45 or 75 min at 100 V. We compared these two time periods of electrophoresis to determine if the generated patterns improved the discriminatory power of the technique. The stained gels were visualized by UV illumination and photographed. Each gel included a DNA molecular weight standard (1-kb ladder; Gibco-BRL, Grand Island, N.Y.). Resulting patterns were compared to each other and to the prototype O157:H7 pattern by visualization.

Serotyping and ELISA for *E. coli* feedlot strains. At Kansas State University, the presence of *E. coli* O157:H7 in environmental samples collected at beef cattle feedyards was determined by using standard procedures, including enrichment, isolation on sorbitol MacConkey agar (Difco), biochemical confirmation by use of API20e strips (BioMierieux Vitek), and serological testing by latex agglutination (Remel). All 54 isolates were identified initially as O157:H7 based on these tests. Subsequently, at U.S. Department of Agriculture (Albany, Calif.), we reanalyzed the isolates in an ELISA using bacterial cells as antigen and anti-O157 and anti-H7 monoclonal antibodies (MAbs). Each of the strains was grown on LB agar overnight at 37°C. Cells were then harvested with a plastic loop and suspended in 10 mM phosphate-buffered saline (pH 7.4) to an OD_{620} of 0.2 to 0.3 ($\sim 10^8$ cells/ml). The suspension was incubated in a 55°C water bath for 30 min, and then 70 μl was added to microtiter plate wells (Maxisorp; Nalge Nunc, Inc., Naperville, Ill.). The plates were incubated overnight (12 to 20 h) at 40°C in a drying oven. The wells were rinsed twice with distilled and deionized (DD) water (18 mohm resistance) to remove salts and unbound cells. Then, 200 μl of a blocking solution (0.5% casein, 10 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween 20, 30 mM sodium azide; pH 7.4) (modified from reference 15) was added to each well, and the plates were incubated for 1 h. The wells were emptied, washed with DD water, and used immediately or dried, placed in a plastic bag, and stored at 4°C for up to 3 weeks. The anti-O157 MAb 13B3 (30) and anti-H7 MAb 2B7 (12) were diluted in 10 mM Tris-HCl–150 mM NaCl–1% bovine serum albumin–0.05% Tween 20 (pH 7.4; TBS-BSA); a 70-μl aliquot of this solution was then added to the wells, and they were incubated for 1 h at room temperature (RT). The wells were washed three times with DD water, and 70 μl of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G (H+L; Zymed, South San Francisco, Calif.) diluted 1:1,000 in TBS-BSA was added to wells; the wells were then incubated 1 h at RT. The wells were washed three times with DD water, and 70 μl of a 1-mg/ml *p*-nitrophenylphosphate substrate mixture (Sigma, St. Louis, Mo.) diluted in 1 M diethanolamine–0.5 mM $MgCl_2$ (pH 9.8) was added to the wells. The OD_{405} was measured after 30 min. Strains producing an OD_{405} of >0.15 at an MAb dilution of 1:4,800 were designated tentatively as positive for that epitope. Of 54 strains (39%), 21 were found to be O157:H7 in the first assay. The H7 antigen may be expressed weakly depending upon growth medium or other conditions (12, 24). Passage of potential $H7^+$ strains on a blood-containing medium can enhance the expression of H7 (24). Each of the 54 strains was passaged three times on successive days on LB medium containing 5% sheep blood and then retested for both O157 and H7 epitope expression as described above. Of 54 strains, 31 strains were found to be $O157^+$ before and after passage on blood medium; no additional $O157^+$ strains were identified. However, seven strains designated as $O157:H7^-$ after the first assay were found to be $H7^+$ after passage on blood (28 of total O157:H7 strains [52%]).

Comparison of RSS-PCR and serological data. All of the assays were performed independent of each other, and the monoclonal serotyping results were not disclosed to those who performed the RSS-PCR procedure. Thus, the study was carried out in a blinded fashion to prevent biased observation of PCR patterns. The RSS-PCR patterns for the 54 feedlot strains were designated as either an O157:H7 pattern, possibly an O157:H7 pattern, or a non-O157:H7 pattern, based on visual comparison of the electrophoretic patterns. Each unique non-O157:H7 pattern was given an alphabetical designation (B to K) to further discriminate the patterns. The RSS-PCR pattern data were compared with the serotyping and ELISA results for concordance.

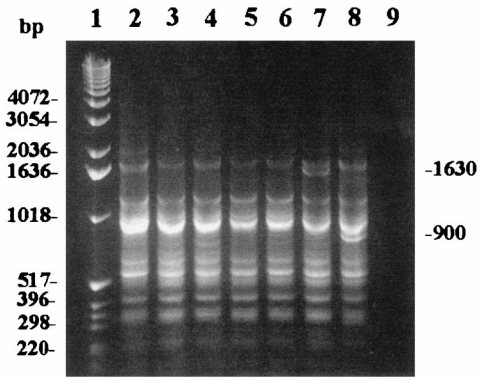

FIG. 1. RSS-PCR patterns of seven different *E. coli* O157:H7 isolates from various domestic outbreaks. Lane 1 is a 1-kb molecular-weight DNA ladder (Gibco-BRL). Lanes 2 to 6 are, in order, *E. coli* O157:H7 strains F4637, F4761, G5244, H2294, and H2493. All represent *E. coli* O157:H7 pattern A. Lane 7 is *E. coli* O157:H7 strain H2548 which represents *E. coli* O157:H7 pattern A2 (extra band of 1,630 bp). Lane 8 shows a pattern for a KSU *E. coli* O157:H7 strain (strain 34) which represents *E. coli* O157:H7 pattern A1. Note the extra band of approximately 900 bp. Lane 9 is a negative control (no template DNA).

RESULTS

Prototype *E. coli* O157:H7 "fingerprint" pattern. The RSS-PCR method with primers EC-1 and EC-2 was applied to six unrelated *E. coli* O157:H7 isolates (strains F4637, F4761, G5244, H2294, H2493, and H2548). PCR amplification of all six strains generated identical band patterns comprising 11 bands for each sample on an agarose gel (Fig. 1, lanes 2 to 7). An extra band of approximately 1,630 bp was observed from

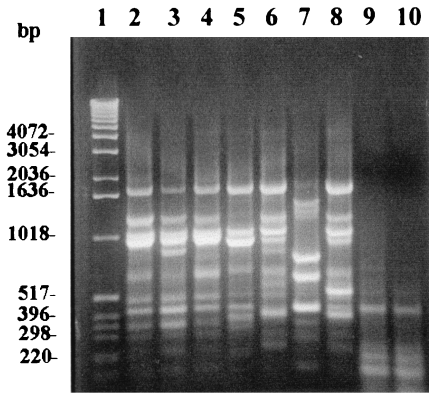

FIG. 2. RSS-PCR patterns of selected diarrheagenic *E. coli* isolates and *Salmonella* serovar Enteritidis strains. Lane 1, 1-kb molecular-weight ladder; lane 2, EHEC (*E. coli* O157:H7 pattern A [F4761]); lane 3, EHEC (*E. coli* O157:H7 pattern A1 [KSU 34]); lane 4, EPEC (O55:H7); lane 5, EPEC (O111:NM); lane 6, ETEC (strain H10407); lane 7, enteroaggregative *E. coli* (strain 25-2); lane 8, enteroinvasive *E. coli* (strain 11); lane 9, *Salmonella* serovar Enteritidis phage type 4; lane 10, *Salmonella* serovar Enteritidis phage type 8. Note the similarity between the patterns for *E. coli* O157:H7 and *E. coli* O55:H7 (lanes 2 and 4).

strain H2548. To determine the stability of these patterns, we performed the PCR on the same six strains multiple times on different days for a period of 6 months. While there appeared to be some day-to-day variation in the intensity of two of the lower-molecular-weight bands (ca. 360 and 320 bp) and a high-molecular-weight band of approximately 2,000 bp on the agarose gel, the patterns remained consistent over multiple tests. The most common pattern associated with *E. coli* O157:H7 was designated pattern A, and the pattern with the extra 1,630-bp band (in strain H2548) was designated pattern A2. In the analysis of 54 test isolates, another variant pattern from RSS-PCR of *E. coli* O157:H7 samples was recognized (Fig. 1, lane 8). This pattern had a band of approximately 900 bp in addition to all of the other bands seen in pattern A and was designated *E. coli* O157:H7 pattern A1. *E. coli* O157:H7 pattern A was hereafter considered the prototype pattern and was used as a positive control for the PCR assays.

RSS-PCR on selected pathogenic *E. coli* serotypes and *Salmonella* serovar Enteritidis. To determine the discriminatory power of the RSS-PCR method in differentiating serotypes, we tested several representative strains from each of the major diarrheagenic *E. coli* groups and two strains of *Salmonella* serovar Enteritidis (phage types 4 and 8). With the exception of *E. coli* O157:H7 and *E. coli* O55:H7, amplification with primers EC-1 and EC-2 generated unique patterns for each different *E. coli* serotype (Fig. 2). Interestingly, *E. coli* O55:H7 generated a pattern that is identical to the *E. coli* O157:H7 pattern A. As expected, both *Salmonella* serovar Enteritidis strains (phage types 4 and 8) did not generate any interpretable patterns.

RSS-PCR of feedlot strains. To validate RSS-PCR as a method for detecting *E. coli* O157:H7, we assayed 54 *E. coli* strains isolated from Kansas cattle feedlot samples and without prior knowledge of their serotypes or other serological characteristics. Based on visual comparison of the generated patterns with the prototype *E. coli* O157:H7 pattern (*E. coli* O157:H7 pattern A), 15 strains were determined to have the O157:H7 pattern, and 21 strains were determined to possibly have the O157:H7 pattern. The designation of "possibly O157:H7" were given to those patterns which looked almost identical to the prototype O157:H7 pattern except for 1 band (*E. coli* O157:H7 pattern A1). The remaining 18 strains were determined to have patterns that appeared to be completely different from the O157:H7 prototype pattern and were thus designated non-O157:H7 patterns ("−" in RSS-PCR result column, Table 1). The non-O157:H7 patterns were further compared to each other, and each unique pattern was given a letter designation (B to K). There were 10 distinct patterns represented among the 18 strains found to have non-O157:H7 patterns. A representative example of each non-O157:H7 pattern among the feedlot strains is shown in Fig. 3.

Serotyping and ELISA of cattle feedlot strains. All 54 feedlot isolates were initially classified as belonging to the O157:H7 serotype by the commercial latex agglutination test based on polyclonal antibodies (Remel). Of the 54 isolates passaged three times on LB 5% blood agar, 28 (52%) reacted strongly with both anti-O157 and anti-H7 MAbs. Four strains reacted strongly with anti-O157 antibody but not with anti-H7 antibody. Four strains reacted strongly with anti-H7 antibody but not with anti-O157 antibody. Eighteen strains reacted with neither anti-O157 antibody nor anti-H7 antibody and were classified as non-O157:H7 strains.

Comparison of RSS-PCR with MAb-ELISA serology results. Of the 54 feedlot strains, 28 strains were found to be of serotype O157:H7, based on ELISA. Among these 28 strains, the RSS-PCR method identified all 28 strains as O157:H7 strains. Thus, the sensitivity of the RSS-PCR method compared to the monoclonal ELISA was 100%. Based on the ELISA serology results, the remaining 26 feedlot strains were determined to be non-O157:H7 strains, or strains that had either the O157 lipopolysaccharide antigen or the H7 flagellar antigen, but not both. The RSS-PCR method identified 18 of these 26 strains as non-O157:H7 strains. However, the remaining eight strains were falsely identified as O157:H7 strains by the RSS-PCR method. Therefore, the specificity of the RSS-PCR method was 69%. Overall, the RSS-PCR method yielded 46 results that were concordant with the serological-ELISA tests. The positive predictive value and negative predictive value of the RSS-PCR method compared with the monoclonal ELISA test as the "gold standard" were 78 and 100%, respectively.

The eight strains for which there was discrepancy between the ELISA and RSS-PCR data were retested by RSS-PCR. The amplified products were electrophoresed for 75 min for better band separation. Seven strains generated patterns that were clearly different from the prototype *E. coli* O157:H7 pattern A (Fig. 4, lanes 3 to 9). One strain which had the O157 antigen but not the H7 flagellar antigen still generated a pattern identical to the prototype O157:H7 pattern (Fig. 4, lane 10). Based on this reevaluation, the sensitivity and the specificity of the RSS-PCR method were 100 and 96%, respectively. The positive predictive value and the negative predictive value were 97 and 100%, respectively.

DISCUSSION

An effective bacterial detection method that relies on molecular techniques can be used to efficiently screen a large number of environmental samples. We have developed a simple diagnostic method that relies on restriction site polymorphisms found in the *E. coli* genome. Other PCR-based methods for the detection of verotoxin-producing *E. coli* exist. Gannon et al. (9) have described a multiplex PCR method, which uses two pairs of primers that are directed toward SLTI and SLTII genes. The method was shown to be specific in that the two primer pairs amplified the respective toxin genes they were designed to target. Another multiplex PCR method described by Fratamico et al. (7) used three pairs of primers

TABLE 1. Results of serology and PCR assays with 54 feedlot strains

Feedlot strain no.	Binding of MAb[a] to bacteria passaged:				H7 serotype changed after passage	MAb-defined serotype[c]	RSS-PCR result (PCR pattern type)	Agreement between ELISA and RSS-PCR
	On LB agar		Three times on 5% blood agar[b]					
	Anti-O157	Anti-H7	Anti-O157	Anti-H7				
1					No	Neg:Neg	− (B)	Yes
2					No	Neg:Neg	− (C)	Yes
3	++++		++++		No	O157:Neg	− (D)	Yes
4					No	Neg:Neg	− (E)	Yes
5		++++		++++	No	Neg:H7	− (E)	Yes
6					No	Neg:Neg	− (F)	Yes
7	++++		++++	+++	Yes	O157:H7	O157:H7	Yes
8	++++		++++	+	Yes	O157:H7	O157:H7	Yes
9	++	++++	+	++++	No	O157:H7	O157:H7	Yes
10					No	Neg:Neg	− (E)	Yes
11		++		++++	No	Neg:H7	− (E)	Yes
12		++++		++++	No	Neg:H7	− (E)	Yes
13					No	Neg:Neg	− (G)	Yes
14		+		+++	No	Neg:H7	− (I)	Yes
15					No	Neg:Neg	− (H)	Yes
16	++++		++++		No	O157:Neg	− (I)	Yes
17					No	Neg:Neg	− (G)	Yes
18	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
19	+++	+	+	++++	No	O157:H7	O157:H7	Yes
20	++++	++	++++	+++	No	O157:H7	O157:H7	Yes
21	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
22	++++		++++	++	Yes	O157:H7	O157:H7	Yes
23	++++		++++	++	Yes	O157:H7	O157:H7	Yes
24	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
25	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
26	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
27	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
28	++	++	+	++++	No	O157:H7	O157:H7	Yes
29	++++		++++	+	Yes	O157:H7	O157:H7	Yes
30	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
31	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
32	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
33	++++		++++	++	Yes	O157:H7	O157:H7	Yes
34	++++	+++	++++	++++	No	O157:H7	O157:H7	Yes
35	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
36	++	+	+	+++	No	O157:H7	O157:H7	Yes
37	++++		++++	+	Yes	O157:H7	O157:H7	Yes
38	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
39	++++	++	++++	++++	No	O157:H7	O157:H7	Yes
40	++++	+++	++++	++++	No	O157:H7	O157:H7	Yes
41					No	Neg:Neg	− (I)	Yes
42	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes
43					No	Neg:Neg	− (I)	Yes
44					No	Neg:Neg	− (I)	Yes
45					No	Neg:Neg	− (I)	Yes
46					No	Neg:Neg	− (I)	Yes
47					No	Neg:Neg	− (J)	Yes
48	++				No	O157:Neg	− (I)	Yes
49					No	Neg:Neg	− (F)	Yes
50					No	Neg:Neg	− (J)	Yes
51					No	Neg:Neg	− (K)	Yes
52	++++		++++		No	O157:Neg[d]	O157:H7	No
53					No	Neg:Neg	− (K)	Yes
54	++++	++++	++++	++++	No	O157:H7	O157:H7	Yes

[a] MAbs were added to wells at a 1:4,800 dilution: negative (i.e., no entry), $OD_{405} < 0.150$; +, $OD_{405} = 0.151$ to 0.400; ++, $OD_{405} = 0.401$ to 0.800; +++, $OD_{405} = 0.801$ to 1.200; ++++, $OD_{405} > 1.200$.

[b] Bacteria were subcultured for 3 successive days on LB agar containing 5% sheep blood.

[c] Neg, designates that the strain did not bind the anti-O157 or the anti-H7 MAb. The strains were not assayed for other O and H serotypes.

[d] This strain was positive for SLTII, intimin, and *flicC* in a multiplex PCR (data not shown).

specific for the *eaeA* gene, conserved regions of the SLTI and SLTII genes, and the 60-MDa plasmid for simultaneous amplification in one PCR reaction. Recently, Gannon et al. (8) introduced a new multiplex PCR method that uses primers directed to the H7 flagellar gene, *flicC*, in addition to primers for the verotoxin and *eaeA* genes to improve specificity for EHEC strains. All of these methods are designed to specifically detect verotoxin-producing *E. coli* strains. However, one com-

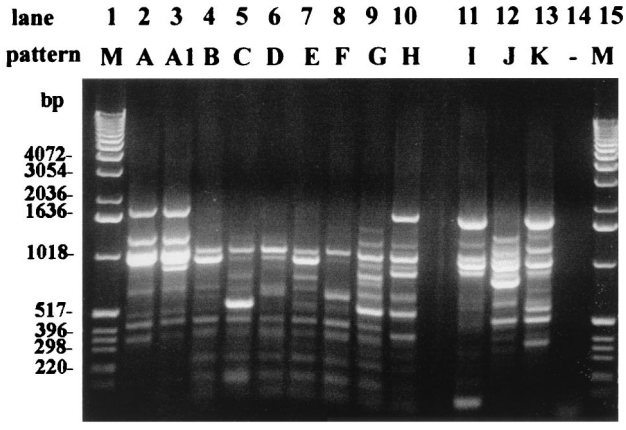

FIG. 3. RSS-PCR patterns of representative non-O157:H7 strains among KSU strains. Lanes 1 and 15 are 1-kb DNA ladders (Gibco-BRL). Lanes 2 and 3 represent *E. coli* O157:H7 patterns A and A1, respectively. Lanes 4 to 13 represent non-O157:H7 patterns B to K, respectively. Lane 14 is a negative control (no template DNA).

mon limitation of all of these methods is that they are not specific enough to differentiate individual serotypes among the verotoxin-producing group of *E. coli*.

The RSS-PCR method described herein is a rapid *E. coli* O157:H7 detection assay that differentiates genotypes on the basis of multiple band patterns. Most bacterial detection methods that have been developed thus far are mainly based on unique biochemical characteristics (26), immunogenic properties (1, 5, 6, 14, 20, 21), or the presence or absence of genes that are unique to the organism of interest (3, 8–10, 33). Although these methods vary significantly in terms of the specificity in identifying the organism, all of them are limited by the fact that they only give either a "positive" or "negative" result (e.g., generating a single PCR amplicon). This can be problematic when certain strains of *E. coli* exhibit similar or identical phenotypes while belonging to different serotypes (i.e., expression of SLTs). One major advantage of the RSS-PCR method is that it generates "fingerprint" patterns that are distinct for different serotypes of *E. coli*. Thus, the method can potentially reduce the ambiguity often encountered in identifying and differentiating serotypes of *E. coli* among clinical isolates using more conventional diagnostic techniques. The detection of electrophoretic patterns rather than a single amplicon helps to reduce false-positive results, increases specificity, and therefore confidence in the interpretation of the results. While other PCR methods to generate "fingerprint" patterns for *E. coli* exist (REP, ERIC, and BOX), they often rely on repetitive DNA elements that are not specific to *E. coli* (28, 29). The RSS-PCR method is based on an outer membrane heme receptor gene *chuA* that is specific to *E. coli* (27). Hence, as shown here (Fig. 2), no discernible pattern was generated with another member of the *Enterobacteriaceae*, *S. enterica* serovar Enteritidis. The genotypic analysis also precludes the problems encountered with serologic tests that require expression of proteins, such as that observed with differential expression of the H7 antigen by *E. coli* O157:H7.

We showed that the RSS-PCR method generates a genotypic pattern for strains of *E. coli* O157:H7 that is distinct from that of other *E. coli* serotypes. As in other tests used to differentiate *E. coli* O157:H7 from other *E. coli* serotypes, this test is designed to differentiate *E. coli* strains only after the bacterial organism has been shown to be *E. coli*. It is not designed to differentiate *E. coli* from non-*E. coli* bacterial organisms. Our RSS-PCR results correlated well with the MAb ELISA results. Of concern is the discrepancy between results obtained with the MAb ELISA and the RSS-PCR results compared to the polyclonal antibody latex agglutination test. All 54 isolates were initially characterized as O157:H7 by the latex agglutination test following the manufacturer's protocol. Both the MAb ELISA and RSS-PCR tests identified only 28 (52%) of the isolates to be O157:H7. Therefore, there was complete agreement between the MAb ELISA test and RSS-PCR results in the differentiation of *E. coli* O157:H7 strains. While the latex agglutination test may be simpler to perform, it appears to give a relatively high rate of false-positive results.

Of the 26 MAb ELISA-confirmed non-O157:H7 strains, 8 strains were initially found to give discordant results by the RSS-PCR method. Because these strains yielded patterns that appeared on first inspection to be similar to the prototype O157:H7 pattern, they were initially classified as O157:H7 strains. Upon further analysis of the serological data, we noticed that two of the eight strains were those that had retained the O157 antigen but had lost the H7 antigen, and 1 strain had the H7 antigen but did not have the O157 antigen. These eight discordant strains were retested with the RSS-PCR method to attempt to generate more discriminating patterns for better

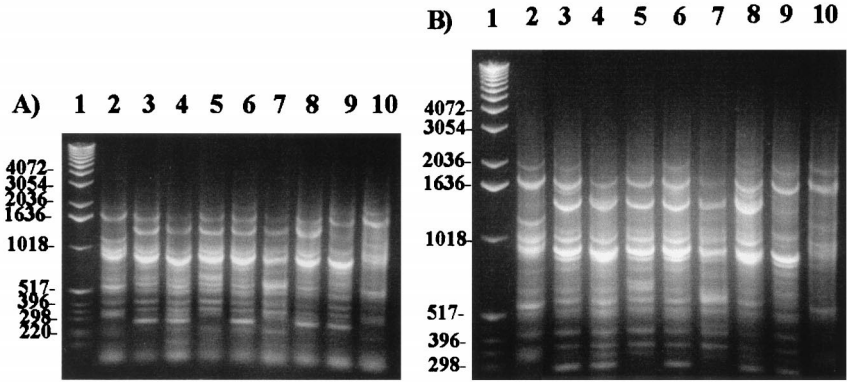

FIG. 4. Electrophoresis for 55 (A) and 75 (B) min. Comparison of the eight KSU strains that gave discrepant results with the RSS-PCR and serological tests. Lane 1 represents the 1-kb DNA ladder (Gibco-BRL). Lane 2 represents the *E. coli* O157:H7 pattern A (positive control). Lanes 3 to 8 represent non-O157:H7 strains with pattern H (KSU strains 14, 41, 43, 45, 46, and 48). Lane 9 represents a non-O157:H7 strain with pattern E (KSU strain 49). Lane 10 represents KSU strain 52 which was found to be missing the H7 antigen but which still gave a pattern similar to that of *E. coli* O157:H7 pattern A. A longer separation time yielded better discrimination of the patterns.

comparison. When we extended the electrophoresis time from 55 to 75 min, the pattern differences became clearly apparent (Fig. 4). This underscores the limitation of visual analysis of PCR patterns. Although we observed gel-to-gel variation in the intensity of a 2,000-bp band and two other low-molecular-weight bands (360 and 320 bp), the patterns overall were shown to remain stable and reproducible when the test was repeated over a period of 6 months (data not shown). We believe that due to the high molecular weight of the 2,000-bp fragment, it is not always efficiently amplified by the conditions we use. As in other PCR-based methods to generate "fingerprint" patterns, this method is likely to show laboratory-to-laboratory variability and, therefore, there is a need to always include in every gel a positive control sample that will generate the expected O157:H7 RSS-PCR patterns for comparison to the test patterns.

The *E. coli* serotype, O157:H7, comprises a group of closely related verotoxin-producing strains, which are widely distributed throughout North America (31). While there has been considerable debate concerning the evolution of these strains, based on genetic studies of *E. coli* O157:H7, it is now widely recognized that O157:H7 serotype represents a group of strains that were derived from a single ancestral clone (31, 32). Multilocus enzyme electrophoresis studies conducted by Whittam et al. have shown that the electrophoretic profiles of four different enzymes among *E. coli* O157:H7 strains are distinctly similar to each other but clearly different from that of other *E. coli* isolates from diverse sources in the natural environment (31). Further studies have suggested that *E. coli* O157:H7 strains are distantly related to other EHEC serotypes and have actually evolved from *E. coli* O55:H7, a serotype classified under the EPEC group (32). Our results of the RSS-PCR on *E. coli* serotypes O157:H7 and O55:H7 show that both organisms generate identical patterns (pattern A) that are distinct from patterns generated by other serotypes, which may indicate that the two are highly similar genetically and support the observation made by Whittam et al. Another study (8) that evaluated an EHEC detection assay also showed identical patterns for both *E. coli* O157:H7 and O55:H7 when the amplified PCR products were treated with a specific restriction enzyme. These observations reflect the clonality of *E. coli* O157:H7 and lend credence to the theory that *E. coli* O157:H7 is derived from *E. coli* O55:H7. They also further support the RSS-PCR technique as a valid method to differentiate *E. coli* serotypes.

RSS-PCR is a simple and rapid method that requires minimal pieces of equipment and time. The entire procedure can be completed in less than 2 days and does not require the laborious extraction and purification of DNA from organisms. Furthermore, unlike conventional restriction fragment length polymorphism analyses, the procedure does not require the use of restriction enzymes. Another major advantage is that the procedure does not require the use of multiple pairs of primers like the multiplex PCR procedures described previously. Hence, this test may serve as a rapid way to test a large number of *E. coli* samples, such as those derived from environmental sources.

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