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# Use of the Flagellar H7 Gene as a Target in Multiplex PCR Assays and Improved Specificity in Identification of Enterohemorrhagic *Escherichia coli* Strains

V. P. J. GANNON,[1]* S. D'SOUZA,[1] T. GRAHAM,[1] R. K. KING,[1] K. RAHN,[2] AND S. READ[2]

*Animal Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada T1J 3Z4,[1] and Animal and Plant Health Laboratory, Agriculture and Agri-Food Canada, Guelph, Ontario, Canada N1G 3W4[2]*

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**PCR products of 1.8 kb were generated with DNAs from all *Escherichia coli* H7 strains tested by using oligonucleotide primers which flank the *fliC* gene. Three *Rsa*I digestion profiles of these PCR products were evident on agarose gels; the first occurred with serotype O55:H7, O157:H7, or nonmotile (NM) strains, the second occurred with serotype O1:H7 and O18:H7 strains, and the third occurred with serotype O?:H7, O19:H7, O121:H7, O88:H7, and O156:H7 strains. Despite these differences, the nucleotide sequences of the *E. coli* E32511 (O157:NM) and U5-41 (O1:H7) *fliC* genes were 97% homologous. Two PCR primer pairs synthesized on the basis of the E32511 H7 *fliC* sequence amplified specific DNA fragments from all *E. coli* H7 strains, but did not amplify DNA fragments from the other bacterial strains. The H7-specific primers were used in combination with other primers which target the Verotoxin 1 (VT1) and VT2 genes and the *E. coli* O157:H7 *eaeA* gene in multiplex PCR assays. In these assays, *vt* and *eaeA* PCR products were observed with DNAs from the majority of EHEC strains and *vt*, *eaeA*, and *fliC* PCR products were observed with DNAs from *E. coli* O157:H7 or NM strains. Only *eaeA* PCR products were present with DNA from enteropathogenic *E. coli*, and only *vt* PCR products occurred with VT-producing *E. coli* which are not EHEC. The multiplex PCR assays described allow for the specific identification of *E. coli* O157:H7 or NM and other EHEC strains.**

Enterohemorrhagic *Escherichia coli* (EHEC) serotypes are associated with hemorrhagic colitis (HC), the hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (23, 33, 55). While several EHEC serotypes have been associated with human infection, recent well-publicized outbreaks of infection with *E. coli* O157:H7 have resulted in a focus on the development of methods for the identification of this specific EHEC serotype. This single serotype-directed effort is in part justifiable, in that the vast majority of HC cases are caused by *E. coli* O157:H7 (32, 57). However, there is also concern that the high selectivity of methods for the detection of *E. coli* O157:H7 has biased cultural procedures for EHEC identification in favor of sorbitol-negative *E. coli* O157:H7 and will continue to do so. This may lead to a failure in the assessment of the prevalence of other EHEC isolates associated with human disease and also may leave us unprepared for the emergence of new clones of these organisms. A future swing in phenotypic and serological characteristics of EHEC may well occur, on the basis of our knowledge of the epidemiology of many other pathogenic microorganisms and, in particular, other species of the family *Enterobacteriaceae.*

All EHEC isolates produce one or more antigenic types of Verotoxin (VT) (also known as Shiga-like toxin) (34, 44) and contain a large (ca. 60-MDa) plasmid encoding factors which may contribute to their virulence, such as fimbria and enterohemolysins (15, 26, 29, 37, 51, 52). Several methods have been developed for the detection of VTs, including tissue culture (34, 45), immunoassay (3, 6, 14, 35), DNA hybridization (22, 31, 43, 50), and PCR assays (19, 24, 28, 30, 38, 47). However, not all VT-producing *E. coli* (VTEC) isolates are EHEC isolates, and many VTEC strains isolated from species such as cattle and pigs have never been associated with human disease (1, 9, 13). Therefore, while VT is an important marker for these pathogenic bacteria, methods with greater specificity are required for the identification of EHEC.

Most EHEC isolates also possess a genetic locus associated with attachment to enterocytes and effacement of their microvilli (27, 42). The latter property is shared with enteropathogenic *E. coli* (EPEC), and it has recently been shown that this activity is mediated by a 35-kb chromosomal region termed the "locus of enterocyte effacement" (41). While this locus contains several genes, one of these, *eaeA*, encodes the protein intimin, which is thought to be responsible for the close association of EHEC and EPEC isolates to the cytoplasmic membrane of cell lines such as HEp-2 in vitro and enterocytes in vivo. The 5′ portion of the *eaeA* gene appears to be relatively well conserved among EHEC and EPEC isolates; however, the 3′ one-third of the gene differs among EHEC and EPEC serotypes (4, 20, 40, 60).

We have previously shown that *vt-1* and *vt-2* genes and a 5′ conserved area of the *eaeA* gene ($eaeA_{GEN}$) can be used in PCR assays as targets for the identification of the majority of EHEC isolates and, in addition, that certain oligonucleotide primers with homology to the 3′ end of the *eaeA* of *E. coli* O157:H7 ($eaeA_{O157}$) are quite specific for this organism in PCR assays (20). In these studies, the $eaeA_{O157}$ oligonucleotide primers were shown to amplify DNAs from all *E. coli* O157:H7 (NM [nonmotile]) strains, an EPEC strain of serotype O55:H7, and an EHEC strain of serotype O145:NM but not DNAs from other *E. coli* strains.

This report describes multiplex PCR assays which employ $vt_{1+2}$ and $eaeA_{GEN}$ primers to aid in the identification of VTEC and the majority of EHEC strains ($vt^+$, $eaeA_{GEN}{}^+$). To provide simultaneous identification of *E. coli* O157:H7, we have used $vt_{1+2}$ and $eaeA_{GEN}$ primers with $eaeA_{O157}$ oligonu-

* Corresponding author. Mailing address: Animal Diseases Research Institute, Health Canada, Box 640, Lethbridge, Alberta, Canada T1J 3Z4. Phone: (403) 382-5514. Fax: (403) 381-1202. E-mail: gannonv@em.agr.ca.

TABLE 1. Summary of PCR results with DNAs from bacterial strains

| Group and origin | *E. coli* O serogroup | No. of strains | No. of strains with specific PCR products with primer: | | | |
|---|---|---|---|---|---|---|
| | | | $vt_{1+2}$ | $eaeA_{GEN}$ | $eaeA_{O157}$ | $fliC_{h7}$ |
| VTEC | | | | | | |
| Human and bovine | 5 | 2 | 2 | 2 | 0 | 0 |
| | 26 | 9 | 9 | 9 | 0 | 0 |
| | 38 | 1 | 1 | 0 | 0 | 0 |
| | 91 | 3 | 3 | 0 | 0 | 0 |
| | 103 | 5 | 5 | 5 | 0 | 0 |
| | 111 | 11 | 11 | 10 | 0 | 0 |
| | 121 | 1 | 1 | 1 | 0 | 0 |
| | 121:H7 | 1 | 1 | 0 | 0 | 1 |
| | 128 | 1 | 1 | 1 | 0 | 0 |
| | 145 | 3 | 2 | 2 | 2 | 0 |
| | 157 | 26 | 26 | 26 | 26 | 26 |
| Porcine | 138, 139, 141 | 4 | 4 | 0 | 0 | 0 |
| Non-VTEC | | | | | | |
| Other O157 | 157:H42 | 3 | 0 | 0 | 0 | 0 |
| EPEC | 26, 86, 111, 119, 125, 126, 127, 128 and 142 | 9 | 0 | 5 | 0 | 0 |
| | 55:H7 | 1 | 0 | 1 | 1 | 1 |
| Enteroinvasive *E. coli* | | 1 | 0 | 0 | 0 | 0 |
| Bovine and porcine ETEC | | 27 | 0 | 0 | 0 | 0 |
| Other *E. coli* strains; other H7 (FMT E)[a] | | 5 | 0 | 0 | 0 | 5 |
| FMT A, B, other E, C, D, F, and U[b] | | 49 | 0 | 2[c] | 0 | 0 |
| *E. coli* K-12 strains | | 3 | 0 | 0 | 0 | 0 |
| Other bacterial species[d] | | 41 | 0 | 0 | 0 | 0 |

[a] Single *E. coli* strains of O1:H7, O18ac:H7, O19:H7, O88:H7, and O156:H7.

[b] *E. coli* strains of FMTs A (H4, H17), B (H5, H25, H33, H38, H39, H42, H47, H52), C (H2, H10, H24, H29, H43, H48, H53), D (H8, H11, H21, H27, H40), E (H1, H12, H23, H34, H45, H49, H51), F (H6, H14, H15, H18, H19, H20, H26, H28, H30, H31, H32, H37, H41, H44, H46), and U (H3, H9, H16, H35, H36).

[c] Two *E. coli* strains of EPEC O serogroup 86; one with H34 and the other with H47.

[d] *Aeromonas hydrophila*, *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia hermanii*, *Klebsiella pneumoniae*, *Pasturella haemolytica*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas testosteroni*, *Salmonella montevideo*, *Salmonella typhimurium*, *Salmonella cholerae-suis*, *Sarcina aurantiaca*, *Serratia marcescens*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Vibrio cholerae*, *Vibrio parahaemolytica*, *Vibrio vulnificus*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Agrobacterium tumefaciens*, *Jonesia denitrificans*, *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Streptococcus faecalis.*

cleotide primers or primers complementary to a portion of the *fliC* gene encoding the H7 flagellar antigen. In addition, the *fliC* genes of a collection of *E. coli* strains with the H7 flagellin were characterized. The *fliC* gene of *E. coli* O55:H7 appears to be most closely related to that found in *E. coli* O157:H7 (NM) strains, based on restriction endonuclease digestion analysis of PCR products of the *fliC* gene.

(A portion of this work was presented at the First International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases, Rapid City, S.D., 27 to 30 September 1995 [18].)

## MATERIALS AND METHODS

**Bacterial strains, culture conditions, and DNA extraction.** The bacterial strains used in this study are listed in Table 1. The VTEC, EHEC, EPEC, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, and *E. coli* K-12 strains and strains of other bacterial species have been described previously (20). *E. coli* strains representative of different *E. coli* H serotypes (46) and flagellar morphotypes (FMTs) A, B, C, D, E, F, and U (36) were obtained from the American Type Culture Collection (Rockville, Md.), R. Wilson (*E. coli* Reference Center, University Park, Pa.), and D. Woodward (National Laboratory for Enteric Pathogens, Health Canada, Ottawa, Ontario, Canada). *E. coli* O?:H7, *E. coli* O88:H7, *E. coli* O121:H7, *E. coli* O156:H7, and *E. coli* O157:H42 strains were isolated from cattle feces or beef by the authors (10, 48). Bacteria were grown in 5 ml of brain heart infusion broth for 16 to 18 h at 37°C. Total bacterial DNA was extracted as described previously (19, 20). Following ethanol precipitation, nucleic acid preparations were dried under vacuum and resuspended in 100 μl of distilled $H_2O$, incubated at 65°C for 30 min, and stored at 4°C until they were used.

**PCR amplification.** The oligonucleotide primers used in the study are listed in Table 2. These were synthesized with a model 391 DNA synthesizer (PCR-Mate; Applied Biosystems Inc., Foster City, Calif.). The *E. coli eaeA* and *vt* oligonucleotide primers used in the study have been described previously (19, 20). The *fliC* gene generic primers (FLIC-F and FLIC-R) are those described by Schoenhals and Whitfield (54). The FLICH7-F and FLICH7-R primers were synthesized on the basis of the nucleotide sequence data from the *fliC* gene of *E. coli* U5-41 O1:K1:H7 (accession number L07388) reported by Schoenhals and Whitfield (54) and the nucleotide sequence data provided in this study for the *fliC* gene of *E. coli* O157:NM E32511.

PCR assays were performed in 100-μl volumes containing 20 ng of DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 μM (each) primer set, 0.2 mM (each) 2′-deoxynucleoside 5′-triphosphate, and 2.5 U of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, Md.). The reactions were carried out with a 9600 Gene Amplification PCR thermal cycler (Perkin Elmer-Applied Biosystems Inc., Foster City, Calif.). Temperature cycles consisted of 15 s at 94°C, followed by 15 s at 65°C and 75 s at 72°C. Each cycle was repeated 35 times, and the final cycle was followed by incubation of the reaction mixture for 5 min at 72°C.

In some experiments, PCR products were digested with restriction endonucleases (Gibco-BRL) according to the manufacturer's instructions. PCR products and restriction endonuclease digests of PCR products were analyzed by electrophoresis with 1.2 to 1.5% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide per ml. These were visualized with UV illumination and photographed. DNA molecular size standards (1-kb ladder; Gibco/BRL) were included in each agarose gel electrophoresis run.

**DNA sequence analysis.** In the DNA sequence analysis of PCR products, DNA was resuspended in 500 μl of 1× TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and a 250-μl aliquot of polyethylene glycol–$MgCl_2$ (40% [wt/vol] polyethylene glycol 8000, 30 mM $MgCl_2$) was added. The mixture was left at room temperature for 10 min and was then centrifuged at 12,000 × *g* for 20 min. The pellet was washed twice with 70% ethanol, dried under a vacuum, resuspended in 200 μl of 1× TE, and desalted through a Sephadex G-100 spin column. The concentration

TABLE 2. Oligonucleotide primers used in the study

| Primer[a] | Oligonucleotide sequence (5′→3′) | Location within gene[b] | Predicted size of amplified product (bp) |
|---|---|---|---|
| $vt_{1+2}$ | | | |
| VT1-F | CATTGTCTGGTGACAGTAGCT | 639–659 | |
| VT1-R | CCCGTAATTTGCGCACTGAG | 1371–1351 | 732 |
| VT2-F | CCATGACAACGGACAGCAGTT | 624–644 | |
| VT2-R | CCTGTCAACTGAGCACTTTG | 1403–1384 | 779 |
| $eaeA_{GEN}$ | | | |
| EAE-F | GTGGCGAATACTGGCGAGACT | 943–963 | |
| EAE-R | CCCCATTCTTTTTCACCGTCG | 1833–1813 | 890 |
| $eaeA_{O157}$ | | | |
| EAE157-F | CAGGTCGTCGTGTCTGCTAAA | 1959–1979 | |
| EAE157-R | TCAGCGTGGTTGGATCAACCT | 3047–3027 | 1,087 |
| *fliC* | | | |
| FLIC-F | CCGAATTCATGGCACAAGTCATTAATAC | 1–20 | |
| FLIC-R | CCGAATTCTTAACCCTGCAGTAGAGACA | 1736–1755 | 1,771 |
| $fliC_{h7}$ | | | |
| FLICH7-F | GCGCTGTCGAGTTCTATCGAGC | 69–91 | |
| FLICH7-R | CAACGGTGACTTTATCGCCATTCC | 671–694 | 625 |

[a] $vt_{1+2}$, $eaeA_{GEN}$, and $eaeA_{O157}$ primers have been described previously (19, 20). The *fliC* generic primers (FLIC-F and FLIC-R) are those described by Schoenhals and Whitfield (54) and contain an 8-base noncomplementary string of nucleotides at their 5′ ends.

[b] The $fliC_{h7}$ primer position numbers are from the nucleotide sequence of the *fliC* gene of *E. coli* E32511 O157:H7 (Fig. 3).

and the purity of the PCR product were determined with a UV spectrophotometer (Pharmacia, Uppsala, Sweden).

For each nucleotide sequencing reaction, a 1.0-μl (0.5-pmol) aliquot of DNA of the PCR product, 9.5 μl of Prism Ready Reaction Dye Deoxy Terminator Mix (Perkin Elmer-Applied Biosystems Inc.), 1.0 μl (1.0 μM) of primers, and 8.5 μl of distilled $H_2O$ were mixed. For DNA sequencing, FLIC-F and FLIC-R primers (Table 1) and other primers synthesized on the basis of the O1:H7 $fliC_{h7}$ nucleotide sequence were used (54). DNA cycle sequencing reactions were performed for 25 cycles, with each cycle consisting of three steps: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. On completion of the sequencing reaction the mixture was desalted with a Sephadex G-50 medium spin column. DNA cycle sequencing reaction mixtures were vacuum dried and stored at −20°C until use. The nucleotide sequence of the insert DNA was determined for five different PCR products, each derived from independent PCR amplification experiments. The nucleotide sequence of the insert DNA was determined with the 373A Automated DNA Sequencer (Perkin Elmer-Applied Biosystems Inc.) according to the manufacturer's instructions. Electrophoresis was carried out with a 6% (vol/vol) polyacrylamide gel with Tris-borate electrophoresis buffer for a run time of 12 h. Nucleotide sequence data were collected and analyzed with the Applied Biosystem 373 Data Collection and Analysis software.

A comparison of nucleotide sequences was carried out with the NALIGN program of PC/GENE, version 6.8 (Intelligenetics Inc., Campbell, Calif.).

**Nucleotide sequence accession number.** The nucleotide sequence of the *E. coli* E32511 (O157:NM) *fliC* gene has been submitted to GenBank and has been assigned the accession number U47614.

## RESULTS

**PCR with *fliC* primers.** The *fliC* generic oligonucleotide primers (FLIC-F and FLIC-R) listed in Table 2 generated single PCR products of various sizes and intensities with DNA from *E. coli* strains representative of FMTs B, C, D, E, F, and U (Fig. 1A). No PCR product was generated with these primers by using DNA from *E. coli* ATCC 23502 O5:H4 (FMT A).

For *E. coli* strains with the H7 antigen, including those of serotypes O?:H7, O1:H7, O18ac:H7, O19:H7, O55:H7, O88:H7, and O156:H7, a PCR product of ca. 1,800 bp was observed (representative results are presented in Fig. 1A). Digestion of the PCR products from these H7 strains with the restriction endonuclease *Rsa*I resulted in three distinct patterns on agarose gels (Fig. 2). The first *Rsa*I profile was common among strains of serotypes O55:H7, O157:H7, and O157:NM, the second was observed with *E. coli* strains belonging to serotypes O1:H7 and O18:H7, and the third was observed with *E. coli* strains belonging to serotypes O?:H7, O19:H7, O88:H7, O121:H7, and O156:H7. The first profile was also observed with this restriction endonuclease following digestion of the *fliC* PCR products generated with DNA from a collection of 24 other *E. coli* O157:H7 strains.

Figure 3 presents the nucleotide sequence alignment of the *fliC* of *E. coli* E32511 (O157:NM) with that of U5-41 (O1:H7), as presented by Schoenhals and Whitfield (54). These nucleotide sequences are 97% homologous, and the *Rsa*I sites identified in the respective sequences are consistent with the respective PCR product digest patterns obtained. The predicted amino acid sequences of the proteins encoded by these two *fliC* genes are 98% homologous, with the E32511 *fliC* gene product consisting of 585 residues and with that of U5-41 consisting of 584 amino acid residues (data not shown). PCR primers syn-

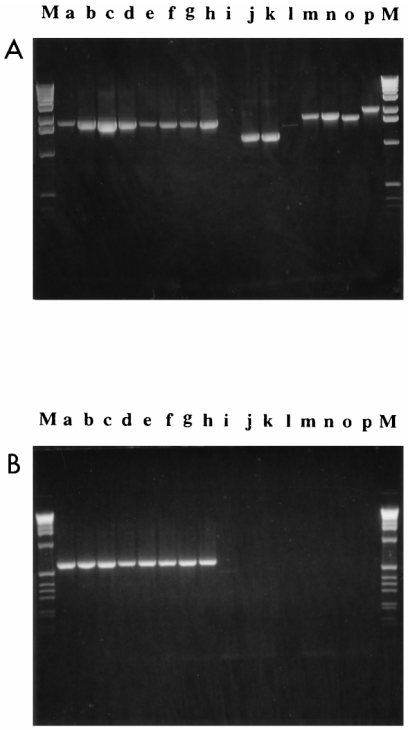


FIG. 1. PCR assays with $fliC_{GEN}$ and $fliC_{h7}$ oligonucleotide primers with DNAs from *E. coli* strains. Following DNA amplification, a 15-μl aliquot of each 100 μl of PCR mixture was analyzed by agarose gel electrophoresis (1.2% agarose containing ethidium bromide). (A and B) Agarose gels of $fliC_{GEN}$ (FLIC-R and FLIC-F) and $fliC_{h7}$ (FLICH7-F and FLICH7-R) PCR products, respectively. Lanes: M, molecular size markers (1-kb ladder; Gibco-BRL); a, E32511 O157:NM (EHEC); b, 319 O157:H7 (EHEC); c, EC322 O55:H7 (EPEC); d, O?:H7; e, U5-41 (ATCC 117551) O1:H7; f, ATCC 23513 O18ac:H7; g, ATCC 23514 O19:H7; h, EC950272 O88:H7; i, ATCC 23502 O5:H4 (FMT A); j, ATCC 23533 O70:H42 (FMT B); k, ATCC 23507 O11:H10 (FMT C); 1, ATCC 23509 O13:H11 (FMT D); m, ATCC 19138 O6:H1 (FMT E); n, ATCC 23505 O9:H12 (FMT E); o, ATCC 23512 O17:H18 (FMT F); and p, ATCC 23526 O36:H9 (FMT U).

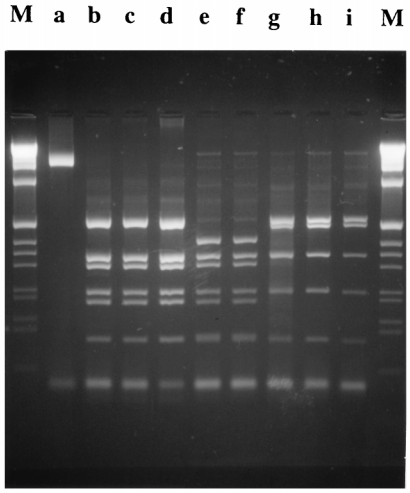


FIG. 2. *Rsa*I digestion profiles of $fliC_{GEN}$ PCR products generated with DNAs from *E. coli* H7 strains. Following DNA amplification, PCR products were digested with *Rsa*I and were then analyzed by agarose gel electrophoresis (1.5% agarose containing ethidium bromide). Lanes: M, molecular size markers (1-kb ladder; Gibco-BRL); a, undigested DNA from E32511 O157:NM (EHEC); b to i, *Rsa*I-digested DNAs from E32511 O157:HM (EHEC), 319 O157:H7 (EHEC), EC322 O55:H7 (EPEC), U5-41 (ATCC 11755) O1:H7, ATCC 23513 O18ac:H7, ATCC 23514 O19:H7, EC950272 O88:H7, and EC930099 O121:H7, respectively.

thesized on the basis of the $fliC_{h7}$ sequence, FLICH7-F and FLICH7-R (Table 2), amplified DNA fragments of the predicted size (ca. 650 bp) from all strains with the H7 antigen which were examined but did not amplify DNA fragments from the other bacterial strains tested, including 49 other *E. coli* strains of other H serotypes (Fig. 1B and Table 1).

**Multiplex PCR assays with *vt*, $eaeA_{O157}$, and $fliC_{h7}$ primers.** The $vt_{1+2}$, $eaeA_{O157}$, and $fliC_{h7}$ oligonucleotide primers used in the multiplex PCR assays generated three PCR product bands with DNAs from all *E. coli* O157:H7 strains tested and from *E. coli* E32511 (O157:NM) (Fig. 4A). Multiplex PCR assays with DNAs from VTEC isolates of human, bovine, and porcine origin generated single bands on agarose gels corresponding in size to those predicted for PCR assays with the $vt_{1+2}$ primers. The multiplex PCR amplified DNA from an O145:NM EHEC strain, with two bands corresponding in size to the sizes of the $vt_{1+2}$ and $eaeA_{O157}$ PCR products but no band corresponding in size to the size of the $fliC_{h7}$ PCR product. The non-VT-producing EPEC *E. coli* strain of serotype O55:H7, in contrast, had two bands on agarose gels corresponding in size to those expected for $fliC_{h7}$ and $eaeA_{O157}$ PCR products but no band corresponding in size to that of the $vt_{1+2}$ PCR product.

The $vt_{1+2}$, $eaeA_{GEN}$, and $fliC_{h7}$ oligonucleotide primers also generated three PCR products of the predicted size with DNAs from all *E. coli* O157:H7 strains tested and the O157:NM strain E32511 when they were used in the multiplex PCR (Fig. 4B). PCR assays with DNAs from EHEC strains of human and bovine origin generated two bands on agarose gels corresponding in size to those predicted for the $vt_{1+2}$ and $eaeA_{GEN}$ PCR products. In contrast, for *E. coli* strains which do not possess *eaeA*, such as *E. coli* strains causing pig edema disease and strain B2F/1 (O91:H21), which was isolated from a patient with HUS, multiplex PCR assays produced only a single band corresponding to the size predicted for that for the $vt_{1+2}$ PCR products. As in the first multiplex PCR described above, DNA was amplified from the O145:NM EHEC strain, with two bands corresponding in size to the sizes expected for $vt_{1+2}$ and $eaeA_{GEN}$ PCR products but had no band corresponding in size to that for the $fliC_{h7}$ PCR product. The non-VT-producing EPEC *E. coli* strain of serotype O55:H7, again, had only two bands, with sizes corresponding to those of the $fliC_{h7}$ PCR product and the $eaeA_{GEN}$ PCR product, but no band corresponding in size to that of the $vt_{1+2}$ PCR product was present. Among single strains of classical EPEC serotypes tested, 6 of 10 had a single band corresponding in size to that of the $eaeA_{GEN}$ PCR product (Table 1). This same band was also present in assays with DNAs from two other *E. coli* strains of EPEC O serogroup 86 which represented H types 34 and 47.

No PCR products were obtained by either multiplex PCR assay with DNAs from any other bacterial strains tested, including 3 *E. coli* O157:H42 strains, 27 porcine and bovine enterotoxigenic *E. coli* strains, 1 enteroinvasive *E. coli* strain, other *E. coli* strains with FMT A, B, C, D, E, F, or U, 3 laboratory *E. coli* K-12 strains, or 41 strains other bacterial species (Table 1).

## DISCUSSION

Schmidt et al. (53) reported that their generic *eaeA* PCR assay allowed for the detection of all (26 of 26) class I EPEC strains, and among 47 EHEC strains positive by the fluorescence actin staining test, 45 were also positive by the *eaeA* PCR. Unfortunately, the specific serotypes of EHEC tested in the latter study were not presented. In the present study, among the EHEC isolates tested, only members of O serogroup 91 were negative by the *eaeA* PCR. Jerse et al. (27) also could not detect *eaeA* in *E. coli* strains of this serogroup using DNA hybridization. Members of O serogroup 91 have been associated with HC and HUS (32, 33) and therefore would be considered EHEC by most workers. Despite the apparent absence of *eaeA* among *E. coli* O91 strains, Lindgren et al. (39) found that strain B2F1 (O91:H21) was able to effectively colonize the intestines of mice and produce renal lesions in these animals. It is therefore possible that these bacteria and other EHEC isolates possess virulence attributes other than *eaeA* or possess a form of the gene which cannot be readily detected by current assays. These *E. coli* strains would, however, be identified as VTEC and would therefore be subject to further examination if they were isolated from patients with clinical disease.

Recently, Cebula et al. (7) and Fratamico et al. (16) have also described PCR-based procedures for the specific identification of *E. coli* O157:H7 (NM) which use *vt* oligonucleotide primers together with oligonucleotide primer pairs which target other genes found in the organism. The assay described by Cebula et al. (7) relies on a 1-base difference between the wild-type *E. coli* β-glucuronidase gene (*uidA*) and the *E. coli* O157:H7 *uidA* for identification of this pathogen. The multiplex PCR assay described by Fratamico et al. (16) uses the $eaeA_{O157}$ oligonucleotide primers that we have described (20), together with *vt* primers and primers complementary to a region present on the *E. coli* O157:H7 60-MDa plasmid, to identify *E. coli* O157:H7. These assays not only provide a means for the relatively specific identification of *E. coli* O157:H7 but also allow for the identification of other VTEC isolates. The multiplex PCR assay described in the study of Fratamico et al. (16) also allows for the identification of certain other EHEC isolates with 60-MDa plasmids related to that of *E. coli* O157:H7.

The PCR assays described in this study are useful for the identification of VTEC, EHEC, and EPEC strains possessing the *eaeA* gene and the specific identification of *E. coli* O157:H7 (and NM variants). This broad-based detection is achieved by targeting conserved regions of two virulence genes, *vt* and *eaeA*. The *vt* genes are present in VTEC and EHEC isolates,

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E32511  - ATGGCACAAGTCATTAATACCAACAGCCTCTCGCTGATCACTCAAAATAATCTCAACAAGAACCAGTCTGCGCTG -75
U5-41   -                                                   A                          -75

E32511  - TCGAGTTCTATCGAGCGTCTGTCTTCTGGCTTGCGTATTAACAGCGCGAAGGATGACGCCGCAGGTCAGGCGATT -150
U5-41   -                                                                G             -150

E32511  - GCTAACCGTTTTACTTCTAACATTAAAGGCCTGACTCAGGCGGCCCGTAACGCCAACGACGGTATTTCTGTTGCG -225
U5-41   -                                         T  A                             A -225

E32511  - CAGACCACCGAAGGCGCGCTGTCCGAAATCAACAACAACTTACAGCGTATTCGTGAACTGACGGTTCAGGCCACT -300
U5-41   -                         T                           C     G             TT  -300

E32511  - ACAGGGACTAACTCCGATTCTGACCTGGACTCCATCCAGGACGAAATCAAATCTCGTCTTGATGAAATTGACCGC -375
U5-41   -   C  A        T     A  T           T                 C                      -375

E32511  - GTATCCGGCCAGACCCAGTTCAACGGCGTGAACGTGCTGGCGAAAGACGGTTCAATGAAAATTCAGGTTGGTGCG -450
U5-41   -                                   __A__    A           G                    -450

E32511  - AATGACGGCGAAACCATCACGATCGACCTGAAAAAAATCGATTCTGATACTCTGGGTCTGAATGGCTTTAACGTA -525
U5-41   -          T     T     T         G                                  T         -525

E32511  - AATGGTAAAGGTACTATTACCAACAAAGCTGCAACGGTAAGTGATTTAACTTCTGCTGGCGCGAAGTTAAACACC -600
U5-41   -                                                                            -600

E32511  - ACGACAGGTCTTTATGATCTGAAAACCGAAAATACCTTGTTAACTACCGATGCTGCATTCGATAAATTAGGGAAT -675
U5-41   -                 G                                                          -675

E32511  - GGCGATAAAGTCACAGTTGGCGGCGTAGATTATACTTACAACGCTAAATCTGGTGATTTTACTACCACTAAATCT -750
U5-41   -              C              ---  TC                                  C     -747

E32511  - ACTGCTGGTACGGGTGTAGACGCCGCGGCGCAGGCTGCTGATTCAGCTTCAAAACGTGATGCGTTAGCTGCCACC -825
U5-41   -                                     A           AA                           -822

E32511  - CTTCATGCTGATGTGGGTAAATCTGTTAATGGTTCTTACACCACAAAAGATGGTACTGTTTCTTTCGAAACGGAT -900
U5-41   -                                                                   T        -897

E32511  - TCAGCAGGTAATATCACCATCGGTGGAAGCCAGGCATACGTAGACGATGCAGGCAACTTGACGACTAACAACGCT -975
U5-41   -                                                                            -972

E32511  - GGTAGCGCAGCTAAAGCTGATATGAAAGCGCTGCTCAAAGCAGCGAGCGAAGGTAGTGACGGTGCCTCTCTGACA -1050
U5-41   -          CG                        T     C                       T         -1047

E32511  - TTCAATGGCACAGAATATACCATCGCAAAAGCAACTCCTGCGACAACCACTCCAGTAGCTCCGTTAATCCCTGGT -1125
U5-41   -            T        T                           T                           -1122

E32511  - GGGATTACTTATCAGGCTACAGTGAGTAAAGATGTAGTATTGAGCGAAACCAAAGCGGCTGCCGCGACATCTTCA -1200
U5-41   -                                                                            -1197

E32511  - ATTACCTTTAATTCCGGTGTACTGAGCAAAACTATTGGGTTTACCGCGGGTGAATCCAGTGATGCTGCGAAGTCT -1275
U5-41   -                                                                            -1272

E32511  - TATGTGGATGATAAAGGTGGTATCACTAACGTTGCCGACTATACAGTCTCTTACAGCGTTAACAAGGATAACGGC -1350
U5-41   -                         T                                                  -1347

E32511  - TCTGTGACTGTTGCCGGGTATGCTTCAGCGACTGATACCAATAAAGATTATGCTCCAGCAATTGGTACTGCTGTA -1425
U5-41   -                                                                            -1422

E32511  - AATGTGAACTCCGCGGGTAAAATCACTACTGAGACTACCAGTGCTGGTTCTGCAACGACCAACCCGCTTGCTGCC -1500
U5-41   -                                                                            -1497

E32511  - CTGGACGACGCAATCAGCTCCATCGACAAATTCCGTTCTTCCCTGGGTGCTATCCAGAACCGTCTGGATTCCGCA -1575
U5-41   -            T                                G                               -1577

E32511  - GTCACCAACCTGAACAACACCACTACCAACCTGTCCGAAGCGCAGTCCCGTATTCAGGACGCCGACTATGCGACC -1650
U5-41   -                                     T                                      -1647

E32511  - GAAGTGTCCAACATGTCGAAAGCGCAGATCATTCAGCAGGCCGGTAACTCCGTGCTGGCAAAAGCTAACCAGGTA -1725
U5-41   -                                T  C             G                   C     -1722

E32511  - CCGCAGCAGGTTCTGTCTCTACTGCAGGGTTAA -1758
U5-41   -                   G           G -1755
```

FIG. 3. Alignment of the nucleotide sequences of the *E. coli* E32511 O157:NM and *E. coli* U5-41 O1:H7 *fliC* genes. The nucleotide sequences are 97% homologous, as determined with the NALIGN program of PC/GENE, version 6.8 (Intelligenetics Inc.). The E32511 sequence is 1,758 bp, and the U5-41 sequence is 1,755 bp. The bases which differ between the *fliC* genes are shown with boldface letters in the E32511 sequence, and the base present in the U5-41 sequence is shown beneath it; blank spaces occur in the U5-41 sequence where the bases in the two sequences are identical. Dashes indicate areas where gaps were introduced into the sequence by the program to optimize the alignment. The areas in the E32511 sequence underlined and in boldface correspond 5′ to 3′ to the FLICH71-F and FLICH71-R binding sites. *Rsa*I sites in the E32511 and U5-41 *fliC* sequences are underlined and in italics. The *E. coli* E32511 O157:NM *fliC* nucleotide sequence data have been submitted to GenBank (accession number U47614), and the *E. coli* U5-41 O1:H7 *fliC* sequence (accession number L07388) is that presented by Schoenhals and Whitfield (54).

and the *eaeA* gene is present in the majority of EHEC and class I EPEC strains. Detection of *E. coli* strains with *eaeA* alone may be helpful not only in the identification of certain EPEC isolates but also for the detection of EHEC isolates which have lost *vt* genes (5).

The PCR assays described in this study may be appropriate for screening colonies (individually or as pools) derived from clinical samples plated onto selective media such as MacConkey agar. We have also found these PCR assays to be very useful for the identification of *E. coli* O157:H7 colonies obtained by selective culture of bovine feces by a procedure similar to that described by Chapman et al. (8) (data not shown). By this procedure, samples are cultured in selective broth, organisms are captured from the broth with anti-O157-coated magnetic beads, the beads are plated onto Sorbitol-MacConkey agar containing cefixime and tellurite, and suspect colonies are tested by slide agglutination with *E. coli* O157 antiserum and then by the multiplex PCR assays described in this study. Recently, similar selective culture and immunocapture procedures have also been applied to human stool samples for the isolation of *E. coli* O157:H7 (12). Presumably, the multiplex PCR assays described in this study would also be

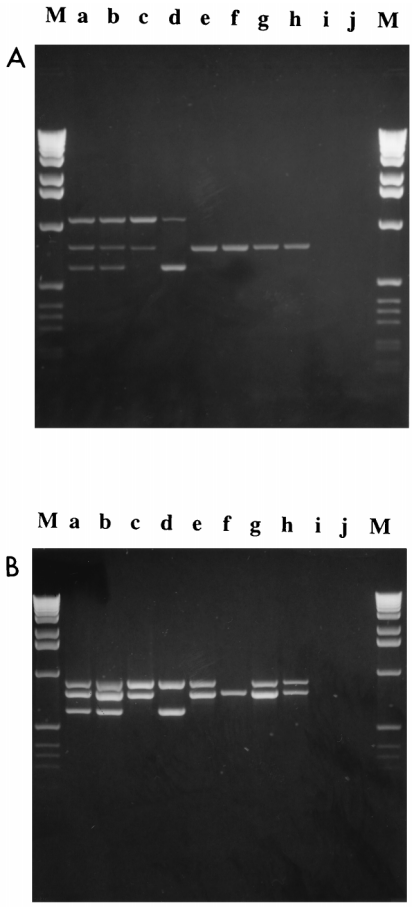


FIG. 4. Multiplex PCR assays with DNAs from VTEC, EHEC, *E. coli* O157:H7, and EPEC strains. (A) PCR assays with $vt_{1+2}$, $eaeA_{O157}$, and $fliC_{h7}$ oligonucleotide primer pairs. (B) Multiplex PCR assays with $vt_{1+2}$, $eaeA_{GEN}$, and $fliC_{h7}$ oligonucleotide primer pairs. Following DNA amplification, a 15-μl aliquot of each 100 μl of PCR mixture was analyzed by agarose gel electrophoresis (1.2% agarose containing ethidium bromide). Lanes: M, molecular size markers (1-kb ladder; Gibco-BRL); a, E32511 O157:NM (EHEC); b, 319 O157:H7 (EHEC); c, CH33264 O145:NM (EHEC); d, EC322 O55:H7 (EPEC); e, 41131 O26:H11 (EHEC); f, EC17258 O38:H21 (VTEC); g, 52050 O111:K58 (EHEC); h, H.I.8 O128:B12 (EHEC); i, DH5α O?R:K12 (laboratory strain); and j, no DNA.

useful in this clinical setting for the identification of *E. coli* O157:H7 colonies.

We have previously shown that the $eaeA_{O157}$ oligonucleotide primers provide a high level of specificity in the identification of *E. coli* O157:H7 (20). In this study, the broad-based assay for the detection of VTEC and EHEC was modified to also provide increased specificity for the identification of *E. coli* O157:H7 (NM). This was done by inclusion of $fliC_{h7}$ oligonucleotide primers together with $vt_{1+2}$ and either $eaeA_{O157}$ or $eaeA_{GEN}$ oligonucleotide primers.

The multiplex PCR procedures described in this study may decrease the need for certain specialized procedures such as the fluorescent actin staining test and VT neutralization assays in tissue culture and, in the case of *E. coli* O157:H7, the need for H serotyping. Despite this, additional laboratory work is still required for complete characterization of EPEC, VTEC, and EHEC strains. This work includes serotyping, characterization of the type(s) of VT produced (19, 22, 28), hybridization studies with EPEC adherence factor or EHEC plasmid probes (26, 37, 52), and assays to detect adhesins such as the EPEC bundle-forming gene (*bfpA*) (21). In addition, techniques such as phage typing and molecular fingerprinting procedures (2, 17, 49, 58) may be required for detailed taxonomic and epidemiological studies.

In this study, the $fliC_{h7}$ gene of *E. coli* O157:H7 (NM) was shown to be highly homologous to that of *E. coli* U41-5 O1:H7 (54). Attempts were made to exploit the few differences in nucleotide sequences between the E32511 and U5-41 *fliC* genes to design oligonucleotide primer pairs with greater specificity for *E. coli* O157:H7 (NM) strains. Two of these different *fliC* primer pairs tested were H7 specific; however, neither was O157:H7 (NM) specific (data not shown). Despite this, sufficient differences were present between the H7 flagellar antigen genes of the *E. coli* H7 strains to give three distinct *Rsa*I profiles for the *fliC* PCR products. Interestingly, one *Rsa*I profile was unique to *E. coli* O157:H7 (NM) strains and the EPEC *E. coli* O55:H7 strain examined. These latter two *E. coli* serotypes have also been shown to possess highly homologous *eaeA* genes (40) and to be closely related to each other on the basis of information from multilocus enzyme electrophoresis studies (58). While the *E. coli* O55:H7 strain examined does not produce VT, VT-producing *E. coli* O55:H7 strains have recently been reported in Chile in association with HC and HUS (11). Since the *vt* genes of many EHEC isolates have been shown to reside on bacteriophages (25, 56, 59), it is easy to see how bacteriophage-mediated acquisition of *vt* genes could lead to the formation of an EHEC strain from an EPEC isolate such as *E. coli* O55:H7.

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