Applied and Environmental Microbiology, Apr. 2011, p. 2275–2281 Vol. 77, No. 7
0099-2240/11/$12.00 doi:10.1128/AEM.02832-10

Identification of Genetic Markers for Differentiation of Shiga Toxin-Producing, Enteropathogenic, and Avirulent Strains of *Escherichia coli* O26▽

Marie Bugarel,[1] Lothar Beutin,[2] Flemming Scheutz,[3] Estelle Loukiadis,[4] and Patrick Fach[1]*

French Agency for Food, Environmental and Occupational Health (Anses), Food Safety Laboratory, 23 Av. du Général De Gaulle, Fr-94706 Maisons-Alfort, France[1]; National Reference Laboratory for Escherichia coli (NRL-E. coli), Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, D-12277 Berlin, Germany[2]; WHO Collaborating Centre for Reference and Researchon Escherichia and Klebsiella, Artillerivej 5, DK-2300 Copenhagen, Denmark[3]; and VetAgro Sup Campus Vétérinaire de Lyon, Unité de Microbiologie Alimentaire et Prévisionnelle (UMAP)/National Reference Laboratory for Escherichia coli, 1 Avenue Bourgelat, Aile 3, Niveau 1, Fr-69280 Marcy L'Etoile, France[4]

Received 3 December 2010/Accepted 4 February 2011

Shiga toxin-producing *Escherichia coli* (STEC) O26 is one of the top five enterohemorrhagic *E. coli* (EHEC) O groups most often associated with hemorrhagic colitis and hemolytic uremic syndrome (HUS) worldwide. STEC O26 is considered to have evolved from enteropathogenic (EPEC) O26 strains through the acquisition of Shiga toxin (Stx)-encoding genes. Our PCR data identified several STEC-like strains expressing all features of STEC except Stx production and carrying remnants of Stx phages that were probably derivatives of EHEC O26. EHEC and EPEC O26 strains phenotypically resemble O26 EHEC-like and apathogenic *E. coli* O26 strains and are therefore undistinguishable by cultural methods. A clear discrimination between the different O26 groups is required for diagnostics in patients and for control of food safety. To develop an assay for specific detection of EHEC and EHEC-like O26 strains, we used a high-throughput PCR approach for selection of discriminative genetic markers among 33 tested genes mostly encoding type III secretion system effector proteins. The genes *ECs1822*, *nleH1-2*, *nleA*, *nleC*, *nleH1-1*, *nleG*, *nleG2*, *nleG6-1*, *nleG6-2*, *espJ*, *espM2*, *nleG8-2*, *espG*, *ent* (or *espL2*), *nleB*, *nleE*, *efa1*, and *espB* were detected at different frequencies in O26 EHEC, EHEC-like, and EPEC strains, indicating the possible role of these genes in virulence of human pathogenic O26 strains. The *espK* and *espN* genes were detected only in EHEC and EHEC-like O26 strains. *espK* was present in 99.14% of EHEC and 91.14% of EHEC-like O26 strains and was hence the best candidate as a genetic marker for characterizing these pathogroups. These data were corroborated by a genotyping real-time PCR test based on allelic discrimination of the *arcA* (aerobic respiratory control protein A) gene. The results indicate that a combination of molecular detection tools for O26 *wzx* (*wzx*$_{O26}$), *eae*-beta, *stx*, *espK*, and *arcA* genotyping is highly discriminative for clear identification of EHEC and EHEC-like *E. coli* O26 strains. This simple diagnostic test might be applicable in hospital service laboratories or public health laboratories to test strains isolated from stools of patients suffering from diarrhea.

Escherichia coli strains of serogroup O26 were originally classified as enteropathogenic *E. coli* (EPEC) due to their association with outbreaks of infantile diarrhea in the 1940s (37). In 1977, Konowalchuk et al. (28) reported that some O26 strains showed toxic activity to Vero cells. Analysis of virulence genes present in *E. coli* O26 strains revealed two groups of pathogens: EPEC O26 strains that were characterized by the presence of the LEE (locus of enterocyte effacement) pathogenicity island and Shiga toxin-producing *E. coli* (STEC) O26 strains that, in addition to LEE, carried phages encoding the genes for Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2) (26). Human infections with Stx-producing *E. coli* O26 were frequently associated with severe diseases such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Accordingly, these strains were assigned to the enterohemorrhagic *E. coli* (EHEC) group together with EHEC O157, O103, O111, and O145 strains (35).

EHEC O26 constitutes the most common non-O157 EHEC group associated with severe diarrhea and HUS in Europe (16, 17). Studies of sporadic cases of HUS in Germany and Austria (19, 20) identified EHEC O26 in 14% of all STEC infections and representing 40% of non-O157 STEC strains isolated from these patients. High prevalence rates of EHEC O26 in patients with HUS and diarrhea were reported from other European countries, such as the Czech Republic (3), Denmark (15), Finland (13), Italy (41), and Spain (7). Reports on an association between EHEC O26 and HUS or diarrhea from North America, including the United States (8, 22, 25), South America (39, 42), Australia (14), and Asia (23, 24), provide further evidence for the worldwide significance of EHEC O26 strains.

Ruminant animals, in particular cattle, were identified as a major reservoir of EHEC and STEC strains (21, 36). More than 85% of human infections with EHEC were associated with consumption of contaminated food (31), and studies from different countries have shown that meat and milk products are frequently contaminated with STEC strains belonging to numerous serotypes (18). A study in Germany and Switzerland showed that strains of serogroup O26 represented about 2% of STEC strains isolated from food samples (1, 34, 45). In contrast to EHEC O157 strains, EHEC O26 strains phenotypically

* Corresponding author. Mailing address: Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), Laboratoire de Sécurité des Aliments, 23 Av. du Général De Gaulle, Fr-94706 Maisons-Alfort, France. Phone: 33 14977 2813. Fax: 33 14568 9762. E-mail: patrick.fach@anses.fr.

▽ Published ahead of print on 11 February 2011.

resemble other STEC and apathogenic *E. coli* strains and are therefore not easily detectable by cultural methods (26). As a consequence, molecular techniques are required for identification of EHEC O26 strains, the second most important EHEC type worldwide (26).

Molecular analysis of O26 EPEC and EHEC strains (6, 32) revealed specific characteristics. Similar intimin (*eae*-beta) and flagellar (antigen H11 *fliC* [*fliC*$_{H11}$]) genes were detected in nonmotile (NM) and motile EPEC and EHEC O26 strains (26). However, analysis of genotypes by multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and multilocus variable-number tandem repeat analysis (MLVA) of a collection of 62 EPEC and EHEC O26 strains revealed two major genetic clusters (33, 44). EHEC O26 could be attributed to a genetic lineage which is characterized by similar PFGE and MLVA profiles and by MLST for allelic type 2 of the *arcA* (aerobic respiratory control protein A) gene (33). EPEC O26 strains were found distributed over both genetic clusters but divided into two subgroups according to some phenotypical and genotypical attributes (6, 32). One group of EPEC O26 strains showed all characteristics of EHEC O26, including virulence markers, the EHEC virulence plasmid, and the MLST, MLVA, and PFGE profiles (30, 33). This group of O26 EPEC was classified as EHEC-like since it expresses all features of EHEC O26 strains except production of Shiga toxins (9, 10). The other group of EPEC O26 strains was different from all other EPEC and EHEC O26 strains by their biofermentative types, the presence of the plasmids encoding alpha-hemolysin, and the *arcA* allelic type 1 gene (11, 33). This group of EPEC strains was found to cluster separately from EHEC and EHEC-like O26 strains when investigated by PFGE and MLVA (33). Similar genotypes, virulence markers, and phenotypes were found in the groups of EPEC, EHEC-like, and EHEC O26 strains irrespective of whether these strains were isolated from humans, animals, or food (9, 10, 33), indicating that these strains are widespread in humans, animals, and the environment.

Rapid detection of EHEC and EHEC-like O26 strains is of diagnostic value since 5% of HUS patients were shown to excrete *stx*-negative derivates of causative EHEC strains (4). Diagnostic screening of clinical samples for Stx only bears the disadvantage that *stx*-negative derivatives of EHEC are not detected. Also, Stx screening of food samples does not alone differentiate between human pathogenic and nonpathogenic STEC that might be present.

In an attempt to develop an assay for specific detection of EHEC and EHEC-like O26 strains, we used a high-throughput PCR approach to select genetic markers among the genes encoding type III secretion system effector proteins. Using this approach, we could identify genetic markers which allow discrimination of EHEC O26 and their *stx*-negative derivatives from EPEC and apathogenic *E. coli* O26 strains.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study (n = 250) were from the collections of the National Reference Laboratory for *E. coli* (NRL-*E. coli*) at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, from the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* in Copenhagen, Denmark, from VetAgro Sup Campus Vétérinaire de Lyon in Marcy l'Etoile, France, and from the French Agency for Food, Environmental and Occupational Health (Anses) in Maisons-Alfort, France. A collection of 62 *E. coli* O26 strains that had previously been characterized by Miko et al. (33) for their serotypes, virulence profiles, PFGE profiles, MLVA profiles, and *arcA* genotypes was used as a reference for testing high-throughput microfluidic real-time PCR. Other *E. coli* O26 strains investigated in this study had their *E. coli* O (lipopolysaccharide [LPS]) and H (flagellar) antigens determined either with specific antisera as previously described (29) or by using *wzx* and *fliC* genotyping (38). The EHEC strain EDL933 (O157:H7) was used as a positive control for the complete set of genes encoding the effector proteins translocated by the type III secretion system. The strain NV254 (O113:H21) was used as a positive control for testing the autoagglutinating adhesin (*saa*) and the subtilase cytotoxin (*subA*).

All bacteria were cultured to single colonies on Luria-Bertani agar plates and grown overnight at 37°C. One colony was picked and DNA extracted using either an InstaGene matrix (Bio-Rad Laboratories, Marnes La Coquette, France) or an EZ1 bio-robot (Qiagen, Coutaboeuf, France) with a DNA tissue kit (Qiagen, France) in accordance with the manufacturer's instructions.

High-throughput real-time PCR system. The BioMark real-time PCR system (Fluidigm, San Francisco, CA) was used for high-throughput microfluidic real-time PCR amplification using either the 96.96 or the 48.48 dynamic arrays (Fluidigm). Amplifications were performed in accordance with the recommendations of the manufacturer, using either the EvaGreen DNA binding dye (Biotium Inc., Hayward, CA) followed by a melting curve analysis or using 6-carboxyfluorescein (FAM)- or HEX (6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein succinimidyl ester)-labeled TaqMan probes with TaqMan universal PCR master mix (Applied Biosystems, Coutaboeuf, France). The BioMark real-time PCR system was used with the following thermal profile: 95°C for 10 min (enzyme activation) followed by 35 cycles of 95°C for 15 s and 60°C for 1 min (amplification step).

PCR amplifications were developed for detecting genes encoding Shiga toxins 1 and 2 (*stx*$_1$ and *stx*$_2$), intimins (*eae*, *eae*-alpha, *eae*-beta, *eae*-gamma, *eae*-ε, and *eae*-theta), the O26 group-associated protein (O26 *wzx* [*wzx*$_{O26}$]), flagellar antigen H11 (*fliC*$_{H11}$), effector proteins translocated by the type III secretion system (EspG [*espG*], EspF1 [*espF1*], EspL2 [*ent* {or *espL2*}], NleB [*nleB*], NleE [*nleE*], NleH1-2 [*nleH1-2*], NleA [*nleA*], EfA1 [*efA1*], EspX1 [*espX1*], NleC [*nleC*], NleH1-1 [*nleH1-1*], EspN [*espN*], EspO1-1 [*EspO1-1*], EspK [*espK*], NleG [*nleG*], NleG2 [*nleG2*], NleG6-1 [*nleG6-1*], EspR1 [*espR1*], NleG5-2 [*nleG5-2*], NleG6-2 [*nleG6-2*], EspJ [*espJ*], EspM2 [*espM2*], NleG8-2 [*nleG8-2*], EspB [*espB*], and EspX5 [*espX5*]), and a hypothetical protein encoded by *ECs1822*. The array was also designed for the detection of other putative EHEC virulence factors, such as the autoagglutinating adhesin (*saa*) and the subtilase cytotoxin (*subA*). The gene *wecA* was used as a reference genetic marker of *E. coli* (Table 1).

Real-time PCR genotyping of the *arcA* gene. Real-time PCR genotyping of the *arcA* (aerobic respiratory control protein A) gene was based on single-nucleotide differences (C/T) at position 430 between the *arcA* sequences published under GenBank accession no. AJ875429 (EHEC O26) and AJ875430 (EPEC O26) (26). The primers arcA-F (5′-GCGCCATGCTTCACTTCTG-3′) and arcA-R (5′-CCGGTCATTTTCTTCAGCAGTT-3′) and the probes arcA-P1 (5′-VIC-C AAAATTCAGTC**T**CGTGC-minor groove binder [MGB]-3′) and arcA-P2 (5′-FAM-CAAAATTCAGTC**C**CGTGC-MGB-3′) (underlining and boldface indicate the position of the single nucleotide difference used to genotype) were selected with the Primer express 3.0 software program (Applied Biosystems, Foster City, CA). Primers and labeled gene probes were purchased from Applied Biosystems. Genotyping experiments were performed with an ABI 7500 instrument (Applied Biosystems) in 25-μl reaction volumes according to the recommendations of the supplier.

PCR detection of *stx* phage-associated genes in *E. coli* O26 strains. PCRs for three different insertion sites of *stx* phages in the chromosomes of EHEC strains were developed by designing forward primers located in the chromosomal genes *wrbA*, *yecE*, and *yjjG* that were combined with backward primers located in the *stx* phage integrase gene, which was located closely to the phage insertion site in the bacterial genome (Table 2). A positive PCR result is obtained only with those strains that carry the phage integrase in the vicinity of the targeted chromosomal genes *wrbA*, *yecE*, and *yjjG*. In addition, we have developed PCRs specific for three different open reading frames (ORFs) (orf189, orf357, and orf588; GenBank accession no. AJ236875) of the *stx*$_1$ prophage that is present in the EHEC O26:[H11] strain H19. The presence of the q gene encoding the O26 *stx* phage putative antirepressor protein was investigated by PCR with primers derived from the total genomic sequence of EHEC O26 strain 11368 (GenBank accession no. AP010953) (Table 2).

Statistical analysis. To analyze the relationship between the genetic markers investigated in this work and the four *E. coli* O26 pathogroups, the frequencies of the genetic markers were calculated per pathogroup (Table 1). In violation of the conditions (small expected frequencies), Fisher's exact test was used for calculation. As a significance level, α was set to 0.05. All P values of $\leq\alpha$ were considered statistically significant.

TABLE 1. Association of the genetic markers with different pathogroups of *E. coli* O26

Genetic marker	% of strains associated with indicated group (95% CI[a])			
	EHEC (n = 117)	EHEC-like (n = 35)	EPEC (n = 87)	Avirulent *E. coli* (n = 11)
stx	100 (94.47–100)	0 (0–8.2)	0 (0–3.38)	0 (0–23.84)
eae	99.14 (95.33–99.98)	100 (91.8–100)	98.85 (93.76–99.97)	0 (0–23.84)
eae-beta	99.14 (95.33–99.98)	100 (91.8–100)	95.55 (90.25–99.28)	0 (0–23.84)
wzx_{O26}	100 (94.47–100)	100 (91.8–100)	100 (96.62–100)	100 (76.16–100)
$fliC_{H11}$	100 (94.47–100)	100 (91.8–100)	95.55 (90.25–99.28)	0 (0–23.84)
espF1	100 (94.47–100)	94.29 (80.84–99.30)	91.95 (84.12–96.70)	100 (76.16–100)
espX1	100 (94.47–100)	100 (91.8–100)	98.85 (93.76–99.97)	90.91 (58.72–99.77)
espR1	99.14 (95.33–99.98)	100 (91.8–100)	100 (96.62–100)	90.91 (58.72–99.77)
espX5	100 (94.47–100)	100 (91.8–100)	100 (96.62–100)	100 (76.16–100)
ECs1822	90.60 (83.8–95.21)	94.14 (84.67–99.93)	90.80 (82.68–95.95)	0 (0–23.84)
nleH1-2	99.14 (95.33–99.98)	100 (91.8–100)	100 (96.62–100)	0 (0–23.84)
nleA	95.72 (90.31–98.60)	94.14 (84.67–99.93)	97.70 (91.94–99.72)	0 (0–23.84)
nleC	94.87 (89.17–98.1)	80 (63.06–91.56)	80.46 (70.57–88.19)	0 (0–23.84)
nleH1-1	99.14 (95.33–99.98)	100 (91.8–100)	98.85 (93.76–99.97)	0 (0–23.84)
nleG	90.60 (83.8–95.21)	94.14 (84.67–99.93)	91.95 (84.12–96.70)	0 (0–23.84)
nleG2	99.14 (95.33–99.98)	100 (91.8–100)	95.55 (90.25–99.28)	0 (0–23.84)
nleG6-1	96.58 (91.48–99.06)	94.14 (84.67–99.93)	94.25 (87.10–98.11)	0 (0–23.84)
nleG6-2	90.60 (83.8–95.21)	85.71 (69.74–95.19)	94.25 (87.10–98.11)	0 (0–23.84)
espJ	96.58 (95.33–99.98)	94.29 (80.84–99.30)	98.85 (93.76–99.97)	0 (0–23.84)
espM2	96.58 (95.33–99.98)	94.14 (84.67–99.93)	97.70 (91.94–99.72)	0 (0–23.84)
nleG8-2	99.14 (95.33–99.98)	94.14 (84.67–99.93)	97.70 (91.94–99.72)	0 (0–23.84)
espG	99.14 (95.33–99.98)	100 (91.8–100)	98.85 (93.76–99.97)	0 (0–23.84)
ent (or *espL2*)	98.30 (93.96–99.79)	100 (91.8–100)	94.25 (87.10–98.11)	0 (0–23.84)
nleB	99.14 (95.33–99.98)	100 (91.8–100)	94.25 (87.10–98.11)	0 (0–23.84)
nleE	99.14 (95.33–99.98)	100 (91.8–100)	94.25 (87.10–98.11)	0 (0–23.84)
efa1	99.14 (95.33–99.98)	100 (91.8–100)	94.25 (87.10–98.11)	0 (0–23.84)
espB	99.14 (95.33–99.98)	100 (91.8–100)	98.85 (93.76–99.97)	0 (0–23.84)
nleG5-2	61.54 (52.09–70.38)	42.86 (26.32–60.65)	4.6 (1.27–11.36)	0 (0–23.84)
espN	89.74 (82.77–94.59)	68.57 (50.71–83.15)	1.15 (0.03–6.24)	0 (0–23.84)
espK	99.14 (95.33–99.98)	94.14 (84.67–99.93)	0 (0–3.38)	0 (0–23.84)
espO1-1	0 (0–2.53)	0 (0–8.2)	0 (0–3.38)	0 (0–23.84)
saa	0 (0–2.53)	0 (0–8.2)	0 (0–3.38)	0 (0–23.84)
subA	0 (0–2.53)	0 (0–8.2)	0 (0–3.38)	0 (0–23.84)

[a] CI, confidence interval.

RESULTS

Association of genes encoding phenotypes and virulence with different groups of *E. coli* O26 issued from the German, Danish, and French strain collections. We have recently investigated STEC and EHEC strains belonging to different serotypes for *nle* (non-locus of enterocyte effector) genes and other virulence factors (9, 10). EHEC O26:H11 and its nonmotile derivative (O26:[H11]) showed characteristic patterns

TABLE 2. PCR primers for detection of *stx* phage-specific sequences in *E. coli* O26 strains

DNA target	GenBank accession no.	Positions in sequence	Primer	Nucleotide sequence (5′–3′)	T_m[a] (°C)	Length of PCR product (bp)
Between *wrbA* and prophage EC26 integrase gene	AP010953	1558679–1558697	ML1	GTGGAACTAAAGACGCTCG	55.0	500
			ML2	ATTTATTGCATCACAGATGGGG		
Between *yecE* and stx_2 prophage ECO111_P11 integrase gene	AP010960	2440340–2440597	ML25	TCAAYGTWTACGGAGCATGGATG	56.0	253
			ML26	AAAACAGAAGCGGAAGTCATCTG		
Between *yjjG* and prophage ECO103_P15 putative integrase gene	AP010958	5363848–5364273	ML29	TGTGTAAACACTGATTGCCTCC	56.0	425
			ML30	AACGCAACCACGCATCAGAC		
orf357 of bacteriophage H19	AJ236875	89–342	Orf357–F	AGGAGAACGAGGATATTGCG	56.0	253
			Orf357-R	TCCTTCAAGCGTCGATTTGG		
Prophage ECO26_P06 putative antirepressor protein	AP010953	1576524–1576723	ML21	TCATCCTCTGGACAGAACG	54.0	200
			ML22	GCGGTTAGCATAATCCCAC		

[a] T_m, melting temperature.

of chromosome (*eae*-beta, *ent* [or *espL2*], *nleB*, *nleE*, *nleF*, *nleH1-2*, and *nleA*)- and plasmid (*e-hlyA*, *katP*, and *espP*)-encoded virulence genes (9, 10, 30, 33). Furthermore, we could identify *stx*-negative derivatives of *E. coli* O26:H11 and O26:NM strains that resembled EHEC O26 for their virulence attributes and have classified these as "EHEC-like" strains (9, 10). EHEC and EHEC-like O26 strains were furthermore characterized by their *arcA* allelic type 2 genes. A reference collection of 62 EHEC, EHEC-like, EPEC, and apathogenic O26 strains, which was previously investigated by genotyping methods (33), was investigated together with 188 additional *E. coli* O26 strains for 33 different genetic markers by high-throughput microfluidic real-time PCR amplification (Table 1). Only 3 of the 33 markers (*espO1-1*, *saa*, and *subA*) were absent in all 250 *E. coli* O26 strains. A combination of molecular detection tools for wzx_{O26}, *eae*-beta, *stx*, and *espK* was found suitable for assignment of the 250 strains in respective groups of EHEC ($n = 117$), EHEC-like ($n = 35$), EPEC ($n = 87$), and avirulent *E. coli* O26 ($n = 11$) strains (Table 1).

All 250 strains tested positive for wzx_{O26}. The 117 O26 EHEC strains were positive for the genes stx_1 ($n = 97$), stx_1-stx_2 ($n = 5$), and stx_2 ($n = 15$) and for the *eae*-beta ($n = 116$) gene. The *eae*-negative EHEC strain (1049.3) was also negative for *map*, which is another LEE genetic marker (data not shown). All 117 O26 EHEC strains tested positive for the $fliC_{H11}$ gene. EHEC-like strains were negative for the *stx* genes but all tested positive for *eae* (*eae*-beta) and $fliC_{H11}$ genes.

Eighty-five of the 87 EPEC O26 strains were characterized as positive for wzx_{O26}, $fliC_{H11}$, *eae*-beta, alpha-hemolysin, and the *arcA* type 1 genotype. One strain (7391) sharing all other characteristics with the O26 EPEC group tested negative for the *eae* gene. This strain was also negative for *map*, which is another LEE genetic marker (data not shown).

Two of the EPEC O26 strains tested positive for the *eae*-ε subtype and carried genes and expressed flagellar antigens H31 (C238-09) and H34 (C257-09). These two strains originated from human patients (a 30-year-old male and a 2-year-old female, both in Denmark) and were positive for the genes *espG*, *espF*, *nleH1-2*, *nleA*, *espX1*, *nleC*, *nleH1-1*, *espR1*, *espJ*, *espM2*, *nleG8-2*, *espB*, and *espX5* but were negative for *espK*. In contrast to all other O26 EPEC strains, they showed *arcA* genotype 2 and did not express alpha-hemolysin.

Eleven *stx*- and *eae*-negative *E. coli* O26 strains that were negative for $fliC_{H11}$ were shown to express the flagellar antigen H32 or were nonmotile. These *nle*-negative strains were assigned to a group of avirulent *E. coli* O26 strains (Table 1).

The relationship between the genetic markers investigated in this work and the four *E. coli* O26 pathogroups is shown in Table 1. The genetic markers *espF1* (91.95 to 100% of strains), *espX1* (90.91 to 100% of strains), *espR1* (90.91 to 100% of strains), and *espX5* (100% of strains) were common in strains of all groups and were thus not suitable for discrimination between O26 pathotypes. Eighteen virulence-associated genes (*ECs1822*, *nleH1-2*, *nleA*, *nleC*, *nleH1-1*, *nleG*, *nleG2*, *nleG6-1*, *nleG6-2*, *espJ*, *espM2*, *nleG8-2*, *espG*, *ent* [or *espL2*], *nleB*, *nleE*, *efa1*, and *espB*) were detected only in strains belonging to EHEC, EHEC-like, and EPEC groups. Two virulence-associated genes (*espN* and *espK*) were present only in O26 EHEC and EHEC-like strains. *espN* was less frequent in EHEC (89.74%) and EHEC-like (68.57%) strains than *espK*, which was present in 116/117 EHEC (99.14%) and 33/34 EHEC-like (91.14%) strains. Strains H19 and CB6706 were the only EHEC and EHEC-like strains, respectively, that tested negative for the gene *espK*.

Presence of EHEC- and EPEC-associated "virulence" genes in *E. coli* O26 reference strains and association with *arcA* alleles. EHEC, EHEC-like, and EPEC strains could be distinguished by their allelic types of the *arcA* gene as tested by nucleotide sequencing (33). For diagnostics, we have developed a rapid *arcA* genotyping assay for detection of EHEC, EHEC-like, EPEC, and apathogenic *E. coli* O26 strains. The reference collection of 62 *E. coli* O26 strains that were previously characterized by Miko et al. (33) was used for evaluation of this assay. The results from the *arcA* genotyping assay were consistent with those obtained from *arcA* sequencing (33). All 22 EHEC and 16 EHEC-like O26 strains were positive for the *arcA* "allele" in the genotyping TaqMan assay. The 18 EPEC O26:[H11] strains were confirmed for their *arcA* allelic type 1 genes by real-time PCR genotyping. The six *eae*- and *stx*-negative O26:H32 strains from the reference collection shared the *arcA* allelic type 2 gene with EHEC and EHEC-like O26 strains.

Our results indicate that a combination of molecular detection tools for wzx_{O26}, *eae*-beta, *stx*, *espK*, and *arcA* genotyping is highly discriminative for identification of EHEC and EHEC-like *E. coli* O26 strains.

PCR detection of *stx* phage-specific sequences in *E. coli* O26 strains. In order to further characterize *stx*-negative *E. coli* O26 strains for their relationship with EHEC O26, we have investigated 44 strains of the *E. coli* O26 reference collection (33) for *stx* phage integration sites, phage-specific antirepressor protein gene (q gene), and phage-integrase genes and for the presence of ORFs specific for the *E. coli* O26 stx_1 phage H19. The results are summarized in Table 3. Phage-specific genes were detected in 18 of 19 (94.7%) EHEC, 7 of 16 (43.8%) EHEC-like, and 1 of 9 (11.1%) EPEC O26 strains. Phage H19-specific orf357 was the most frequent gene, present in 23 strains (Table 3). Similar results were obtained for phage H19-specific ORFs orf199 and orf588 (data not shown). The nine tested EPEC O26 strains were negative for all *stx* phage-specific gene sequences except strain CB277, which was positive for H19-specific ORFs orf199, orf357, and orf588.

Stx phage integrase genes were found in the chromosome of 10/19 EHEC and 6/16 EHEC-like O26 strains. In contrast, the phage q gene which was present in 10/19 EHEC strains was absent in the 16 EHEC-like and the nine EPEC O26 strains.

Only two *stx*-positive strains (D618/98 and CB5805) were negative for all *stx* phage-associated sequences that were investigated in this study (Table 3). These showed remarkable differences in regard to the other EHEC O26 strains. D618/98 shows all characteristics of EHEC O26 except for its stx_{2d} (activatable) subtype (33). CB5805 (stx_2) was grouped as EPEC O26 on the basis of the *arcA* type 1 allele, production of alpha-hemolysin, and its *nle* genotype (this work, 33).

DISCUSSION

To date, EHEC O26 constitutes the most common non-O157 EHEC strain associated with diarrhea and HUS worldwide. EHEC O26:H11 and its nonmotile derivatives were

TABLE 3. PCR detection of *stx* phage-specific sequences in *E. coli* O26 strains

Strain	Type	*stx* gene	Length (bp) of *stx* phage-specific sequence detected by PCR				
			ML1/2	ML25/26	ML29/30	ML21/22	orf357-1
CB10782	EHEC	stx_1					250
CB11578	EHEC	stx_1		300			
CB8236	EHEC	stx_1-stx_2		300		200	250
CB6996	EHEC	stx_1-stx_2	500	300		200	250
CB8474	EHEC	stx_1-stx_2	500				250
D618/98	EHEC	stx_{2d}					
CB8112	EHEC	stx_1				200	250
CB8240	EHEC	stx_2		300			250
CB9410	EHEC	stx_1	450			200	250
RL0083/05	EHEC	stx_1	450			200	250
CB9271	EHEC	stx_1	450			200	250
CB9703	EHEC	stx_2		300			250
D613/98	EHEC	stx_1		300			250
331/02	EHEC	stx_1				200	250
D316/04	EHEC	stx_1				200	250
CB7505	EHEC	stx_1				200	250
H19	EHEC	stx_1					250
CB8962	EHEC	stx_1					250
CB282	EHEC	stx_1				200	250
C2810/67	EHEC-like						
CB1025	EHEC-like						
CB278	EHEC-like		400				
IP2844	EHEC-like						
C3888	EHEC-like						
CB9853	EHEC-like				500		250
C4006	EHEC-like			300	500		250
C3096/70	EHEC-like						
CB9855	EHEC-like				500		250
DG237/1	EHEC-like			300			
DG501/5	EHEC-like						
CB6706	EHEC-like						
CB9862	EHEC-like						250
DG223/5	EHEC-like						
CB9857	EHEC-like				500		250
C1970-1	EHEC-like						
CB5805	EPEC	stx_2					
C3322/73	EPEC						
KK111/1	EPEC						
CB1030	EPEC						
CB277	EPEC						250
DG113/5	EPEC						
CB1027	EPEC						
CB286	EPEC						
691/83	EPEC						

found to be positive for Stx1, Stx2, or both toxins and share similarities with EPEC O26 for intimin-β (*eae*-beta), the long polar fimbriae ($lpfA_{O26}$), an iron-repressible protein (*irp-2*), the porcine-attaching and -effacing protein (*paa*) gene (2, 26), and many chromosomally encoded effector proteins translocated by the type III secretion system (2, 9, 10). EPEC O26 and *stx*-negative derivatives of EHEC O26:H11/NM cannot be easily and rapidly distinguished based on their biochemical profiles.

A rapid and clear discrimination between O26 EPEC or apathogenic strains and EHEC/EHEC-like strains is needed for clinical diagnostics and for control of food safety. The presence of *stx*-negative derivatives of EHEC O26 in stool could indicate an infection with Stx-producing EHEC O26 in the patient and is an indicator of possible progress of infection into HUS (6). In human infections with EHEC, (bloody) diarrhea often precedes HUS (20, 27, 40). The combination of molecular detection tools for wzx_{O26}, *eae*-beta, *stx*, *espK*, and *arcA* genotyping was found suitable for a rapid and specific identification of EHEC as well as EHEC-like O26 strains. This method could be used as a simple and rapid diagnostic approach applicable in hospital, public health, and food control laboratories for performance of the appropriate clinical management, epidemiological investigations, and food safety measures.

We showed by PCR that *stx* phage-specific sequences were present in 94.7% of O26 EHEC and in 43.8% of O26 EHEC-like strains but only in 11.1% of EPEC O26 strains. This indicates that most of the O26 EHEC-like strains are derivatives of EHEC O26 that have lost their *stx* genes. Also, previous findings showed that *stx*-negative/*eae*-positive *E. coli* O26:H11/NM (EHEC-like) can be converted to EHEC by transduction with *stx* phages (5). The finding that 6/16 EHEC-like strains were positive for phage integrase genes but that

none of these strains was positive for *stx* phage q genes supports these suggestions. The integrase gene is closely located to the chromosomal integration site of *stx* phages, and the q gene is located in the vicinity of the *stx* gene on the phage genome. Deletions of the *stx* genes could more frequently extend into the q gene than into the integrase genes, which could explain our findings. Similar findings have been made for stx_{2e} phage P27, which is present in truncated forms in many Stx2e-producing STEC strains. In addition, our suggestion that EHEC-like O26 is a derivative of EHEC O26 is supported by clinical studies. Conversion of EHEC to EHEC-like strains over time has been observed in follow-up studies for patients with bloody diarrhea (6) and HUS (4), and the necessity of an *stx*-independent diagnostic strategy was, therefore, strongly indicated (4).

Only two stx_2-positive strains (D618/98 and CB5805) were negative for all tested *stx* phage-associated gene sequences (Table 3). D618/98 was the only O26 EHEC strain that showed the activatable (stx_{2d}) genotype. CB5805 shared all characteristics of EPEC O26:[H11] strains but expressed Stx2. It is possible that these strains have acquired their *stx* genes by a phage-independent mechanism or harbor *stx* phages that are different from those commonly found in EHEC O26 strains.

The *espK* gene was the unique genetic marker found only in EHEC (99.14%) and EHEC-like (91.14%) strains of O26. The only exceptions were strains H19 (EHEC) and CB6706 (EHEC-like). We do not know if these strains were originally *espK* positive and have mutated or have lost their *espK* genes. Nevertheless, the close association of *espK* with EHEC and EHEC-like O26 strains makes *espK* a suitable candidate for detection of EHEC and EHEC-related O26 strains. In a recent study, Creuzburg et al. (12) investigated 136 STEC strains for the presence of 16 *nle* genes, including *espK*, and showed that 10 of 12 (83%) of EHEC O26 strains tested PCR positive for the gene *espK*. The fact that *espK* is highly specific for EHEC and EHEC-like O26 strains provides evidence to support the association of *espK* with the virulence. This corroborates the work of Vlisidou et al. (43), which indicated that *espK* was involved in the persistence of EHEC O157:H7 in the intestines of orally inoculated calves and had a role in virulence of EHEC O157:H7.

Most strains belonging to a given pathogroup were very similar to each other in their virulence genes and phenotypical traits. However, a few exceptions were observed. In the EHEC group, one food-borne strain (1049.3) which was negative for LEE-associated genes *eae* and *map* was detected. It is possible that this strain has lost the complete LEE island. Another *eae*-negative strain was found to be associated with the group of O26 EPEC strains (7391). In contrast to these strains, which are supposed to be mutants, two other EPEC O26 strains that are genetically more distant from the rest of the EPEC O26 strains since they were showing altered signatures for their *fliC*, *arcA*, and *eae* genes were identified. These strains belonged to serotypes O26:H31 and O26:H34. EPEC strains from these serotypes have been described as causes of diarrhea and characterized as atypical EPEC (6).

In conclusion, this study demonstrates that a high-throughput PCR approach can be used for selection of a highly specific genetic marker characterizing EHEC and EHEC-like O26 strains. The virulence of pathogenic O26 *E. coli* strains is associated with a number of well-characterized and putative factors with *espK* as a candidate. Hence, several other genes encoding effector proteins secreted through the type III secretion system were detected only in EHEC, EHEC-like, and EPEC pathogroups, not in avirulent *E. coli* O26 strains. These genes, which were found to be present in both EHEC, EHEC-like, and EPEC strains but with various distributions might be associated with virulence. Additional work is still needed to substantiate this hypothesis, but simultaneous detection of these markers in combination with wzx_{O26}, *eae*-beta, *stx*, *espK*, and *arcA* alleles provides data suitable for molecular risk assessment of the potential virulence of *E. coli* O26 isolates.

ACKNOWLEDGMENT

We are grateful to the Plateforme Génomique Génopole Toulouse/Midi-Pyrénées for technical assistance in high-throughput microfluidic real-time PCR amplifications.

REFERENCES

1. **Beutin, L., et al.** 2007. Identification of human-pathogenic strains of Shiga toxin-producing Escherichia coli from food by a combination of serotyping and molecular typing of Shiga toxin genes. Appl. Environ. Microbiol. **73:** 4769–4775.
2. **Bielaszewska, M., W. Zhang, A. Mellmann, and H. Karch.** 2007. Enterohaemorrhagic Escherichia coli O26:H11/H-: a human pathogen in emergence. Berl. Munch. Tierarztl. Wochenschr. **120**(7–8)**:**279–287.
3. **Bielaszewska, M., et al.** 1996. Verocytotoxin-producing *Escherichia coli* in children with hemolytic uremic syndrome in the Czech Republic. Clin. Nephrol. **46:**42–44.
4. **Bielaszewska, M., et al.** 2007. Shiga toxin–mediated hemolytic uremic syndrome: time to change the diagnostic paradigm? PLoS One **2:**e1024.
5. **Bielaszewska, M., et al.** 2007. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. Appl. Environ. Microbiol. **73:**3144–3150.
6. **Bielaszewska, M., et al.** 2008. Shiga toxin-negative attaching and effacing *Escherichia coli*: distinct clinical associations with bacterial phylogeny and virulence traits and inferred in-host pathogen evolution. Clin. Infect. Dis. **47:**208–217.
7. **Blanco, J. E., et al.** 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J. Clin. Microbiol. **42:**311–319.
8. **Brooks, J. T., et al.** 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. J. Infect. Dis. **192:**1422–1429.
9. **Bugarel, M., L. Beutin, and P. Fach.** 2010. Low-density macroarray targeting non-locus of enterocyte effacement effectors (nle genes) and major virulence factors of Shiga toxin-producing Escherichia coli (STEC): a new approach for molecular risk assessment of STEC isolates. Appl. Environ. Microbiol. **76:**203–211.
10. **Bugarel, M., L. Beutin, A. Martin, A. Gill, and P. Fach.** 2010. Micro-array for the identification of Shiga toxin-producing Escherichia coli (STEC) seropathotypes associated with hemorrhagic colitis and hemolytic uremic syndrome in humans. Int. J. Food Microbiol. **142:**318–329.
11. **Burgos, Y. K., K. Pries, A. F. Pestana de Castro, and L. Beutin.** 2009. Characterization of the alpha-haemolysin determinant from the human enteropathogenic Escherichia coli O26 plasmid pEO5. FEMS Microbiol. Lett. **292:**194–202.
12. **Creuzburg, K., et al.** 2011. Evolutionary analysis and distribution of type III effector genes in pathogenic *Escherichia coli* from human, animal and food sources. Environ. Microbiol. **13:**439–452.
13. **Eklund, M., F. Scheutz, and A. Siitonen.** 2001. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. J. Clin. Microbiol. **39:**2829–2834.
14. **Elliott, E. J., et al.** 2001. Nationwide study of haemolytic uraemic syndrome: clinical, microbiological, and epidemiological features. Arch. Dis. Child. **85:**125–131.
15. **Ethelberg, S., et al.** 2004. Virulence factors for hemolytic uremic syndrome, Denmark. Emerg. Infect. Dis. **10:**842–847.
16. **European Food Safety Authority (EFSA).** 2007. Scientific opinion of the panel on biological hazards on a request from EFSA on monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic types. http://www.efsa.europa.eu/en/efsajournal/doc/579.pdf.
17. **European Food Safety Authority (EFSA).** 2009. Scientific report of EFSA: technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food). EFSA J. **7**(11)**:**1366. http://www.efsa.europa.eu/en/efsajournal/doc/s1366.pdf.

18. **European Food Safety Authority (EFSA).** 2009. The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. http://www.efsa.europa.eu/en/efsajournal/doc/223r.pdf.
19. **Friedrich, A. W., et al.** 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74–84.
20. **Gerber, A., H. Karch, F. Allerberger, H. M. Verweyen, and L. B. Zimmerhackl.** 2002. Clinical course and the role of Shiga toxin-producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997–2000, in Germany and Austria: A prospective study. J. Infect. Dis. **186:**493–500.
21. **Geue, L., et al.** 2009. Analysis of the clonal relationship of serotype O26:H11 enterohemorrhagic *Escherichia coli* isolates from cattle. Appl. Environ. Microbiol. **75:**6947–6953.
22. **Gilmour, M. W., et al.** 2005. Multilocus sequence typing of *Escherichia coli* O26:H11 isolates carrying *stx* in Canada does not identify genetic diversity. J. Clin. Microbiol. **43:**5319–5323.
23. **Hiramatsu, R., et al.** 2002. Characterization of Shiga toxin-producing *Escherichia coli* O26 strains and establishment of selective isolation media for these strains. J. Clin. Microbiol. **40:**922–925.
24. **Hoshina, K., et al.** 2001. Enterohemorrhagic *Escherichia coli* O26 outbreak caused by contaminated natural water supplied by facility owned by local community. Jpn. J. Infect. Dis. **54:**247–248.
25. **Jelacic, J. K., et al.** 2003. Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. J. Infect. Dis. **188:**719–729.
26. **Jenkins, C., J. Evans, H. Chart, G. A. Willshaw, and G. Frankel.** 2008. Escherichia coli serogroup O26—a new look at an old adversary. J. Appl. Microbiol. **104:**14–25.
27. **Karch, H., P. I. Tarr, and M. Bielaszewska.** 2005. Enterohaemorrhagic Escherichia coli in human medicine. Int. J. Med. Microbiol. **295:**405–418.
28. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18:**775–779.
29. **Kozub-Witkowski, E., et al.** 2008. Serotypes and virutypes of enteropathogenic and enterohaemorrhagic *Escherichia coli* strains from stool samples of children with diarrhoea in Germany. J. Appl. Microbiol. **104:**403–410.
30. **Leomil, L., A. F. Pestana de Castro, G. Krause, H. Schmidt, and L. Beutin.** 2005. Characterization of two major groups of diarrheagenic Escherichia coli O26 strains which are globally spread in human patients and domestic animals of different species. FEMS Microbiol. Lett. **249:**335–342.
31. **Mead, P. S., et al.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
32. **Mellmann, A., et al.** 2008. Analysis of collection of hemolytic uremic syndrome-associated enterohemorrhagic Escherichia coli. Emerg. Infect. Dis. **14:**1287–1290.
33. **Miko, A., B. A. Lindstedt, L. T. Brandal, I. Lobersli, and L. Beutin.** 2010. Evaluation of multiple-locus variable number of tandem-repeats analysis (MLVA) as a method for identification of clonal groups among enteropathogenic, enterohaemorrhagic and avirulent Escherichia coli O26 strains. FEMS Microbiol. Lett. **303:**137–146.
34. **Miko, A., et al.** 2009. Assessment of Shiga toxin-producing Escherichia coli isolates from wildlife meat as potential pathogens for humans. Appl. Environ. Microbiol. **75:**6462–6470.
35. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
36. **O'Reilly, K. M., et al.** 2010. Associations between the presence of virulence determinants and the epidemiology and ecology of zoonotic *Escherichia coli*. Appl. Environ. Microbiol. **76:**8110–8116.
37. **Orskov, F.** 1951. On the occurrence of *E. coli* belonging to O-group 26 in cases of infantile diarrhoea and white scours. Acta Pathol. Microbiol. Scand. **29:**373–378.
38. **Perelle, S., F. Dilasser, J. Grout, and P. Fach.** 2004. Detection by 5′-nuclease PCR of Shiga toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. Mol. Cell. Probes **18:**185–192.
39. **Rivas, M., et al.** 2006. The case-control study group. Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. Foodborne Pathog. Dis. **3:**88–96.
40. **Tarr, P. I., C. A. Gordon, and W. L. Chandler.** 2005. Shiga toxin-producing Escherichia coli and the haemolytic uraemic syndrome. Lancet **365:**1073–1086.
41. **Tozzi, E., et al.** 2003. Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome in Italy 1988–2000. Emerg. Infect. Dis. **9:**106–108.
42. **Vaz, T. M. I., et al.** 2004. Virulence properties and characteristics of Shiga toxin-producing *Escherichia coli* in Sao Paulo, Brazil, from 1976 through 1999. J. Clin. Microbiol. **42:**903–905.
43. **Vlisidou, I., et al.** 2006. Identification and characterization of EspK, a type III secreted effector protein of enterohaemorrhagic Escherichia coli O157:H7. FEMS Microbiol. Lett. **263:**32–40.
44. **Whittam, T. S., et al.** 1993. Clonal relationships among Escherichia coli strains that cause hemorrhagic colitis and infantile diarrhea. Infect. Immun. **61:**1619–1629.
45. **Zweifel, C., et al.** 2010. Characteristics of Shiga toxin-producing Escherichia coli isolated from Swiss raw milk cheese within a 3-year monitoring program. J. Food Prot. **73:**88–91.