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Detection of Shiga Toxin-Producing *Escherichia coli* Serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in Raw-Milk Cheeses by Using Multiplex Real-Time PCR▽

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains are a diverse group of food-borne pathogens with various levels of virulence for humans. In this study, we describe the use of a combination of multiple real-time PCR assays for the screening of 400 raw-milk cheeses for the five main pathogenic STEC serotypes (O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7). The prevalences of samples positive for *stx*, intimin-encoding gene (*eae*), and at least one of the five O group genetic markers were 29.8%, 37.3%, and 55.3%, respectively. The H2, H7, H8, H11, and H28 *fliC* alleles were highly prevalent and could not be used as reliable targets for screening. Combinations of *stx*, *eae* variants, and O genetic markers, which are typical of the five targeted STEC serotypes, were detected by real-time PCR in 6.5% of the cheeses (26 samples) and included *stx*–*wzx*$_{O26}$–*eae*-β*1* (4.8%; 19 samples), *stx*–*wzx*$_{O103}$–*eae*-ε (1.3%; five samples), *stx*–*ihp1*$_{O145}$–*eae*-γ*1* (0.8%; three samples), and *stx*–*rfbE*$_{O157}$–*eae*-γ*1* (0.3%; one sample). Twenty-eight immunomagnetic separation (IMS) assays performed on samples positive for these combinations allowed the recovery of seven *eae*β*1*-positive STEC O26:H11 isolates, whereas no STEC O103:H2, O145:H28, or O157:H7 strains could be isolated. Three *stx*-negative and *eae*β*1*-positive *E. coli* O26:[H11] strains were also isolated from cheeses by IMS. Colony hybridization allowed us to recover STEC from *stx*-positive samples for 15 out of 45 assays performed, highlighting the difficulties encountered in STEC isolation from dairy products. The STEC O26:H11 isolates shared the same virulence genetic profile as enterohemorrhagic *E. coli* (EHEC) O26:H11, i.e., they carried the virulence-associated genes EHEC-*hlyA*, *katP*, and *espP*, as well as genomic O islands 71 and 122. Except for one strain, they all contained the *stx1* variant only, which was reported to be less frequently associated with human cases than *stx2*. Pulsed-field gel electrophoresis (PFGE) analysis showed that they displayed high genetic diversity; none of them had patterns identical to those of human O26:H11 strains investigated here.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (Stx)-producing *E. coli* (STEC) strains that are isolated from human patients and are responsible for severe clinical symptoms, such as hemorrhagic colitis (HC) and the potentially lethal hemolytic uremic syndrome (HUS) (28, 29). Although most outbreaks of HC and HUS have been attributed to serotype O157:H7/H$^-$, an increasing number of human infections are caused by other serotypes, such as O26:H11/H$^-$, O103:H2, O111:H8/H$^-$, and O145:H28/H$^-$ (13, 20, 28). O157:H7/H$^-$ and these four serotypes were classified into seropathotypes A and B, respectively, which occur most frequently in human diseases and outbreaks and in patients with severe symptoms (30).

Shiga toxins, the main virulence factors contributing to pathogenicity, consist of two major types, Stx1 and Stx2, each including several variants (47). In addition to Stx, typical EHEC strains carry on their chromosomes the locus for enterocyte effacement (LEE), a large pathogenicity island that is shared with enteropathogenic *E. coli* (EPEC). The LEE is responsible for attaching and effacing (A/E) lesions on enterocytes (34); it encodes several virulence factors, including the outer membrane adhesin intimin and its translocated receptor Tir, as well as components of a type III secretion machinery and its effector proteins. Intimin is involved in the tight attachment of bacteria to the enterocytes. It is encoded by the *eae* gene, 18 types and 9 subtypes of which have been described on the basis of its variable 3′ region (25). The EHEC serotypes O157:H7 and O145:H28 are known to be associated with the *eae*-γ*1* subtype, whereas EHEC O26:H11, O103:H2, and O111:H8 harbor the *eae*-β*1*, *eae*-ε, and *eae*-γ*2*/θ subtypes, respectively (7, 11, 37, 51). Additional candidate pathogenicity islands, or "O islands" (OI), that may contribute to virulence have been identified. They include OI-71 and OI-122, which contain a number of type III non-LEE-encoded effector (*nle*) genes whose presence correlates with outbreak and HUS potential (17). Finally, EHEC O157:H7 contains a large plasmid with putative virulence genes encoding the EHEC-hemolysin (EHEC-*hlyA*), a serine protease (*espP*), and a catalase peroxidase (*katP*). However, strains lacking one or more of these genes have been involved in HUS, and high variability in the

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gene composition of this large plasmid has also been reported in various STEC serotypes (14).

Domestic ruminants have long been identified as a major reservoir for STEC (29). Although STEC transmission to humans is frequently associated with consumption of raw or undercooked meat, raw milk and dairy products have also been implicated in human disease (4). Detection and isolation of STEC in foodstuffs by traditional culture methods is rather laborious and time-consuming and is complicated by the lack of common biochemical characteristics distinguishing most STEC from other *E. coli* strains. Development of rapid methods for the detection of the most pathogenic STEC strains is essential to ensure the safety of food products. A sequential approach based on real-time PCR assays specific for *stx*, wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, and $rfbE_{O157}$ genetic markers has been described for the detection of STEC O26, O103, O111, O145, and O157 in foods (3, 40). A similar strategy that includes an additional *eae* amplification step has been proposed by working group 6 of the European Committee for Standardization (CEN) TC275 for a technical specification (currently under International Organization for Standardization [ISO] evaluation) regarding the detection of these five STEC serogroups in foods (21).

Recently, we developed real-time PCR assays for detection of the *eae* variants and *fliC* alleles associated with the five most common EHEC serotypes (32). The aim of the present study was to investigate whether these newly developed assays could further refine the diagnostic result obtained from PCR analysis of foods. To this end, the prevalence of *stx*, O group genetic markers, *eae* variants, and *fliC* alleles typical of EHEC O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 was investigated in 400 raw-milk cheeses. STEC and *E. coli* strains belonging to the five targeted serotypes were then isolated for confirmation of the PCR results, and their virulence traits were characterized. As the term EHEC includes a clinical connotation (28, 29), the *stx*-positive *E. coli* isolates recovered from food are designated STEC (not EHEC) here, irrespective of the serotype.

MATERIALS AND METHODS

Food samples, enrichment, and DNA extraction. Soft cheeses and smear semihard uncooked cheeses produced from raw cow's milk (n = 265) and unpasteurized goat's milk cheeses (n = 135) were collected over a 5-month period (March to August 2009) in various French retail stores. They were immediately analyzed or held at 4°C before use. Twenty-five grams of cheese was subjected to enrichment, and bacterial DNAs were extracted as described elsewhere (32). DNA extracts (100 μl) were stored at 4°C before PCR analysis.

Screening of cheeses for EHEC-associated genetic markers. Two-microliter aliquots of DNA extracts were subjected to the following real-time PCR assays: an internally controlled PCR for the detection of the *stx* gene (2), a simplex PCR for the detection of *eae* (35), a quadruplex PCR for the detection of four *eae* variants (*eae*-β*1*, *eae*-γ*1*, *eae*-ε, and *eae*-γ*2*/θ), and simplex and multiplex PCR assays for the detection of five *fliC* alleles ($fliC_{H2}$, $fliC_{H7}$, $fliC_{H8}$, $fliC_{H11}$, and $fliC_{H28}$) (32). A multiplex real-time PCR was used for the detection of the O group markers as described by Perelle et al. (40) with the following modifications: the O103-specific primers and probe targeted wzx_{O103} (39), and the primers and probes specific for $rfbE_{O157}$ and $wbdI_{O111}$ were not incorporated in the reaction mixture and were used in two separate simplex PCR assays. All primers and probes specific for *stx*, *eae*, and the five O genetic markers were those specified in the CEN technical specification project (21). All the PCRs were performed on a LightCycler 480 instrument (Roche Diagnostics). The cycle threshold (C_T) value was defined as the PCR cycle at which the fluorescent signal exceeded the background level. The C_T was determined automatically by the Lightcycler 480 software by the second derivative maximum method.

Isolation of STEC strains from naturally contaminated cheeses and characterization of their virulence gene profiles. Isolation of STEC strains was performed within 1 day after PCR analysis of cheeses. Colony hybridization was carried out as described previously (22). As previous analyses of food products showed that no STEC strains could be isolated by colony hybridization from enriched broths with high *stx* C_T values corresponding to low STEC concentrations (2), only cheese samples with *stx* C_T values of <30 were subjected to this technique. For isolation of STEC O26, O103, O111, O145, and O157, immunomagnetic separation (IMS) (Dynabeads, Invitrogen, Cergy Pontoise, France) was performed manually as recommended by the manufacturer. IMS-concentrated bacteria were plated onto TBX agar (Bio-Rad Laboratories, Marnes-la-Coquette, France), cefixime-tellurite-sorbitol-MacConkey agar (CT-SMAC), and a recently described selective differential medium for *E. coli* O26, O103, O111, and O145 (41). Suspected colonies were confirmed as *E. coli* by using an API 20E test (bioMérieux). Strains were further characterized for their O:H antigens by agglutination assays (36, 46) and by real-time PCR as described above. Conventional PCR was used for detection of the virulence markers *katP* (15); *espP* (14); OI-122-associated *pagC*, *ent*, and *efa1* (30); and OI-71-associated *nleA* (17). Real-time PCR was used for detection of *stx*, *eae*, and *eae* variants as described above. Subtypes of *stx* were identified by PCR (F. Scheutz, L. D. Teel, L. Beutin, D. Piérard, G. Buvens, H. Karch, A. Mellmann, A. Caprioli, R. Tozzoli, A. D. O'Brien, A. R. Melton-Celsa, S. Persson, and N. A. Strockbine, unpublished data), according to the subtyping nomenclature established at the 7th International Symposium on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections (Buenos Aires, 10 to 13 May 2009). The presence of EHEC-*hlyA*, *saa*, *bfpA*, and EPEC adherence factor (EAF) plasmid was determined by DNA dot blot hybridization as previously described (45). The hemolysis phenotype and Shiga toxin production were assessed on red blood cell agar (8) and with a Vero cell assay (36), respectively. The alpha-hemolytic phenotype was further confirmed by PCR detection of the α-*hlyA* gene as described previously (7).

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed as described by Ribot et al. (43). DNAs were digested with XbaI and BlnI enzymes (Roche Diagnostics), and the restriction fragments were resolved at 14°C on 1% Seakem gold agarose gels (Cambrex, Emerainville, France) on a Chef-DR-III system (Bio-Rad Laboratories, Germany). Pulse times were ramped with 2 s and 2.2 s at the beginning and 64 s and 63.8 s at the end for separation of DNA fragments digested with XbaI and BlnI, respectively. XbaI-digested *Salmonella enterica* serovar Braenderup H9812 DNA was used as a molecular size standard. After being stained with ethidium bromide (10 μg ml^{-1}), gels were visualized on GelDocEQ (Bio-Rad Laboratories), and the PFGE profiles were analyzed using Bionumerics software (Applied Math, Sint-Martens-Latem, Belgium). The PFGE typing scheme included seven human O26:H11 isolates (CB6307, ED-21, VTH7, EH284, EH324, 5917/97, and 6061/96) and 11 O26:H11 strains recovered at the French National Reference Laboratory for *E. coli* (VetAgro Sup) from various types of samples, i.e., cattle feces collected in two French dairy farms in 2009 (FFL1-1, FFL2-6, FFL3-12, FV2-33, FV3-11, FV4-14, and FV5-36); cheeses analyzed in 2005 (FR14-18), 2008 (51-2) and 2009 (F15-313); and ewe's milk analyzed in 2007 (L23A).

RESULTS

Monitoring of EHEC-associated genetic markers in raw-milk cheeses. A total of 400 raw-milk cheeses were screened for the presence of genetic markers associated with EHEC O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 (Table 1). The *stx* gene was detected in 29.8% of the samples. More than half (55.3%) of the samples contained at least one of the five EHEC O group genetic markers; the most frequent O group marker was $ihp1_{O145}$ (45.8%), followed by wzx_{O103} (23.8%), wzx_{O26} (11.3%), and $rfbE_{O157}$ (5.8%). Only one sample (0.3%) contained $wbdI_{O111}$. Many of the samples (87.3%) contained at least one of the five EHEC *fliC* alleles (Table 1), and the prevalences of the *fliC* alleles individually ranged from 38.5% ($fliC_{H28}$) to 65.5% ($fliC_{H8}$). The *eae* gene was present in 37.3% of the samples. At least one of the four variants *eae*-β*1*, *eae*-γ*1*, *eae*-ε, and *eae*-γ2/θ was detected in 32.3% of the samples; the most frequent variants were *eae*-β*1* (21.8%) and *eae*-

TABLE 1. Detection of EHEC-associated genetic markers in 400 raw-milk cheese samples

Genetic markers targeted by real-time PCR (either alone or in combination with each other)	Positive samples/ 400 tested	
	No.	%
Individual markers		
stx	119	29.8
eae[a]	149	37.3
eae-β*1*	87	21.8
eae-γ*1*	5	1.3
eae-ε	11	2.8
eae-γ2/θ	62	15.5
eae-β*1*, *eae*-γ*1*, *eae*-ε, *eae*-γ2/θ[b]	129[c]	32.3
wzx_{O26}	45	11.3
wzx_{O103}	95	23.8
$wbdI_{O111}$	1	0.3
$ihp1_{O145}$	183	45.8
$rfbE_{O157}$	20	5
wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, $rfbE_{O157}$[d]	221	55.3
$fliC_{H2}$	244	61.0
$fliC_{H7}$	234	58.5
$fliC_{H8}$	262	65.5
$fliC_{H11}$	197	49.3
$fliC_{H28}$	154	38.5
$fliC_{H2}$, $fliC_{H7}$, $fliC_{H8}$, $fliC_{H11}$, $fliC_{H28}$[e]	349	87.3
Combinations of markers		
stx-eae	68	17
stx-wzx_{O26}-*eae*	22	5.5
stx–wzx_{O26}–*eae*-β*1*	19	4.8
stx–wzx_{O26}–*eae*-β*1*–$fliC_{H11}$	19	4.8
stx-wzx_{O103}-*eae*	31	7.8
stx–wzx_{O103}–*eae*-ε	5	1.3
stx–wzx_{O103}–*eae*-ε–$fliC_{H2}$	5	1.3
stx-$wbdI_{O111}$-*eae*	0	0
stx–$wbdI_{O111}$–*eae*-γ2/θ	0	0
stx-$ihp1_{O145}$-*eae*	51	12.8
stx–$ihp1_{O145}$–*eae*-γ*1*	3	0.8
stx–$ihp1_{O145}$–*eae*-γ*1*–$fliC_{H28}$	3	0.8
stx-$rfbE_{O157}$-*eae*	4	1
stx–$rfbE_{O157}$–*eae*-γ*1*	1	0.3
stx–$rfbE_{O157}$–*eae*-γ*1*–$fliC_{H7}$	1	0.3
stx-(wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, $rfbE_{O157}$)[f]	89	22.3
stx-(wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, $rfbE_{O157}$)-*eae*[g]	61	15.3
stx-(wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, $rfbE_{O157}$)-(*eae*-β*1*, *eae*-γ*1*, *eae*-ε, *eae*-γ2/θ)[h]	54	13.5
stx–wzx_{O26}–*eae*-β*1*, *stx*–wzx_{O103}–*eae*-ε, *stx*–$wbdI_{O111}$–*eae*-γ2/θ, *stx*–$ihp1_{O145}$–*eae*-γ*1*, *stx*–$rfbE_{O157}$-*eae*-γ*1*[i]	26[j]	6.5

[a] Detection of the *eae* gene with universal primers/probe.

[b] Detection of at least one of the four *eae* variants *eae*-β*1*, *eae*-γ*1*, *eae*-ε, and *eae*-γ2/θ.

[c] Twenty-two samples contained *eae*-β*1* and *eae*-γ2/θ; two samples contained *eae*-β*1* and *eae*-ε; two samples contained *eae*-ε and *eae*-γ2/θ; one sample contained *eae*-β*1* and *eae*-γ*1*; two samples contained *eae*-β*1*, *eae*-γ2/θ, and *eae*-ε; and two samples contained *eae*-β*1*, *eae*-γ2/θ, and *eae*-γ*1*.

[d] Detection of at least one of the five serogroup genetic markers wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$, and $rfbE_{O157}$.

[e] Detection of at least one of the five *fliC* alleles specific for H2, H7, H8, H11, and H28 flagellar antigens.

[f] Detection of *stx* and at least one of the five serogroup genetic markers.

[g] Detection of *stx*, at least one of the five serogroup genetic markers and *eae*.

[h] Detection of *stx*, at least one of the five serogroup genetic markers, and at least one of the four *eae* variants.

[i] Detection of at least one of the five *stx*-serogroup-*eae* variant marker combinations.

[j] Two samples contained two combinations of markers each (i.e., *stx*–wzx_{O26}–*eae*-β*1* plus *stx*–$ihp1_{O145}$–*eae*-γ*1* and *stx*–$ihp1_{O145}$–*eae*-γ*1* plus *stx*–$rfbE_{O157}$–*eae*-γ*1*).

γ2/θ (15.5%), followed by *eae*-ε (2.8%) and *eae*-γ*1* (1.3%) (Table 1). The concomitant presence of several *eae* variants was observed for 31 (7.75%) samples. The presence of both *stx* and *eae* was observed for 68 (17%) samples, and the simultaneous presence of *stx*, *eae*, and at least one of the five EHEC O group markers was detected in 61 (15.3%) samples (Table 1). Twenty-six (6.5%) samples were positive for both *stx* and at least one of the four main EHEC associations of markers wzx_{O26}–*eae*-β*1*, wzx_{O103}–*eae*-ε, $ihp1_{O145}$–*eae*-γ*1*, and $rfbE_{O157}$–*eae*-γ*1* (Table 1). The most frequent combination identified was *stx*–wzx_{O26}–*eae*-β*1* (19 samples; 4.8%), followed by *stx*–wzx_{O103}–*eae*-ε (5 samples; 1.3%), *stx*–$ihp1_{O145}$–*eae*-γ*1* (3 samples; 0.8%), and *stx*–$rfbE_{O157}$–*eae*-γ*1* (one sample; 0.3%). The prevalences of these four combinations remained the same if a *fliC* allele was included in each of the combinations (Table 1). None of the samples were positive for the combination *stx*–$wbdI_{O111}$–*eae*-γ2/θ.

Isolation of STEC strains from raw-milk cheeses and characterization of their virulence profiles. All 26 samples that tested positive by real-time PCR for *stx*–wzx_{O26}–*eae*-β*1*, *stx*–wzx_{O103}–*eae*-ε, *stx*–$ihp1_{O145}$–*eae*-γ*1*, and/or *stx*–$rfbE_{O157}$–*eae*-γ*1* were subjected to IMS assays. As two samples contained two combinations each (Table 1), a total of 28 IMS assays were performed. Despite frequent nonspecific binding of bacteria to the IMS particles (data not shown), 10 *E. coli* strains were isolated out of 19 IMS O26 assays; they corresponded to seven STEC O26:H11/[H11] (the brackets represent strains whose serotype could be determined only by real-time PCR and not by conventional serotyping) strains and to *stx*-negative *E. coli* O26:[H11] (306.D and 315.2) and O26:H32 (355.2) (Table 2). No *E. coli* O103, O145, or O157 strains could be isolated from the 5 IMS O103, 3 IMS O145, and 1 IMS O157 assays performed, respectively. Eleven additional IMS assays (7 O26, 1 O103, 2 O145, and 1 O157) performed on cheese samples that were negative for *stx* but positive for serogroup/*eae* variant associations allowed us to isolate two *stx*-negative *E. coli* strains belonging to O26:[H11] (04.2) and O103:H25 (167.2) serotypes (Table 2).

Colony hybridization was applied to 45 *stx*-positive samples with low *stx* C_T values (see Materials and Methods), including 8 out of the 26 samples containing *stx*-serogroup-*eae* variant marker combinations. This allowed the recovery of one STEC O26:H11 isolate that showed the same characteristics as the STEC O26:H11 isolate obtained by IMS from the same sample (Table 2), and both isolates were therefore considered the same strain. Fifteen additional STEC strains were recovered by colony hybridization from *stx*-positive samples that were not investigated by IMS. They belonged to nine different serotypes, i.e., O2:H27 ($n = 3$), O nontypeable (ONT)/O rough:H10 ($n = 3$), O8:H19 ($n = 2$), O175:H16 ($n = 2$), O91:H10, O128ab:[H2], O166:[H28], O174:H2, and O178:H19 (one strain each) (Table 2). One *E. coli* O133:H29 strain was also isolated, but it was not confirmed as *stx* positive (275.1 [Table 2]). It was recovered from an *stx-eae-*$ihp1_{O145}$-positive cheese sample and tested positive for $ihp1_{O145}$, as was also the case for the STEC O166:[H28] strain 124.1 (Table 2).

All the isolates were characterized for the presence of the genes *stx1*, *stx2*, *eae*, EHEC-*hlyA*, *katP*, *espP*, *saa*, *nleA*, *pagC*, *ent*, and *efa1*, as well as for the presence of the EAF plasmid. Twenty-two *E. coli* strains possessed at least one *stx* gene (Ta-

TABLE 2. Characterization of the 28 *E. coli* strains isolated from raw-milk cheeses

Strain	Isolation[a]	Serotype[b]	Presence of[f]:												
			stx (subtype[c])	*eae* (type)	Hemolysis[e]	EHEC-*hlyA*	*katP*	*espP*	*nleA*	*pagC*	*ent*	*efa1*	*saa*	EAF	*bfpA*
STEC															
33.1	C	O2:H27	+ (*stx2a*)	−	−	+	−	−	−	−	−	−	−	−	−
162.5	C	O2:H27	+ (*stx2a*)	−	−	+	−	−	−	−	−	−	−	−	−
246.3	C	O2:H27	+ (*stx2a*)	−	−	+	−	−	−	−	−	−	−	−	−
104.5	C	O8:H19	+ (*stx2a* + *stx2d*)	−	E-hly	+	−	−	−	−	−	−	−	−	−
303.1	C	O8:H19	+ (*stx2*ND)	−	−	−	−	−	−	−	−	−	−	−	−
170.2	I, C	O26:H11	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
238.2	I	O26:H11	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
129.2	I	O26:[H11]	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
245.3	I	O26:[H11]	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
260.3	I	O26:[H11]	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
283.4	I	O26:[H11]	+ (*stx1a*)	+ (β*1*)	E-hly	+	+	+	+	−	+	+	−	−	−
277.2	I	O26:[H11]	+ (*stx1a* + *stx2a*)	+ (β*1*)	E-hly	+	+	−	+	−	+	+	−	−	−
161.2	C	O91:H10	+ (*stx2d*)	−	−	−	−	−	−	−	−	−	−	−	−
282.2	C	O128ab:[H2]	+ (*stx1c*)	−	E-hly	+	−	−	−	−	−	−	−	−	−
124.1	C	O166:[H28][d]	+ (*stx2b* + *stx2c*)	−	−	−	−	−	−	−	−	−	−	−	−
91.1	C	O174:H2	+ (*stx1a*)	−	E-hly	+	−	+	−	+	−	−	+	−	−
299.3	C	O175:H16	+ (*stx2a*)	−	E-hly	+	−	−	−	−	−	−	+	−	−
311.2	C	O175:H16	+ (*stx2d*)	−	−	−	−	−	−	−	−	−	−	−	−
381.1	C	O178:H19	+ (*stx2c*)	−	−	−	−	−	−	+	−	−	−	−	−
225.4	C	ONT:H10	+ (*stx1c*)	−	−	−	−	−	−	−	−	−	−	+	−
47.1	C	Or:H10	+ (*stx1c*)	−	−	−	−	−	−	−	−	−	−	+	−
180.E	C	Or:H10	+ (*stx1c*)	−	−	−	−	−	−	−	−	−	−	+	−
Non-STEC															
04.2	I	O26:[H11]	−	+ (β*1*)	α-hly	−	−	−	+	−	+	+	−	−	−
315.2	I	O26:[H11]	−	+ (β*1*)	α-hly	−	−	−	+	−	+	+	−	−	−
306.D	I	[O26]:[H11]	−	+ (β*1*)	α-hly	−	−	−	+	−	+	+	−	−	−
355.2	I	O26:H32	−	−	−	−	−	−	−	−	−	−	−	−	−
167.2	I	O103:H25	−	+ (γ*2*/θ)	E-hly	+	−	−	−	+	+	+	−	−	−
275.1	C	O133:H29[d]	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] C, colony hybridization; I, immunomagnetic separation.
[b] Or, O rough. O and H types in brackets represent strains whose serotype could be determined by real-time PCR only and not by conventional serotyping.
[c] *stx* subtypes refer to prototype organisms (GenBank accession numbers in parentheses) as follows: *stx1a*, O157-EDL933 (M19473); *stx1c*, O174-DG131-3 (Z36901); *stx2a*, O157-EDL933 (X07865); *stx2b*, O118-EH250 (AF043627); stx_{2c}, O157-E32511 (M59432); *stx2d*, O73-C165-02 (DQ059012); ND, not determined.
[d] These strains tested positive for $ihp1_{O145}$ by real-time PCR.
[e] E-hly and α-hly, EHEC hemolytic and alpha-hemolytic phenotypes on red blood cell agar, respectively.
[f] +, present; −, absent.

ble 2) and were cytotoxic to Vero cells (data not shown). All the STEC O26:[H11] strains shared the same virulence gene profile (*stx1a eae*-β*1* EHEC-*hlyA katP espP nleA ent efa1*), except for one strain (277.2) that carried an additional *stx2a* gene and was *espP* negative (Table 2). The three *E. coli* O26:[H11] isolates 04.2, 306.D, and 315.2 carried the same virulence genes as STEC O26:H11 except that they lacked an *stx* gene and the plasmid-associated genes EHEC-*hlyA*, *katP*, and *espP* (Table 2). They also differed from STEC O26:H11 by the production of an α-hemolysin phenotype on red blood cell agar (Table 2). Among the non-O26 STEC strains isolated, none belonged to the four other main EHEC serotypes targeted here, and none contained the *eae* gene (Table 2). No strains contained the bundle-forming pilus *bfpA* gene, and only three STEC ONT/O rough:H10 (*stx1c*-positive) strains carried the EAF plasmid (Table 2). Two STEC O174:H2 (91.1) and O175:H16 (299.3) isolates contained the EHEC-*hlyA* gene and the autoagglutinating adhesin-encoding gene *saa* found in many *eae*-negative STEC strains (27); the former also carried the *espP* and *pagC* genes (Table 2). The *stx*-negative *E. coli* O103:H25 isolate (167.2) possessed the *eae*-γ*2*/θ variant and the three OI-122-encoded virulence markers tested (Table 2).

Typing of *E. coli* O26 strains by PFGE. To examine the relatedness among the *E. coli* O26 isolates recovered from cheeses, their XbaI and BlnI restriction patterns were analyzed by PFGE. This typing scheme was also applied to 18 additional O26:H11 strains from various origins (see Materials and Methods). PFGE produced 13 to 22 fragments ranging in size from ca. 40 to 700 kb. A dendrogram analysis of PFGE patterns showed high heterogeneity among the 29 *E. coli* O26 strains examined, with 21 distinct XbaI-BlnI patterns obtained (Fig. 1). Two clusters designated A (11 strains; >56% similarity) and B (17 strains; >58% similarity) were identified (Fig. 1). Cluster A comprised four PFGE patterns. The first pattern was common to the α-hemolysin-producing *stx*-negative O26:H11 strain 315-2 recovered here and to one cheese (FR14-18) and four cattle (FFL1-1, FFL2-6, FFL3-12, and FV5-36) *stx*-negative O26:H11 isolates recovered elsewhere. This pattern showed >90% similarity to that of the α-hemolysin-producing *stx*-negative O26:H11 strain 306D. A third and a fourth pattern were found in the O26:H32 strain (355.2) recovered here and in three cattle O26:H11 strains (FV2-33, FV3-11, and FV4-17), respectively. Cluster B included 7 and 10 *stx*-positive O26:H11 strains of human and cheese origins, respectively. Two cheese

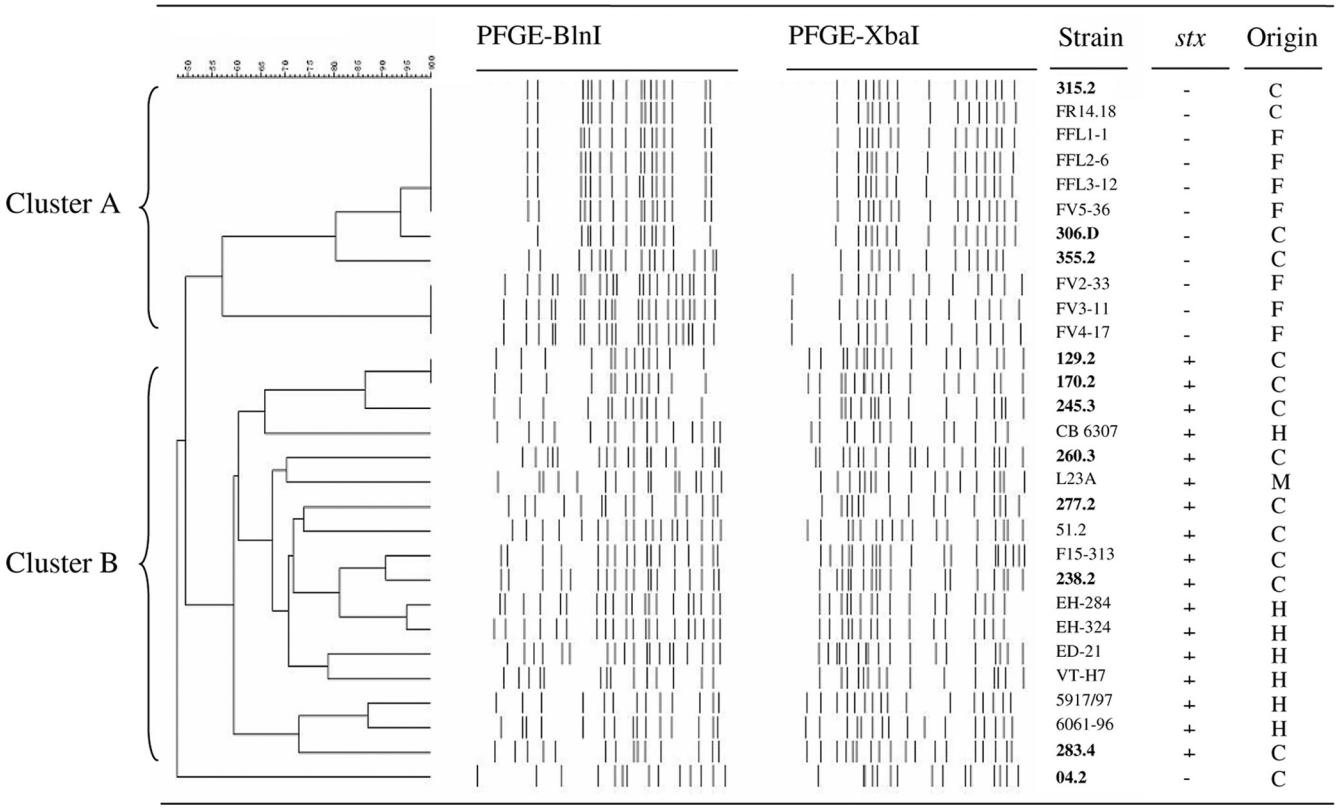

FIG. 1. Pulsed-field gel electrophoresis patterns of 29 *E. coli* strains isolated from raw-milk cheese (C), humans (H), cattle feces (F), and raw-milk (M) samples. The dendrogram was constructed using the Dice coefficient with a 2% band position tolerance and the unweighted pair group method using arithmetic averages. The names of strains isolated in this study are indicated in boldface. −, negative; +, positive.

isolates (129.2 and 170.2) shared a single PFGE pattern, and two human isolates (EH284 and EH324) were 95% similar. All the other strains had more distantly related patterns. Finally, the α-hemolysin-producing and *stx*-negative O26:H11 isolate 04.2 showed a PFGE pattern with less than 50% similarity to the others and thus clustered separately.

DISCUSSION

The prevalence of the *stx*-positive cheeses analyzed here was similar to those reported previously (2, 22) but higher than those found in other studies (5.7% to 13%) (42, 50, 53). However, direct comparison of the results is difficult, as the last studies were based on the use of distinct *stx* detection methods (i.e., conventional PCR) and included analysis of hard cheeses. In contrast, samples selected for this study were uncooked and soft cheeses, all made from raw milk and thus associated with higher risk of STEC infection than other types of cheeses derived from manufacturing processes more effective in eliminating STEC. Only 15 STEC strains could be isolated by colony hybridization, confirming the difficulties frequently encountered in STEC isolation from food (2, 3, 22, 50). Several hypotheses have been proposed to explain this outcome, including the presence of high levels of competing microflora or of natural inhibitors within dairy products that interfere with STEC isolation (22, 50). STEC might also be present in a stressed or injured state that prevents its isolation.

When the presence of the five main pathogenic STEC serotypes was investigated in cheeses based on the simultaneous detection of *stx*, *eae*, and at least one of the five STEC O group markers, the prevalence of samples with a presumptive positive result was 15.3%. However, this prevalence was lowered to 6.5% when the associations between the *eae* variants and the STEC serotypes were taken into account, highlighting the interest of the use of combinations of related STEC genetic alleles or variants for food screening. The most prevalent combination was *stx*–wzx_{O26}–*eae*-β*1* (4.8%), and the presumptive presence of STEC O26:H11 in *stx*–wzx_{O26}–*eae*-β*1*-positive cheeses could be confirmed by IMS for 7 out of 19 samples. In contrast, no STEC O103:H2, O145:H28, or O157:H7 isolates could be recovered by IMS from the 9 samples that tested positive by PCR, again illustrating the problems associated with STEC isolation. Due to their high prevalence in cheeses, the five STEC *fliC* alleles were not selective enough to be used as reliable markers for food screening. The prevalence of the $ihp1_{O145}$ gene was also high, suggesting that, although proposed for specific detection of STEC O145 (38), this marker could be associated with other *E. coli* serogroups commonly present in raw-milk cheeses. Indeed, $ihp1_{O145}$ was detected in non-O145 *E. coli* (i.e., O133 and O137) (6), as was also shown here for STEC O166:[H28] and *E. coli* O133:H29 isolates. Other real-time PCR assays, such as that targeting the O145-specific O antigen gene cluster (23), might therefore be used as alternative tests in screening for STEC O145 in foods.

E. coli O26:H11 has been frequently detected in calves and cattle (26), and contamination of raw milk and raw-milk cheeses with STEC O26:H11 has also been described (1, 18). The STEC O26:H11 strains isolated here tested positive for *stx1* only, except for one strain that also carried *stx2*. They contained genomic islands OI-71 and OI-122 and showed high genetic diversity by PFGE, as reported previously (17, 30, 52). According to their virulence genetic profiles, these strains should be considered pathogenic for humans. However, their ability to cause severe disease and their potential to cause outbreaks could be questioned. The last outbreak of STEC O26:H11 infection in France occurred in 2005 and was caused by *stx2*-positive strains (18). The virulence of *stx2*-positive O26:H11 might differ from that of *stx1*-positive O26:H11, which seems to be associated with milder clinical presentation even in an outbreak situation (19). Furthermore, the *stx2* variant is known to be the most clinically important *stx* subtype (24), and HUS-associated STEC O26:H11 harboring *stx2* (alone or in combination with *stx1*) has been more frequently isolated in central Europe since 1996 than strains containing *stx1* only (52). The fact that *stx*-negative *E. coli* O26:[H11] strains were isolated from *stx*-positive samples also raises some questions about the diagnostic result. As loss of the bacteriophage-associated *stx* gene was shown to occur frequently both *in vitro* and *in vivo* (9), it is tempting to speculate that these strains are derivatives of STEC that have lost their *stx* genes during the enrichment or isolation procedure. Alternatively, the *stx* gene detected in cheese samples might have been carried by other bacterial strains. Although colony hybridization could have been useful to test this hypothesis, it was not performed on these samples, as they showed high *stx* C_T values indicative of low STEC concentrations. When isolated from human patients, such *stx*-negative *E. coli* O26:H11 strains are referred to as atypical EPEC, as they lack the EAF plasmid (10). Interestingly, these strains were found to produce α-hemolysin and did not cluster with any human strains in the PFGE analysis, as was also reported by others (31, 33). Although α-hemolysin-producing *E. coli* O26:H11 strains have been isolated from children with diarrhea in the past (8), their virulence potential remains to be clearly defined.

Among the non-O26 STEC strains isolated here, none carried the *eae* gene, which is a virulence trait of typical EHEC strains. Most of them belonged to serotypes previously found in cheese and cattle, such as O2:H27, O8:H19, and O175:H16 (5, 44, 49, 50). One STEC strain belonged to O91:H10, a serotype of atypical (*eae*-negative) EHEC strains known to be associated with human disease (12). Finally, one *E. coli* isolate belonged to O103:H25, a serotype that was responsible for an outbreak in Norway linked to the consumption of contaminated fermented sausages (48). This isolate was *eae*-γ2/θ positive, as reported for another O103:H25 strain (16), and harbored OI-122. However, as reported for several human O103:H25 isolates (48), the strain was *stx* negative.

In conclusion, the molecular approach based on real-time PCR assays targeting specific EHEC combinations of genetic markers (such as *stx*, O groups, and *eae* variants) represents an interesting and valuable strategy for rapid screening of the most pathogenic STEC strains within food products. In the particular case of STEC O157:H7, this approach could be further evaluated by comparison with standardized or validated methods available for this serotype. In terms of risk to human health, the significance of the prevalence found for typical EHEC combinations of markers in raw-milk cheeses (i.e., 6.5%) deserves further investigation. In particular, the pathogenic potential of cheese STEC isolates belonging to EHEC serotypes, such as O26:H11 and O91:H10, needs to be further examined. The hypothetical loss of *stx* genes during STEC isolation from foods should also be investigated, as loss of *stx* could result in foodstuffs being falsely considered uncontaminated by pathogenic STEC and therefore safe.

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