Prevalence of Carriage of Shiga Toxin-Producing *Escherichia coli* Serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among Slaughtered Adult Cattle in France

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The main pathogenic enterohemorrhagic *Escherichia coli* (EHEC) strains are defined as Shiga toxin (Stx)-producing *E. coli* (STEC) belonging to one of the following serotypes: O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28. Each of these five serotypes is known to be associated with a specific subtype of the intimin-encoding gene (*eae*). The objective of this study was to evaluate the prevalence of bovine carriers of these "top five" STEC in the four adult cattle categories slaughtered in France. Fecal samples were collected from 1,318 cattle, including 291 young dairy bulls, 296 young beef bulls, 337 dairy cows, and 394 beef cows. A total of 96 *E. coli* isolates, including 33 top five STEC and 63 atypical enteropathogenic *E. coli* (aEPEC) isolates, with the same genetic characteristics as the top five STEC strains except that they lacked an *stx* gene, were recovered from these samples. O157:H7 was the most frequently isolated STEC serotype. The prevalence of top five STEC (all serotypes included) was 4.5% in young dairy bulls, 2.4% in young beef bulls, 1.8% in dairy cows, and 1.0% in beef cows. It was significantly higher in young dairy bulls ($P < 0.05$) than in the other 3 categories. The basis for these differences between categories remains to be elucidated. Moreover, simultaneous carriage of STEC O26:H11 and STEC O103:H2 was detected in one young dairy bull. Lastly, the prevalence of bovine carriers of the top five STEC, evaluated through a weighted arithmetic mean of the prevalence by categories, was estimated to 1.8% in slaughtered adult cattle in France.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are responsible for severe clinical symptoms, such as hemorrhagic colitis (HS) and hemolytic uremic syndrome (HUS) (1). EHEC are of serious public health concern because HUS is the leading cause of acute renal failure in children that is potentially fatal (2). Cattle are known to be the reservoir of EHEC strains, and bovine carriers do not show signs of clinical disease (3). Human infection occurs mainly through consumption of contaminated food and water (4). Monitoring EHEC strains is required at all steps along the food chain, from the area of primary production through the area of food production and handling and to foodstuffs, in order to identify and prevent contamination of food. Moreover, quantitative data on the prevalence of EHEC strains should be provided for quantitative microbial risk assessment, which should help in risk management. The identification of EHEC strains during the bacterial examination of food or environmental samples, conducted outside a clinical context involving humans, is challenging. In fact, it is the detection of the different virulence factors within the same strain that enables to estimate its pathogenicity.

The main virulence factor of EHEC contributing to pathogenicity is Shiga toxin (Stx) (5). But not all Shiga toxin-producing *E. coli* (STEC) strains are able to induce illness, as accessory EHEC genes may also contribute to human disease. Besides the *stx* gene, typical EHEC strains possess the *eae* gene, coding for the intimin protein, implicated in attaching and effacing lesions in the intestinal cells (6). Moreover, epidemiological studies have shown that five serotypes are more frequently involved in outbreaks than others (7). Therefore, the French Agency for Food Safety defined five

major EHEC strains as STEC, belonging to serotypes O157:H7, O26:H11, O145:H28, O103:H2, and O111:H8 (8). More precisely, the serotypes O157:H7 and O145:H28 are known to be associated with the *eae*-γ1 subtype, whereas STEC O26:H11, O103:H2, and O111:H8 harbor *eae*-β1, *eae*-ε, and *eae*-θ subtypes, respectively (9, 10). When isolated from the food chain, these "top five" STEC are considered to be highly pathogenic for humans.

The ISO 13136:2012 technical specification (TS) describes a real-time PCR-based approach for the detection of the top five STEC, which should be used to monitor these STEC along the food chain, in order to harmonize the results (11). At the farm level, few studies have focused on the specific detection of the five main pathogenic STEC. Regarding the literature on the prevalence of STEC in cattle, the majority of studies focused only on serotype

TABLE 1 Distribution of sampled cattle by campaign, abattoir, and category

Sampling campaign ID	Abattoir ID	No. of animals in category			
		Young dairy bulls ($n = 291$)	Young beef bulls ($n = 296$)	Dairy cows ($n = 337$)	Beef cows ($n = 394$)
B	2	0	5	3	112
C	1	8	0	88	24
D	3	1	21	32	66
E	4	7	52	1	3
F	5	19	64	9	33
G	6	24	47	46	13
H	5	28	70	6	26
I	1	57	18	28	27
J	3	0	14	46	70
K	1	115	5	0	0
L	5	32	0	78	20

O157:H7 (12, 13). Recent studies focused on the specific detection of the top five STEC in cattle feces using PCR screening approaches. Nevertheless, the low number of cattle screened and the fact that serogroup-specific strain isolation was restricted to only a few samples did not allow to obtain a reliable estimate of the prevalence of the top five EHEC (14–18).

Therefore, the objective of the present study was to evaluate the prevalence of the five main pathogenic STEC in the different categories of slaughtered cattle used for the production of ground beef in France (young dairy and beef bulls and dairy and beef cows). The aims of our study were (i) to isolate and characterize the top five STEC strains from fecal samples containing EHEC-associated genetic markers, (ii) to evaluate the prevalence of bovine carriers of STEC per cattle category at the time of their slaughter, and finally (iii) to estimate the prevalence of the top five STEC bovine carriers in slaughtered adult cattle in France.

MATERIALS AND METHODS

Sampling plan. Samples were collected from cattle slaughtered in six of the French abattoirs with the highest slaughter capacities for adult cattle. They produced from 20,000 to 46,000 tons carcass weight equivalent of adult cattle per year. They were selected for inclusion in this study on the basis of their geographical location covering the French cattle production area. Eleven sampling campaigns were conducted from 19 October 2010 to 28 June 2011. The sampling plan was devised in order to enable a good estimation of prevalence among the four categories of slaughtered cattle used for the production of ground beef in France, i.e., young dairy bulls, young beef bulls, dairy cows, and beef cows. Young bulls are defined by the European legislation (Council Regulation [EC] No. 1234/2007) as animals slaughtered before the age of 24 months. A minimum sample size of about 300 animals by category was calculated with the assumption of a 2% prevalence of carriage of the top five STEC and a targeted precision of 1.6%. This assumption was based on data published for STEC O157:H7 shedding in French and European cattle (19–21). Cattle was randomly sampled throughout the slaughter period to avoid as much as possible the sampling of animals from the same batch/herd or farm and to include the requested number of animals in each of the four categories. We checked before the analysis the animal origin and considered only a single positive animal per farm for prevalence calculation if several were detected. In all, feces were collected from 1,318 animals, including 291 young dairy bulls, 296 young beef bulls, 337 dairy cows, and 394 beef cows (Table 1). Fecal samples were obtained by opening the terminal rectum after evisceration.

They were kept chilled and sent to the laboratory by overnight courier for analysis.

E. coli **control strains.** Seven reference strains were used as positive controls in PCR analysis: Sakaï (O157:H7, stx_1, stx_2, eae-γ1, ehxA, espK), PMK5 (O103:H2, stx_1, eae-ε), H19 (O26:H11, stx_1, eae-β1), 95NR1 (O111:H8, stx_1, stx_2, eae-θ), ED-28 (O145:H28, stx_1, eae-γ1), E2348/69 (O127:H6, bfpA, EAF), and EDL933 (O157:H7, stx_2, eae-γ1, pagC, nleB, efa1) (22–25). ED-28 was provided by the Istituto Superiore di Sanità (Rome). Laboratory nonpathogenic *E. coli* strain MG1655 was used as a negative control for all virulence factors investigated.

Sample enrichment and DNA extraction. Upon arrival, each sample (10 g) was diluted 10-fold (wt/vol) in 90 ml of modified tryptone soya broth (Oxoid, Dardilly, France) supplemented with novobiocin (Oxoid, Dardilly, France) at 16 mg · liter^{-1} and incubated overnight at 37°C. Bacterial DNA was extracted from 1 ml of each enriched broth using lysis tubes (Pall GeneDisc Technologies, Bruz, France), as already described (26).

PCR-based screenings for EHEC-associated genetic markers. DNA extracts were subjected to a sequential real-time PCR-based approach for the detection of EHEC-associated genetic markers. This PCR-based strategy used to detect suspect samples was the same as the one described in the ISO 13136:2012 technical specification, to which a screening of the eae subtypes associated with the five major EHEC was added (11, 26). An initial screening step was performed for the detection of stx, eae genes, and eae subtypes γ1, β1, ε, and θ. The detection of stx_1, stx_2, and an internal control was performed as already described (27), and two additional assays allowed the detection of the eae gene and eae subtypes as previously published (28, 29). A second screening step was performed on stx- and eae-positive samples for the detection of the five O group markers. This second step included two simplex real-time PCR assays to detect $rfbE_{O157}$ and $wbd1_{O111}$ genes and one triplex real-time PCR assay for the screening of wzx_{O26}, wzx_{O103}, and $ihp1_{O145}$ genes, with primers and probes described elsewhere (30, 31). All the PCR experiments were performed using a CFX96 instrument (Bio-Rad), except those targeting eae subtypes, which were performed on a LightCycler 480 instrument (Roche Diagnostics).

Isolation procedures. Isolations of *E. coli* strains belonging to the five targeted serotypes were performed for samples that tested positive by PCR for the simultaneous presence of an stx gene, an eae subtype, and its associated O group marker. Three isolation procedures were used in parallel, except for two campaigns, in order to maximize the recovery of strains. The first isolation procedure consisted of immunomagnetic separation (IMS) assays using Dynabeads (Invitrogen, Cergy Pontoise, France), as recommended by the manufacturer. Ten microliters of immunoconcentrated bacteria was plated onto cefixime-tellurite-sorbitol-MacConkey agar (Oxoid, Dardilly, France) for *E. coli* O157, O103, and O111, onto cefixime-tellurite-rhamnose-MacConkey agar for *E. coli* O26, and onto cefixime-tellurite-raffinose-MacConkey agar for *E. coli* O145 (32). A total of 10 plates were used for each serogroup, and all media were incubated for 18 to 24 h at 37°C. Up to 10 suspect colonies were tested by slide agglutination with serogroup-specific antisera (Statens Serum Institut, Copenhagen, Denmark), and each serogroup was confirmed by real-time PCR as described above. In the second procedure, *E. coli* O157 strains were isolated using the Vidas Immuno-Concentration *E. coli* O157 (ICE) kit (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Automated immunoconcentration of *E. coli* O26 was performed using the *E. coli* Serogroup (EES) kit (bioMérieux), adapted for immunoconcentration. IMS-based isolations (Dynabeads; Invitrogen, Cergy Pontoise, France) were performed to isolate *E. coli* strains belonging to serogroups O103, O111, and O145 according to the manufacturer's instructions. In the third procedure, isolation of *E. coli* strains belonging to serogroups O157, O26, O103, O111, and O145 was performed using the Vidas UP *E. coli* serogroups (ESPT) kits under development (bioMérieux). For the second and third procedures, 50 μl of immunoconcentrated bacteria was inoculated on two selective isolation media, Rapid'*E. coli*

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TABLE 2 Detection of stx_1, stx_2, eae, and eae subtypes in cattle categories

EHEC marker targeted by real-time PCR	No. (%) of samples positive for EHEC-associated genetic markers in:			
	Young dairy bulls ($n = 291$)	Young beef bulls ($n = 296$)	Dairy cows ($n = 337$)	Beef cows ($n = 394$)
stx^a	245 (84.2)	208 (70.3)	235 (69.7)	278 (70.6)
stx_1	122 (41.9)	81 (27.4)	136 (40.4)	165 (41.9)
stx_2	219 (75.3)	182 (61.5)	201 (59.6)	247 (62.7)
eae^b	199 (68.4)	190 (64.2)	155 (46.0)	229 (58.1)
eae-β1	100 (34.4)	98 (33.1)	70 (20.8)	111 (28.2)
eae-γ1	34 (11.7)	21 (7.1)	12 (3.6)	28 (7.1)
eae-ε	33 (11.3)	40 (13.5)	23 (6.8)	52 (13.2)
eae-θ	80 (27.5)	71 (24.0)	76 (22.6)	101 (25.6)
stx-eae	177 (60.8)	145 (49.0)	125 (37.1)	182 (46.2)

^{a} Samples positive for stx were positive for stx_1 and/or stx_2.
^{b} Detection of the eae gene with the universal primers/probe.

O157:H7 (Bio-Rad, Marne la Coquette, France) and ChromoID O157:H7 (bioMérieux), supplemented with cefixime and tellurite for *E. coli* O157, with sorbitol-MacConkey agar and rhamnose-MacConkey agar (Oxoid) for *E. coli* O26, or with sorbitol-MacConkey agar and a selective differential medium for *E. coli* O103, O111, and O145 (32). All media were incubated for at 18 to 24 h at 37°C. Up to 10 suspect colonies per each serogroup screened were tested by real-time PCR, as described above.

For the three isolation procedures, once the targeted serogroup was confirmed by PCR, isolates were characterized for the presence of *stx* genes and *eae* subtypes by real-time PCR, as described above. The presence of the *fliC*_H alleles (*fliC*_H2, *fliC*_H7, *fliC*_H8, *fliC*_H11, and *fliC*_H28) was also investigated as previously described (29). Isolates were also confirmed as *E. coli* using an API 20E test (bioMérieux, Marcy l'Etoile, France). Based on PCR results, *E. coli* isolates positive for *stx* genes were classified as Shiga toxin-producing *E. coli* (STEC). *E. coli* isolates positive for *eae* gene and negative for *stx* genes were classified as enteropathogenic *E. coli* (EPEC).

Virulence profiles. Subtyping of *stx*_1 and *stx*_2 genes was performed as described previously (33). The presence of additional EHEC virulence markers (*ehxA* gene and OI-122-associated genes, namely, *pagC*, *nleB*, and *efa1* genes) was screened by PCR as described previously (34, 35). The presence of the virulence marker *espK* was screened by PCR for STEC O26:H11 (36). The presence of typical EPEC markers, i.e., *bfpA* and EPEC adherence factor (EAF) genes, was also tested by PCR (37, 38). *E. coli* isolates positive for the *eae* gene and negative for the *bfpA* gene and EAF plasmid were classified as atypical enteropathogenic *E. coli* (aEPEC) (1).

PFGE typing of the top five STEC strains. STEC strains were typed using the Standard PulseNet pulsed-field gel electrophoresis (PFGE) protocol for *E. coli* O157 (39). Agarose-embedded DNAs were digested overnight at 37°C with 20 U of XbaI enzyme (Promega Corp., Madison, WI, USA). XbaI-digested DNA of *Salmonella enterica* serotype Braenderup strain H9812 (Centers for Disease Control and Prevention, Atlanta, GA) was used as a universal molecular size marker. Restriction fragments were resolved at 14°C in 0.5× Tris-borate-EDTA (TBE) buffer on 1% Seakem gold agarose gels (FMC Bioproducts, Rockland, ME, USA) using a pulsed-field Chef-DR-III system (Bio-Rad laboratories, Munich, Germany). After being stained with ethidium bromide (10 μg ml⁻¹), gels were visualized on gel image digitization by Easy RH equipment (Herolab GmBH, Germany) and an image analyzer (VisioCapt-Bio1D; Fisher Bioblock Scientific, Illrisch, France), and the PFGE profiles were analyzed using GelCompar II software version 6.5 (Applied Maths, Ghent, Belgium). A dendrogram was generated using the band-based Dice similarity coefficient with a 1.5% band position tolerance and the unweighted pair group method with arithmetic mean clustering.

Statistical analysis. Statistical analyses were conducted using R software (R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org/]). Comparisons of detection of EHEC-associated genetic markers among the different cattle categories were done using logistic models (the response variable was presence or absence of the specific gene; the explicative variable was animal category). The prevalence of bovine carriers of the top five STEC per cattle category was calculated considering in the numerator one single positive animal per farm (as the animals of a same farm are correlated). Comparisons of prevalences between cattle categories were done using the chi-square test or Fisher's exact test if the number of animals was too small. For all the analyses, the significance level was set to 0.05. The prevalence of the top five STEC (all serotypes included) among French slaughtered adult cattle was calculated through the weighted mean prevalence, using the proportion of animals of each category slaughtered in France as weights. These proportions were given by the French Cattle and Meat Association (Interbev) for the 2010-2011 period.

RESULTS

Detection of EHEC-associated genetic markers in bovine feces. A total of 1,318 fecal bovine samples were screened for the presence of stx_1, stx_2, eae, and eae subtypes β1, ε, γ1, and θ (Table 2). Whatever the category studied, the most frequently detected EHEC marker was stx. The percentage of stx-positive fecal samples was significantly higher in young dairy bulls than in the three other categories ($P < 0.001$). Moreover, the percentage of eae-positive fecal samples was significantly higher in young dairy bulls and in young beef bulls than in dairy and beef cows ($P < 0.001$). For the four cattle categories, the most frequently detected eae subtypes were eae-β1 and eae-θ, followed by eae-γ1 and eae-ε. Regarding the association of EHEC markers, samples positive for both stx and eae genes were more frequently detected in young dairy bulls than in the three other categories ($P < 0.001$).

In all, 629 samples were stx and eae positive (47.7%) and were subjected to a screening for the five EHEC O group markers. The results showed that 555 (42.1%) of the samples were positive for at least one of the five stx-eae-O group marker combinations. The stx-eae-O group marker combinations were detected 1,217 times, as several samples contained more than one O group marker, and the most frequently detected combination was stx-eae-$ihp1$_{O145} (Table 3). When taking into account the results of the detection of the eae subtypes, 235 (17.8%) samples were positive for at least one of five targeted stx-eae subtype-O group marker combinations. The most frequently identified combinations were stx–eae-β1–wzx_{O26}, stx–eae-ε–wzx_{O103}, and stx–eae-γ1–ihp_{O145} (Table 3). In all, the stx-eae subtype/associated O group marker combinations were detected 363 times, as several samples contained more than one combination of EHEC-associated genetic markers.

Isolation of STEC and aEPEC strains belonging to the five EHEC serotypes. Samples positive for one or more stx-eae sub-

TABLE 3 Number of combinations of the top five EHEC-associated genetic markers detected in 1,318 bovine fecal samples and results of isolation assays targeting the top five EHEC serogroups

Combinations of EHEC markers	No. of combinations (% of positive samples)	Results of isolation assays		
		No. of strains isolated	No. of STEC	No. of aEPEC
stx-eae-O group marker	1,217 (NA[a])			
stx-eae subtype/associated O group marker	363 (NA)	96	33	63
stx-eae-rfbE$_{O157}$	216 (16.4)			
*stx–eae-γ*1–*rfbE*$_{O157}$	47 (3.6)	20	18	2
stx-eae-wzx$_{O26}$	202 (15.3)			
*stx–eae-β*1–*wzx*$_{O26}$	129 (9.8)	37	3	34
stx-eae-wzx$_{O103}$	262 (19.9)			
stx–eae-ε–wzx$_{O103}$	93 (7.1)	27	8	19
stx-eae-wbd1$_{O111}$	27 (2.0)			
stx–eae-θ–wbd1$_{O111}$	14 (1.1)	2	2	0
stx-eae-ihp1$_{O145}$	510 (38.7)			
*stx–eae-γ*1–*ihp1*$_{O145}$	80 (6.1)	10	2	8

[a] NA, not applicable, as several samples contained more than one combination of EHEC-associated genetic markers.

type/associated O group marker combinations were subjected to isolation assays using three procedures in parallel (see Materials and Methods). An *E. coli* strain belonging to one of the five targeted EHEC serotypes was isolated for 96 of the 363 assays (Table 3). Of the 96 strains isolated, 33 were the top five STEC and belonged to serotypes O157:H7 (*n* = 18), O26:H11 (*n* = 3), O103:H2 (*n* = 8), O111:H8 (*n* = 2), and O145:H28 (*n* = 2). The 63 other *E. coli* strains isolated belonged to serotypes O157:H7, O103:H2, O145:H28, and O26:H11 and harbored the corresponding *eae* subtype but lacked an *stx* gene. These strains were classified as aEPEC, as they were negative for the *bfpA* gene and the EAF plasmid (1). No aEPEC was isolated for the serotype O111:H8. Finally, the proportion of STEC among the obtained *E. coli* isolates varied between the five serotypes. Concerning the O157:H7 serotype, isolation procedures led to the isolation of STEC strains rather than aEPEC strains, whereas the reverse was observed for serotypes O26:H11 and O103:H2.

Virulence profiles of the top five STEC strains. The 33 STEC belonging to the top five serotypes were further characterized (Table 4). All these isolates harbored the *eae* subtype known to be specifically associated with its serotype. Concerning the 18 STEC O157:H7 strains, they were all positive for the *stx*$_2$ gene, and the *stx*$_{2c}$ subtype was the most frequently detected (*n* = 14). The *stx*$_{2a}$ subtype was detected in 3 STEC O157:H7, and the simultaneous presence of *stx*$_{2a}$ and *stx*$_{2c}$ subtypes was detected in one STEC O157:H7 strain (B3-O157-1). In addition to the *stx*$_2$ gene(s), an *stx*$_1$ gene (*stx*$_{1a}$ subtype) was present in two STEC O157:H7 strains, isolated from cows (C61-O157-1 and B3-O157-1). The *stx*$_{1a}$ subtype was detected in all the non-O157 STEC strains, whereas the *stx*$_{2a}$ subtype was detected in only one STEC O103:H2 strain (L154-O103-3). The screening of additional EHEC virulence markers showed that all STEC strains possessed the *ehxA* gene, except for the O26:H11 strain I92-O26-1. Moreover, all STEC strains of serotypes O157:H7, O103:H2, and O111:H8 were positive for *pagC*, *nleB*, and *efa1* genes. STEC strains of serotype O26:H11 were positive only for *nleB* and *efa1* genes, and STEC strains of serotype O145:H28 were negative for these

three OI-122-associated genes. Finally, all STEC O26:H11 strains harbored the *espK* gene.

Origin and genetic diversity of the top five STEC strains. The 33 STEC strains were isolated from 32 distinct animals, as the young dairy bull K106 harbored both STEC O26:H11 and STEC O103:H2. Besides, it was noteworthy that young dairy bulls K143 and K146, coming from the same farm (farm 24), carried STEC O157:H7 and STEC O103:H2, respectively (Table 4). In order to explore the genetic relatedness of STEC within a same serotype, the 33 top five STEC strains were subjected to PFGE analysis. The strain L71-O157-1 could not be typed. The dendrograms of STEC O157:H7 and STEC O103:H2 are shown in Fig. 1.Within the same serotype, a high diversity was observed. Nevertheless, on two occasions, STEC O157:H7 strains isolated from animals coming from different farms but sampled during the same campaign showed an identical PFGE pattern (PFGE patterns A and K). A unique PFGE pattern was also observed for the two STEC O145:H28 carried by two young dairy bulls coming from the same farm and sampled at the same campaign (data not shown). In contrast, it was noteworthy that STEC O157:H7 strains isolated from cattle coming from the same farm (farms 3 and 15), sampled or not during the same campaign, showed different PFGE patterns.

Prevalence of bovine carriers of the top five STEC per cattle category. The prevalence rates of the top five STEC were 4.5%, 2.4%, 1.8%, and 1.0% in young dairy bulls, young beef bulls, dairy cows, and beef cows, respectively (Table 5). Young dairy bulls harbored significantly more STEC strains than other categories (*P* < 0.01). The prevalence of STEC serotype O157:H7 was significantly higher in young dairy bulls than in other categories (*P* < 0.05). Finally, taking into account the proportion of animals of each category slaughtered in France during the sampling period, the weighted mean prevalence of STEC with the five targeted serotypes combined was estimated at 1.8% in adult slaughtered cattle. It was estimated at 1.2% for STEC O157:H7.

DISCUSSION

The main objective of our study was to evaluate the prevalence of the five main pathogenic STEC in French cattle per category of cattle that are slaughtered for the production of ground beef. The PCR-based strategy used to detect suspect samples was that described in the ISO 13136:2012 technical specification, to which we added a screening of the *eae* subtypes associated with the five major EHEC (11, 26). Indeed, we previously showed that this additional screening step helped to be more discriminating for the specific detection of suspect samples likely to contain the five major STEC in cattle feces. As confirmed in this study, identification of samples positive for *stx-eae* subtype/associated O group marker combinations rather than *stx-eae*-O group marker combinations allowed to narrow down the number of potential positive samples that should be subjected to isolation assays for confirmation. The proportion of isolation assays leading to the isolation of one of the top five STEC strains was relatively low, as previously observed in various studies (14, 15, 17). The reasons for this discrepancy, including PCR-based strategy and isolation assays limitations, have been already discussed elsewhere (26, 40–42). In this study, three isolation procedures were used in parallel and allowed us to improve the isolation fraction of the top five STEC strains. In addition to STEC strains, we isolated *stx*-negative aEPEC belonging to the same top five serotypes and harboring *eae* subtypes associated

TABLE 4 Origin and characterization of STEC O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 isolated from bovine feces

Serotype[a]	Origin	Strain[b]	Presence[c] of gene:								Farm ID
			stx_1 (subtype)	stx_2 (subtype)	eae (subtype)	$ehxA$	$pagC$	$nleB$	$efa1$	$espK$	
O157:H7	Young dairy bull	C117-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	4
O157:H7	Young dairy bull	H105-O157-1	−	+ (stx_{2a})	+ (γ1)	+	+	+	+	ND	10
O157:H7	Young dairy bull	I113-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	15
O157:H7	Young dairy bull	I114-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	15
O157:H7	Young dairy bull	K118-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	23
O157:H7	Young dairy bull	K143-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	24
O157:H7	Young dairy bull	L81-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	27
O157:H7	Young beef bull	G15-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	6
O157:H7	Young beef bull	H13-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	9
O157:H7	Young beef bull	H37-O157-1	−	+ (stx_{2a})	+ (γ1)	+	+	+	+	ND	11
O157:H7	Young beef bull	J28-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	16
O157:H7	Dairy cow	C29-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	2
O157:H7	Dairy cow	C61-O157-1	+ (stx_{1a})	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	3[d]
O157:H7	Dairy cow	J49-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	3bis[d]
O157:H7	Dairy cow	L71-O157-1	−	+ (stx_{2a})	+ (γ1)	+	+	+	+	ND	26
O157:H7	Beef cow	B3-O157-1	+ (stx_{1a})	+ ($stx_{2a, 2c}$)	+ (γ1)	+	+	+	+	ND	1
O157:H7	Beef cow	G79-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	8
O157:H7	Beef cow	J76-O157-1	−	+ (stx_{2c})	+ (γ1)	+	+	+	+	ND	18
O26:H11	Young dairy bull	I92-O26-1	+ (stx_{1a})	−	+ (β1)	−	−	+	+	+	14
O26:H11	Young dairy bull	K106-O26-1	+ (stx_{1a})	−	+ (β1)	+	−	+	+	+	22
O26:H11	Beef cow	J77-O26-1	+ (stx_{1a})	−	+ (β1)	+	−	+	+	+	19
O103:H2	Young dairy bull	K56-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	21
O103:H2	Young dairy bull	K106-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	22
O103:H2	Young dairy bull	K146-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	24
O103:H2	Young dairy bull	L24-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	25
O103:H2	Young beef bull	F63-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	5
O103:H2	Young beef bull	G22-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	7
O103:H2	Young beef bull	H115-O103-1	+ (stx_{1a})	−	+ (ε)	+	+	+	+	ND	13
O103:H2	Dairy cow	L154-O103-3	+ (stx_{1a})	+ (stx_{2a})	+ (ε)	+	+	+	+	ND	28
O111:H8	Young dairy bull	K50-O111-1	+ (stx_{1a})	−	+ (θ)	+	+	+	+	ND	20
O111:H8	Dairy cow	J43-O111-1	+ (stx_{1a})	−	+ (θ)	+	+	+	+	ND	17
O145:H28	Young dairy bull	H99-O145-1	+ (stx_{1a})	−	+ (γ1)	+	−	−	−	ND	12
O145:H28	Young dairy bull	H101-O145-1	+ (stx_{1a})	−	+ (γ1)	+	−	−	−	ND	12

[a] The serotype was determined by PCR.
[b] The first letter of the name of the strain corresponds to the sampling campaign.
[c] +, detected by PCR; −, not detected by PCR; ND, not determined.
[d] Fecal samples were collected from cattle coming from farm 3 on two different campaigns (3bis, second campaign).

with the targeted serotypes. It was noteworthy that the proportion of STEC among the recovered *E. coli* strains depended on the targeted serotype and was high for serotype O157:H7 but much lower for serotypes O26:H11 and O103:H2. These results are in agreement with previous studies evaluating *stx* carriage in top five serogroups of *E. coli* strains isolated from Irish beef slaughter chains and Scottish and Swiss cattle (14, 16, 43). Whether these results reflect a higher stability of *stx* bacteriophages within strains of serotype O157:H7 remains to be investigated.

In all, 33 STEC strains belonging to the top five serotypes were isolated from the 1,318 bovine fecal samples screened. They all harbored the expected *eae* subtypes specifically associated with the five targeted serotypes. According to their virulence genetic profiles, these top five STEC should be considered highly pathogenic for humans (8, 9, 23, 44). All STEC O157:H7 strains were positive for the *stx*₂ gene. The stx_{2c} subtype was the most frequently detected in those strains. This is in agreement with previous studies showing that STEC O157 isolated from cattle were dominated by subtype stx_{2c} (45–47). In contrast, the non-O157:H7 STEC strains were all stx_{1a} positive. Another epidemiological study also showed

that stx_1 predominated in STEC O26 isolated from Scotland cattle (43). Moreover, stx_1 predominated in STEC O26, O111, and O103 isolated from humans, food, and cattle in Belgium, whereas isolates of STEC O145 displayed a heterogeneous distribution of *stx* genes (48). Finally, EHEC additional virulence genes *ehxA* and OI-122-associated genes were detected in the top five STEC strains isolated from cattle feces, as well as *espK* in the case of STEC O26:H11. These EHEC virulence markers have already been shown to be associated with the top five STEC strains that cause severe diseases and outbreaks (36, 49–51).

When looking at STEC bovine carriers, one of the most striking features is the fact that a young dairy bull carried STEC of two different serotypes. Moreover, the identification of farms harboring STEC bovine carriers highlighted the fact that STEC of a given serotype could be carried by several animals belonging to the same farm. We also identified two young bulls that came from the same farm and carried STEC of different serotypes. Within each serotype, PFGE analysis showed that the genetic diversity of the top five STEC was high, as already observed by others (52, 53). STEC of a given serotype carried by cattle coming from the same farm

A

B

FIG 1 XbaI PFGE patterns and origins of 17 STEC O157:H7 strains (A) and 8 STEC O103:H2 strains (B) isolated from 1,318 bovine feces, in France, in 2010-2011. The dendrograms were generated using the band-based Dice similarity coefficient with a 1.5% band position tolerance and the unweighted pair group method with arithmetic mean clustering.

were either genetically related (for serotype O145:H28) or not (for serotype O157:H7). Moreover, within a same sampling campaign, identical PFGE patterns could be observed for STEC O157:H7 harbored by cattle coming from different farms. We can assume that the same clone might be present at a given time in several farms. Alternatively, we can also assume that these bovine carriers

of a same clone might have been batched into the same facilities for fattening before slaughter.

Finally, the main objective of the present study was to obtain a reliable estimate of the prevalence of bovine carriers of the top five STEC in slaughtered adult cattle in France and thus new elements for assessment of human exposure to the top five STEC through consumption of beef. It is noteworthy that cattle prevalence studies have rarely addressed the question of the reliability of their results. Indeed, screening strategies, analytical methods, and sampling strategies might lead to certain limitations. The limitations of the screening strategy and the analytical method used in the present study have been discussed above. Concerning the sampling strategy, it was optimized in order to obtain a reliable estimate of the prevalence of each of the five STEC serotypes per slaughtered cattle category. This allowed to identify differences in carriage between categories. All serotypes combined, young dairy bulls harbored significantly more STEC strains than other categories. The prevalence of STEC O157:H7 was also significantly higher in this category. These results are in agreement with the results of PCR screenings showing that the simultaneous presence of *stx* and *eae* genes was significantly more frequently detected in feces from young dairy bulls than in other categories. Overall, these results are consistent with the results of previous studies

TABLE 5 Prevalence of bovine carriers of STEC O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 per cattle category

STEC serotype(s)	Prevalence (%) of bovine carriers of top five STEC in:			
	Young dairy bulls ($n = 291$)	Young beef bulls ($n = 296$)	Dairy cows ($n = 337$)	Beef cows ($n = 394$)
Top five STEC	4.5	2.4	1.8	1.0
STEC O157:H7	2.1	1.4	1.2	0.8
STEC O26:H11	0.3	0.0	0.0	0.3
STEC O103:H2	1.0	1.0	0.3	0.0
STEC O111:H8	0.3	0.0	0.3	0.0
STEC O145:H28	0.3	0.0	0.0	0.0
STEC O26:H11 and O103:H2	0.3	0.0	0.0	0.0

evaluating the influence of type of production and age of animal on STEC fecal shedding. Concerning the effect of type of production on the prevalence of STEC, a survey conducted on 180 Belgium farms showed that the highest prevalence of *E. coli* O157:H7 was found in dairy cattle farms (61.2%) compared to beef farms (22.7%) or mixed dairy and beef farms (44.4%) (54). Concerning the effect of age on STEC shedding, a Scotland investigation on 14,856 cattle fecal samples showed that an increased probability of a sampling group containing a STEC O157:H7-shedding animal was associated with larger numbers of 12- to 30-month-old finished cattle (55). They also showed that a higher maximum age of animals in the sampling group was significantly associated with a lower prevalence of STEC O157:H7. Moreover, a review of published farm prevalence surveys had already shown that 0.5 to 1% of sampled animals were *E. coli* O157:H7 carriers, and this proportion was raised to 5% for later-weaned calves and heifers (56). Nevertheless, it should be noted that a seasonality of production exists for young bulls, with a peak of production observed around the summer, and this seasonality has been observed in France (http://www.agrireseau.qc.ca/bovinsboucherie/documents/pdf_D379-v.pdf). Consequently, in the present study, the feces of this cattle category was largely sampled during this period. It is also worth noting that the season had been shown to have an influence on STEC shedding, the warmer months being associated with a peak in the prevalence of STEC (57). Anyway, the biological basis for either age-related or rearing conditions or seasonal peak shedding by cattle is unknown and remains to be elucidated. Various hypotheses have been advanced to try to explain these variations in STEC carriage; and among them, the seasonal presence of increased numbers of young high shedders might explain a seasonal peak in the prevalence of STEC (58).

Lastly, the prevalence per category was weighted by the number of slaughtered cattle within each category, and the prevalence of the top five STEC (all five serotypes included) was estimated to 1.8% in slaughtered adult cattle in France. The weighted mean prevalence of the most prevalent serotype, O157:H7, was estimated at 1.2%. STEC O157:H7 was detected in the four cattle categories. These values concerning STEC O157:H7 shedding are in agreement with previous results of European prevalence studies. The average proportion of STEC O157-positive samples, based on the investigation of a high number of feces or hides from animals sampled either at the farm or at slaughter, ranged from 0.2% to 2.3% for the 2009-2011 period (59, 60). Concerning the non-O157:H7 STEC serotypes, prevalence data directly comparable to our results are lacking in the literature. Recent studies focused on the detection of the top five STEC in cattle feces, but none of them led to the estimation of their prevalence, due either to a limited number of serogroup-specific strain isolations performed (14, 15) or to a sampling strategy that did not allow a national estimation of the prevalence (16, 18). In our study, the four non-O157:H7 STEC serotypes were detected in slaughtered categories at a low prevalence, ranging from 0.0% to 1.0%.

In conclusion, an estimation of a reliable value of the prevalence of STEC bovine carriers in slaughtered adult cattle in France was attempted here. To this end, the top five STEC strains considered highly pathogenic were isolated, and prevalence weighted by the number of slaughtered animals within each category was calculated. This study also allowed to identify differences in STEC carriage, which need to be clarified. Factors affecting STEC carriage are under investigation; notably, in the farms from which the bovine carriers came.

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