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First isolation of Shiga toxin 1d producing Escherichia coli variant strains in shellfish from coastal areas in France

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Abstract: Aims: This study was carried out to evaluate the presence of Shiga toxin-producing Escherichia coli (STEC) and E. coli O157:H7 in shellfish from French coastal environments.
Methods and Results: Shellfish were collected in six growing areas or natural beds (B-category) and non-farming areas (D-category) from July 2002 to August 2004. PCR-detection of stx genes was performed on homogenized whole shellfish and digestive gland tissues enrichments. STEC strains were detected by colony DNA hybridization using a stx-specific gene probe and E. coli O157 strains were additionally searched by immunomagnetic separation with O157 specific magnetic beads. Stx genes were detected in 40 of 144 (27.8%) sample enrichments from mussels, oysters or cockles, 32 of 130 enrichments (24.6%) were from B-category areas and eight of 14 (57.1%) from the D-category area. Five strains carrying stx1 or stx1d genes and one stx-negative, eae & ehxA-positive E. coli O157:H7 were isolated from six of 40 stx-positive enrichments. No relation was found between the total E. coli counts in shellfish and the presence of STEC strains in the samples.
Conclusions: STEC strains of different serotypes and stx-types are present in shellfish from French coastal environments. It is the first isolation of STEC stx1d strains in France.
Significance and Impact of the Study: Shellfish collected in coastal environments can serve as a vehicle for STEC transmission.

Keywords: Shiga-like toxin producing Escherichia coli (STEC), E. coli O157, shellfish, coastal environment, shellfish-farming areas

INTRODUCTION

Shiga-like toxin producing *Escherichia coli* (STEC) are a known cause of serious illness in humans and a major public health concern (Paton and Paton 1998a). Infections due to STEC can result in severe bloody diarrhoea (haemorrhagic colitis, HC) which may evolve towards the life-threatening haemolytic-uraemic syndrome (HUS). *Escherichia coli* O157:H7 was first recognised as a human pathogen in 1982, and was linked with two outbreaks associated with consumption of contaminated hamburgers in the United States (Riley *et al.* 1983). STEC of different serotypes are related to both outbreaks and sporadic cases of infection and food-borne outbreaks associated with STEC were well documented world-wide. Human infections were reported from over 30 countries on six continents (Mead and Griffin 1998) and result mainly from eating raw or undercooked contaminated foods. The minimal infective dose is very low for STEC O157 compared to other food poisoning organisms and was reported as <100 cells (Griffin and Tauxe 1991).

A major virulence factor of STEC is the production of one or more Shiga toxins (Stx). The production of Stx1 and Stx2 in *E. coli* is conferred by toxin-converting lysogenic bacteriophages (O'Brien *et al.* 1984). The involvement of these phages could explain the production of Shiga toxins in more than 150 different serotypes of *E. coli* (Schmidt 2001) and in other bacteria such as *Citrobacter freundii* or *Enterobacter cloacae* (Schmidt *et al.* 1993; Paton and Paton, 1997). Only few Stx1 variants and more than 20 Stx2 variants have so far been reported (Bastian *et al.* 1998; Nakao *et al.* 2002; Bürk *et al.* 2003). Other virulence factors include enterohaemolysin encoded by the *ehxA* gene and the product of the *eae* gene, intimin, which is involved in the enterocyte attaching and effacing phenotype (A/E lesion) of bacteria.

The natural hosts for STEC are ruminants like sheep, goats, and in particular, bovines (Zschock *et al.* 2000; Hancock *et al.* 2001). Other animals such as pigs and dogs can also harbor STEC strains (Beutin *et al.* 1993; Bouvet *et al.* 2001). After excretion, these strains can persist for months in the environment (for a review see Duffy 2003). Another environmental source of STEC strains is sewage and STEC including O157 were isolated from wastewater from sewage treatment plant (Höller *et al.* 1999; Vernozy-Rozand *et al.* 2002). The contamination of the environment via these sources signifies a risk for the human health since STEC infections through consumption of contaminated water after bathing in contaminated freshwater were reported (Ackman *et al.* 1997; Olsen *et al.* 2002).

The coastal environment is a receptacle of agricultural and urban waste water effluents, and STEC could be present in coastal areas which are particularly exposed. Because of their filter-feeding activities, shellfish can concentrate and retain pathogenic microbes present in waters of their environment. The risk to public health associated with the consumption of shellfish, traditionnally consumed raw or undercooked, is well documented (Lipp and Rose 1997; Feldhusen 2000; Potasman *et al.* 2002). Pathogenic micro-organisms such as human gastrointestinal viruses (norovirus, hepatitis A virus), enterobacteria such as *Salmonella* or marine bacteria such as pathogenic halophytic vibrios species were yet implicated in shellfish foodborne diseases (Wallace *et al.* 1999; Potasman *et al.* 2002). In Europe, to prevent shellfish consumer health from enteric microbial pathogens, shellfish production areas were classified on the basis of the shellfish microbial contamination by the faecal indicator, *E. coli* (European Directive 91/492/EEC; Anon. 1991). This reglementation determines the level of treatment such as depuration or relay which needs to be applied prior to the commercialisation of shellfish. Shellfish to go for human consumption must not contain more than 230 *E. coli* per 100 g of shellfish flesh (shellfish from A-category production areas). Those with higher numbers of *E. coli*, corresponding to shellfish from B-category (<4 600 *E. coli* per 100 g in 90 % of samples) have to be depurated for at least 48 hours. Those ones corresponding to shellfish from C-category (<46 000 *E. coli* per 100 g in 90 % of samples) have to be relayed in special relaying area for at least 2 months prior to sale for consumption. Shellfish in D-category (>46 000 *E. coli* per 100 g) is not allowed for commercialisation.

Among bacterial pathogens, the prevalence of *E. coli* O157:H7 and other STEC in shellfish from farming areas in France was not yet investigated. Therefore, we have explored shellfish from representative coastal areas in France for the presence of STEC and have characterized the STEC strains for serotypes and their virulence properties.

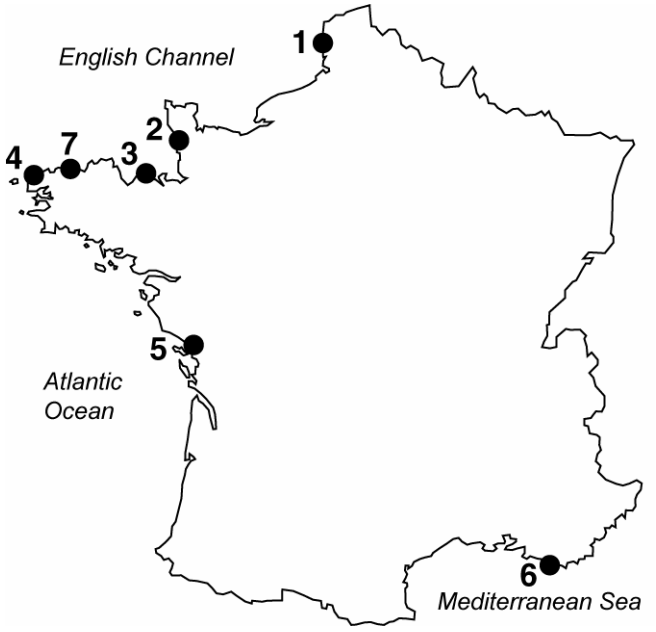
MATERIALS AND METHODS

Origin and collection of shellfish and water samples

Seventy-two batches of mussels (*Mytilus edulis* and *M. galloprovincialis*) (n=50 to 250), oysters (*Crassostrea gigas*) (n=20 to 25) and *cockles* (*Cerastoderma edul*e); (n=150) were collected from French coastal and estuarine environments (Fig. 1). Each shellfish batch was divided into three equal parts of live bivalve molluscs. These were analyzed as follows: part one: for STEC and *E. coli* O157 from total shellfish flesh (SF),

part two: for STEC and *E. coli* O157 from dissected hepato-pancreas (Hp), and part three: enumeration of *E. coli* counts from total shellfish flesh.
A part of the batches (sixty-five) originated from six sampling stations of areas of B-category (see above for the description of such a category; European Directive 91/492/EEC). The remaining shellfish originated from a D-area which is considered to be highly contaminated with microorganisms. The sampling station is located at the outlet of a sewage treatment plant and close to agricultural activities. Sampling was carried out for several times from July 2002 until August 2004 (Fig. 1).
Mussel samples were collected from the coasts of the English Channel (site 1, 2, 7), Atlantic Ocean (site 5), and Mediterranean Sea (site 6). Oysters (sites 3, 4, 7) and cockles (site 3) were collected from the English Channel coastal area.
Since *stx* genes or STEC strains were detected in several shellfish batches collected at site 2 (shellfish-growing area; English Channel), we became interested to study the impact of upstream waters on shellfish contamination. For this, upstream waters from site 2 were collected at the same time as the shellfish samples (November 2003 and June 2004).

Figure 1. Map of French coastal areas. Sites where mussels (1, 2, 5, 6), oysters (3, 4, 7) or cockles (3) were collected. Sites 1, 2, 3, 4, 5, 6 correspond to areas in B-category. Site 7: D-category contaminated area at the outlet of a sewage treatment plant.



Sample preparation for detection of STEC

Shellfish batches were transported immediately (from sites 4 and 7) or within 24 hours (from sites 1, 2, 3, 5 and 6; by road carriers) to the laboratory in insulated coolers. The samples were analysed immediately upon arrival, or held at 4°C for no longer than 48 h before analysis. Shellfish were scrubbed under running tap water to remove debris and attached algae and opened aseptically. Analyses were performed on total shellfish flesh or on hepato-pancreas dissected.
Total shellfish flesh (more than 100 g) was diluted in buffered peptone water (BPW) (1:2) and blended with a Waring commercial blender (Waring Products Division, New Hartford, CT, USA; at high speed for 90 s). Then, 50 ml of homogenized broth (containing 25 g of shellfish flesh) were diluted in more BPW to obtain a 1:10 dilution. The digestive gland was dissected from more than 100 g of total shellfish flesh and around 10 g of hepato-pancreas were diluted at 1:3 in BPW, homogenized (Polytron ; Kinematica AG, Luzern , Switzerland) for 1 min and placed on ice until analysis. This suspension was, then diluted in prewarmed BPW to obtain a 1:10 dilution.
From each homogenized shellfish flesh or digestive gland BPW broth, one part was incubated 6 h at 37°C without agitation for the detection of *E. coli* O157 by IMS (immuno magnetic separation) technique (see below) and another volume was incubated for 20-24 h at 37°C for the detection of STEC by PCR technique.

As the preliminary assays have shown the presence of *stx*-PCR inhibitors in shellfish tissues and a negative effect of shellfish components on the IMS, different purification steps were tested before DNA extraction and IMS was performed. In order to study the effect of these procedures on the *stx*-gene specific PCR, shellfish flesh was contaminated with an *E. coli stx2e*-positive strain (EVL.02.1) at the final concentration of 3 800 or 3.8 X 10^6 colony-forming units (CFU) per 100 g. Enrichment cultures were grown as described above and subjected to different purification treatments as 1) a filtration through the filter of a stomacher bag, 2) a 1 000 g-2 min centrifugation, or 3) an 8-0.22 µm filtration of shellfish enrichment broths. The latter procedure was performed as follows: the enrichment broths were filtered through an 8 µm – membrane, followed by filtration through a 0.22 µm–membrane and the bacteria retained on the 0.22 µm filter were resuspended in phosphate-buffered saline (PBS). DNA extraction and PCR *stx* detection were performed on these suspensions as described below.
Water samples were transported within 24 hours to the laboratory in insulated coolers and a part (250 ml or one liter in November and in June, respectively) was filtered on Nucleopore 0.22 µm (Dominique Dutscher S. A., Brumath, France) and retained bacteria were resuspended in BPW. Enrichment broths were incubated 20-24 h at 37°C. *Stx* genes and STEC strains detection were then performed as described for shellfish.

E. coli enumeration

The faecal contamination (*E. coli*) of shellfish was estimated by the AFNOR standardized five-tube MPN method (Anon. 2000) or impedance measurement (Anon. 2002; Dupont *et al.* 2004). Water *E. coli* enumerations were performed by microplate methods (EN ISO 9308-3; Anon. 1998).

DNA extraction from enriched samples

For each sample, DNA was extracted from enrichment broths before or after an 8-0.22 µm filtration step. A modified method from Fagan *et al.* (1999) was used for DNA sample preparation. One millilitre of the 20-24 h-sample broth was centrifuged at 12 000 g for 3 min. The pellet was washed twice with PBS with Tween 20 (2%) and three times in PBS alone. After centrifugation, 100 µl or 400 µl of InstaGene Matrix (Bio-Rad, Hercules, USA) were added to the pellet from filtered broths or not, respectively. The mixture was incubated at 56°C for 30 min, vortexed for 15 s and then incubated at 100°C for 15 min. After incubation, these suspensions were vortexed for 15 s, centrifuged at 12 000 g for 10 min and the supernatant was conserved at –80°C for PCR analysis.

PCR detection of _stx_ genes

Detection of *stx* genes was performed with degenerated primers ES149 and ES151 as described by Read *et al.* (1992). These primers amplify a DNA region which is conserved in the *stx1* and *stx2* genes, generating a 323-bp fragment (Table 1). Ten microliters of the DNA supernatant were added to a reaction mixture containing 5 µl of 10X PCR buffer (supplied with the enzyme; Tris-HCl 100 mmol l^{-1}, $MgCl_2$ 15 mmol l^{-1}; KCl 500 mmol l^{-1}, pH 8.3; Roche Diagnostics, Meylan, France); 200 µmol l^{-1} of each deoxynucleoside triphosphate; 1.6 µmol l^{-1} of each primer and 1 U Taq DNA polymerase (Roche Diagnostics) for a final volume of 50 µl. The amplification were performed in a Peltier Thermal Cycler (PTC 200; MJ Research, Waltham, MA, USA) with the following cycle conditions: initial denaturation at 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 s, primer annealing at 49°C for 30 s and elongation at 72°C for 30 s, followed by a 7 min-extension at 72°C and holding at 4°C. Amplified products (15 µl) were visualised by ethidium bromide staining of 1.5% (w/v) agarose gels after electrophoresis.

Table 1. PCR primers used in this study.

Primers	Primer sequences (5' →3')*	Gene	Amplicon size (bp)	References
ES149	CGAAATYCYCTCTGTATYTGYC	*stx*	323	Read *et al.* 1992
ES151	GARCRAAATAATTTATATGT			
LP30	CAGTTAATGTGGTGGCGAAGG	*stx1*	348	Cebula *et al.* 1995
LP31	CACCAGCAATGTAACCGCTG			
LP43	ATCCTATTCCCGGGAGTTTACG	*stx2*	584	Cebula *et al.* 1995
LP44	GCGTCATCGTATACACAGGAGC			
stx1cF	TTTTCACATGTTACCTTTCCT	*stx1c*	498	Zhang *et al.* 2002
stx1cR	CATAGAAGGAAACTCATTAGG			
Vt1AvarF	CTTTTCAGTTAATGCGATTGCT	*stx1d*	192	Bürk *et al.* 2003
Vt1AvarR	AACCCCATGATATCGACTGC			
VS1	CATAGTGGAACCTCACTGACGCT	*stx1*	88	Sharma *et al.* 1999
VS2	TTTGCCGAAAACGTAAAGCTTCA			
Stx1F	GACTGCAAAGACGTATGTAGATT CG	*stx1*	150	Ibekwe *et al.* 2002
Stx1R	ATCTATCCC TCTGACATCAACTGC			
Vt1F	GGATAATTTGTTTGCAGTTGATGT C	*stx1*	107	Nielsen and Andersen 2003
Vt1R	CAAATCCTGTCACATATAAATTAT TTCGT			
VTEa	CCTTAACTAAAAGGAATATA	*stx2*e	230	Johnson *et al.* 1990
VTEb	CTGGTGGTGTATGATTAATA			
PT2	GCGAAAACTGTGGAATTGGG	*uidA*	252	Cebula *et al.* 1995
PT3	TGATGCTCCATAACTTCCTG			
hlyAF	GCATCATCAAGCGTACGTTCC	*ehxA*	534	Paton and Paton 1998b
hlyAR	AATGAGCCAAGCTGGTTAAGCT			
eaeP1	CTGAACGGCGATTACGCGAA	*eae*	917	Reid *et al.* 1999
eaeP2	CCAGACGATACGATCCAG			
VT1AF	TCGTATGGTGCTCAAGGAGT †	*stx1*	1335	Bürk *et al.* 2003 (VT1AF)
VT1BR2var	CAGGCAACTGTCAACTGACT †			This study

Isolation of *E. coli* O157

Escherichia coli O157 was isolated using the IMS method (ISO 16654; Anon. 2001) from 6 h-enrichment broths prepared with homogenized whole shellfish and digestive gland tissues. One hundred and eight enrichment broths prepared with total shellfish flesh or hepato-pancreas were tested directly and after a filtration step. Bacteria were recovered from cefixine tellurite sorbitol MacConkey (CT-SMAC) and O157:H7 ID media (BioMérieux, Marcy l'Etoile, France). Suspected colonies were tested with a latex agglutination *E. coli* O157 kit (Oxoid Ltd., Basingstoke, England) and with biochemical API 20E strip (BioMerieux, Marcy l'Etoile, France).

Isolation of STEC strains

Escherichia coli O157:H7 strain ATCC 43895 which carries a *stx1* and a *stx2* gene was used for generating the *stx* gene probe. DNA was extracted from strain culture and PCR for *stx* genes, using degenerated

primers ES149 and ES151 (Table 1) was performed as described previously (Bouvet *et al.* 2002). PCR products were separated on and excised from a 2% (w/v) agarose gel and purified using the Agarose Gel DNA Extraction Kit (Roche Applied Science, Meylan, France). Digoxigenin labelling was performed with DIG-High Prime kit (Boehringer Mannheim GmbH, Germany) as recommended by the manufacturer.
Samples that gave a positive result with the *stx*-PCR were investigated by colony DNA-hybridization. For this, 100 µl of the 10^{-4} and 10^{-5} dilution of enriched samples were spread onto MacConkey (bioMérieux, Marcy l'Etoile, France) or trypticase soya agar plates (AES, Combourg, France). After a 24 h-incubation at 37°C, the plate with the highest number of isolated colonies was selected for colony hybridization and precooled at 4°C for 30 min. Prehybridization was performed with 10 ml of hybridization solution (5X SSC, blocking reagent, 1.5% (w/v), N-lauroylsarcosine, 0.1% (w/v), SDS, 0.02% (w/v) at 60°C for 2 h. Hybridization was carried out for 18-20 h at 60°C with 5 ml of hybridization solution containing 100 ng of freshly heat-denaturated DIG labelled probes. The membranes were washed twice for 5 min in 2X SSC, SDS, 0.1 % (w/v) at room temperature and twice for 20 min in 0.1X SSC, SDS, 0.1 % (w/v) at 60°C. The immunological detection with alkaline phosphate-conjugated antibody was performed according to manufacturers (Dig-High Prime, Boehringer Mannheim GmbH, Germany). Hybridizing colonies were isolated from the master plate and further investigated as described below.

Bacterial strain identification and characterisation

All isolates were confirmed biochemically as *E. coli* using the API 20E test (bioMérieux, Marcy l'Etoile, France). The presence of *stx* genes in these strains was, first, investigated by PCR with primers ES149 and ES151 (Table 1). Stx-positive strains were further tested for the presence of the following genes: *stx1*, *stx2* and *uidA* (Cebula *et al.* 1995), *eae* (Reid *et al.* 1999), *ehxA* (Paton and Paton 1998b) and *stx2e* (Johnson *et al.* 1990; Table 1). Some *stx* probe positive-strains were tested positive with common *stx* primers ES149 and ES151 but were negative for *stx1* and *stx2* genes using the PCRs described by Cebula *et al.* (1995, Table 1). For testing the presence of *stx*-variants which would possibly not be detected with these primers we used other *stx1* or *stx1*-variant specific primers to characterize these *stx*-positive strains as described by Sharma *et al.* (1999), Ibekwe *et al.* (2002), Nielsen and Andersen (2003) for *stx1*; Zhang *et al.* (2002) for *stx1c* and Bürk *et al.* (2003) for *stx1d* (Table 1).
All STEC strains were investigated for motility and biochemical properties (fermentation of sorbitol in 24 h, β-D-glucuronidase activity and enterohemolytic phenotype) and were serotyped for their O and H antigens as previously described (Orskov and Orskov 1984; Beutin *et al.* 1996; Machado *et al.* 2000). The Vero cell toxicity assay was performed according to a protocol described previously (Beutin *et al.* 1998; Beutin *et al.* 1999).

DNA sequencing

First amplified products obtained by *stx* PCR with primers ES149 and ES151 (Table 1) from enrichment broths prepared with shellfish flesh from naturally-contaminated shellfish were sequenced to know if the protocol was suitable for shellfish matrix. PCR products obtained with primers VT1AvarF-VT1AvarR (Table 1) from some *stx*-positive strains (IF Ec LR2, IF Ec AB8SF, IF Ec AB8Hp) were also sequenced in both directions. Then, PCR products obtained with primers VT1AF-VT1BR2var (complete sequence for *stx1*; Table 1) were sequenced in both directions by primer walking. The amplified products were pooled (275 µl) and used for sequencing. Separation was performed in a 2% (w/v) low-melting agarose gel (NuSieve GTG agarose; BMA, Rockland, ME, USA) in TBE buffer at 110 v for 2 h. Fragments of the expected size were cut, weighed and purified using Qiagen columns (QIAquick Extraction Kit; Qiagen S.A., Courtaboeuf, France), according to the supplier's recommendations. Nucleotide sequences of purified PCR products were determined using an Applied Biosystems 3730 XL automated DNA sequencer (Genome Express, Meylan, France).

Nucleotide sequence accession number

The nucleotide sequence of the *stx1* variant genes present in strains IF Ec AB8SF, IF Ec AB8Hp and IF Ec LR2 were entered into GenBank database under accession numbers AY986980, AY986981 and AY986982.

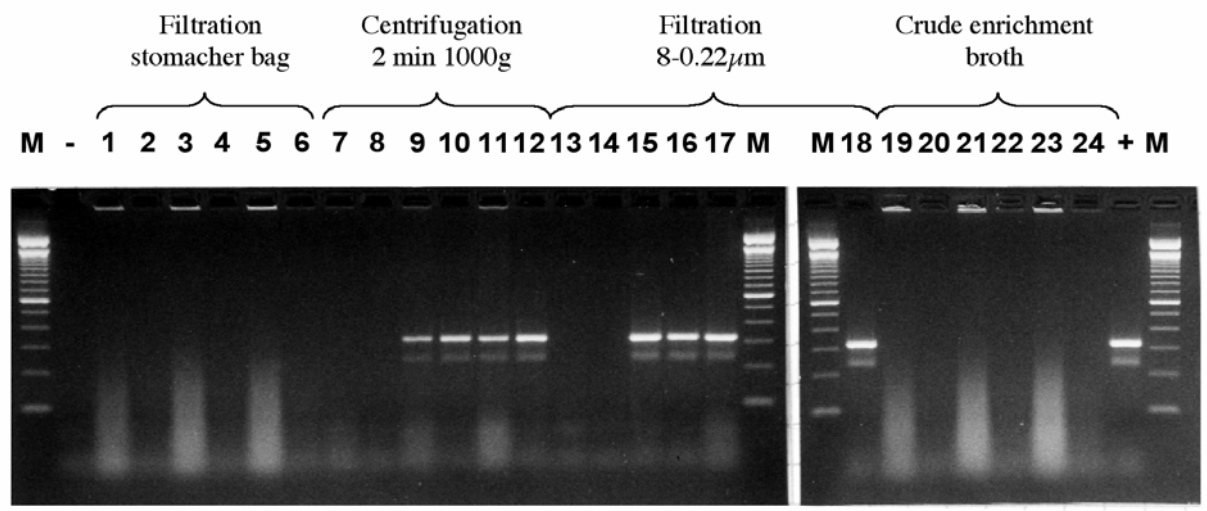
RESULTS

Improvement of PCR and IMS protocols for detection of STEC and *E. coli* O157 in shellfish

The PCR-protocol used for detection of STEC was previously tested on food- and on samples from animals and allowed the detection of 1 to 10 CFU of STEC per sample (Bouvet *et al.* 2001). In this study, the

assays performed on seeded shellfish flesh showed that additional centrifugation (2 min-1 000 g) or filtration (8 µm and 0.22 µm pore size membranes) of the enrichment broths before nucleic acids extraction increased the sensitivity of the PCR (Fig. 2).
These purification steps allowed the elimination of most of shellfish debris which disturbed the *stx*-PCR and which produced non-specific interactions with O157-beads (data not shown). The investigation of STEC and *E. coli* O157 in naturally contaminated shellfish confirmed a more sensitive detection of *stx* genes by PCR, and a more specific detection of *E. coli* O157 with IMS, using an 8-0.22 µm filtration of the enrichments before performing the analysis. Fourteen (35%) of the 40 enrichment cultures from shellfish which were positive for *stx* genes, were only detectable when the filtration step was performed before PCR.

Figure 2. Effect of centrifugation and filtration of shellfish enrichment broths on PCR sensitivity. Lanes: M, molecular weight size marker (100-bp DNA ladder); -, PCR-negative control; 1-6, enrichment broths filtered (stomacher bag); 7-12, centrifugation (2 min, 1 000g) of enrichment broths; 13-17, 18, enrichment broths filtered on 8 and 0.22 µm; 19-24, crude enrichment broths; +, PCR-positive control (strain *stx2e* positive, EVL 02. 1).Lanes 1-2, 7-8, 13-14, 19-20: DNA extracts from shellfish flesh (SF) without *E. coli*, 3-4, 9-10, 15-16, 21-22: shellfish flesh with 3 800 CFU per 100 g of SF; 5-6, 11-12, 17-18, 23-24: shellfish flesh seeded with 3.8 X 10^6 CFU per 100 g of SF. DNA extracts were tested not diluted (ND; odd numbers) and diluted at 1/10 (even numbers).



Detection of s*tx* genes

Stx genes were detected in 40 of 144 (27.8%) enrichments from shellfish flesh or hepato-pancreas. They were present in 32 of 130 enrichment broths (24.6%) from shellfish batches collected from B-category shellfish-growing areas or natural beds and in eight of 14 (57.1%) from D-category area.
Stx genes were detected in all shellfish species analysed and at least once in each of the seven sites investigated (Table 2) and positive samples were detected during the whole year. At site 2, monthly analyses were performed between July 2002 and August 2004 (16 sampling of shellfish). *Stx*-genes were detected in samples taken at different times and not only during the summer months when the excretion of STEC seemed to be more important (Chapman *et al.* 1997).

Table 2. Detection of STEC and *stx* genes in shellfish from coastal environment

Collection sites (Area*)	Shellfish species	Stx-positive SF† enrichments / total no. of SF† enrichments	Stx-positive HP‡ enrichments / total no. of HP‡ enrichments	Stx-positive enrichments / total no. enrichments (% *stx*-positive enrichments)	*E. coli* count per 100 g of SF			Isolation of STEC (no. of STEC strains)§
					Geometric mean	Range	No. of samples	
Site 1 (B)	Mussel	4/8	3/8	7/16 (43.8)	619.2	<100 - 14 000	8	N
Site 2 (B)	Mussels	5/16	7/16	12/32 (37.5)	372.4	<100 - 4 900	16	P (2)
Site 3 (B	Oysters	1/10	1/10	2/20 (10)	324.6	<100 – 2 800	10	N
	Cockles	2/4	2/4	4/8 (50)	964.2	200 – 4 100	4	N
Site 4 (B)	Oysters	2/11	1/11	3/22 (13.6)	498.3	<100 – 1 400	11	P (2)
Site 5 (B)	Mussels	2/8	1/8	3/16 (18.8)	116.1	<18 – 1 300	8	P (1)
Site 6 (B)	Mussels	1/8	0/8	1/16 (6.3)	267.8	<100 – 4 900	8	N
Site 7 (D)	Oysters	4/7	4/7	8/14 (57.1)	2 419.3	<100 – 160 000	7	P (1)

*Shellfish from B-category were collected in growing areas or natural beds farmed or not;

†SF, shellfish flesh;

‡Hp, hepato-pancreas;
§N, negative; P, positive

Isolation and characterization of STEC from shellfish

Five *stx*-positive *E. coli* non O157 strains were isolated from five of the 40 (12.5%) *stx* gene-positive enrichments. The strain characteristics are presented in Table 3. The strains showed a common biotype profile of *E. coli* and were positive for fermentation of sorbitol, motility, and for β glucuronidase. All five strains tested *stx*-positive with the common *stx*-primers of Read *et al.* (1992) and shown to produce cytotoxins by the Vero cell assay. Two of these strains (IF Ec PB1; IF Ec PB4) were positive for *stx1* with primers LP30-LP31 and negative for *stx2* with primers LP43-LP44 of Cebula *et al.* (1995). The remaining three strains (IF Ec LR2; IF Ec AB8SF; IF Ec AB8Hp) were negative with both (LP30-LP31 and LP43-LP44) primer sets.
As the total *stx* gene of these three strains was sequenced, we observed that LP30 and LP31 primer has four (positions 5, 8, 10 and 12; 3'-terminus) and one (first position; 3'-terminus) mismatches, respectively. This could explain that no product was obtained by LP30-LP31 primer pair with these three strains. However, these ones were found to carry *stx1* genes using other PCR primers: VS1-VS2, Stx1F-Stx1R, Vt1F-Vt1R and stx1cF-stx1cR (Table 1) and found to carry *stx1d* genes using specific primers Vt1AvarF-Vt1AvarR (Table 1). All *stx*-positive strains from our study were negative for *eae* and *ehxA* genes.

Table 3. Phenotypic and genotypic characterisation of *E. coli* strains isolated from shellfish.

			Description strain-positive sample									genes		
Date of isolation	Site	Strain number	Shellfish	SF or Hp	*E. coli* count *	Serotype	SOR	Hemolysin phenotype	GUD	mobility	VCA	*stx (type)*	*eae*	*ehxA*
23.09.02	2	IF Ec PB1	Mussels	SF	189	O38:H26	P	N	P	P	P	P (*stx1*)	N	N
06.01.03	5	IF Ec LR2	Mussels	SF	1 300	O100:H21	P	N	P	P	P	P (*stx1d*)	N	N
17.07.03	2	IF Ec PB4	Mussels	SF	120	O38:H26	P	N	P	P	P	P (*stx1*)	N	N
02.10.03	4	IF Ec AB8SF	Oysters	SF	1 400	O149:H1	P	N	P	P	P	P (*stx1d*)	N	N
02.10.03	4	IF Ec AB8Hp	Oysters	Hp	1 400	O149:H1	P	N	P	P	P	P (*stx1d*)	N	N
08.09.03	7	IF Ec SP5	Oysters	SF	3 100	O157:H7	N	P (Ehly)	N	P	N	N	P	P

**E. coli* count per 100 g of shellfish flesh;

SOR, Sorbitol fermentation; GUD, ß-glucuronidase activity; VCA, Vero cell assay; Hp, hepato-pancreas; SF, total shellfish flesh; N, negative; P, positive; Ehly, hemolysis after a 22h incubation step.

Analysis of the sequence data of the *stx1d*-positive strains (IF Ec AB8SF, IF Ec AB8Hp, IF Ec LR2) showed that they are identical (100% nucleotide homology) among each other and show 99% nucleotide homology with *stx1d* (strain MHI 813; Bürk *et al.* 2003), *stx1v52* and *stx1v51* genes (strains 92-1251 and 92-1252; Ohmura-Hoshino *et al.* 2003). The comparison of nucleotide and translated sequences of the A-subunit and the B-subunit of the Shiga-toxin of the strains isolated in this study and those described by Bürk *et al.* (2003) and Ohmura-Hoshino *et al.* (2003) shows that: 1) the nucleotide sequence of *stx1A* differed by 1 bp from *stx1dA*, 1 bp from *stx1v52A* and 3 bp from *stx1v51A* (positions 357; 477; 477, 546 and 693 from 5'-terminus, respectively), 2) the nucleotide sequence of *stx1B* by 2 pb from *stx1dB*, by 1 pb from *stx1v52B* and by 1 pb from *stx1v51B* (positions 122 and 221; 122; 122 from 5'-terminus, respectively) and 3) while the aminoacids deduced from these sequences were identical (Bürk *et al.* 2003 and Ohmura-Hoshino *et al.* 2003).

Detection of *E. coli* O157

Only one strain of *E. coli* O157, which was *stx*-negative, but *eae* and *ehxA*-positive, was isolated by IMS method from one of 108 enrichments from SF or hepato-pancreas tested. *Escherichia coli* O157-positive shellfish were collected at the outlet of a sewage treatment plant (site 7; September 2003; Table 3). This strain was isolated from a total shellfish flesh and did not show toxicity on Vero cells.
Other *E. coli* strains, *Citrobacter freundii* and *Enterobacter cloacae* were also isolated on CT-SMAC or O157:H7 ID media after IMS on 6 h-shellfish enrichment broths. They were all found *stx*-negative by PCR. In a small number of samples, bacteria such as *Aeromonas* spp. or *Shewanella putrefaciens*, present in the shellfish background flora, grew in shellfish enrichment broths. After IMS, these bacteria were isolated as characteristic non-sorbitol fermenting colonies on CT-SMAC and latex positive colonies. They were distinguished from *E. coli* O157 only by API20E test.

***E. coli* as indicator of faecal contamination of shellfish**

The total *E. coli* counts in shellfish batches were determined. The geometric means and range of shellfish *E. coli* concentrations measured, from July 2002 to August 2004, are given in Table 2, site by site. They show an important heterogeneity depending on shellfish sampling. In B-category areas, shellfish *E. coli* concentrations mainly vary from <100 to 4 600 *E. coli* per 100 g of SF. However, higher concentrations could also be observed. For example, a concentration of 14 000 *E. coli* per 100 g of SF were once recorded at the site 1. This result leads to particularly high range for *E. coli* concentrations at this site.

Stx-positive PCR results were obtained in 11.7% of the enrichments from weakly contaminated (<230 *E. coli* per 100 g SF; seven of the 60 enrichment broths tested) shellfish batches, in 33.8% of enrichments from moderately (between 230 and 4 600 *E. coli* per 100 g of SF; 25 of the 74 enrichments broths tested) and in 70% of the enrichments from highly contaminated shellfish batches (> 4 600 *E. coli* per 100 g of SF; seven of the 10 tested). Thus, the probability to obtain *stx*-positive results correlated with the total numbers of *E. coli* found in shellfish. However, *stx*-positive samples were detected even among the weakly contaminated ones.
The strains which were isolated in this study came from weakly (IF Ec PB4, IF Ec PB1) and moderately contaminated (IF Ec LR2, IF Ec AB8SF, IF Ec AB8Hp, IF Ec SP5; Table 3) shellfish samples. So, there was no relation between the presence of viable STEC strains and total *E. coli* count in shellfish.

Stx gene detection and STEC isolation in waters upstream shellfish (site 2)

In order to identify the origin of *stx* genes and STEC strains detected in shellfish, upstream water and seawater was investigated in spring and in winter at site 2 shellfish-growing area. *Stx* genes were detected in all of the 8 water samples (sewage, brackisch water, freshwater or seawater) which were collected in November 2003, and in seven of the eight water samples (only seawater W4b was *stx*-negative) collected in June 2004 (Table 4). STEC strains could not be isolated from the water samples. *Escherichia coli* concentrations varied remarkably from one water sample to another and bacterial numbers were about 10-fold higher in November 2003 than in June 2004 (Table 4).

Table 4 *E. coli* concentration and *stx* genes detection by PCR after enrichment of waters collected upstream from shellfish-farming area or of shellfish collected in November 2003 and June 2004. Water temperature varied from 10.2 to 13.4°C in November and from 14.1 to 16.9°C in June.

Water or shellfish sample	Date	Type of water or of shellfish*	*E. coli* concentration per 100 ml of water or per 100 g of SF†	*Stx*-PCR results‡
W1a	November 26, 2003	Freshwater	4 669	P
W2a		Brackish water	1 450	P
W3a		STP outfall (beach)	3 530	P
W4a		Seawater	676	P
W5a		Inlet of STP	$2.11\ 10^7$	P
W6a		Freshwater	577	P (weak)
W7a		Freshwater	57 100	P
W8a		Freshwater	20 270	P
S1a		Mussels	4 900	P
W1b	June 1, 2004	Freshwater	200	P
W2b		Brackish water	1 010	P
W3b		STP outfall (beach)	410	P (weak)
W4b		Seawater	127	N
W5b		Inlet of STP	$7.7\ 10^6$	P
W6b		Freshwater	80	P
W7b		Freshwater	860	P
W8b		Freshwater	350	P (weak)
S1b		Mussels	1 400	N
S2b		Mussels	2 300	P

*STP, sewage treatment plant;

†SF, shellfish flesh;

‡P, positive; N, negative.

DISCUSSION

One of the objectives of this study was to improve current protocols for the detection of *stx* genes in shellfish collected from the coastal environment. The protocol was based on the detection of *stx* genes in enrichments from total shellfish flesh or from dissected hepato-pancreas by increasing the sensitivity of the detection methods by filtration of enrichment cultures before DNA extraction. Although analyses concerned generally the total shellfish flesh of live bivalve molluscs (Guyon *et al.* 2000; Dupont *et al.* 2004), other pathogens such as vibrios or viruses, were searched in dissected hepato-pancreas rather than in whole shellfish tissue in previous studies (Le Guyader *et al.* 2000; Hervio-Heath *et al.* 2002). In fact, many pathogens were shown to be concentrated in these tissues and less PCR-inhibitors were expected to be present in hepato-pancreas than in whole tissues (Le Guyader *et al.* 2000). In this study, similar results were obtained in the analysis of total SF or Hp [*stx* genes were detected in 21 of 72 (29.2%) enrichment broths from SF and in 19 of 72 (26.4%) enrichments from Hp]. In only nine of 72 (12.5%) shellfish batches, both total shellfish flesh and hepato-pancreas enrichments were *stx* genes-positive. These results could be attributed to the heterogeneity of live bivalve molluscs from a same batch (presence of contaminated and not contaminated shellfish in the same batch) rather than to an influence of the organ part of shellfish analysed.

The results obtained in this study show that shellfish collected in coastal or estuarine environments can be positive for *stx* genes as indicator for STEC or *stx*-encoding phages. Stx-positive samples were obtained from all investigated shellfish species. When oysters and cockles were sampled at the same site and date, *stx* genes were more often found in cockles than in oysters (data not shown). The fact that cockles are burrowing animals, and that oysters are living in water could explain this difference. The sandy or muddy sediments could be more favourable for the survival of STEC than water. Furthermore, with cockles more organisms are needed to obtain a same weight of shellfish flesh than from oysters. Stx genes could be detected in shellfish collected in various areas (at least one positive result was obtained for shellfish from the 7 sites) and at different time periods (positive results were obtained at all seasons).

The frequency of *stx*-positive samples varied depending on the activities upstream and the weather conditions; i. e. abundant rainfall, before sampling, by increasing the run-off was a favourable factor to obtain a faecal bacterial shellfish contamination downstream (this study; Lee and Morgan 2003).

The *stx* genes present in shellfish samples could originate from STEC or from *stx*-bacteriophages. Muniesa and Jofre (1998) have evaluated the prevalence of Shiga toxin-converting phages in waste waters from two origins; the number of phages infectious for *E. coli* O157:H7 and carrying the *stx2* gene was in the range of 1 to 10 per ml of sewage. According to Muniesa and Jofre (2000), Stx-encoding bacteriophages are common in sewage from industrialized countries.

Five *stx1*-positive STEC strains were isolated from oysters and mussels from B-category areas. Three strains from two locations (sites 4 and 5) carried a *stx1d* gene and this is the first report on occurrence of *stx1d*-positive STEC from shellfish. The virulence of these strains for humans is probably limited because major virulence markers such as *stx2* and *eae* genes are absent in these strains.

On the other hand, we isolated one *E. coli* O157:H7 strain which resembled EHEC O157 for its virulence markers and phenotypical traits. This strain was negative for a *stx* gene which might have got lost upon passage from its original source to shellfish. Frequent loss of *stx* genes in EHEC O157 was reported (Karch *et al.* 1992) and *stx*-negative *E. coli* O157 strains have already been isolated in raw cow's milk cheese in Italy (Conedera *et al.* 2004), in heifer faecal samples (Vernozy-Rozand *et al.* 2000) or from human feces (Schmidt *et al.* 1999). The virulence of a *stx*-negative *E. coli* O157:H7 strain is likely to be the same as the one of enteropathogenic *E. coli* such as *E. coli* O55:H7 known to be a major cause of infantile diarrhoea world-wide (Rodrigues *et al.* 1996; Schmidt *et al.* 1999).

Few studies focused on the detection of STEC or *E. coli* O157 strains in shellfish. Moreover, researchers have used different approaches from a study to another. Rampersad *et al.* (1999) and Kumar *et al.* (2001-2004) isolated *E. coli* strains which were then tested for the O157 antigen and other serotypes and for virulence genes. Guyon *et al.* (2000) and MacRae *et al.* (2003) limited their study only to STEC O157 strains searched by the IMS method. Samadpour *et al.* (1994) investigated STEC strains by colony DNA-hybridization from different food enrichments. Dupray *et al.* (1999) detected *stx* genes in shellfish enrichments but no attempt was made to isolate STEC strains from positive samples. The low recovery rates for *E. coli* O157:H7 for STEC strains from *stx*-PCR positive enrichments in our study is in agreement with other reports on STEC in shellfish (Guyon *et al.* 2000; Kumar *et al.* 2001-2004; MacRae *et al.* 2003). The low frequency of isolated STEC strains from shellfish could be explained by: 1) low numbers of these bacteria in coastal environment, 2) the difficulty to isolate these strains from shellfish samples with an important background flora, 3) the presence of viable but non culturable STEC due to environnental stress in the marine environment (Troussellier *et al.* 1998; Rozen and Belkin 2001), 4) the presence of free *stx*-bacteriophage or phage DNA in samples.

Serotyping STEC strains from shellfish showed new serotypes such as O38:H26 and O149:H1, and serotype O100:H21 which is associated with STEC from pigs (L. Beutin, pers. comm.). These results confirm that

environment is a reservoir in which new STEC strains can emerge (Vernozy-Rozand *et al.* 2004) and point to domestic animals as possible contamination source. As shellfish came from coastal environment and were analysed just after collection, the STEC contamination observed in this study which occurred in shellfish-farming areas is probably due to upstream waters of agricultural or urban origin. Two strains with the same serotype and the same genetic profile were isolated in shellfish collected in September 2002 and July 2003 from the same area (site 2). A better evaluation of potential sources in this area could give some useful information about the origin of these strains. The first assays performed on the upstream waters and shellfish failed to isolate STEC strains in any of the samples. However, *stx* genes were detected in most of the water samples from various origins and in shellfish. These results underline the existing difficulty to identify the potential source of these *stx* genes and indicate that shellfish contamination could result from several sources at the same time.

As we detected, one strain *E. coli* O157 *stx*-negative and *stx* genes in the same shellfish enrichment, it would be useful to know if conversion of *E. coli* strains by *stx* bacteriophages encoding *stx* gene could occur in coastal environment especially in shellfish. To this date, no study has focused on detection of *stx* bacteriophages in coastal environment. The conversion of *E. coli* strains by phages in the environment has not been observed yet but it would be an important question to elucidate (Schmidt 2001).

In shellfish, we could not find any relation between the presence of STEC and the total count of *E. coli* as indicator for faecal contamination. This result is in accordance with previously published data on other products (Chapman *et al.* 2001; Conedera *et al.* 2004). If for the most highly contaminated shellfish batches *stx* gene was mostly present (however no strain was isolated), five batches with *E. coli* concentration weaker than 230 bacteria per 100 g of SF were also found positive for *stx* gene and allowed the isolation of two STEC strains. It is noteworthy that 1) shellfish were collected in B-category shellfish growing areas or natural beds and were under possible influence of various faecal inputs, and 2) even if shellfish enrichments were found *stx*-PCR positive, STEC strains could be present at low concentration in shellfish before enrichment.

The risk of a human infection by STEC due to shellfish consumption seems to be limited for two reasons. First, a 48 h-depuration step has to be performed before commercialisation of shellfish from B-category. Second, STEC were present in low numbers in the samples and the STEC isolates lacked genes associated with high human virulence such as *eae* and *stx2*. However, the presence of *stx* genes in 27.8% of enrichments from shellfish collected and the positive isolation of STEC strains lead to recommend: 1) a tracking of agricultural and urban sources upstream shellfish-farming areas and 2) a limitation of their input to prevent a shellfish contamination by STEC strains.

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