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Successful detection of pathogenic Shiga-toxin-producing *Escherichia coli* in shellfish, environmental waters and sediment using the ISO/TS-13136 method

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Abstract :

The presence of highly pathogenic Shiga-toxin-producing *Escherichia coli* (STEC) in shellfish, upstream waters and sediment from coastal shellfish sites was evaluated using the ISO/TS-13136 method. Shellfish (oysters, mussels and cockles), water and sediment samples were collected monthly over a period of 1 year. The method used real-time PCR detection of stx1, stx2 and eae genes and genetic markers corresponding to the five major serogroups (O157, O26, O103, O111 and O145) on enrichment broths and the identification of STEC when these genes and markers were detected. stx genes were detected in the broth of 33% of shellfish batches (n = 126), 91% of water samples (n = 117) and 28% of sediment (n = 39). One stx1+, eae+ O26:H11 strain was isolated from a shellfish batch, and O26:H11, O145:H28 and O103:H2 strains without the stx gene (n = 9) were isolated from shellfish and waters. In conclusion, this study shows the suitability of the ISO/TS-13136 method to assess the presence of highly pathogenic E. coli strains in shellfish farming areas. It also highlights a low prevalence of STEC and consequently suggests a reduced corresponding human health risk.

Keywords : eae, sediment, shellfish, Shiga-toxin-producing *Escherichia coli*, stx, water

Significance and Impact of the Study

(STEC) infections have been reported following ingestion of contaminated food or water or after bathing in contaminated waters. However, to date, few studies concerning their detection in coastal environment and shellfish have been reported. The aim of this work was to assess the presence of STEC in three shellfish-harvesting areas by the ISO/TS-13136 method, which has recently been used for STEC detection in food.

INTRODUCTION

Pathogenic Shiga-toxin-producing *E. coli* (STEC) may cause human illnesses such as haemorrhagic colitis and life-threatening haemolytic-uraemic syndrome. Although a wide range of serotypes have been implicated in human STEC infections, five major serotypes (O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28) are responsible for the vast majority of sporadic cases and outbreaks in Europe (Beutin and Fach 2014). Pathogenic STEC are characterized by the production of Shiga-toxin, a bacteriophage-encoded cytotoxin (O'Brien *et al*. 1984) and, in addition, are often shown to produce attaching and effacing lesions on intestinal mucosa. This latter property is encoded by genes, including *eae*, grouped together in a pathogenicity island referred to as the "locus of enterocyte effacement" (Paton and Paton 1998). Human infection occurs through ingestion of contaminated food or water or by contact with animals. The main reservoir for STEC is domestic ruminants, especially cattle (Pradel *et al.* 2000). Other animals, such as sheep, goats, pigs, birds, other wild animals, as well as humans, can also harbour STEC (Gyles 2007). STEC contamination of the environment may occur through the spreading of livestock manure, animal waste on pastures, via wastewaters from slaughterhouses, from treatment plant effluents and by wildlife (Ogden, *et al*. 2001; Vernozy-Rozand *et al.* 2002; Loukiadis *et al.* 2006). Coastal environments, including shellfish, may therefore become contaminated with STEC. Because of their filter-feeding behaviour and because they are traditionally consumed raw or undercooked, shellfish may present a potential risk to public health (Potasman *et al*. 2002). However, to our knowledge, while they have previously been detected in shellfish, STECs have not yet been reported as being involved in shellfish-borne outbreaks (Guyon *et al.* 2000; Gourmelon *et al.* 2006).

To date, few studies concerning the detection and isolation of pathogenic STEC strains in coastal environments and shellfish have been reported and, of those that have, none has

66 focused on the prevalence of the five major STEC serotypes. Recently, a horizontal PCR-
67 based method has been developed for the detection of STEC (including the five major
68 serotypes associated with human infection) in foods and animal feed. This method,
69 recommended by EFSA (2009), is described in the ISO-13136 Technical Specification
70 (ISO/TS-13136:2012). It has already been applied in France for monitoring control in minced
71 meat frozen beef (DGAL 2013) and to evaluate the prevalence of STEC from their
72 corresponding serotypes in cattle faeces (Bibbal *et al.* 2014).
73 The present study describes the first application of the ISO/TS-13136 protocol to shellfish,
74 environmental waters and sediment in order to evaluate the presence of highly-pathogenic
75 STEC in shellfish-harvesting areas.
76

RESULTS AND DISCUSSION

Level of contamination in water and shellfish

E. coli counts were determined for all shellfish and water samples. Widely varying *E. coli* concentrations were found in shellfish, with concentrations ranging from <67 to 71,000 per 100 g. Thirty-seven samples presented a concentration of less than or equal to 230 *E. coli* per 100 g, 64 contained between 230 and 4,600 *E. coli* per 100 g and 25 presented a concentration greater than 4,600. Among shellfish, cockles were the most contaminated (geometric mean of 2,440 *E. coli* per 100 g *versus* 1,070 for mussels, and 364 for oysters). In waters, *E. coli* concentrations ranged from <38 to 190,530 per 100 ml, with a geometric mean of 1,224.

Detection of *stx1, stx2* and *eae* and prevalence of the five major serogroups

Real-time PCRs enabled detection of *stx* genes from enrichment broths of 44 shellfish batches (34.9%), 107 water samples (91.4%) and 11 sediment samples (28.2%) (Table 1). In correlation with the level of contamination of shellfish, *stx* genes were more frequently detected from cockles and mussels (50.0% and 36.5% stx^+, respectively) than from oysters (22%). The presence of *stx* gene was previously investigated in shellfish batches collected between 2002 and 2004 from across France (Gourmelon *et al.* 2006). In this earlier study, in which 72 samples of mussels (41) oysters (27) and cockles (4) were analyzed, the *stx* gene was also more frequently detected from cockles (100%) than from mussels (43.9%) or oysters (33.3%). The *stx2* gene was slightly more frequently found than *stx1* in enrichment broths from water and sediments, while *stx1* was more frequently detected from shellfish. As with *stx*, *eae* gene was more frequently detected in water than in shellfish or sediments. Indeed, 116 water samples (99.1%), 92 shellfish batches (73.0%), and 19 sediments (48.7%) were

100 *eae*$^{+}$ (Table 1). *eae* was more frequently detected in cockles and mussels (87.5% and 82.7%,
101 *eae*$^{+}$, respectively) than in oysters (56.0% *eae*$^{+}$). A total of 152 samples (37 shellfish, 107
102 waters, and 8 sediments) were positive for at least one *stx* gene and the *eae* gene. Real-time
103 PCRs were performed to detect genetic markers associated with the five major serogroups
104 from the 152 corresponding enrichment broths (Table 2). One or several serogroups were
105 detected from 28 samples of shellfish, 106 waters and 7 sediments. Consequently, 22.2% of
106 shellfish samples, 90.6% of water samples and 17.9% of sediment samples were positive for
107 *stx*, *eae* and one of the O-group markers, with a higher percentage of positive samples among
108 environmental water samples than among cattle faeces collected at slaughterhouse (58% in
109 cattle faeces [Bibbal *et al.* 2014]). Regardless of the sample type, serogroups O103 and O145,
110 followed by O26 and O157 were the most frequently detected.
111
112 **Isolation and characterization of strains belonging to serogroup O157, O26, O103, O111**
113 **or O145**

114 Ten strains (five from water and five from shellfish) were isolated using two complementary
115 methods: direct streaking and IMS (Immuno-Magnetic Separation). Their characterization
116 revealed that six belonged to the O26:H11 serotype, while two were identified as O103:H2
117 and two as O145:H28 (Table 3); all 10 isolates contained the *eae* gene. These strains were
118 isolated from highly contaminated samples (*E. coli* geometric mean of 14,550 per 100 g for
119 shellfish and 4,490 per 100 ml for waters). Only one strain possessed a *stx* gene (*stx1*)
120 whereas the remaining corresponded to potentially enteropathogenic *E. coli* (EPEC). The
121 STEC strain belonged to the O26:H11 serotype and was isolated from mussels. Only 9 of the
122 141 enrichment broths that were positive for *stx, eae* and one of the major serotypes (6%)
123 resulted in STEC or EPEC isolation. We have to consider the possibility that the targeted *stx*,

eae and serotypes markers could also be detected in the absence of culturable STECs. Indeed, their detection can be due to the presence free bacteriophages, dead or viable but non-culturable bacteria, or other bacteria such as *Shigella* and *Citrobacter*, which are also known to carry these genes (Muniesa *et al*. 1999; Martinez-Castillo *et al.* 2013). A poor isolation rate of highly pathogenic STEC was also previously described for other matrices, such as cattle faeces (Bibbal *et al.* 2014), retail-minced beef (Auvray *et al.* 2007) and raw-milk cheeses (Madic *et al.* 2011) from which six (n=150), zero (n=164) and zero (n=400) strains were isolated, respectively. In the present study, EPEC were more frequently isolated than STEC. This may be the consequence of the loss of *stx* genes by STEC discharged in the environment. Indeed, previous studies have demonstrated the capacity of STEC to lose *stx* genes in bovine, avian human and environmental sources (Feng *et al.* 2001; Wetzel and LeJeune 2007).

A low detection rate of STEC in shellfish is in agreement with previous studies. Indeed, three studies previously described STEC belonging to the O157-serogroup in shellfish using an immunomagnetic method. One, conducted in France on 150 oyster samples, led to the isolation of only one O157-*E. coli* strain (O157:H- *stx1*$^{+}$, *stx2c*$^{+}$, *eae*$^{+}$) (Guyon *et al.* 2000), a second failed to isolate O157:H7 strains from 192 batches of different shellfish species in the UK (MacRae *et al.* 2005) and the third described the isolation of one EPEC strain (O157:H7) from 72 shellfish batches collected in France (Gourmelon *et al.* 2006). Other approaches focused on the isolation of *E. coli* strains followed by investigation for the presence of *stx* genes, which demonstrated low percentages of STEC among the isolates (6.2% in shellfish from Morocco [Bennani *et al*. 2011] and 4.1% in clams from India [Sanath Kumar *et al*. 2001]). Furthermore, five STEC strains not belonging to the five major serogroups have previously been isolated in France from shellfish by analyzing STEC by hybridization, following culture enrichment (Gourmelon *et al.* 2006).

These results suggest that STEC and EPEC are occasionally present in the environment and can contaminate shellfish. According to our results, the risk of human infection by highly pathogenic STEC resulting from the consumption of shellfish from the investigated areas seems to be limited as only one STEC strain was isolated from 126 batches taken from the area and shellfish which are from category B or C areas, which are depurated or which undergo protracted relaying prior to sale. As with the *E. coli* indicators, we may expect STEC to be eliminated during the depuration or relaying step. However, further assays are currently being undertaken in our laboratory to compare STEC behavior with that of *E. coli* indicators in shellfish.

CONCLUSION

In conclusion, this study shows that the ISO/TS-13136 protocol effectively detects STEC or EPEC strains belonging to the five major serotypes in shellfish and environmental samples. Even though *stx* and *eae* genes were often detected, the presence of STEC in such environments appears to be limited, as only one STEC and nine EPEC were isolated from water or shellfish samples. Furthermore, the human health risk associated with consuming the shellfish collected from these sites should be reduced as they are depurated or relayed prior to consumption.

MATERIALS AND METHODS

Origin and collection of samples

Three French shellfish sites from the English Channel coastal area were studied. One is located in the “Cotes d’Armor” department (Brittany region) while others are in the department “La Manche” (Lower-Normandy region). All three sites are characterised as

receiving inputs from agricultural catchments with high stocking densities (cattle, sheep, pigs, or poultry) but are also impacted by microbial pollution of human origin. The bays are the focus of extensive shellfish gathering activities. According to the European classification of shellfish harvesting areas (Anonymous 2004), these shellfish sites are classified as category B (*E. coli* <4,600 CFU per 100 g total flesh for 90% of the samples) for mussels and oysters and as category C (*E. coli* <46,000 CFU per 100 g) for cockles. Shellfish from category B areas are only suitable for human consumption following depuration, relaying or cooking by an approved method whereas shellfish from category C areas can only be consumed after relaying for at least two months or cooking by an approved method. For each site, mussels, oysters and cockles were collected monthly, from February 2013 to February 2014. A total of 126 batches of shellfish (52 batches for mussels [n≈70], 50 for oysters [n≈14] and 24 for cockles [n≈80]) were analysed. In parallel, 117 water samples from nine different points located upstream of the shellfish areas and 39 samples of sediment were collected. Samples, which were transported in insulated cooler boxes, were analysed within 24h. Before analysis, shellfish were scrubbed under running tap water to remove debris and algae.

E. coli **enumeration**

E. coli enumeration was performed using the Most Probable Number method in MUG-microtitre plates according to the EN/ISO-9308-3 method (Anon 1998) and using an impedance method according to NF/V-08-106 for shellfish (Anon 2000).

E. coli **enrichment**

After opening, shellfish were crushed in a Warring blender for 60 seconds. Twenty-five grammes of crushed flesh and intravalvular liquid were introduced into 225 ml of buffered peptone water (BPW). For sediment, 10 g were introduced into the same quantity of BPW

while for water samples, 1 litre was filtered using 0.45 µm membranes and the filter placed in 225 ml of BPW. Incubation was performed at 37°C, for 24h.

Detection of *stx* and *eae* genes and markers associated with the five major serotypes

DNA was extracted from 1 mL of enrichment cultures in BPW using an automatic method (EZ1-biorobot, and DNA tissue card kit [Qiagen®, Cournaboeuf, France]). *stx* and *eae* genes were detected by real-time PCR, according to ISO/TS-13136. For samples positive for *stx* and *eae*, genetic markers associated with the major serogroups O157, O26, O103, O111, and O145 *(rfbEO157, wzxO26, wzxO103, wbd1O111,* and *ihp1O145* genes, respectively) were detected by real-time PCR, also according to ISO/TS-13136 and using primers and probes published previously (Nielsen and Andersen 2003; Perelle *et al.* 2007).

Isolation of Shiga toxin-producing *E. coli*

The isolation of STEC strains was performed only for the positive samples for which the targeted combination of genetic marker was detected, *i.e.*, *stx*, *eae* and at least one O group marker. From the positive BPW broths, direct streaking and streaking after immuno-magnetic separation (IMS), were performed on the following media. The O157 serogroups were searched by direct streaking onto chromID™ O157:H7+cefixime-tellurite (BioMérieux®) and streaking after the IMS onto cefixime-tellurite-Sorbitol-MacConkey agar (biokar, Beauvais, France) and chromID™ O157 (bioMérieux, Marcy l'Etoile, France). Streakings for O26 serogroups were performed onto Sorbitol-MacConkey agar (SMAC) (bioMérieux, Marcy l'Etoile, France) and after onto SMAC and chromID™ O26 (bioMérieux, Marcy l'Etoile, France) after the IMS, while serogroups O103, O145 and O111 were directly detected using Posse media (Posse *et al.* 2008) or using SMAC and chromID™ after the IMS. All media were incubated for 18 to 24 h at 37°C. Confirmation of the clones belonging to a particular

serotype and presence of *stx* and/or *eae* genes were then tested by PCR on presumptive colonies as described in ISO/TS-13136. Characterization of the *fliCH* alleles (*fliCH2*, *fliCH7*, *fliCH8*, *fliCH11*, and *fliCH28*) was investigated by PCR, as described by Madic *et al*. (2011).

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CONFLICT OF INTEREST

No conflict of interest declared

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TABLES

Table 1. Prevalence of *stx1*, *stx2* and *eae* genes in enrichment broths.

Sample (n)	*stx1*[*] n (%)	*stx2*[†] n (%)	*stx1* and *stx2* n (%)	Total *stx1* and/or *stx2* n (%)	*eae* n (%)
Cockles (24)	3 (12.5)	3 (12.5)	6 (25.0)	12 (50.0)	21 (87.5)
Mussels (52)	12 (23.1)	8 (15.4)	1 (1.9)	21 (40.4)	43 (82.7)
Oysters (50)	7 (14.0)	2 (4.0)	2 (4.0)	11 (22.0)[‡]	28 (56.0)[§]
Total Shellfish (126)	22 (17.5)	13 (10.3)	9 (7.1)	44 (34.9)	92 (73.0)
Waters (117)	10 (8.5)	21 (17.9)	76 (65.0)	107 (91.4)[¶]	116 (99.1)[**]
Sediment (39)	3 (7.7)	6 (13.4)	2 (5.1)	11(28.2)	19 (48.7)

[*] *stx1* was found but not *stx2*.
[†] *stx2* was found but not *stx1*.
[‡] P value (Khi2 test) between oysters and other shellfish = 0.0136
[§] P value (Khi2 test) between oysters and other shellfish = 3.23×10^{-5}
[¶] P value (Khi2 test) between waters and other samples = 2.33×10^{-22}
[**] P value (Khi2 test) between waters and other samples = 4.87×10^{-11}

371 **Table 2.** Prevalence of strains of the five highly pathogenic serogroups in the BPW broths
372 and number of isolated strains belonging to the STEC or EPEC pathotype.

Sample (n)	*stx*$^+$ and *eae*$^+$	O157$^+$ (%)*	O26$^+$ (%)*	O103$^+$ (%)*	O111$^+$ (%)*	O145$^+$ (%)*	*stx*$^+$, *eae*$^+$ and serotype$^{+\,\dagger}$ (%)‡	isolated strains
Shellfish (126)	37	7 (18.9)	13 (35.1)	20 (54.1)	3 (8.1)	15 (40.5)	28 (22.2)	5
Waters (117)	107	65 (60.7)	67 (62.6)	93 (86.9)	30 (28.0)	83 (77.6)	106 (90.6)§	5
Sediment (39)	8	1 (12.5)	1 (12.5)	5 (65.5)	0 (0.0)	4 (50.0)	7 (17.9)	0

373 * % calculated based on the number of samples *stx*$^+$ and *eae*$^+$.
374 † *stx*$^+$, *eae*$^+$ and at least one of the major serotypes
375 ‡ % calculated based on total number of samples.
376 § P value (Khi2 test) between waters and other samples = 1.62×10^{-30}
377

378 **Table 3.** Characterisation of STEC and EPEC strains isolated from shellfish and water.

Strain	Sample type	*E. coli* count	Serotype	*stx* (type)	*eae*
10	Mussel	15,000*	O26:H11	+ (*stx*1)	+
9	Cockle	12,000*	O26:H11	-	+
7	Mussel	15,000*	O26:H11	-	+
4	Water	3,570†	O26:H11	-	+
5	Water	5,200†	O26:H11	-	+
8	Water	16,620†	O26:H11	-	+
2	Cockle	22,000*	O145:H28	-	+
3	Mussel	11,000*	O145:H28	-	+
1	Water	357†	O103:H2	-	+
6	Water	16,620†	O103:H2	-	+

379 * *E. coli* count per 100 g
380 † *E. coli* count per 100 ml