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Isolation and characterisation of Shiga toxin-producing *Escherichia coli* from Norwegian bivalves

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ABSTRACT

Only a few studies concerning Shiga toxin-producing *E. coli* (STEC) detection in bivalves and their harvesting areas have been reported, and to the best of our knowledge there are no outbreaks associated with STEC from bivalves described. The aim of the present study was to investigate the occurrence of STEC in Norwegian bivalves, and to characterize potential STEC isolated from the samples. A total of 269 samples of bivalves were screened for the presence of *stx* and *eae* genes, and markers for the serogroups O26, O103, O111, O145 and O157 by using ISO TS 13136 (2012). The screening returned 19 samples that were positive for *stx* and *eae*, and attempts of isolation of STEC were made from these samples. Presumptive STEC were obtained from three samples, and three isolates (one from each sample) were subjected to whole-genome-sequencing (WGS). The WGS revealed that one of the isolates did not carry the *stx* genes, while the other two were identified as stx_{2i} positive *E. coli* O9:H19 and stx_{2g} positive *E. coli* O96:H19. Neither of the two STEC isolates were positive for virulence markers such as *eae* and *ehx*. The results suggest that the occurrence of STEC in Norwegian bivalves is low.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic pathogens that have emerged as an important cause of food-borne infections and consequently have become an important public health problem worldwide. In 2016, 6,378 confirmed cases of STEC infections were reported in the EU (EFSA, 2017). The main virulence genes of STEC are the *stx* genes encoding the Shiga-toxins and the *eae*, encoding a 90 kDa intimin protein, involved in the attaching and effacing mechanism of adhesion. The Shiga toxins genes are divided into stx_1 and stx_2, and further subtyping schemes exist (Scheutz et al., 2012). Shiga toxins and intimin represent two of the major virulence attributes of typical STEC strains causing disease in humans, and the presence of these genes are considered trademarks of STEC as a preliminary identification of the pathogenicity of *E.coli* (Paton and Paton, 1998). The severity of an STEC-induced disease vary from mild infections with few symptoms to severe and life-threatening conditions, such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS). In addition to cause such serious infections, the infectious dose for STEC has been described to be as low as 1 to 100 bacteria (Paton and Paton, 1998).

The principal reservoir of STEC is the gastrointestinal tract of ruminant animals, particularly of cattle (Caprioli et al., 2005; Persad and LeJeune, 2015), and since STEC can persist in the environment for a prolonged period of time at different conditions (Bolton et al., 2011), they may as any other *E.coli*, enter the marine environment through sewage and runoff from land.

The areas were bivalves are cultivated and/or collected *e.g.* bivalve harvesting areas, can be influenced by such faecal contamination from land, depending on their proximity to effluent sources, as well as weather and water conditions. Since bivalves filter water as part of their feeding mechanism, they also accumulate particles and microorganisms, including bacteria, from the water column (Cranford et al., 2011). Contaminated food and water are recognised as vehicles for STEC, and although there are no reported STEC outbreaks linked to shellfish (Balière et al., 2016), any edible marine species exposed to faecal contamination could act as potential STEC carriers.

Cultivation of edible bivalves is an important and well-established industry along the coast of Norway. In 2016, totally 2213 tons of cultured and harvested shellfish were landed for sale, and of this, the most common species, the blue mussel (*Mytilus edulis*), comprised 2178 tons (Directorate of Fisheries, 2017). The EU has established regulations for bivalve harvesting areas (Regulation (EC) No 853/2004, 2004), and

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these areas are classified as Class A, B or C according to their level of contamination where *E.coli* function as the faecal indicator organism examined by standardised Most Probable Number (MPN) method (Donovan et al., 1998). According to EU's microbiological criteria (Regulation (EC) No 2073/2005, 2005), the limit for bivalves for direct human consumption is 230 *E.coli*/100g of flesh and intra-valvular liquid of bivalves. The sanitary classification of shellfish-harvesting areas in Europe is an important measure that helps to prevent shellfish food-borne outbreaks (Balière et al., 2016). The Norwegian Food Safety Authority (NFSA) runs an annual Norwegian surveillance program where bivalve harvesting areas are monitored for *E.coli*. There are currently no food safety criteria for STEC in bivalves. Only a few studies have focused on STEC detection and isolation from bivalves (Balière et al., 2015; Bennani et al., 2011; Gourmelon et al., 2006; MacRae et al., 2005; Sanath Kumar et al., 2001), and these studies from France, Morocco and India, all showed a low occurrence of STEC.

The aim of the current study was to investigate the occurrence of STEC in bivalves harvested along the Norwegian coast and to characterize possible STEC isolates, by examination with respect to the presence of *stx* and *eae* genes and/or the genes for the five serogroups of most concern (O26, O103, O111, O145 and O157), and to attempt to isolate and characterize STEC from PCR positive samples.

2. Materials and methods

2.1. Sampling areas

Sampling of marine bivalves included in this study was conducted as part of the Norwegian surveillance program of shellfish, which the Institute of Marine Research run on behalf of the NFSA. In this program bivalves from harvesting areas are routinely monitored for the level of *E. coli* to determine faecal contamination by standardised methods (ISO 16649-3). Sampling was conducted from 67 harvesting areas at 50 locations distributed along the Norwegian coast (Fig. 1) in the period from February to December 2016.

2.2. Sample preparation and pre-enrichment

Each batch sample comprised 10 to 15 individual marine bivalves. Each bivalve was rinsed on the outside in running tap water before they were aseptically opened using sterile knifes. Soft tissues and intra-valvular liquid were collected into sterile stomacher bags with filter to obtain 25 g of sample material and homogenized for 2 min 30 s in a Stomacher ® 400 Circulator (Seward, UK). The homogenate was subsequently diluted with 225 ml of Buffered Peptone Water (VWR International Inc., USA) and further homogenized for 30 s. The enrichment was performed at 37 °C ± 1 °C for 18 h–24 h. Aliquots of 1.5 ml from the enriched homogenate was mixed with 0.5 ml glycerol (85%) prior to storage at − 80 °C.

2.3. Screening methodology

Screening of the enriched samples was carried out in accordance with ISO/TS 13136 (2012). DNA was extracted using the DNeasy ® Blood & Tissue test kit (Qiagen, Germany), following the instructions from the manufacturer. The DNA was subsequently screened for the major virulence genes of STEC (*stx*$_1$, *stx*$_2$ and *eae*) and genes associated with the serogroups O26, O103, O111, O145 and O157, using primers and probes described in ISO/TS 13136, with the exception for O145 primers and probe (Fratamico et al., 2009). The master mix contained 12.5 µl TaqMan ® Universal (Applied Biosystems, USA), 0.5 µM of each forward and reverse primer, 200 nM of probe, 4.5 µl of water and 5 µl of DNA template. The PCR assays were run with C1000 Touch Thermal Cycler, CFX384 Real-Time System instrument (Bio-Rad, USA). The PCR program included a 50 ˚C for 2 min decontamination step, 95 ˚C for 10 min (enzyme activation), followed by 45 consecutive cycles of 95 ˚C for 15 s and 60 ˚C for 1 min (annealing of primers and amplification step). Positive controls were included in all analysis, as well as negative controls comprising milliQ water. Data acquisition and analysis of PCR assays were interpreted by the Bio-Rad CFX Manager 3.1 Software.

2.4. Isolation of STEC strains

The isolation of STEC strains was applied only to the samples that were PCR-positive for the targeted genes, *stx*$_1$ and/or *stx*$_2$ and *eae*. The samples were considered PCR-positive when the cycle threshold (C_t) value was below 35. For the attempted isolation of STEC strains, the ISO 13136 method was applied (ISO/TS 13136, 2012) with some amendments described below. Isolation was carried out from the frozen enriched samples. The enrichment broths were rapidly thawed in a water bath (GRANT, UK) at 50 °C until the ice disappeared, following a 1 h resuscitation period at room temperature. A total of 1 ml was transferred into 9 ml buffered peptone water and further incubated for 3 h at 37 °C ± 1 °C (Ternent et al., 2004). A loopful of the broths (10 µl) were streaked onto selective media plates, specifically CHROMagar O157 (CHROMagar Microbiology, France) and Sorbitol MacConkey agar (SMAC, Oxoid CM813, Thermo Fisher Scientific, USA) and incubated overnight at 37 °C ± 1 °C.

A selection of 50 colonies with presumptive *E.coli* morphology were point-inoculated on blood agar and incubated overnight at 37 °C ± 1 °C. From each sample, five pools (each with material from ten colonies) were produced and screened for *stx*$_1$ and/or *stx*$_2$ and *eae* genes. If a pool was positive for at least *stx*$_1$ and/or *stx*$_2$, individual colonies from the positive pool were tested in order to identify a single positive colony. Presumptive STEC colonies were confirmed as *E.coli* by MALDI-TOF-MS (Bruker, Germany) and serogroup was checked if the sample was positive for a serogroup-specific PCR (by real-time PCR).

2.5. Characterisation of STEC strains

The STEC isolates were characterized by whole genome sequencing (WGS) analysis. DNA from the STEC isolates were extracted using QIAamp DNA Mini kit (Qiagen, Germany) according to the protocol of the manufacturer with some minor changes, including eluting DNA in 100 µl 10 mM Tris pH 8.0 and a RNase A treatment step. DNA from the selected isolates were submitted for whole genome sequencing to GATC Biotech (Konstanz, Germany) for paired-end sequencing of 150 bp using Illumina technology. The WGS sequence data were trimmed using Trimmomatic (Bolger et al., 2014) and *de novo* assembled using Spades 3.11 (Bankevich et al., 2012). For annotation of the bacterial genomes Prokka was used (Seemann, 2014). MLST was identified uploading the WGS sequence data to Enterobase using the Achtman 7 Gene MLST scheme (www.enterobase.warwick.ac.uk/species/ecoli).

Characterisation of the *E.coli* isolates was performed by using BioNumerics vers 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and the application *E.coli* genotyping, which uses public databases for serotype, virulence and resistance prediction as well as plasmid and prophage detection. In addition, CGE webtools were used for virulence prediction and manual inspection and Blast search for the Shiga toxin subtypes. WGS data are available at the European Nucleotide Archive as a project PRJEB29102 and each sample are registered with accession number ERS2782944-ER2782946.

3. Results & discussion

The screening of 269 bivalve enrichment broths by the real-time PCR approach, returned 19 positive samples harbouring both *stx* and *eae* virulence genes. These positive samples comprised 17 from blue mussels (*Mytilus edulis*) and two from oysters (*Ostrea edulis*), deriving from different harvesting areas distributed in six counties of Norway (Table 1). A complete overview of the initial real-time PCR results for the detection of virulence genes and O-serogroups for all samples

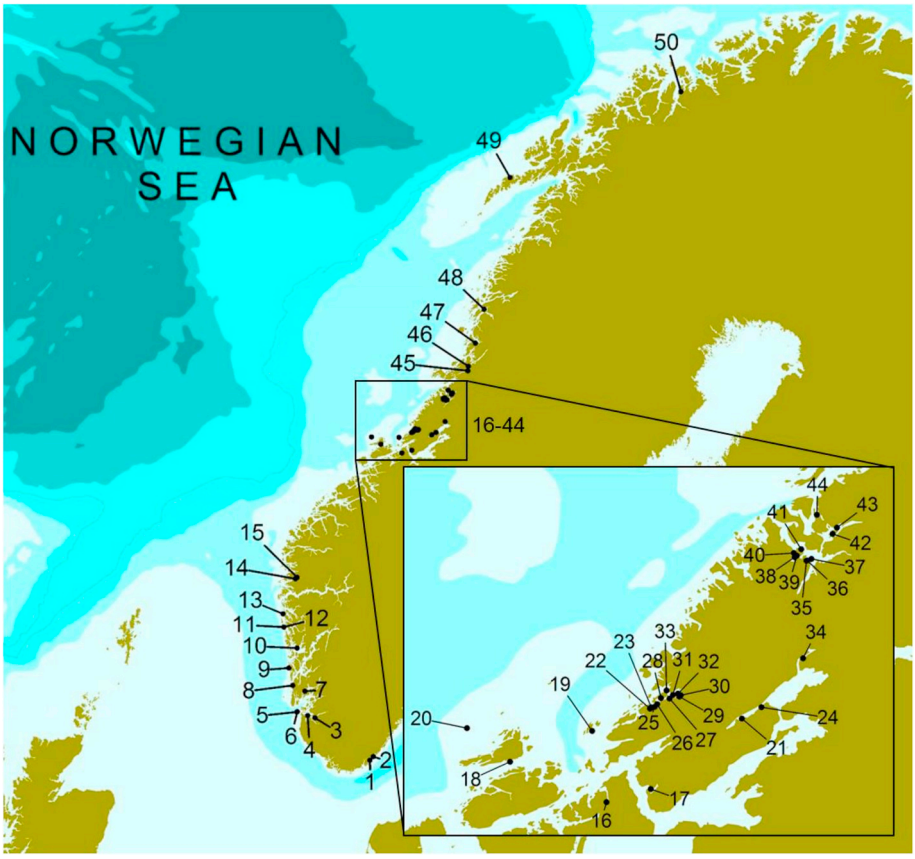

Fig. 1. Map of Norway showing the location of harvesting areas along the Norwegian coast from where the examined bivalve samples were collected by the Norwegian Food Safety Agency during the months February to December 2016.

Table 1
Summary of the screening results for the 19 positive real-time PCR samples for *stx* and *eae* genes. Sample category: BM: Blue mussel (*Mytilus edulis*) and OY: Oysters (*Ostrea edulis*).

Sample	Sample category	County	stx_1	Stx_2	*eae*
561	BM	Hordaland	+	–	+
635	BM	Sør-Trøndelag	–	+	+
732	BM	Nord- Trøndelag	+	+	+
733	BM	Sør-Trøndelag	+	+	+
734	BM	Sør-Trøndelag	+	+	+
735	BM	Sør-Trøndelag	+	+	+
737	BM	Sør-Trøndelag	+	+	+
738	BM	Sør-Trøndelag	+	–	+
809	BM	Sogn og Fjordane	+	+	+
811	OY	Rogaland	–	+	+
1041	BM	Nord- Trøndelag	–	+	+
1200	BM	Nord-Trøndelag	+	–	+
1218	BM	Sør-Trøndelag	–	+	+
1239	BM	Sogn og Fjordane	–	+	+
1246	BM	Sør-Trøndelag	–	+	+
1329	OY	Hordaland	+	+	+
1330	BM	Nordland	–	+	+
1332	BM	Sør-Trøndelag	+	+	+
1373	BM	Nord-Trøndelag	+	+	+

analyzed in this study can be found in the supplemental data (Table S1). Direct plating was performed on the 19 samples in order to isolate STEC from the samples, and presumptive STEC was isolated from three samples. The obtained isolates were positive for stx_2, but negative for stx_1 and *eae*, and did not belong to any of the major O-groups tested for in the present study (O26, O103, O111, O145 and O157).

The prevalence of STEC in this study was low, and positive isolates were obtained from only three of the 269 samples (1.1%). The three samples from which STEC was isolated were collected from localities classified as class B (> 230 MPN *E. coli*/100 g) and had numbers of *E. coli* between 300 and 700 MPN/100 g (unpublished results). Products originating from class B localities must be purified by relaying/re-suspension in a class A area for a period of time or heat-treated before distribution. *E. coli* are found in the faeces of warm-blooded animals, including humans, in high and relatively stable concentrations. The density of *E. coli* in human faeces normally varies from 10^6 to 10^7 cells g^{-1} (Forsythe, 2010). With respect to STEC there is less knowledge of the levels of bacteria present in faeces from animals. Cattle are known to intermittant shedders, and may excrete > 10^4 cells g^{-1} (super shedders) of STEC O157 during their colonization period (Persad and LeJeune, 2015). Bacteria from animals and humans are transported to the sea by land runoff, or from the sewage systems. In periods with high rainfall, especially following dry periods, it is expected that increased amounts of faecal material from land living animals will reach the sea. In addition, heavy rain or high amounts of melting snow may give an overload and possible leakage in drains and sewage systems (Lunestad el al., 2016). Shellfish harvesting farms can be influenced by sewage discharges or exposed in any other way to fecal contamination from land runoff, resulting in an impact to the shellfish by the change in microbiological quality of the water (Balière et al., 2015). The level of STEC in the present study is lower than what other studies have reported, where STEC was isolated from five out of 144 samples of shellfish (3.5%) in France (Gourmelon et al., 2006) and from five of 82 shellfish samples (6.1%) in Morocco (Bennani et al., 2011). A study in India reported a prevalence of 5% non-O157 STEC in clams (Sanath Kumar et al., 2001).

A possible explanation for the low isolation rate from samples that tested positive for the virulence genes, could be that some strains were viable but non-culturable due to environmental stress, such as

Table 2
Characterisation of isolates based on whole genome sequence data.

Sample ID	663_811_23	666_1246_14	667_1239_4
Source	Oyster	Blue mussels	Blue mussels
MLST	88	1611	7428
MLST clonal complex	–	–	ST23 Cplx
Serotype	O9:H10	O96:H19	ONT:H21
Predicted virulence	*gad, iss, lpfA, stx*$_2$	*gad, stx1, astA, lpfA, stx*$_2$	*gad, lpfA*
Stx2 subtype	*stx*$_{2i}$	*stx*$_{2g}$	–
Plasmids	IncFIB (AP001918), IncFIC(FII), IncFII, IncFII(pSE11)	Col156, IncFIB (AP001918)	IncFIB (AP001918), IncFII(29), IncI2
Aquired resistance	–	–	–

previously described (Rozen and Belkin, 2001). *E.coli* can enter a dormancy state where they lose culturability, but remain viable and potentially pathogenic (Grimes et al., 1986). Virulence genes can be present in *stx*-encoding bacteriophages, and the *stx* bacteriophages can enhance the stability of intact *stx* genes allowing extended persistence outside their host cells (Bergan et al., 2012). The bacteriophages can exist freely in the environment (Martinez-Castillo et al., 2013) and can remain for long periods of time (Muniesa et al., 1999). The *stx*-encoding bacteriophages play an important role in the evolution of STEC strains as they possess the ability to transfer the *stx* genes and consequently convert non-pathogenic strains into STEC. *E.coli* also has the ability to lose its *stx* genes, and this has previously been observed (Karch et al., 1992). In such cases, *E.coli* may become harmless after the loss of the *stx*-encoding bacteriophage genome (Bielaszewska et al., 2007; Feng et al., 2001), and since the *stx* genes can be gained or lost, the pathogenicity of the different strains may change over time. Other bacteria, which are not *E.coli*, can also harbour Shiga toxins and have previously been recorded (Gray et al., 2015; Schmidt et al., 1993). In the current study, two isolates were identified by MALDI-TOF as being non-*E.coli.* These strains were identified as *Citrobacter freundii* and *Enterobacter cloacae,* respectively. However, whether they encoded *stx* genes were not further examined.

One *stx*$_2$ positive isolate from each positive sample, three isolates in total, were selected for further characterisation by whole genome sequencing (WGS) and an overview of results is presented in Table 2. Raw data and quality control data from the WGS is listed in Supplementary data Table S2. When analysing the WGS data the *stx*$_2$ genes were not identified in strain 667_1239_4. Whether this is due to loss of *stx* genes or a contaminated isolate is not clear, and this sample will not be included in further discussions. The two other isolates; 663_811_23 and 666_1246_14 lack virulence-associated genes such as *eae* and *ehx*A, which are commonly seen in human pathogenic STEC. The lack of these genes might indicate a lower health risk. The lack of the intimin gene has been observed previously in STEC isolated from shellfish in Morocco (Bennani et al., 2011).

The WGS results revealed that one STEC isolate, 663_811_23, deriving from a batch of flat oysters, harboured a newly described Shiga-toxin variant; *stx*$_{2i}$ (Lacher et al., 2016) and was identified as serotype O9:H11 and ST-88. Information on this new *stx*$_2$ variant, *stx*$_{2i}$, is published in Genbank (FN252457-1) and is also further described in Lacher et al. (2016). The isolate 663_811_23 has five nucleotide differences from the *stx*$_{2i}$ variant described in Genbank with a similarity of 99.6% and will, based on this be identified as *stx*$_{2i}$. To the best of the authors' knowledge, this is not a commonly identified *stx*$_2$ variant and since the isolate does not contain any other virulence-associated genes of concern, it is less likely that this isolate will cause severe disease in humans. The second STEC isolate, 666_1246_14, derived from a batch of blue mussels, harboured *stx*$_{2g}$ which has been described in STEC and *stx*-encoding bacteriophages isolated from aquatic environments as well as wastewater, leafy greens, cattle and humans (Feng and Reddy, 2013; Granobles Velandia et al., 2012; Leung et al., 2003; Vu-Khac and Cornick, 2008). A study from France (Balière et al., 2016) described a *stx*$_{2g}$-encoding STEC originating from a shellfish with similar virulence profile, but of another serotype. The *stx*$_{2g}$ subtype is usually not associated with severe disease in human, but has been identified in patients with diarrhoea (Prager et al., 2011). Our isolate also encoded the heat-stable enterotoxin ST-Ia which has been described together with *stx*$_{2g}$ in other STEC isolates previously (Nyholm et al., 2015; Prager et al., 2011). Such isolates with this combination of virulence-associated genes are described as hybrids of STEC and enterotoxigenic *E. coli* (ETEC). The isolate 666_1246_14 was identified as serotype O96:H19 and ST-1611. *E. coli* O96:H19 has also been described as an emerging enteroinvasive *E. coli* (EIEC) and described from two recent EIEC outbreaks in the UK (Michelacci et al., 2016; Newitt et al., 2016; Pettengill et al., 2015). However, these outbreaks strains had a similar, but not identical sequence type. *E. coli* with identical sequence type; ST-1611, has been identified from different sources, but according to Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) most of these isolates have a different serotype than our isolate. Even though STEC O96 has been described from deer in Japan with the *stx*$_{2d}$ subtype (Kabeya et al., 2017), to the best of the authors' knowledge, *E. coli* O96:H19 (ST-1611) has not been identified as a hybrid STEC/ETEC before.

4. Conclusion

In conclusion, the current study is the first to address isolation and characterisation of STEC strains from bivalves originating from bivalve harvesting areas along the Norwegian coast. This study reported a < 2% prevalence of STEC in these bivalves, and the results suggest that STEC are rarely present in the Norwegian bivalve cultivation areas and imply a low risk of STEC infection after consuming shellfish from these designated areas. Nevertheless, this study demonstrated the presence of STEC in bivalves, which could emerge as being pathogenic to humans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103268.

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