ISOLATION AND CHARACTERISATION OF SHIGA TOXIN-PRODUCING ESCHERICHIA COLI FROM NORWEGIAN BIVALVES

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ABSTRACT

Only few studies concerning Shiga toxin-producing *E. coli* (STEC) detection in coastal environments and bivalves have been reported and there have been no reported outbreaks by STEC from bivalves in the world.

The aim of this thesis was to investigate the occurrence of STEC in Norwegian bivalves, and to characterize potential STEC isolates obtained from the samples.

To improve our understanding of STEC, the occurrence was investigated in 269 bivalves collected from harvesting areas along the Norwegian coast in 2016/17.

Microbial enrichment of the samples followed by DNA extraction with subsequent screening of STEC-associated genes was performed as described in ISO/TS -13136. Real-time PCR assays were conducted for genes encoding Shiga toxin (*stx_1 and stx_2*), intimin (*eae*) and the five major serogroups of concern (O157, O26, O111, O145 and O103). The screening results revealed the presence of the virulence genes (*eae* and *stx*) in 19 of the 269 samples. These 19 samples were selected for isolation of STEC. Colonies obtained from enrichment were screened for presence of *stx* and positive isolates were further characterized to determine their serotype and virulence profile. For two samples, automated immuno-magnetic separation (AIMS) was performed to facilitate isolation of STEC associated serogroups. Presumptive positive colonies from different serogroups were isolated by AIMS and the serogroup O157 was confirmed by real-time PCR but lacked the virulence genes. A total of three samples from 269 analyzed harbored STEC isolates, therefore, there seems to be a low risk of human infection by STEC in Norwegian bivalves.

Contents

1. INTRODUCTION

1.1 BIVALVES

Bivalves are mollusks that have laterally compressed bodies enclosed by a shell consisting of two hinged parts (Figure 1). Bivalves are opportunistic feeders that exploit the diverse nature of suspended particulate matter (Cranford et al., 2011). They are filter-feeders and therefore filter water as a feeding mechanism, removing bacteria and toxins from the water column. The retention efficiency of suspension-feeding bivalve mollusks depends on particle size, it is assumed that effective retention of particles is reached when particles are larger than 3-7µm (Cranford et al., 2016).

The most common bivalve species harvested in Norway are the blue mussels. This edible marine bivalve mollusk is in the family *Mytilidae* and they live in intertidal areas and attach themselves with byssal threads to hard substrates. Blue mussels are found along the entire Norwegian coast from the Swedish to the Russian borders.

These organisms have a great potential for bioaccumulation and have the ability to concentrate microorganisms, such as *Escherichia coli,* to a much higher level than that of the surrounding seawater.

Figure 1. Blue mussels, oysters and scallops presented as meals. These shellfish are commonly consumed worldwide (DISHIN&DISHES, 2012 and SEAFOOD AND RAW BAR, 2017).

1.2 PRODUCTION AND CONSUMPTION OF SHELLFISH IN NORWAY

Bivalves are consumed by the Norwegian population, are commonly harvested for food in Norway, from both wild and farmed sources and there are many farms in the country responsible of the growth of blue mussels (*Mytilus edulis)* for commercial use. Cultivation of bivalves in Norway is an established industry all along the coast and bivalves are grown on

horizontal systems of ropes suspended in the water by buoys, pipes or floats. The cultivated bivalves are not fed, but they rather filter water. After harvest they are transported in net bags. In 2014, 2 016 tons of shellfish were sold in aquaculture, and 1 983 tons were of mussels (Directorate of Fisheries, 2015). 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 (Baliere et al., 2015). Bacteria from animals or humans can wash off into the water, be accumulated by bivalves, and result in closure or downgrading of shellfish classification in harvesting farms.

1.3 FOOD SAFETY ASPECTS OF BIVALVES

The ability of bivalves to accumulate microorganisms from the water column is of concern as it can potentially lead to outbreaks of food poisoning from the consumption of shellfish. Several species of bivalves are preferably consumed live or raw (e.g. oysters), or lightly cooked (e.g. mussels). Bivalves could be responsible for the transfer of toxic substances, viruses and pathogenic bacteria along the food chain considering the importance of this food source in the diet of humans. There is an on-going surveillance program run by the National Institute of Nutrition and Seafood Research (NIFES) on behalf of the Norwegian Food Safety Authority (NFSA), where the harvesting areas of bivalves are monitored. This Norwegian surveillance program for shells started under the direction of the Directorate of Fisheries in 1999 as a follow-up of the EU Council Directives 91/492 EEC and 79/923 EEC. Under the establishment of the NFSA in 2004, the program was continued, but changed its name to "Monitoring for shells harvested and traded commercially". The purpose of the program is to control and monitor production areas for shells and the quality of shells produced for human consumption in EU countries.

The shells are checked for a range of parameters, including the contents of fecal indicator bacteria and *Salmonella*, as well as undesirables as heavy metals, dioxins, brominated flame retardants, poly-chlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (Duinker et al., 2015).

The EU has established regulations for cultivation of bivalves (854/2004/EC, 2004), and farm localities are classified according to their water quality. All shellfish-harvesting farms are classified as Class A, B or C and this is defined by the *E. coli* concentrations in the harvested bivalves (Table 1). The sanitary classification of shellfish-harvesting areas in Europe is an important measure that helps to prevent shellfish food-borne outbreaks (Baliere et al., 2016).

Table 1. Classification of farming localities according to E. coli concentration in bivalves (854/2004/EC, 2004).

Class	Microbiological standard	Treatment after harvesting
A	Live bivalve mollusks must not contain > 230 MPN *E. coli* per 100 g of flesh and intravalvular liquid	None
B	Live bivalve mollusks must not contain > 4600 MPN *E. coli* per 100 g of flesh and intravalvular liquid	Purification, relaying in A-area or boiling by approved procedure
C	Live bivalve mollusks must not contain > 46 000 MPN *E. coli* per 100 g of flesh and intravalvular liquid	Relaying in A-area for a long period of time or boiling by approved procedure

1.4 *ESCHERICHIA COLI*

E. coli is a gram-negative, facultative anaerobic, rod-shaped bacterium of the family *Enterobacteriaceae* (Figure 2). It is usually a commensal bacterium which can be found in the intestinal microbiota of warm-blooded animals. *E. coli* is a member of the fecal coliform group and is often referred to as “indicator organism”. The presence of *E. coli* in the environment can indicate fecal contamination. Most strains are harmless, but some can be pathogenic and therefore present a concern to human health. This study focused on Shiga-toxin producing *E. coli* (STEC).

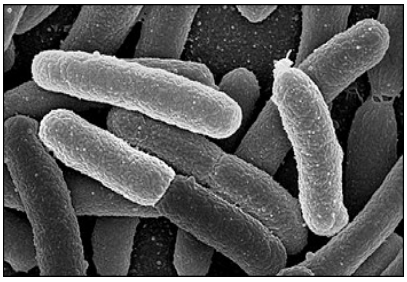


Figure 2. Electron microscopy of several E. coli cells (Genzer, 2009).

1.5 SHIGA TOXIN-PRODUCING *E. COLI* (STEC)

Shiga toxin-producing *E. coli* (STEC) is a zoonotic pathogen transferred from animals to humans that has become an important public health problem worldwide. The Shiga toxin name is derived from its source, the dysentery bacillus *Shigella dysenteriae*, which was first described by Kiyoshi Shiga in 1898 (Shiga, 1898). The nomenclature of these toxins varies, and they are also referred to as verotoxins. In this study we will use Shiga toxin and the term STEC. The main pathogenic property of STEC strains is the production of Shiga toxins (stx) (Perelle et al., 2004). STEC are *E. coli* strains possessing the *stx* encoding genes making them pathogenic, with the ability to cause severe diseases in humans. There are dangerous sequelae associated with STEC disease, the hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and hemorrhagic colitis (HC). HC can cause gastrointestinal bleeding by an inflammation of the colon. HUS is characterized by acute renal failure, this is a very serious disease as it can be fatal (Bergan et al., 2012). TTP is a hematologic emergency fatal without prompt treatment characterized by clotting in small blood vessels of the body (Khatun and Morshed, 2015). Any human being can become infected by STEC but young children and immunocompromised people have a higher risk of developing severe illness. The severity of the disease can vary, some infections can be very mild (with self-limiting watery diarrhea) while other infections can be severe (with hemorrhagic diarrhea) and result to be life-threatening. STEC might have a low infectious dose, as low as the order of 1 to 100 CFU (Paton and Paton, 1998b).

1.5.1 VIRULENCE GENES

The major virulence genes of STEC are the *stx* genes encoding the Shiga toxins and the *eae* gene, encoding the intimin protein, which is responsible for adhesion. This protein is essential for the tight binding of bacteria to target cells and is encoded on a chromosomal pathogenicity island termed the locus for enterocyte effacement (LEE) (Paton and Paton, 1998b). The LEE has an attaching and effacing (A/E) lesion which allows the binding of the toxins and hence results in an infection. The vast majority of virulence factors are encoded in mobile elements of the DNA: pathogenicity islands, transposons, plasmids and phages (Brussow et al., 2004). Shiga genes are encoded in *stx* phages. There are many other genes

associated with STEC virulence, such as *saa, paa, ehaA, iha*, *sab* and more (Baliere et al., 2016), but these are not further discussed here. Two main Shiga toxins have been described: stx_1 and stx_2. Within each group, there have been several variants identified throughout the years, stx_1 comprising 3 subtypes (stx_{1a}, stx_{1c} and stx_{1d}) and stx_2 with seven variants (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, stx_{2g}) (Scheutz et al., 2012). From these subtypes, some specific ones are closely associated with human infections (stx_{2a}, stx_{2c} and stx_{2d}) while others affect animals ($stx_{2e,}$ related with edema disease in pigs). If the type of variant is known, this can give an idea of the origin of that specific strain and the pathogenicity it may have (Vernozy-Rozand et al., 2004). Strains harboring the stx_2 appear to be more commonly responsible for severe human infections rather than strains harboring only the stx_1 (Boerlin et al., 1999, Paton and Paton, 1998a).

1.5.2 SEROGROUPS

STEC belong to a diverse number of serogroups (bacteria containing a common antigen) and there are some specific ones that have been linked to severe diseases in humans, such as O157, O26, O111, O103 and O145. These are the five main serogroups of concern in Europe, whereas in the USA they have two additional serogroups of concern (O45 and O121).
The O157:H7 serotype is the predominant serotype implicated in food-borne infections worldwide, and most frequently associated with HUS. *E. coli* O157:H7 was the first to be described as an STEC and has been implicated in serious diseases since the early 1980s (Baliere et al., 2016). Compared with O157 STEC infections, the identification of non-O157 STEC infections is more complicated and many laboratories typically cannot identify them.

1.5.3 RESERVOIRS

STEC live in the gastrointestinal tract of ruminant animals, including cattle, goats, sheep, deer, and elk. The main reservoir for STEC and therefore the major source for human illnesses is cattle (Mora et al., 2012). Swine and birds can pick up STEC from the environment and may spread it. STEC can persist in the environment for a period of time, at different temperatures and environments (Bolton et al., 2011).

1.5.4 STEC OUTBREAKS

STEC infections have been reported after ingestion of contaminated food/water or contact with animals as many different vehicles can transmit this pathogen to people. In 1993, there was a hamburger outbreak in the USA, Washington, where 477 cases of O157:H7 infection were reported (O'Brien et al., 1993). Radish sprouts were the vehicle of a large outbreak of *E. coli* O157:H7 infections in Sakai city, Japan, in 1996 (NIID, 1997). In 2006, 17 cases were reported in Norway, associated with a traditional cured sausage by a rare STEC variant (O103:H25) (Schimmer et al., 2008). In 2011, one of the largest documented outbreaks of STEC infection worldwide occurred in Germany by an *E. coli* strain of serotype O104:H4, which was transmitted to humans through the consumption of contaminated sprouts (EFSA, 2011). To date, no shellfish outbreak involving STEC strains has been described (Baliere et al., 2016).

1.6 METHODS FOR IDENTIFICATION AND ISOLATION OF STEC

Detecting and isolating STEC is a challenge and laborious using conventional methods. Screening for *E. coli* serogroups depends on isolation of the bacteria, confirmation of *E. coli* and identification of the O-antigen using serotyping methods and this is time consuming (Perelle et al., 2005). No quick or easy methods are available nowadays to isolate STEC strains frequently at laboratories. Automated immuno-magnetic separation is a traditional method, which focuses on serogroups but it is very time consuming. Current methods are more focused on real-time PCR which is widely used for the detection of virulence genes at different levels in the detection methodology.

Isolation of STEC and subsequent strain characterization is conducted to ensure that the detected genes are present on the same bacteria. The disadvantage with this DNA based method is that it cannot differentiate between DNA from viable or non-viable cells. A standardized method has been developed to detect STEC in food and includes pre-enrichment and real-time PCR. Environmental samples usually contain low numbers of STEC together with background flora and an enrichment step is usually required. It is important to obtain a bacterial isolate to be able to further characterize and thereby assess the virulence potential of the organism (Nielsen and Andersen, 2003).

2. MATERIALS & METHODS

2.1 STUDY SITE

Sampling was coordinated with the Norwegian Food Safety Authority (NFSA). Bivalves are routinely monitored for the presence of *E. coli* to check for fecal contamination on the production areas. The bivalves analyzed in this study were collected from 67 harvesting farms along the coast of Norway (Figure 3), distributed in 26 municipalities by 13 different local offices of NFSA.

Figure 3. Map representing the harvesting farm location sites from where the different bivalve samples were obtained for this study.

2.2 STUDY DESIGN

A total of 269 samples of bivalves were examined in this study. The examined material comprised 218 samples of blue mussels (*Mytilus edulis*), 28 samples of oysters (*Ostrea edulis*), 15 samples of great scallops (*Pecten maximus*), five samples of horse mussels (*Modiolus modiolus*), two samples of clams (*Arctica islandica*) and one sample of banded carpet shell (*Politapes rhomboides*). Samples arrived once a week to NIFES, in intact bags in a box with cooling elements and were taken to the laboratory for examination. The temperature was kept at 4°C and the microbiological analysis was initiated within 24 h.

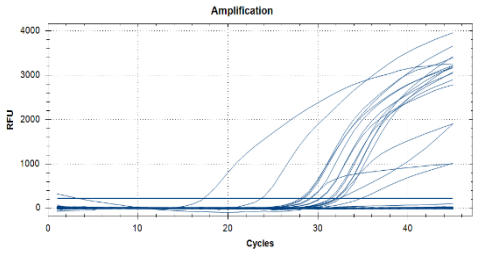
This study applied the ISO/TS 13136:2012 methodology for detection and isolation of STEC. This Technical Specification has a protocol which uses real-time PCR as the reference technology for detection of the virulence and serogroup associated genes. Figure 4 shows a summary of the steps followed in this study, which consists of an enrichment step, DNA extraction, real-time PCR analysis for the detection of the toxin and intimin genes (*stx*$_1$, *stx*$_2$ and *eae* genes), serogroup determination by real-time PCR (only in case the *stx*/*eae* PCR is positive), cultivation and isolation of suspected colonies from the enrichment broth, and confirmation of the presence of virulence genes by screening the colony itself (Kagkli et al., 2011). Figure 5 represents a schematic diagram with the steps from ISO/TS 13136:2012 methodology followed in this study.

SAMPLING OF BIVALVES

ENRICHMENT

DNA EXTRACTION

REAL-TIME PCR



ISOLATION, CHARACTERIZATION

Figure 4. Flow chart of steps followed in this study. (FISHERIES 2017, STOMACHERS 2012, LABEQUIM 2005 and FOOD CONSULTING SERVICES 2017)

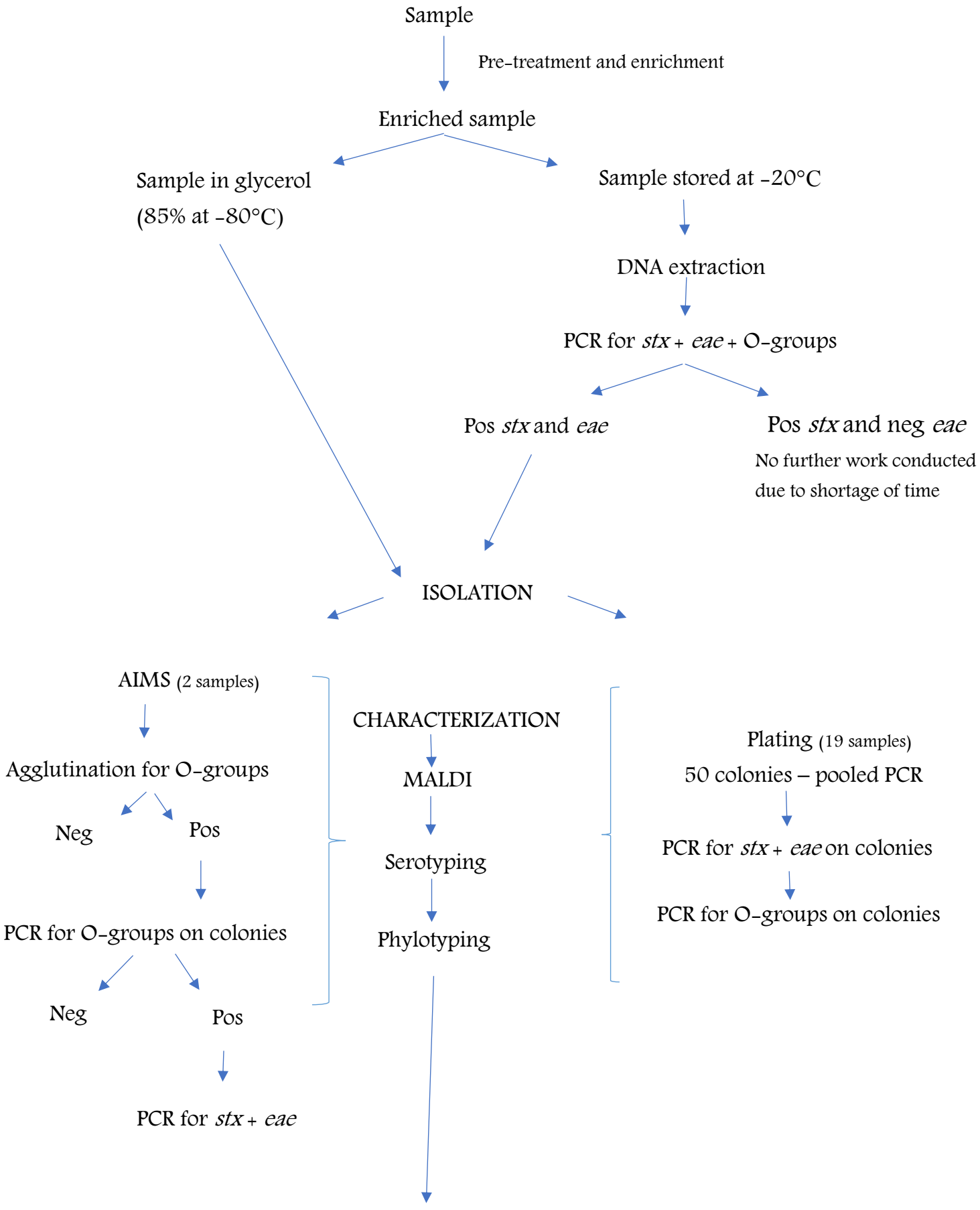


Figure 5. Schematic diagram showing the methodology steps followed in this study.

2.3 SCREENING FOR VIRULENCE FACTORS AND SELECTED SEROGROUPS

2.3.1 SAMPLE PREPARATION AND ENRICHMENT

Different bivalve species required different approaches and tools to be opened. The shells with signs of damage were discarded. Before opening the bivalves, they were rinsed under cold, running tap water to remove sediment adhered to the shell. Mussels were opened with a regular kitchen knife, which was inserted between the shells, and with a twisting motion separated them open and mantel water along with the tissue were emptied into a separate sterilized bag (Standard 400, GRADE) with mesh to minimize particles clogging during subsequent pipetting. A specific knife with a thick blade was used to withstand the pressure applied when opening the oysters without bending. The oysters were placed on a flat surface and the knife was pushed into the shell, forcing it open. The tissue was removed and placed into a sterilized bag together with intravalvular liquid. One sample represented material from 10-15 shells, as a minimum of 25g was required from each sample.

The samples were homogenized using a Stomacher 400 CIRCULATOR (Seward) for 2min and 30secs. The enrichment broth was prepared by adding 225ml of Buffered Peptone Water (BPW) to the shell homogenate and this mix was homogenized again in the stomacher for 30secs. The homogenate was incubated aerobically at 37°C ± 1°C for 18-24h. Two aliquots from the enrichment broth were withdrawn after incubation, one for DNA extraction (1ml, frozen at -20°C) and the other for possible further isolation of STEC. Enrichment broth (1.5ml) was transferred to glycerol (0.5ml) (85%) in a 2ml Eppendorf tube and stored at -80°C for further analysis.

2.3.2 DNA EXTRACTION BY COMMERCIAL KIT

DNA extraction breaks the bacterial cell wall, removes inhibitors and release DNA. DNA extraction from the tissue of the samples was performed using the DNeasy ® Blood & Tissue test kit (Qiagen, Germany). From the enrichment broth, 200µl was pipetted into an Eppendorf tube, followed by 180µl of buffer ATL and 20µl of Proteinase K, for tissue lysis and optimal DNA-binding conditions to the DNeasy membrane. The mixture was vortexed to yield a homogenous solution and incubated at 56°C in a block heater for 30min until achieving the breakdown of the cell wall and membrane. To purify the DNA, 200µl buffer AL and 200µl of ethanol (96-100%) were added followed by vortexing. The full mixture (800µl) was pipetted into a DNeasy Mini spin column placed in a 2ml collection tube. This was then centrifuged at 6 000 relative centrifugal force (rcf) for 1min. The spin column was placed in a new collection tube and 500µl of Buffer AW1 was added. This tube was centrifuged at 6 000rcf for 1min to remove any contaminants and enzyme inhibitors, and furthermore to bind the DNA to the DNeasy membrane. The spin-column was carefully removed once more and placed in a clean collection tube and 500µl of Buffer AW2 was added. This was followed by centrifugation at 20 000rcf for 3min to dry the DNeasy membrane. The last step is important as residual ethanol could interfere with subsequent reactions. This step ensured that no ethanol would be carried over during the following elution.

The spin-column was put into an Eppendorf tube and 50µl Buffer AE was carefully added directly on top of the DNeasy membrane. The sample was incubated 1min at room temperature before centrifuging one last time at 6 000rcf for 1min. Purified DNA was eluted from the spin column in 50µl buffer AE giving results of high amounts of DNA binding to the DNeasy membrane. Approximately 60–80% of the DNA will elute in the first elution. This buffer is composed of 10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0. The DNA concentration and purity was measured using a Nanodrop ND/1000 3.8.1 Spectrophotometer (Thermo Fisher, USA). All DNA preparations were then stored at -20°C until further examination.

2.3.3 POLYMERASE CHAIN REACTION (PCR)

PCR detects and multiplies specific areas of DNA (fragments). The reaction starts with samples heated to denature (separate into single strands) the target DNA. The temperature is lowered to allow the primers to anneal to their complementary sequences. The temperature is raised again and polymerase can attach at each priming site and synthesize a new DNA strand.

2.3.3.1 REAL-TIME PCR

In the present study, real-time PCR was used and this system relies on the detection from a fluorescent reporter. Probes are fluorescently labelled DNA oligonucleotides and bind to the DNA strand during the PCR reaction to give a fluorescent signal. The primers are designed to specifically bind to DNA region to be amplified. One of the advantages of the real-time PCR is that is produces a final result within 2h. The 5' end of the probe is labelled with a fluorescent reporter molecule and on the 3' end of the probe is a quencher molecule. The cycle threshold (Ct) value was defined as the PCR cycle at which the fluorescent intensity raised above the threshold and became detectable. Any negative results obtained by the PCR method indicated the absence of the corresponding target in the sample. Positive controls were included in all analysis, whereas a sample comprising milliQ water was included as negative controls. Samples were screened for virulence genes and amplifications using a master mix containing 12.5µl TaqMan® Universal (Applied Biosystems, UK), 0.5µM of each forward and reverse primer, 200nM probe, 4.5µl of water and 5µl of DNA template. The PCR assays were run with C1000 Touch Thermal Cycler, CFX384 Real-Time System instrument (Bio-Rad Laboratories) (Figure 6) using the program described in Table 2 and data acquisition and analysis of the PCR assays were handled by the Bio-Rad CFX Manager 3.1 software.

Table 2. Real-time PCR program description used at NIFES laboratory to screen for virulence genes and O-serogroups. Details on PCR steps with time and temperature, estimated run time 1h and 43 min.

Target gene	PCR program		
		Time (seconds)	**Temperature (°C)**
stx$_1$, *stx*$_2$, eae, O157, O145, O26, O103, O111	Pre-PCR: Decontamination	120	50
	Polymerase activation and template denaturation	600	95
	PCR 45 consecutive cycles:		
	Denaturation	15	95
	Annealing and DNA synthesis	60	60

2.3.3.2 PROBES AND PRIMERS FOR REAL-TIME PCR

The set of probes and primers used were obtained from the literature and standard methods to detect the specific serogroups and virulence genes (Table 3). The probe targeting *stx*$_1$ contained the FAM dye at the 5'end and a non-fluorescent quencher (BHQ1) at the 3'end. The probe targeting *stx*$_2$ contained the HEX dye at the 5'end and BHQ1 at the 3'end.

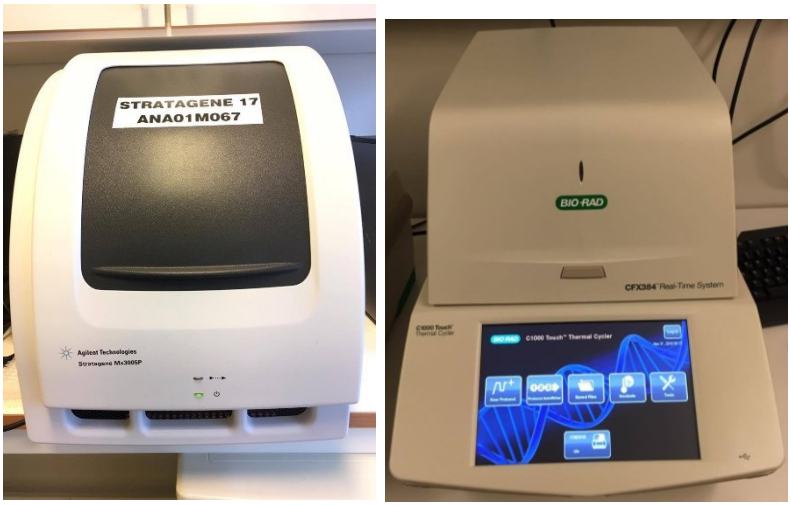


Figure 6. Real-time PCR instruments, to the left the Strategene Mx3005P QPCR systems (Agilent Technologies, Germany) used at VI, Oslo. To the right, the C1000 Touch Thermal Cycler, CFX384 Real-Time System instrument (Bio-Rad Laboratories, United Kingdom) used at NIFES, Bergen.

Table 3. List of primers and probes used for detection of virulence genes/O-serogroups for real-time PCR assays. In the sequence, F is forward primer, R is reverse primer and P is probe. In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).

Target gene	Primer and probe	Sequence (5' - 3')	Location within sequence	Amplicon size (bp)	Reporter dye	GenBank accession no.	References
	stx-F	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	878-906				
				131	FAM	M16625	ISO/TS 13136:2012
stx$_1$	*stx*-R	CCC CAG TTC ARW GTR AGR TCM ACR TC	983-1008				
stx$_2$	*stx*$_1$-P	FAM-CTG GAT CTC AGT GGG CGT TCT TAT GTA A-BHQ1	941-971				(Perelle et al., 2004)
	stx$_2$-P	HEX-TCG TCA GGC ACT GTC TGA AAC TGC TCC-BHQ1	838-864	128	HEX	X07865	
	eae-F	CAT TGA TCA GGA TTT TTC TGG TGA A	899-924				
eae				102	FAM-TAMRA	Z11541	ISO/TS 13136:2012
	eae-R	CTC ATG CGG AAA TAG CCG TTA	1000-979				(Nielsen and Andersen, 2003)
	eae-P	FAM-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-TAMRA	966-936				
	O145wzy2-F	ATA TTG GGC TGC CAC TGA TGG GAT	6052-6075				
wzy O145				310	FAM	AY863412	(Fratamico et al., 2009)
	O145wzy2-R	TAT GGC GTA CAA TGC ACC GCA AAC	6361-6338				
	O145wzy-P	FAM-AGC AGT GGT TCG CGC ACA GCA TGG T-BHQ1	6215-6238				
	rfbE0157-F	TTT CAC ACT TAT TGG ATG GTC TCA A	348-372				
rfbE O157				88	FAM-TAMRA	AF163329	
	rfbE0157-R	CGA TGA GTT TAT CTG CAA GGT GAT	412-435				
	rfbE0157-P	FAM-AGG ACC GCA GAG GAA AGA GAG GAA TTA AGG-TAMRA	381-410				
	wbdl0111-F	CGA GGC AAC ACA TTA TAT AGT GCT TT	3464-3489				ISO/TS 13136:2012
wbdl O111				146	FAM-TAMRA	AF078736	
	wbdl0111-R	TTT TTG AAT AGT TAT GAA CAC CTT GTT TAG C	3579-3609				(Perelle et al., 2004)
	wbdl0111-P	FAM-TTG AAT CTC CCA GAT CAA CAT CGT GAA-TAMRA	3519-3548				
	wzx026-F	CGC GAC GGC AGA GAA AAT T	5648-5666				
wzx O26				135	FAM-TAMRA	AF529080	
	wzx026-R	AGC AGG CTT TTA TAT TCT CCA ACT TT	5757-5782				
	wzx026-P	FAM-CCC CGT TAA ATC AAT ACT ATT TCA CGA GGT TGA-TAMRA	5692-5724				
	wzx0103-F	CAA GGT GAT TAC GAA AAT GCA TGT	4299-4323				
wzx O103				99	FAM	AY532664	
	wzx0103-R	GAA AAA AGC ACC CCC GTA CTT AT	4397-4375				
	wzx0103-P	FAM-CAT AGC CTG TTG TTT TAT-MGB	4356-4373				

2.4 ISOLATION OF STEC BY CULTIVATION

2.4.1 PLATING AND AGAR PLATES

The samples which were PCR-positive for *stx*, *eae* and O-groups were selected for isolation of STEC. For the isolation of STEC strains, ISO/TS 13136:2012 was used with some modifications. Prior to isolation, tubes with enrichment broth were thawed in a water bath (GRANT) at 50°C until the ice had melted (2-3 min), followed by 1h incubation at room temperature. The sample was transferred (1ml) into a tube with 9ml of fresh buffered peptone water (BPW) pre-warmed to 37°C. These tubes were further incubated for 2-3h at 37°C. After incubation, the cultures were mixed using a vortexer (IKA®M53 basic, USA) and a 1:10 dilution of the culture in BPW was prepared. Undiluted and diluted (1:10), volume of 10µl, cultures were plated onto agar media plates by a streaking technique, in order to obtain well isolated colonies. The media plates used were CHROMagar™ O157 (CHROMagar Microbiology, Paris, France) and Sorbitol MacConkey agar (SMAC, Oxoid CM813) (Media production, NVI). These plates were incubated overnight at 37°C.

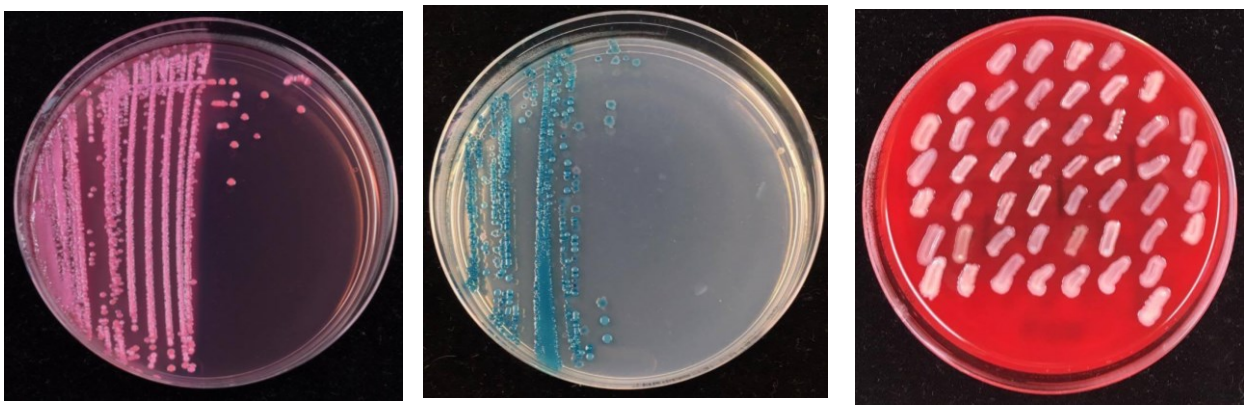


Figure 7. E. coli colonies growing on different selective media plates. To the left, MacConkey agar with sorbitol (SMAC) plate, in the middle, blue CHROMagar plate containing a chromogenic mix with chromogenic substrates and to the right, blood agar plate (BAP) containing mammalian blood.

A selection of 50 colonies with typical or suspicious *E. coli* morphology from the four plates (2 CHROM plates and 2 MacConkey plates, each with one diluted and one undiluted) were point inoculated on blood agar plates (BAP, Media production, NVI) and incubated overnight at 37°C. From each sample, five pools were produced, and each pool included material from 10 colonies. The colony morphology was registered for all individual selected colonies.

DNA from the pooled samples was extracted by the boiling method (see 2.5.2) and used in real-time PCR for detection of virulence genes (*stx*$_1$, *stx*$_2$, *eae*) and O-serogroups (O145, O157, O111, O103 and O26). Whenever a positive pool was detected, DNA were extracted once more from the 10 single colonies comprising the positive pool, and re-tested for the presence of these target genes/serogroups to obtain information on exact colony carrying the genes and to have an isolate for further characterization.

2.4.2 AUTOMATED IMMUNO-MAGNETIC SEPARATION (AIMS)

In this study, automated immuno-magnetic separation (AIMS) was used for rapid and selective concentration of *E. coli* serogroups (O111, O145, O157, O103 and O26). The AIMS were performed applying the BeadRetriever instrument, and during the process most of the background flora from a pre-enriched sample aliquot was removed. Two samples from the 19 analyzed samples from which isolation was attempted, were selected for this methodology. Sample 1246 was tested for serogroup O157 and sample 734 for O26, O111, O145 and O157.
Dynabeads, which are paramagnetic and can be extracted by a magnet, wash buffers and samples were aseptically loaded into the tube-strips, placed into the rack and inserted into the instrument. The program (EPEC/VTEC) automatically performed the entire AIMS process. During incubation, the antibodies coated onto the beads would bind with the target bacteria. The bead-bacteria complexes were subsequently separated from the enrichment broth by a magnet, washed, and followed by final re-suspension into the last tube for further processing to detect and/or isolate the target organisms.

The AIMS method on BeadRetriever comprised the following steps: mixing beads, sample incubation, collecting, first wash, second wash and then releasing the beads with bacteria attached. After running AIMS, for the isolation of *E. coli*, all re-suspended bead-bacteria complex from the 5th tube was plated onto CHROMagar® and CT-SMAC plates (with Cefixime Tellurite selective supplement, Oxoid SR0172E) and incubated at 37°C overnight. The swab-streak technique was used when plating, as this resulted in better isolated pure colonies on the culture media (Figure 8). Typical and suspicious colonies were tested with the respective agglutination sera (SIFIN, Berlin, Germany) recommended for use with the kit.

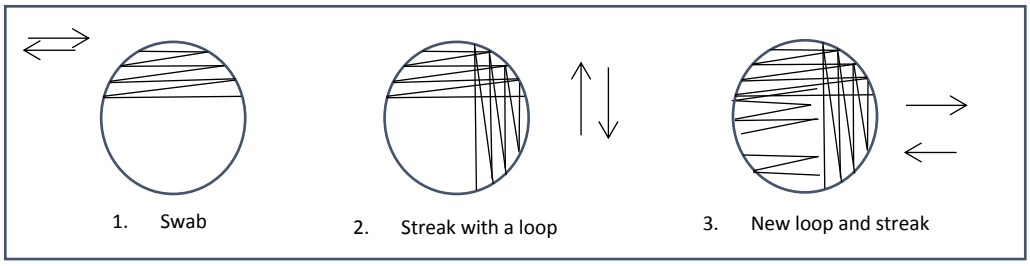


Figure 8. Swab-streak plating technique represented graphically. The bead-bacteria complex was spread over one half of the plate with a sterile swab to ensure the break-up of the complex. This was then diluted further by streaking with an inoculating loop.

The test sera were for pre-testing the serogroup of presumptive *E. coli* strains isolated by using slide agglutination. If the strain possessed an antigen covered by the test serum, this antigen became bound when mixed with the specific antibody. Small amounts of bacterial mass from typical and suspicious colonies was transferred onto a slide and mixed with one drop of the specific test serum as shown in Figure 9. The result was read with the naked eye by holding in front of a light source against a dark background tilting it back and forth. A positive result would be seen as visible agglutination, confirming antigen-antibody reaction, whereas a negative result would be seen as cloudy. The agglutination positive colonies were then plated for purity and confirmed or rejected by using the PCR approach as described in 2.3.3.

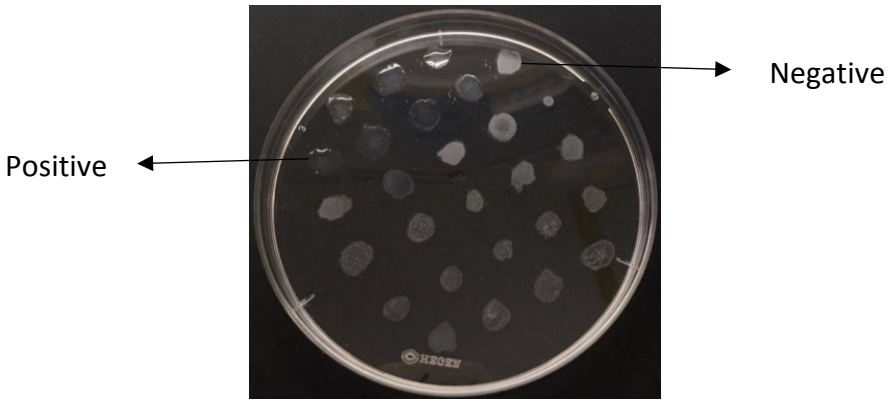


Figure 9. Plate with droplets of antiserum (Statens Serum Institut, Denmark) mixed with bacterial mass showing agglutination analysis.

2.5 CHARACTERIZATION OF COLONIES

2.5.1 *E. COLI* CONFIRMATION BY MALDI-TOF

For the identification of organisms, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was used. This is a well-established approach for rapid species classification from microbial cultures. For the analysis, one isolated colony was picked with a sterile wooden stick and smeared on a MALDI steel target plate (Figure 10) in its corresponding sample position (previously labelled on the MALDI-TOF spreadsheet). A droplet of 1µl of 70% formic acid was added on each position and left to dry at room temperature. Portions of 1µl of the matrix solution (HCCA) were added to extract the proteins that mainly constituted ribosomal proteins found in high concentration.

The plate was placed in the MALDI Biotyper mass spectrometer (Bruker, Germany, Figure 11) and a laser irradiated the spot. This caused evaporation of the matrix and intact proteins into the vacuum, resulting in the release of positively charged proteins and peptides. The mass spectrometer measured the time between pulsed acceleration and the corresponding detector signal, and the speed was converted into an exact molecular mass.
The MALDI-TOF software used to interpret the results was the MBT compass (Bruker, Germany) and it showed the best-matching species to that specific bacterium tested at the time.

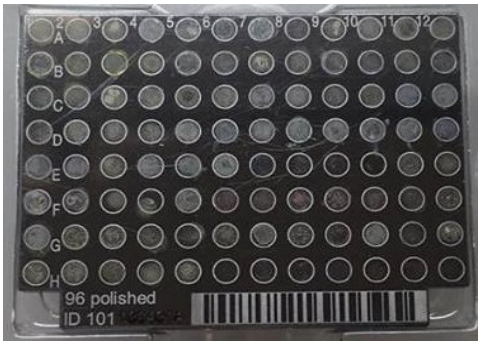


Figure 10. MALDI target plate, where bacteria is loaded before inserting into MALDI-TOF instrument

Figure 11. MALDI-TOF instrument used at VI for E. coli confirmation (Microflex, Maldi Biotyper)

2.5.2 DNA EXTRACTION BY BOILING

Material from bacterial colonies was suspended in Eppendorf tubes containing 100µl of milliQ water. These tubes were heated in a heating block (TECHNE, Dri-Block® DB·2D, Tamro Lab, Figure 12) at 100°C for a duration of 15m. Subsequently, the suspensions were centrifuged (VWR, Microstar 17) at 10 000rcf for 10m. The supernatants were used as template for the PCR analysis.

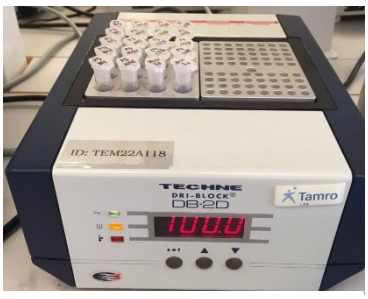


Figure 12. Heating block (TECHNE) used for boiling DNA

2.5.3 REAL-TIME PCR

Real-time PCR was used to detect for virulence genes and O-serogroups, as described in 2.3.3. PCR set up was prepared in the DNA/RNA UV-Cleaner Box (UVT-B-AR, Grant-Bio). The master mix used was Brilliant III Ultra-Fast QPCR (Agilent Technologies, USA). The instrument was Strategene Mx3005P QPCR systems (Agilent Technologies, Germany) which was associated with the software MxPro Mx3005P to register and analyze the results.

2.5.4 CONVENTIONAL PCR

2.5.4.1 SUBTYPING OF *stx*$_2$

Conventional PCR is an end-point detection method while real-time PCR will not only detect but also display the data of amplification during the run after each cycle.
Subtyping of *stx*$_2$ genes, with pure cultures of STEC, was conducted by conventional PCR with the BioRad T100 Thermal cycler instrument, and followed by gel electrophoresis (see 2.5.4.3). The seven subtypes (a-g) of *stx*$_2$ were analyzed (Scheutz et al., 2012). Amplification reaction mixtures containing the respective primers (0.75µl of each), 12.5µl HotStar Taq® and milliQ water were added to make a master mix of 23µl and 2µl of DNA template were used.

2.5.4.2 PHYLOGENETIC ANALYSIS

Conventional multiplex PCR was used to divide the *E. coli* strains into four phylogenetic groups (A, B1, B2, and D). This approach used the phylogenetic markers; *chuA, yjaA*, TSPE4.C2 and *gadA (Clermont et al., 2000, Doumith et al., 2012)*. *E. coli* Bæ14 was used as a positive control for the four genes. A primer mix was made by using 5µl of each corresponding primer (Table 4) added to 20µl milliQ water. For the PCR reactions, 12.5µl of 2x Qiagen mastermix (QIAGEN ®, Germany), 0.5µl of the primer mix and 10µl of milliQ water was produced with the addition of 2µl of DNA template. The program run is described in Table 5 and the result interpretation is described in Table 6. Agarose gel was run as described accordingly in 2.5.4.3.

Table 4. Description of primers used for subtyping of stx_2 ($_{a-g}$) and phylotyping of E. coli

Target gene	Primer	Sequence	Location within sequence	Amplicon size (bp)	References
stx_{2a}	vtx2a-F2	GCGATACTGRGBACTGTGGCC	754-774		(Scheutz et al., 2012)
	vtx2a-R3	CCGKCAACCTTCACTGTAAATGTG	1079-1102	349	
	vtx2a-R2	GGCCACCTTCACTGTGAATGTG	1079-1100	347	
stx_{2b}	vtx2b-F1	AAA-TAT-GAA-GAA-GAT-ATT-TGT-AGC-GGC	968-994		
	vtx2b-R1	CAG-CAA-ATC-CTG-AAC-CTG-ACG	1198-1218	251	
stx_{2c}	vtx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTA	926-955		
	vtx2c-R2	CCGGCCACYTTTACTGTGAATGTA	1079-1102	177	
stx_{2d}	vtx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG	927-955		
	vtx2d-R1	TTYCCGGCCACTTTTACTGTG	1085-1105	179	
	vtx2d-R2	GCCTGATGCACAGGTACTGGAC	1184-1206	280	
stx_{2e}	vtx2e-F1	CGG-AGT-ATC-GGG-GAG-AGG-C	695-713		
	vtx2e-R2	CTT-CCT-GAC-ACC-TTC-ACA-GTA-AAG-GT	1080-1105	411	
stx_{2f}	vtx2f-F1	TGG-GCG-TCA-TTC-ACT-GGT-TG	451-475		
	vtx2f-R1	TAA-TGG-CCG-CCC-TGT-CTC-C	856-874	424	
stx_{2g}	vtx2g-F1	CAC-CGG-GTA-GTT-ATA-TTT-CTG-TGG-ATA-TC	203-231		
	vtx2g-R1	GAT-GGC-AAT-TCA-GAA-TAA-CCG-CT	771-793	573	

Multiplex: *gadA, chuA,yjaA,* TSPE4.C2	gadA.F gadA.R	GATGAAATGGCGTTGGCGCAAG GGCGGAAGTCCCAGACGATATCC		373	(Doumith et al., 2012) (Clermont et al., 2000)
	chuA.F chuA.R	ATGATCATCGCGGCGTGCTG AAACGCGCTCGCGCCTAAT		281	
	yjaA.F yjaA.R	TGTTCGCGATCTTGAAAGCAAACGT ACCTGTGACAAACCGCCCTCA		216	
	TSPE4.C2 F TSPE4.C2 R	GCGGGTGAGACAGAAACGCG TTGTCGTGAGTTGCGAACCCG		152	

Table 5. Description of conventional PCR program for subtyping of stx$_2$ (a-g) and phylogenetic analysis of E. coli

Target gene	**Conventional PCR program**		
		Time (seconds)	**Temperature (°C)**
Subtyping: *stx$_{2a}$*,*stx$_{2b}$*,*stx$_{2c}$*, *stx$_{2d}$*,*stx$_{2e}$*,*stx$_{2f}$*, *stx$_{2g}$*		900	95
	PCR 35 cycles	50	95
		40	64/66*
		60	72
		180	72
		∞	4
BAKT/FYLOGR program			
Multiplex: *gadA, chuA, yjaA,* TSPE4.C2		900	95
	PCR 30 cycles	30	95
		30	60
		30	72
		300	72
		∞	8

- Annealing temperature was different between the subtypes; *stx$_{2a}$*, *stx$_{2b}$*, *stx$_{2e}$*, *stx$_{2f}$*, *stx$_{2g}$* was set to 64°C and stx$_{2c}$ and *stx$_{2d}$* was set to 66°C to avoid cross-reactions.

Table 6. Interpretation of results from phylogenetic analysis.

Phylogroup	***gadA***	***chuA***	***yjaA***	**TSPE4.C2**
A	**+**	**-**	**+/-**	**-**
B1	**+**	**-**	**-**	**+**
B2	**+**	**+**	**+**	**+/-**
D	**+**	**+**	**-**	**+/-**

2.5.4.3 AGAROSE GEL ELECTROPHORESIS

The products from the conventional PCR assays were detected by gel electrophoresis, which is a well-established method used to separate, detect and visualize DNA or RNA fragments by size. This is achieved by moving negatively charged nucleic acid molecules from negative to positive pole through an agarose matrix with an electric field. Agarose DNA pure grade (Electron, VWR Chemicals) was weighed to 2g and mixed with 200ml of TBE buffer (Tris/Borate/EDTA) giving a 1% agarose gel. This was heated to boil in the microwave and then checked the solution was clear with no signs of threads floating. The mix cooled before adding 20µl of GelRed (Fermentas, Life Sciences, USA) in the agarose and mixed thoroughly. This was poured into a mold with combs responsible for making the well spaces. The gel was left to solidify in the mold and was placed in the electrophoresis chamber, where TBE buffer was poured until gel was barely covered and the comb was then removed.

From each PCR product, 25µl were mixed with 5µl of loading Dye (Thermo Fisher Scientific, Canada) where an aliquot of 12µl were loaded into each well. The size marker "O'Gene Ruler 50 bp DNA Ladder" (Fermentas, Life Sciences, USA) was transferred in the first and in the last well (3µl). This was done to create a reference ladder on each side of the gel to then compare and refer to the sizes in the results.

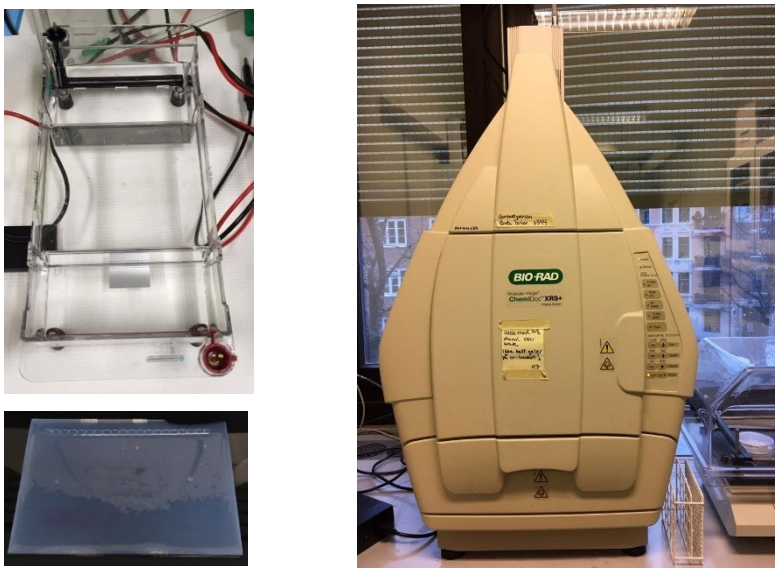


Figure 13. Top left is the gel setup and below is the final matrix with wells. To the right, the molecular imager instrument (Bio-Rad, UK) used to take images from gel.

The instrument used was the PowerPac™ Basic (BioRad, UK) power supply ran at 90 volts for 1h. The bands were visualized under UV light to check that they were sufficiently separated. The Bio-Rad Molecular Imager® ChemiDoc ™ XRS+ Imaging System (Figure 13) was used to analyze the final result and the gel images were acquired and analyzed by the software Image Lab 5.1 (BioRad, UK).

2.5.5 CONVENTIONAL SEROGROUPING

Serogrouping cultures with O-antisera (Statens Serum Institut, Denmark) was conducted for 13 different O-serogroups (Table 7). When a bacterial culture is mixed with a specific antiserum directed against bacterial surface components, the cells are bound together through antigen-antibody bonds to form aggregates. A colony was picked for inoculation and incubated overnight at 37°C. The cultures were boiled for 1h, allowing sedimentation of bacterial debris, and left at room temperature for another hour. O-antiserum was mixed (80µl) in microtiterplates with 80µl of the corresponding sample and incubated at 50°C overnight. The results were read with a black background and against light to distinguish between negative and positive outcomes. If it was positive, a “grey carpet” would be observed covering the bottom of the well, whereas if it was negative, the bacterial suspension would be seen as a small white spot centered in the bottom of the well (Figure 14).

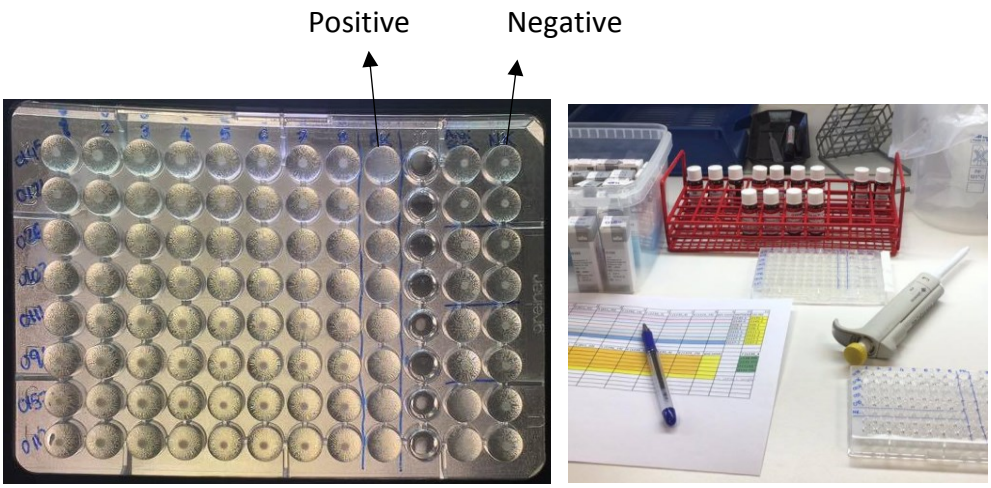


Figure 14. To the left, the mictotiterplate with round bottom used for O-group typing. To the right, the set-up with the dropper bottles belonging to the E. coli antisera.

2.6 REFERENCE STRAINS

Positive *E. coli* controls were used in each of the tests conducted in this study to ensure reliable results. The reference strains for each of the genes/serogroups were from the reference laboratory in Oslo and are described in Table 7 below. The negative controls were milliQ or distilled water.

Table 7. List of control strains used in this study, possessing relevant genes.

Approach	Target gene/serogroup	Control strains	Reference
Real-time PCR	*stx_1*	VI51046 / VI51048 / EDL-933	(Perelle et al., 2004)
	stx_2	VI51048 / VI51049 / EDL-933	
	eae	VI51048 / VI51049 / EDL-933	(Nielsen and Andersen, 2003)
	rfbE (O157)	VI51277 / VI51049	(Perelle et al., 2004)
	wbdI (O111)	VI51048	
	wzx (O26)	VI51052	
	wzy(O145)	VI51046	(Fratamico et al., 2009)
	wzx (O103)	VI51050	(Perelle et al., 2004)
Conventional PCR	*stx_{2a}*	94C	(O'Brien et al., 1984)
	stx_{2b}	EH250	(Pierard et al., 1998)
	stx_{2c}	031	(Paton et al., 1992)
	stx_{2d}	C165-02	(Persson et al., 2007)
	stx_{2e}	S1191	(Weinstein et al., 1988)
	stx_{2f}	T4/97	(Schmidt et al., 2000)
	stx_{2g}	7v	(Leung et al., 2003)
	gadA, chuA, yjaA, TSPE4.C2	Bæ14	(Clermont et al., 2000, Doumith et al., 2012)
Serological detection - Serotyping by *E. coli* antisera	*O145*	A08	European Union Reference Laboratory VTEC (EURL VTEC)
	O121	B08	
	O111	C08	
	O157	D08	
	O103	E08	
	O91	F08	
	O26	G08	
	O113	CRL-464	
	O128	T4/97 (D3546)	
	O146	EC_Norway_NVI_257	
	O104	H519	
	O55	Su 3912-41	
	O45	EU-RL-VTEC-EF-129	

3. RESULTS

3.1 SCREENING OF SAMPLES

A total of 269 bivalve samples collected from harvesting farms distributed across the coast of Norway were analyzed for STEC. The samples were divided into their respective species categories, blue mussels comprising the largest category. A breakdown of how many virulence genes and how often they were detected by screening of enrichment broth by real-time PCR is included in Table 8. Two samples of clams and one banded carpet shell have not being included in this table as the real-time PCR detected no virulence genes or O-serogroups. The appendices shows the complete screening results obtained by real-time PCR for all the samples analyzed in this study.

Table 8. Number of samples divided into their corresponding species categories, with the number of samples positive for virulence genes and/or O-serogroups detected in the screening by real-time PCR.

Sample category	Samples	Virulence genes and O-serogroups							
		***stx*$_1$**	***stx*$_2$**	***eae***	**O145**	**O157**	**O111**	**O26**	**O103**
Blue mussels	218	17	16	68	17	15	15	17	11
Oysters	28	1	2	4	1	1	1	3	1
Scallops	15	-	1	-	-	-	1	1	-
Horse mussels	5	1	-	1	-	-	2	1	-
	Total	19	19	73	18	16	19	22	12

The screening of 269 bivalve enrichment broths by real-time PCR returned 105 samples positive for at least one or more of the eight targeted genes tested. The *stx* genes (*stx*$_1$ and/or *stx*$_2$) were detected in 28 samples, and 19 of these samples contained *stx* and *eae* (some also harbored O-serogroups), hence were selected for attempted isolation of STEC and characterization of isolates obtained.

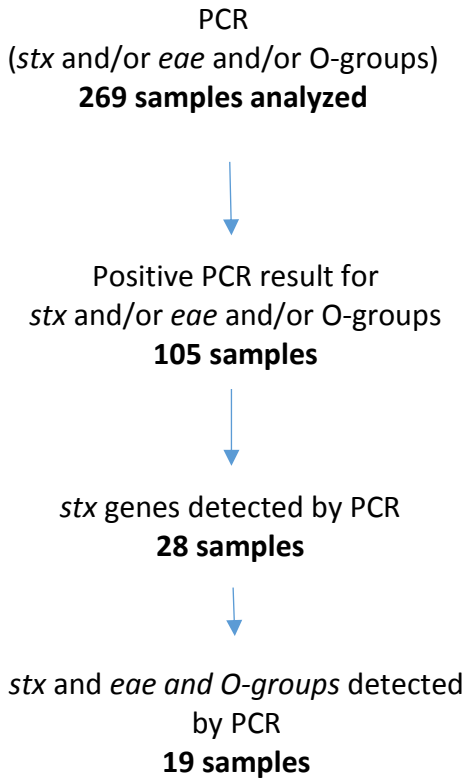


Figure 15. Real-time PCR steps followed for sample analysis. Showing the initial screening for marker genes in all the samples, the number of positive PCR results obtained, and the breakdown of how many samples were detected with stx, eae and O-groups.

The 19 samples selected for attempted strain isolation comprised 17 blue mussels and two oysters. They derived from different shellfish harvesting farms, distributed in six counties. These 19 samples harbored *stx* and *eae* genes and also relevant O-serogroups were detected in the first real-time PCR experiment performed when screening at NIFES as described in Table 9.

Table 9. Description of the stx and eae positive samples, (blue mussel (BM) and oyster) with their corresponding origin by county and the total number of virulence genes & O-groups detected by real-time PCR.

Sample	Sample category	County	Genes targeted	Virulence genes & O-serogroups							
				***stx*$_1$**	***stx*$_2$**	***eae***	**O145**	**O157**	**O111**	**O26**	**O103**
561	BM	Hordaland	2	+	-	+	-	-	-	-	-
635	BM	Sør-Trøndelag	5	-	+	+	+	+	-	+	-
733	BM	Sør-Trøndelag	7	+	+	+	+	+	+	+	-
738	BM	Sør-Trøndelag	4	+	-	+	-	+	-	+	-
735	BM	Sør-Trøndelag	7	+	+	+	+	+	+	+	-
734	BM	Sør-Trøndelag	7	+	+	+	+	+	+	+	-
732	BM	Nord-Trøndelag	7	+	+	+	+	+	+	+	-
737	BM	Sør-Trøndelag	5	+	+	+	-	+	-	+	-
809	BM	Sogn og Fjordane	5	+	+	+	-	+	-	+	-
811	Oysters	Rogaland	2	-	+	+	-	-	-	-	-
1041	BM	Nord-Trøndelag	3	-	+	+	-	-	-	-	+
1246	BM	Sør-Trøndelag	3	-	+	+	-	+	-	-	-
1218	BM	Sør-Trøndelag	2	-	+	+	-	-	-	-	-
1200	BM	Nord-Trøndelag	4	+	-	+	+	-	-	+	-
1239	BM	Sogn og Fjordane	4	-	+	+	+	-	-	-	+
1330	BM	Nordland	3	-	+	+	-	-	-	+	-
1373	BM	Nord-Trøndelag	3	+	+	+	-	-	-	-	-
1329	Oysters	Hordaland	7	+	+	+	+	+	-	+	+
1332	BM	Sør-Trøndelag	5	+	+	+	-	+	-	-	+

3.2 ISOLATION OF STEC

3.2.1 DIRECT PLATING

Direct plating was performed on the 19 samples in order to pick-and-pool 50 colonies. Typical and suspicious colonies were obtained for all the samples. The pools were tested for *stx*$_1$ and *stx*$_2$ genes by PCR, and the results revealed a total of four samples harboring the *stx*$_2$ gene in one or more pools (Table 10). The *stx*$_1$ genes were not detected in any of the 19 samples analyzed by real-time PCR.

Table 10. Samples of colony pools analyzed for virulence genes (stx$_1$, stx$_2$ and eae) by real-time PCR. NA is not available. Positive sample results marked in red.

Sample No.	Genes detected		
	stx$_1$	***stx$_2$***	***eae***
561	-	-	NA
635	-	-	NA
733	-	-	NA
738	-	-	NA
735	-	-	NA
734	-	-	NA
732	-	-	NA
737	-	-	NA
809	-	-	NA
811	-	+	-
1041	-	-	NA
1246	-	+	-
1218	-	-	NA
1200	-	-	NA
1239	-	+	-
1330	-	-	NA
1373	-	-	NA
1329	-	+	+
1332	-	-	NA

From the corresponding positive pools, all ten single colonies comprising the pool, were analyzed separately by PCR to identify which colony harbored the *stx*$_2$ gene. From the pools of the four samples analyzed, eight colonies were detected as *stx*$_2$ positive (Table 11). The *eae* gene from the eight colonies was also tested by real-time PCR, showing only one *eae*-positive isolate (sample 1329, colony number 29). These colonies were identified as presumptive STEC, further subjected to identification by MALDI-TOF (see section 2.5.1). Real-time PCR was used to check for O-serogroups (O145, O157, O103, O111 and O26) in these eight isolates, revealing that none of the isolates belonged to any of the serogroups tested for.

Table 11. Eight isolates belonging to the four positive samples, showing screening results as detected by real-time PCR. Morphology of the colonies is also noted.

Sample	Positive pools	Positive colonies	Colony n°	Morphology	Ct-values from screening results	
					***stx*$_2$**	***eae***
811	4	4	20	BLUE	14.69	-
			23	PINK	16.23	-
			35	PINK	13.88	-
			49	PINK	16.55	-
1246	1	1	14	BLUE	17.00	-
1239	1	2	3	PINK	30.85	-
			4	PINK	17.94	-
1329	1	1	29	PINK	34.00	32.17

3.2.2 AUTOMATED IMMUNOMAGNETIC SEPARATION (AIMS)

The phenotypical method, AIMS, was used for two samples (734 and 1246) to test an alternative isolation method which focuses on O-serogroups.
Screening of the enrichment broth indicated presence of O-groups O26, O111, O145 and O157 in sample 734, while sample 1246 was positive only for O157. AIMS was used as a tool to help with isolation, testing separately for each serogroup. Colonies were tested by agglutination to determine the presence of serogroups.

In sample 734, there were no colonies present on the plates for the O157 serogroup.
The presumptive colonies were screened by real-time PCR to test for four O-serogroups (O26, O157, O145 and O111). This was done to confirm the results obtained from slide-agglutination with the antiserums. Three serogroups were not detected, therefore considered negative (O26, O111 and O145).
Real-time PCR confirmed the agglutination results from sample 1246, showing all 23 colonies to be O157 positive (Table 12). For sample 734, however, the real-time PCR results were not in accordance with the agglutination results from AIMS.

Table 12. Agglutination results for the two samples (734 and 1246) tested with the AIMS approach.

Sample	O-serogroups	CHROMagar®	CT-SMAC
734	O157	0	0
	O26	22	24
	O111	2	8
	O145	1	18
1246	O157	23	0

From picking 50 colonies

From AIMS

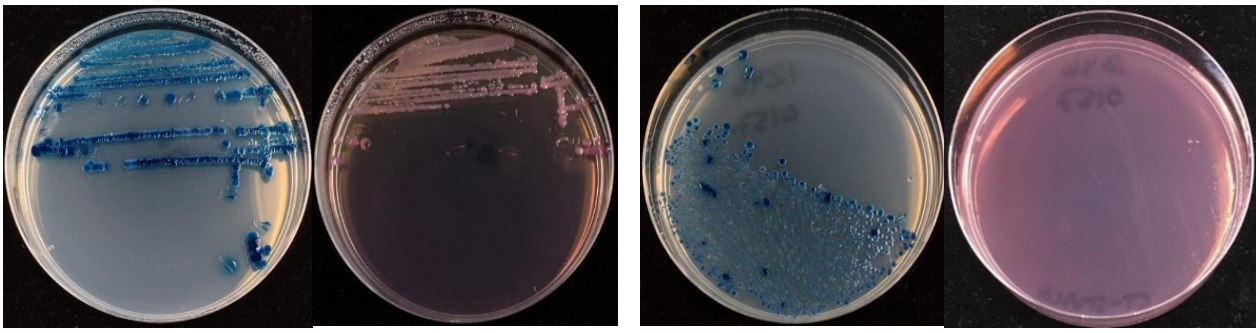


Figure 16. Sample 1246 showing colonies growing on different selective media (CHROMagar and CT-SMAC) from two different isolation approaches. Note there is no colony growth on CT-SMAC plate from AIMS compared to the same plate with the different approach of isolation by direct streaking on plate.

3.3 CHARACTERIZATION OF ISOLATES

3.3.1 MALDI-TOF

The eight presumptive STEC isolates (as indicated from colony morphology and presence of *stx₂* gene), from the direct plating method, were further characterized by the MALDI-Biotyper and six isolates were identified as *E. coli* (Table 13). Samples 1239 and 1329 were detected as *Citrobacter freundii* and *Enterobacter cloacae,* respectively. The samples analyzed had score values between 2.10 and 2.50*,* showing high confidence identification.

Table 13. MALDI-TOF identification results overview for the eight isolates

Sample	Organism (best match)	Score Value	Organism (second best-match)	Score Value
811_20	*Escherichia coli*	2.31	*Escherichia coli*	2.28
811_23	*Escherichia coli*	2.43	*Escherichia coli*	2.35
811_35	*Escherichia coli*	2.33	*Escherichia coli*	2.32
811_49	*Escherichia coli*	2.29	*Escherichia coli*	2.29
1246_14	*Escherichia coli*	2.36	*Escherichia coli*	2.33
1239_3	*Citrobacter freundii*	2.26	*Citrobacter braakii*	2.24
1239_4	*Escherichia coli*	2.32	*Escherichia coli*	2.32
1329_29	*Enterobacter cloacae*	2.37	*Enterobacter cloacae*	2.27

3.3.2 VIRULENCE GENE CHARACTERIZATION

The colonies isolated by AIMS from sample 1246, (23 O157 isolates) were tested for *stx*$_1$, *stx*$_2$ and *eae* genes by using real-time PCR in order to be able to consider it a STEC or not. The results revealed the absence of these virulence genes, therefore these isolates do not belong to the STEC group.

Subtyping of six STEC strains positive for *stx*$_2$ was performed and the variants *stx*$_{2a}$, *stx*$_{2e}$ and *stx*$_{2g}$ were detected (alone and not in combination with other *stx*$_2$ subtypes) amongst five of the six isolates respectively (Table 14). Three strains from the same sample (811) were positive for the *stx*$_{2a}$ subtype, while the remaining strain from this sample gave no outcome. One strain from sample 1246 was *stx*$_{2g}$ and one strain from sample 1239 was *stx*$_{2e}$ (see appendix). No isolates carried the *stx*$_2$ subtypes of *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$ or *stx*$_{2f}$ (see summary Table 16).

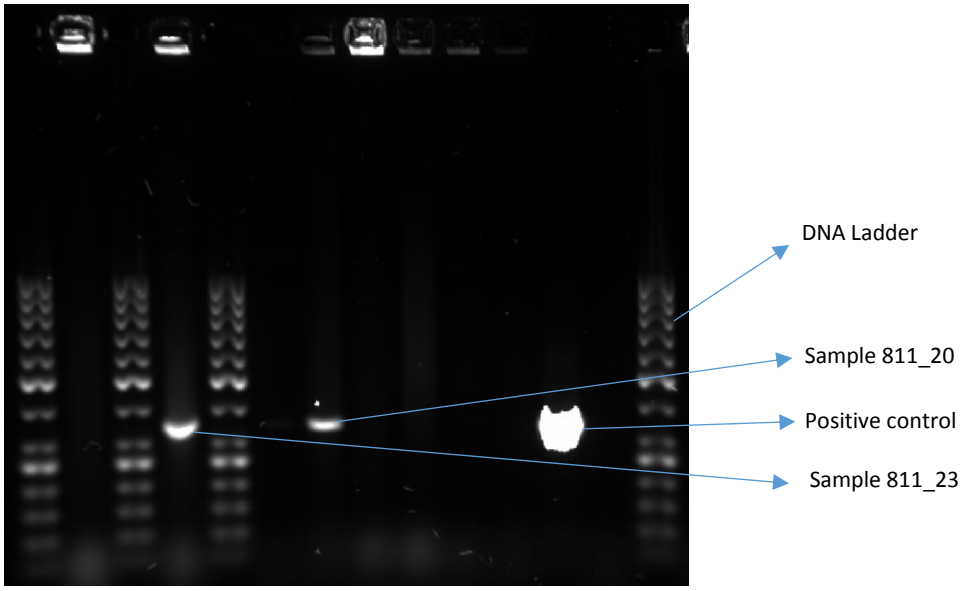


Figure 17. Agarose gel image showing two stx$_{2a}$ *positive strains from the sample 811 for* stx$_{2a}$.

Table 14. Results of the six confirmed E. coli isolates tested by multiplex PCR to identify the stx_2 variant

Sample	stx_{2a}	stx_{2b}	stx_{2c}	stx_{2d}	stx_{2e}	stx_{2f}	stx_{2g}
811_20	+	-	-	-	-	-	-
811_23	+	-	-	-	-	-	-
811_35	+	-	-	-	-	-	-
811_49	-	-	-	-	-	-	-
1246_14	-	-	-	-	-	-	+
1239_4	-	-	-	-	+	-	-
Pos control	+	+	+	+	+	+	+
Neg control	-	-	-	-	-	-	-

3.3.3 SEROTYPING

The six isolates were tested by serotyping, as this identified the somatic antigens. O-agglutination was performed for the following O-groups; O145, O121, O26, O103, O111, O91, O157, O113, O128, O146, O104, O55 and O45.

The serotyping results were non-typable (NT) for all strains, tested for all 13 O-serogroups. As *stx_{2e}* is associated with disease in pigs (Beutin et al., 2008) and usually associated to specific serogroups, four additional O-serogroups were tested (O138, O139, O141abc and O149). Results were negative for all four groups, therefore this isolate was also classified as NT (Summary Table 16).

3.3.4 PHYLOTYPING

Phylogenetic analysis obtained from gel electrophoresis can be observed in Figure 18. The gene *chuA* did not show a positive outcome in the positive control well, therefore there are two sets of possible results. Table 15 shows results 1 (B1/A) with the outcome if the *chuA* gene was absent from the strains analyzed, hence was a true negative. Results 2 (B2/D) would determine the phylogroup if the *chuA* gene was present, therefore positive. Either way, the isolates would belong to two different groups, B1/A or B2/D. Due to time limitations, the analysis could not be run again to obtain one set of results.

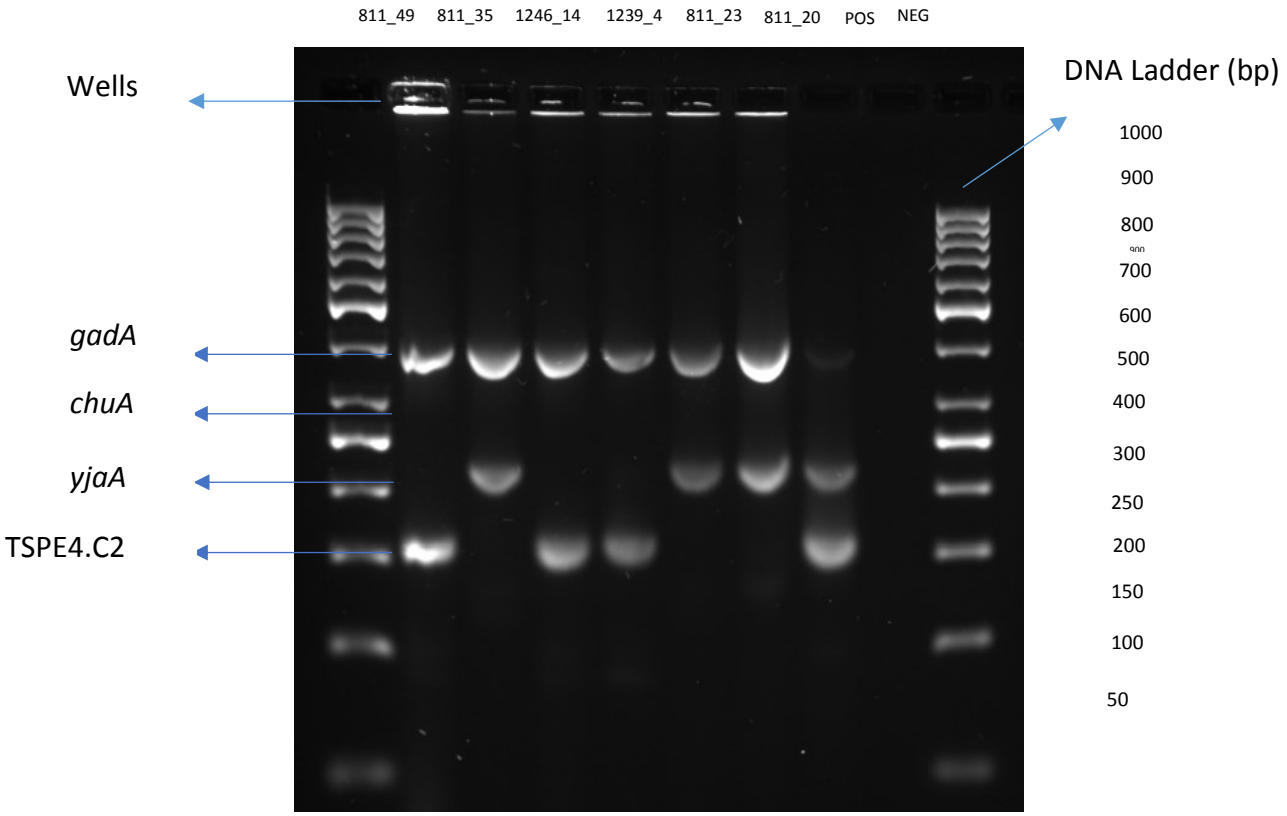


Figure 18. Image obtained from gel electrophoresis run for the phylogenetic analysis

Table 15. Results from phylotyping of the six STEC strains obtained in the present study.

Sample	gadA 373 bp	chuA 281 bp	yjaA 216 bp	TSPE4.C2 152 bp	Results 1	Results 2
811_20	+	-	+	-	A	B2
811_23	+	-	+	-	A	B2
811_35	+	-	+	-	A	B2
811_49	+	-	-	+	B1	D
1246_14	+	-	-	+	B1	D
1239_4	+	-	-	+	B1	D
Pos control	+	**-**	+	+	B2	B2
Neg control	-	-	-	-	-	-

3.4 SUMMARY

The results from the isolation and characterization of STEC from 269 samples of bivalves are summarized in Table 16.

Table 16. Summary of results with information on STEC strains detected in this study. NT is Non-typable.

Sample	Sample type	Virulence gene	*E. coli* pathogroup	MALDI	Ct value from stx_2 PCR	stx_2 variant	Phylogroup	Serotype
811_20	Oysters	stx_2	STEC	*E. coli*	14.69	stx_{2a}	A/B2	NT
811_23	Oysters	stx_2	STEC	*E. coli*	16.23	stx_{2a}	A/B2	NT
811_35	Oysters	stx_2	STEC	*E. coli*	13.88	stx_{2a}	A/B2	NT
811_49	Oysters	stx_2	STEC	*E. coli*	16.55	NT	B1/D	NT
1239_4	Blue mussels	stx_2	STEC	*E. coli*	17.94	stx_{2e}	B1/D	NT
1246_14	Blue mussels	stx_2	STEC	*E. coli*	17.00	stx_{2g}	B1/D	NT

4. DISCUSSION

The current study is the first to address the isolation and characterization of STEC strains from bivalves originating from different shellfish-harvesting farms scattered along the Norwegian coast. The aims of this study were to examine the presence of STEC, by detecting presence of toxin encoding genes, adherence genes and/or the genes for the five serogroups of most concern (O157, O145, O111, O103 and O26). Very few studies have focused on STEC detection and isolation from bivalves (Baliere et al., 2015, Bennani et al., 2011, Sanath Kumar et al., 2001, Gourmelon et al., 2006, MacRae et al., 2005).

OCCURRENCE OF STEC IN BIVALVES

STEC detection started with initial screening of virulence genetic markers (*stx*$_1$, *stx*$_2$, *eae*) and O-serogroups (O157, O145, O111, O103 and O26) with the real-time PCR approach to identify the presence or absence of these marker genes which belong to strains associated with human infections. The screening resulted in an indication that 7.1% of the samples (19 of 269 samples), were harboring virulence genes (both *stx* and *eae*). By the end of the study, after further examination and isolation of STEC was attempted, it was confirmed that three samples contained STEC.

ISOLATION

Shiga toxins and intimin represent two of the major virulence attributes of typical STEC strains (Paton and Paton, 1998b). The presence/absence of these genes are considered trademarks of STEC as a preliminary identification of the pathogenicity of *E. coli*.

From all the bivalve samples analyzed in this study (n=269), three samples contained in total six verified STEC isolates. Four of the isolates were from the same sample. All the six isolates harbored the *stx*$_2$ gene, however, they all revealed the absence of the *eae* gene. The lack of this intimin gene is in agreement with Bennani et al. (2011) in the study of STEC from Moroccan shellfish.

LOW DETECTION OF STEC

The low number of isolated STEC isolates (three) compared to the high numbers of genetic markers detected in the analyzed samples is a common finding, previously observed in other studies. Gourmelon (2006) studied STEC prevalence in French shellfish and *stx* genes were detected by PCR in 40 from 144 samples. STEC isolates were further detected by colony DNA hybridization and isolated five STEC strains. These strains belonged to the serogroups O38, O100 and O149 and harbored the stx_1 genes. Bennani (2011) revealed the first detection in Morocco of STEC in shellfish by PCR but also found low numbers of these (5 from 82). A study in India by Sanath Kumar (2001) looked at the prevalence of STEC in seafood by PCR and reported a 5% non-O157 STEC discovery in clams. This current study reported <2% prevalence.

When trying to explain the low detection of STEC, some possibilities need to be considered. When initial screening by PCR is performed on samples, targeted *stx, eae* and serotypes markers could give a positive outcome because they can be detected by viable but non-culturable or dead bacteria being present. This can result from the stressful conditions these bacteria can experience in the environment (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).

Another possibility is that the real-time PCR detected these genes encoded in *stx* bacteriophages. These are mobile genetic elements that play an important role in the evolution of STEC strains. *E. coli* can shift from being harmless to pathogenic from gaining genetic mobile elements from bacteriophages, pathogenicity islands and plasmids (Baliere et al., 2016).These *stx*-encoding bacteriophages can exist freely in the environment (Martinez-Castillo et al., 2013), and they possess the ability to transfer the *stx* genes and consequently convert nonpathogenic strains into STEC. The *stx*-phages are capable of acting as survival capsules for *stx* genes allowing them to persist outside their host cells (Bergan et al., 2012) in the natural environment. These phages seem to be stable in the environment for long periods of time, therefore, because of their numbers and persistence, they may be an important source of new toxigenic strains in the environment (Muniesa et al., 1999).

They originate from fecal contamination that contains both free *stx*-phages and STEC strains (Allison, 2007). The ability to lose stx genes could also occur. The *stx* genes can be gained or lost giving pathogenicity changes to the strain. The ability to lose *stx* genes has been previously observed by (Karch et al., 1992). *E. coli* may become harmless by losing the *stx*-converting bacteriophage genome (Bielaszewska et al., 2007, Feng et al., 2001).

Other bacteria, which are not *E. coli*, can also harbor these genes. *Shigella*, *Enterobacter* and *Citrobacter* have previously been discovered to possess Shiga toxins (Gray et al., 2015, Paton and Paton, 1997, Schmidt et al., 1993). In this current study, a non-*E. coli* isolate from a blue mussel sample harbored *stx* genes, specifically stx_2, showing a high Ct value (30.85). This isolate was analyzed by MALDI-TOF and was identified as *Citrobacter freundii*. Another isolate from an oyster sample was detected by real-time PCR as containing stx_2 genes, with a high Ct value (34), and when analyzed by MALDI-TOF, the isolate was classified as *Enterobacter cloacae*. This could be due to having a mixed culture as the Ct values are quite high for a pure culture and could be considered negative, therefore these two results have some uncertainties that could be clarified by whole genome sequencing.

Other bacteria can grow in shellfish enrichment broths and suppose a challenge in the isolation of the samples. The AIMS approach deals with background flora as it contains serotype specific beads which increases the chances for recovery of STEC. The plating on different agar plates is also helpful, allowing the target to be distinguished from the numerous background flora present.

PRESENCE OF STEC IN THE ENVIRONMENT

The existence of STEC in bivalves is due to their introduction in the water column from an animal or human reservoir. STEC strains are commonly found in the intestine of cattle and other ruminants, cattle are considered the principal reservoir of STEC (Brussow et al., 2004). These bacteria can enter the marine environment by runoff from land or via the sewage.

STEC could also be temporarily present in surface sediments and can be re-introduced in water, followed by accumulation and filtering behavior from bivalves. Fremaux (2010) studied the fate of STEC strains in soil and concluded that they were able to disseminate down through the soil but were not able to survive for extended periods. Persistence of strains can vary between different serotypes as Ma (2014) studied the differences and observed a non-O157 STEC persistence of up to 3 months in agricultural soil.
Berthe (2013) showed distinct generic *E. coli* survival types and coexistence in bodies of estuarine water in France.

SURVIVAL OF *E. COLI* IN THE MARINE ENVIRONMENT

When bacteria enter the marine environment, they experience several stress factors that they need to overcome with their adaptation capacity in order to survive.
Several studies have investigated the factors that can alter *E. coli* concentrations in seawater. Rainfall is recognizably one of the main factors (Lunestad et al., 2016) affecting the increased exposure of bacterial pathogens in bivalves (Campos et al., 2011). During rainfall, the water washes animal wastes (containing *E. coli*) from contaminated areas and finds its way into water bodies. However, seawater pH normally ranges between 7.5 and 8.5 and this contributes to a lower *E. coli* survival, as an acidic pH was found to be more favorable for generic *E. coli* survival (Rozen and Belkin, 2001). Temperature is an important factor affecting *E. coli* survival, they can survive at lower temperatures encountered in the seawater, even though their optimal growth is usually 37°C. Sunlight can also affect the bacteria as light is considered to contribute to bacterial die-off in the sea. Some biotic factors can also influence the survival of *E. coli* in the marine environments such as predation (commonly by protozoa) and competition (Rozen and Belkin, 2001).

PHYLOGROUP

The STEC strains in this study were classified into either A and B1, or D and B2.
The reason for these two sets of results is due to the failure in one of the phylogenetic markers (*chuA*). The positive control used for the phylogroup analysis was Bæ14, which worked efficiently for all the genes except for *chuA*. If *chuA* represents a true negative then the results of this study would be in agreement with (Garcia-Aljaro et al., 2005) who also discovered the predominance of the phylogroups A and B1 in the environmental STEC strains isolated from urban sewage and animal wastewaters in Spain.
The discovery of environmental samples belonging more frequently to the B1 phylogroup was also observed by a recent study from aquatic environments in France (Berthe et al., 2013).

ISOLATES OF *stx*$_2$

The STEC strains which are highly pathogenic to humans, commonly harbor *stx*$_1$ and/or *stx*$_2$ as well as the intimin coding gene, *eae* (Kagkli et al., 2011).
The latter is lacking from the strains isolated in this study, hence this could indicate a lower virulence to humans as the *eae* gene is considered an important virulence factor.
The most important virulence gene associated with severe human disease has proven to be *stx*$_2$ (Boerlin et al., 1999, Paton and Paton, 1998a). It has been observed that strains associated with HUS often harbor the *stx*$_2$ gene alone or together with *stx*$_1$ (Gerber et al., 2002).

The six isolated strains in this study lacked the *eae* gene, but it is not verified that these strains do not have the genetic ability to adhere to host cells through other structures because they could possess other genes that contain the adhesion factors such as *saa, paa, ehaA, lpfA, espP, iha* and *sab*. These genes were not studied in this study, therefore a whole-genome sequencing analysis for three strains belonging to three different samples is being done to obtain the information needed to classify the strains. Unfortunately, the results are not available within the timeframe of this thesis.

Among the STEC strains discovered in this study, five of six could be pathogenic to humans and cause infection because they revealed the presence of *stx*$_2$ variants, *stx*$_{2a}$, *stx*$_{2e}$ and *stx*$_{2g}$. *stx*$_{2a}$ was the most frequently found variant (present in three strains) and STEC harboring this *stx* subtype have been associated with clinical symptoms, such as HUS and HC (Baliere et al., 2016).

The subtype *stx*$_{2e}$ has been associated with swine edema disease, and this *stx*$_2$ variant has been proven to be less pathogenic to humans (Beutin et al., 2008) The subtype *stx*$_{2g}$ could be associated with cattle sources, and a recent study from Baliere (2016) classified a sample originating from shellfish as *stx*$_{2g}$ positive, which is in agreement with the findings of this study. Leung (2003) discovered the *stx*$_{2g}$ variant in bovine strains.

The knowledge of the type of variant of the toxin is important to not only have an understanding of the origin of the strains but also the virulence of the STEC strain.

The isolates in this study can be somewhat compared to a study by Baliere (2016). The discovery of strains belonging to *stx*$_{2a}$, *stx*$_{2e}$ and *stx*$_{2g}$ were isolated from shellfish, mussels specifically. These French STEC strains subtypes are in agreement with the Norwegian STEC isolates in this study and they also showed the absence of *stx*$_1$ and *eae*. The discrepancy between them is however the phylogroups. The corresponding phylogroups for the variants *stx*2$_a$, *stx*2$_e$ and *stx*2$_g$ from the other study was B1, A and A accordingly. In this study, the groups are either A/B2, B1/D and B1/D respectively. The virulence factors are similar, but the phylogroups are different, thus they are different strains.

A study by Beutin (2007) found 42 strains of *stx*$_{2e}$ in pork samples and 6 strains of *stx*$_{2g}$ from 219 samples analyzed from meat, milk and cheese in Germany. The role of *stx*$_{2g}$ as agents of disease is not very clear yet. A study on French environmental samples by Vernozy-Rozand (2004) found 15 positive samples for *stx*$_{2e}$ originating from wastewater treatment plant, pig farms and dairy cattle herd. The data in this study also suggests the spread of STEC from pigs to cattle. The pathogenicity of *stx*$_{2e}$ STEC strains for humans is regarded as low (Beutin et al., 2008).

METHODOLOGICAL CONSIDERATIONS

PCR is usually considered to be the main approach to screen for specific genes required or typical for STEC. The PCR-based approach used to detect the virulence genes in the shellfish samples was followed from the ISO13136:2012 technical specification.
The enrichment broth was produced in order to increase the bacterial growth and therefore the number of copies of the target sequence (Paton and Paton, 1998a).

The interpretation of the presumptive results from the AIMS approach depends on the skill of the person carrying out the analysis to correctly identify and differentiate the isolated colonies based on typical *E. coli* morphology.
The use of two different culture media (CT-SMAC and CHROMagar®) was used to increase the chances of detecting suspect colonies that have distinct differential features on each media. On the CT-SMAC, suspicious colonies would appear colourless and on CHROMagar® they would appear pink-mauve.
Indeed, antigenically similar organisms (e.g. *E. hermannii, Proteus spp*.) can cross-react and bind, however this will not affect the binding of *E. coli* O157 to the beads.
There is an increasing demand for improved diagnostic procedures for the detection of STEC in food samples.

5. CONCLUSION

Our knowledge about Shiga toxins in bacteria and how they affect humans has expanded in the last few years, but there is still more research that needs to be done in order to better understand the risk STEC presents.
STEC have emerged as an important cause of food-borne infections, therefore the objective of this study was to investigate and characterize STEC in Norwegian commercially farmed bivalves.

In total, six strains were identified as STEC (as they harbored the *stx*$_2$ genes) from three of the 269 samples examined. This represents a low number, hence a low occurrence of STEC strains is seen in shellfish originating from Norwegian harvesting farms. The risk of STEC infection after consuming shellfish from these designated areas can be considered as low.
Rapid methods need to be implemented in food microbiology. The ISO/TS-13136 methodology has proved to be suitable in this study to detect STEC strains.
The incorporation of whole genome sequencing methods in STEC surveillance aim to improve the tracking of infections and gain more knowledge on the biology of this group of bacteria.

When food poisoning by pathogenic *E. coli* occurs, it is of great importance to be able to rapidly detect and type the *stx* and O-antigen genes of STEC strains from humans and suspected food samples. This early detection has priority for public health for several reasons: could prevent the advancement into life-threatening infections such as HUS and to be able to trace back the source of infection to then further prevent outbreaks.

All of the STEC strains isolated in this study lacked the *eae* gene which is strongly associated with high virulence to humans, hence reinforcing the conclusion that the potential risk of STEC infection for shellfish consumers is limited. Nevertheless, this study demonstrated the presence of STEC in bivalves, which could emerge as being pathogenic to humans.

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Carlota Cedillo Martin

Bergen, June 2017

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APPENDIX

The following pictures show the gel images of two samples positive for *stx*$_{2e}$ and *stx*$_{2g}$

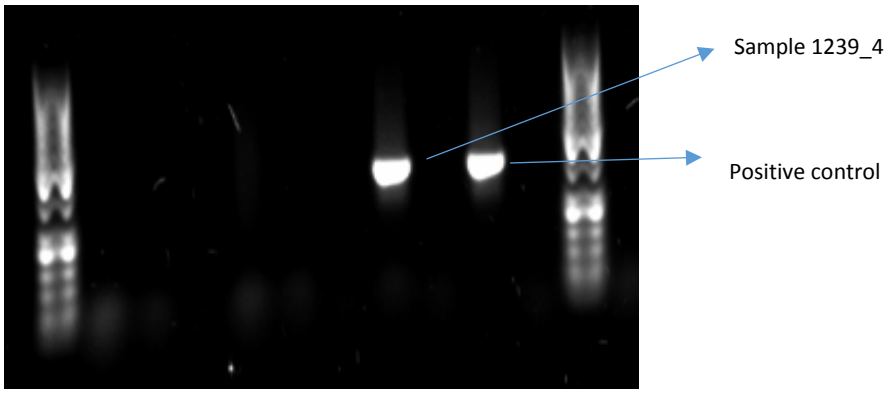

Figure 19. Gel image showing positive sample 1239 for stx$_{2e}$

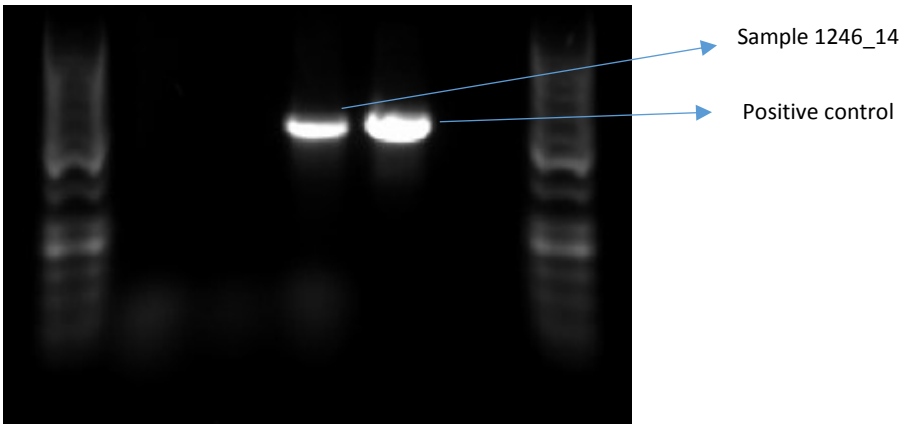

Figure 20. Gel image showing positive sample 1246 for stx$_{2g}$

Below is a complete overview of the real-time PCR results for detection of virulence genes and O-serogroups for all the samples analyzed in this study

The results obtained are shown as "+" when the DNA sample gave a signal of gene amplification, and as blank when no signal was detected.

Sample	Category	Municipality	Screening							
			stx1	stx2	eae	O145	O157	O111	O26	O103
2016-1402	Blue mussel	BRØNNØY			+					
2016-1401	Blue mussel	LILLESAND								
2016-1400	Blue mussel	BJUGN								
2016-1399	Blue mussel	BJUGN								
2016-1398	Blue mussel	ÅFJORD								
2016-1397	Blue mussel	ÅFJORD								
2016-1396	Blue mussel	ÅFJORD								
2016-1395	Blue mussel	FJALER								
2016-1394	Blue mussel	FJALER								
2016-1393	Blue mussel	ÅFJORD			+					
2016-1379	Blue mussel	ÅFJORD								
2016-1378	Blue mussel	ÅFJORD			+					
2016-1377	Blue mussel	ÅFJORD			+					
2016-1366	Oysters	SVEIO								
2016-1367	Blue mussel	NORDREISA			+					
2016-1369	Blue mussel	BØMLO		+						
2016-1370	Blue mussel	VESTVÅGØY								
2016-1371	Scallops									
2016-1372	Blue mussel	MOSVIK FØR 2012			+					
2016-1373	Blue mussel	INDERØY	+	+	+					
2016-1374	Blue mussel	ÅFJORD			+		+			
2016-1375	Blue mussel	ÅFJORD			+					
2016-1376	Blue mussel	ÅFJORD								
2016-1332	Blue mussel	ÅFJORD	+	+	+		+			+
2016-1331	Blue mussel	MOSVIK FØR 2012			+					
2016-1330	Blue mussel	BINDAL		+	+				+	
2016-1329	Oysters	SVEIO	+	+	+	+	+		+	+
2016-1297	Oysters	SVEIO								
2016-1296	Blue mussel	MOSVIK FØR 2012								
2016-1295	Blue mussel	INDERØY								
2016-1293	Blue mussel									
2016-1292	Blue mussel	RISSA			+					
2016-1291	Blue mussel	FOSNES								
2016-1290	Blue mussel	FOSNES								
2016-1256	Blue mussel	NAMDALSEID								
2016-1255	Blue mussel	NAMDALSEID								
2016-1254	Blue mussel	ÅFJORD								
2016-1253	Blue mussel	GRIMSTAD								
2016-1252	Blue mussel	MOSVIK FØR 2012								
2016-1251	Oysters	SVEIO								
2016-1250	Blue mussel	TYSVÆR								
2016-1249	Blue mussel	BJUGN								+
2016-1248	Blue mussel	BJUGN			+					+
2016-1246	Blue mussel	ÅFJORD		+	+		+			

2016-1245	Blue mussel	ÅFJORD			+							
2016-1244	Blue mussel	ÅFJORD			+							
2016-1243	Blue mussel	KVITSØY										
2016-1242	Oysters	KVITSØY										
2016-1241	Scallops	KVITSØY										
2016-1240	Blue mussel	FJALER			+							+
2016-1239	Blue mussel	FJALER		+	+		+					+
2016-1238	Blue mussel	ÅFJORD										
2016-1218	Blue mussel	ÅFJORD		+	+							
2016-1217	Blue mussel	BRØNNØY										
2016-1216	Blue mussel	LEIRFJORD										
2016-1215	Oysters	OS I HORDALAND										
2016-1214	Blue mussel	VERRAN			+							
2016-1213	Blue mussel	INDERØY										
2016-1212	Scallops											
2016-1211	Blue mussel	NORDREISA			+		+					
2016-1210	Blue mussel	BØMLO										
2016-1202	Blue mussel	ÅFJORD										
2016-1201	Blue mussel	VERRAN			+							
2016-1200	Blue mussel	FOSNES	+		+		+				+	
2016-1199	Blue mussel	FOSNES										
2016-1198	Blue mussel	BINDAL			+							
2016-1197	Blue mussel	VESTVÅGØY										
2016-1179	Blue mussel	MOSVIK FØR 2012							+			
2016-1178	Blue mussel	VERRAN										
2016-1177	Blue mussel	INDERØY										
2016-1176	Oysters	OS I HORDALAND										
2016-1175	Scallops	KVITSØY										
2016-1174	Oysters	KVITSØY							+			
2016-1173	Blue mussel	BJUGN										
2016-1172	Blue mussel	ÅFJORD			+							
2016-1171	Blue mussel	BJUGN										
2016-1170	Blue mussel	ÅFJORD										
2016-1169	Blue mussel	ÅFJORD										
2016-1168	Blue mussel	RISSA			+							
2016-1167	Oysters	SVEIO										
2016-1148	Blue mussel											
2016-1147	Blue mussel	FJALER			+							
2016-1146	Blue mussel	FJALER			+							
2016-1145	Blue mussel	ÅFJORD										
2016-1140	Blue mussel	NAMDALSEID			+							
2016-1139	Blue mussel	NAMDALSEID										
2016-1138	Blue mussel	NAMSOS										
2016-1137	Blue mussel	NAMSOS			+							
2016-1136	Blue mussel	NAMSOS										
2016-1132	Blue mussel	VERRAN										

2016-1131	Blue mussel	MOSVIK FØR 2012							
2016-1129	Blue mussel	VESTVÅGØY							
2016-1127	Blue mussel	NORDREISA		+	+				+
2016-1076	Blue mussel	VERRAN							
2016-1075	Blue mussel	NAMDALSEID							
2016-1074	Blue mussel	NAMDALSEID							
2016-1073	Scallops								
2016-1072	Blue mussel	RISSA		+					
2016-1069	Scallops	KVITSØY						+	
2016-1065	Oysters	KVITSØY		+					
2016-1064	Blue mussel	KVITSØY		+					
2016-1063	Oysters	SVEIO							
2016-1042	Blue mussel	NAMSOS							
2016-1041	Blue mussel	NAMSOS	+	+					+
2016-1040	Blue mussel	ÅFJORD							
2016-1039	Blue mussel	ÅFJORD							
2016-1038	Blue mussel	ÅFJORD							
2016-1037	Blue mussel	ÅFJORD							
2016-1036	Blue mussel	ÅFJORD							
2016-1035	Blue mussel	BJUGN		+					
2016-1034	Blue mussel	BJUGN		+					
2016-1033	Blue mussel	ÅFJORD							
2016-1032	Blue mussel	ÅFJORD							
2016-1031	Blue mussel	ÅFJORD							
2016-1030	Blue mussel	ÅFJORD							
2016-1029	Blue mussel	FJALER							
2016-1028	Blue mussel	FJALER							
2016-1026	Blue mussel	VESTVÅGØY						+	
2016-954	Blue mussel	NORDREISA							
2016-946	Blue mussel	NAMDALSEID							
2016-945	Blue mussel								
2016-944	Blue mussel	ÅFJORD							
2016-943	Blue mussel	ÅFJORD							
2016-942	Blue mussel								+
2016-941	Blue mussel	BØMLO							
2016-930	Blue mussel			+					
2016-925	Blue mussel	LILLESAND							
2016-923	Oysters	OS I HORDALAND		+				+	
2016-922	Oysters	ØYGARDEN						+	
2016-921	Oysters	SVEIO							
2016-884	Blue mussel	NÆRØY							
2016-883	Blue mussel								
2016-882	Blue mussel	NAMDALSEID							
2016-881	Blue mussel	FOSNES							+
2016-880	Blue mussel	FOSNES							
2016-879	Blue mussel	BINDAL							

2016-878	Blue mussel	RISSA							+		
2016-874	Blue mussel	LILLESAND									
2016-873	Blue mussel	GRIMSTAD									
2016-836	Blue mussel	NAMDALSEID			+						
2016-835	Blue mussel	GRIMSTAD									
2016-834	Blue mussel	LILLESAND									
2016-833	Blue mussel				+					+	+
2016-832	Blue mussel	ÅFJORD									
2016-831	Blue mussel	ÅFJORD									
2016-830	Blue mussel	BJUGN								+	
2016-829	Blue mussel	BJUGN									
2016-828	Blue mussel	ÅFJORD									
2016-827	Blue mussel	ÅFJORD									
2016-815	Horse mussel				+						
2016-814	Blue mussel	FORSAND									
2016-813	Blue mussel	KVITSØY									
2016-812	Scallops	KVITSØY		+							
2016-811	Oysters	KVITSØY		+	+						
2016-810	Blue mussel	FJALER				+				+	
2016-809	Blue mussel	FJALER	+	+	+		+			+	
2016-808	Horse mussel	No origin info	+							+	
2016-743	Blue mussel	GRIMSTAD	+	+		+	+	+		+	
2016-738	Blue mussel	ÅFJORD	+		+		+			+	
2016-737	Blue mussel	ÅFJORD	+	+	+		+			+	
2016-735	Blue mussel	ÅFJORD	+	+	+	+	+	+		+	
2016-734	Blue mussel	ÅFJORD	+	+	+	+	+	+		+	
2016-733	Blue mussel	ÅFJORD	+	+	+	+	+	+		+	
2016-732	Blue mussel	NAMDALSEID	+	+	+	+	+	+		+	
2016-731	Blue mussel	NAMSOS			+						
2016-730	Blue mussel	NAMDALSEID									
2016-729	Blue mussel	NAMDALSEID			+						
2016-728	Oysters	OS I HORDALAND									
2016-727	Blue mussel										
2016-725	Blue mussel	VESTVÅGØY									
2016-724	Scallops										
2016-722	Oysters	SVEIO									
2016-720	Blue mussel	NORDREISA					+				
2016-717	Blue mussel	BØMLO									
2016-687	Blue mussel	GRIMSTAD									
2016-686	Blue mussel	NAMDALSEID									
2016-685	Blue mussel	NAMSOS									
2016-684	Blue mussel	NAMSOS	+								
2016-683	Blue mussel	FOSNES									
2016-682	Blue mussel	FOSNES									
2016-681	Blue mussel	ÅFJORD	+								
2016-680	Blue mussel	BINDAL	+								

2016-679	Oysters						
2016-678	Blue mussel	RISSA		+			
2016-651	Scallops	KVITSØY					
2016-650	Oysters	KVITSØY					
2016-649	Blue mussel	KVITSØY				+	
2016-644	Blue mussel	NAMSOS					
2016-643	Sand shell (Banded carpet shells)	Ulvsundet					
2016-642	Arctica islandica	Ulvsundet					
2016-641	Scallops	No origin info					
2016-639	Blue mussel	ÅFJORD					
2016-450	Oysters	OS I HORDALAND					
2016-449	Blue mussel	BINDAL					
2016-447	Blue mussel	NAMSOS					
2016-445	Blue mussel						
2016-444	Blue mussel	BØMLO					
2016-443	Oysters	BØMLO					
2016-442	Blue mussel	FJALER					
2016-441	Blue mussel	FJALER					
2016-439	Scallops						
2016-380	Blue mussel	ÅFJORD		+	+		
2016-379	Blue mussel	ÅFJORD		+			
2016-377	Blue mussel	ÅFJORD		+	+		
2016-376	Blue mussel	ÅFJORD					
2016-375	Blue mussel	ÅFJORD		+	+		
2016-374	Oysters	KVITSØY					
2016-373	Blue mussel	KVITSØY					
2016-371	Scallops	KVITSØY					
2016-370	Blue mussel						
2016-368	Blue mussel	NORDREISA					
2016-367	Blue mussel	FOSNES			+		+
2016-366	Blue mussel	FOSNES					
2016-357	Arctica islandica	ØYGARDEN					
2016-356	Scallops	ØYGARDEN					
2016-326	Sea urchin						
2016-324	Blue mussel	FORSAND					
2016-322	Horse mussel						
2016-321	Blue mussel	RISSA		+			
2016-320	Scallops						
2016-319	Oysters	SVEIO					
2016-265	Oysters	SVEIO					
2016-264	Blue mussel	BJUGN					
2016-263	Blue mussel	ÅFJORD	+				+
2016-262	Blue mussel	BJUGN					
2016-261	Blue mussel	ÅFJORD					
2016-260	Blue mussel	ÅFJORD		+			
2016-259	Blue mussel	ÅFJORD					

2016-204	Oysters	OS I HORDALAND								
2016-202	Blue mussel	FJALER								
2016-201	Blue mussel	FJALER								
2016-199	Oysters	BØMLO								
2016-638	Blue mussel	ÅFJORD								
2016-637	Blue mussel	ÅFJORD								
2016-636	Blue mussel	BJUGN								
2016-635	Blue mussel	ÅFJORD		+	+	+	+		+	
2016-633	Blue mussel	BJUGN								
2016-632	Blue mussel	FJALER								
2016-631	Blue mussel	FJALER								
2016-630	Blue mussel	GRIMSTAD								
2016-570	Oysters	OS I HORDALAND								
2016-568	Oysters	GRIMSTAD								
2016-567	Blue mussel	NAMSOS								
2016-566	Blue mussel	NAMDALSEID			+					+
2016-565	Blue mussel	NAMDALSEID			+					
2016-564	Blue mussel	NAMSOS								
2016-563	Blue mussel				+	+	+	+		
2016-561	Blue mussel	BØMLO	+		+					
2016-560	Scallops							+		
2016-559	Horse mussel							+		
2016-558	Blue mussel	FORSAND			+			+		
2016-557	Blue mussel	NORDREISA			+					
2016-540	Blue mussel	GRIMSTAD						+		
2016-539	Blue mussel	LILLESAND			+					
2016-538	Scallops	No origin info								
2016-537	Blue mussel	BJUGN						+		
2016-536	Blue mussel	ÅFJORD								
2016-535	Oysters	SVEIO								
2016-534	Blue mussel	VESTVÅGØY								
2016-510	Blue mussel	GRIMSTAD	+					+		
2016-509	Blue mussel	LILLESAND						+		
2016-508	horse mussel	NAMSOS						+		
2016-507	Blue mussel	NAMSOS					+			
2016-506	Blue mussel				+	+				
2016-505	Blue mussel	RISSA			+					
2016-504	Blue mussel	BINDAL			+					
2016-503	Blue mussel	ÅFJORD								
2016-502	Blue mussel	BJUGN								
2016-501	Blue mussel	ÅFJORD								
2016-500	Blue mussel	ÅFJORD			+					
2016-499	Blue mussel	FOSNES						+		
2016-498	Blue mussel	FOSNES								