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Isolation and characterization of Shiga toxin 2a- producing *Escherichia coli* from Norwegian cattle

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Food Safety, -quality and -hygiene

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Infinite love and gratitude to Gard and Sebastian, by your side every dream becomes true!

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Abstract

Shiga toxin-producing *Escherichia coli*, STEC, also called Verocytotoxin-producing *E. coli* (VTEC) is a pathogroup that include all *E. coli* harboring Shiga toxin genes (*stx*). STEC have emerged as a group of foodborne pathogens that cause disease of varying severity in humans. Particularly, the association of STEC with potential fatal disease outcomes such as Hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) represents a public health concern. Cattle are recognized as the main reservoir of STEC, and bovine fecal contamination as the vehicle of transmission. Yet, not all STEC are human pathogens. Some virulence factors such as Shiga toxin subtype 2a (*stx2a*) and the adhesin intimin (encoded by the *eae*-gene) have been more often associated with severe human disease.

In this study, fecal samples from 178 Norwegian cattle herds were examined for the presence of *stx* genes in general and the HUS-associated subtype *stx2a* in particular. *stx2a*-positive ($stx2a^+$) STEC were further isolated and characterized to determine the potential pathogenicity of the isolates. The results indicated a surprisingly high prevalence of *stx2a*-genes (16.9%) in fecal samples. A broad distribution of *stx* was also observed with a total of 96% of the herds positive for one or both of the *stx* variants (*stx1*/*stx2*). This is the first study on prevalence of *stx* genes in Norwegian cattle. A total of 86 $Stx2a^+$ isolates were recovered from 50% of the PCR -positive fecal samples and 25 isolates were further characterized.

The 25 selected $stx2a^+$STEC belonged to two phylogenetic groups with 52% in the A-group and 48% in the B1-group. Within phylogroup A, the isolates presented very similar virulence profiles, while more diverse profiles were distributed among phylogroup B1. Two isolates were O26 and two O113, both serogroups associated with severe disease. The rest of the isolates did not belong to any of the serogroups tested. The most distributed virulence gene among the isolates, additional to the selected virulence marker *stx2a*, was *ehxA*. Intimin encoding *eae* was also present in some isolates. Comparison of virulence profiles indicates both similarities and differences between the strains isolated from human patients and from cattle. However, based on their virulence profiles, the potential pathogenicity of these strains cannot be discarded. Further analysis of the whole genome sequences can contribute to insight into the real pathogenicity of $stx2a^+$ STEC from cattle.

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1. Introduction

1.1. *Escherichia coli*

Escherichia coli, commonly known as *E. coli*, is a member of the *Enterobactereaceae* family and one of the most common habitants of the intestinal tract of healthy mammals. *E. coli* are Gram-negative, non-sporulating, facultative anaerobic bacteria that show great genotypic and phenotypic diversity (Kaper et al., 2004). Approximately 42% of the genes are “conserved” in all *E. coli* variants, while the remaining 58% is variable (Tenaillon, 2010)

E. coli can be classified in different ways: Phylogenetically, eight groups have been described and most of *E. coli* falls into the groups: A, B1, B2, C, D, E, F and Clade I (Clermont et al., 2000). Serologically, *E. coli* is divided according to antigens O-antigens (lipopolysaccharide chain), K-antigen (capsular membrane polysaccharide) and H-antigen (flagellar proteins) (Tenaillon, 2010,). Nowadays, in addition to the above mentioned classifications, molecular methods are used to compare and differentiate *E.coli* strains.

The *E. coli* genus is highly diverse as many strains are harmless and play an important role in the maintenance of the healthy gut microflora while other strains are highly pathogenic. Pathogenic strains of *E.coli* are likewise very diverse and can roughly be divided into intestinal and extra-intestinal pathogens, reliant on the location of the infection. Extra-intestinal *E. coli* pathogens (ExPEC) trigger infections outside the gastrointestinal tract as, for instance, urinary tract infection, sepsis or meningitis, while the second group of pathogen *E.coli*: diarrhoeagenic *E. coli* (DEC), represents globally one of the most important causes of bacterial gastroenteritis (Kaper et al., 2004).

DEC is further divided into various intestinal “pathogroups” based on their capacity to cause disease using virulence factors typical for the group. These are: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Shiga toxin-producing *E. coli* (STEC) (Tozzoli&Scheutz, 2014). The focus of this study is the STEC pathogroup.

1.2. Shiga toxin-producing *E. coli* (STEC)

Among DEC, STEC includes the most virulent strains (Tozzolli&Scheutz, 2014). STEC, also known as Verocytotoxin-producing *E. coli* (VTEC), is a pathogroup that shares, as common feature, the presence of genes encoding for the production of Shiga toxins (Stx) in their genomes (Kaper et al,. 2004).

Although Shiga toxin genes (*stx*) are the differentiating mark of STEC, several other virulence factors can also be present in their genomes. This genomic diversity is responsible for the varying degrees of pathogenicity reported in humans. While some human infections with STEC are without complications, other advance to hemorrhagic colitis (HC) with bloody diarrhea and a portion of cases develop severe life threatening complications like hemolytic uremic syndrome (HUS) (Karmali, 1989).

More than 400 serotypes are identified as STEC, however only a subset of them has been associated with human disease (Blanco et al., 2004; Hussein, 2007). Historically, the most frequently reported cause of severe STEC-associated human disease and consequently the most studied worldwide is the O157:H7 serotype. However, also non-O157 STEC serogroups: O26, O103, O111 and O145 are reported globally because of their association with severe human disease, as well as 50 other non-O157 serogroups (Scheutz, 2014).

The distribution of *stx* genes among diverse *E.coli* serotypes is mainly due to their location on bacteriophages, mobile elements that can be transferred from the host STEC to other *E. coli* strains, independent of the serotype or pathogroup they belong to (James et al., 2001). This process plays an important role in dynamics and evolution of *E.coli* and can give rise to hybrid strains with unknown virulence profiles (Leopold et al., 2014), as was clearly demonstrated during a large outbreak of STEC O104:H4 in Germany in 2011, where the infecting organism was a member of the EAEC harboring *stx* genes (Navarro-Garcia, 2015).

In USA *E. coli* O157:H7 infection became nationally notifiable in 1995. Since the year 2000 all STEC infections are notifiable in USA. In Europe, STEC cases have been reported to European Centre from Disease Prevention and Control (ECDC) since its stablishment in 2007. In Norway, STEC-infections were made mandatory notifiable to the Norwegian Surveillance System for Communicable Diseases (MSIS) in 1995 (Brandal et al., 2015a; http://ww.msis.no).

1.3. STEC virulence factors

Several virulence genes have been identified to play central roles in STEC pathogenesis, among them: Shiga toxins, the Locus for enterocyte effacement (LEE) Pathogenicity Island and the 60-MDa plasmid encoding for haemolysin. Many other virulence-associated genes are also described, all of them encoded within mobile elements. A short description of the three virulence factors used for characterization of STEC in the present study follows below.

1.3.1. Shiga toxins

Shiga toxins are the fundamental factor for development of disease and HUS. *stx* -genes are carried in bacteriophages integrated in the STEC chromosome. Bacteriophages can enter the lytic cycle in the course of the disease, lyse the host STEC strain and potentially infect other *E. coli* strains. The *stx* genes are expressed when the bacteriophages enter the lytic cycle and the toxin is released when the bacterial cell is lysed.

The virulence of Stx resides in their cytotoxicity, which gives them the ability to induce cell-death. They accomplish this by blocking the capacity of cells to synthetize proteins (O'Brien & Holmes, 1987). The figure 1.1 from Pacheco & Sperandio (2012), shows the mechanism by which Stx induces eukaryotic cell-death.

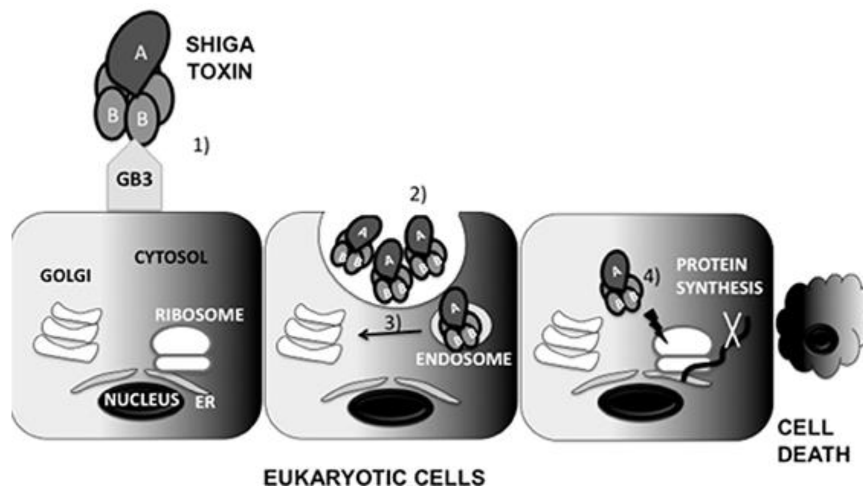

Figure 1.1 Mechanism of action of Shiga toxin.1) Stx bind to globotriaosylceramide (Gb3) expressed by some eukaryotic cells. 2) Stx is internalized by endocytosis. 3) Subsequently, Stx undergoes retrograde transport to the trans-Golgi network. 4) The toxin passes to the endoplasmic reticulum (ER) where it encounters its target, the ribosome, inactivating it. As a consequence, Stx inhibits protein synthesis, causing cell death by apoptosis (Pacheco & Sperandio, 2012). Reprinted with permission from the authors.

The Shiga toxin family comprises two antigenically distinct variants: Stx1 and Stx2. They are genetically different, but with similar biological properties, such as polypeptide subunit structure, enzymatic activity and binding to specific glycolipid receptors (Scheutz et al., 2012b). STEC may produce Stx1 or Stx2, or combinations of both in different amounts/combinations of types/subtypes (Karch et al., 2005). However, within these two variants, there are subtypes which differ in amino acid sequences, phenotypes and specific association with different disease outcomes.

Three different subtypes have been described within the Stx1 group, namely Stx1a, Stx1c and Stx1d (Scheutz, 2012b). Among these, Stx1a is linked to serious human disease, while the others are immunologically distinct and less potent (Melton-Celsa, 2015). Seven subtypes of Stx2, named from a-g have been described. Shiga toxin 2 is more frequently associated with disease than Shiga toxin 1 (Scheutz, 2012b). Among Stx2, the subtypes a, c and d are closely related and more frequently associated with bloody diarrhea and HUS (Fuller et al., 2011; Haugum et al., 2014b, Betzen et al. 2015), while the remaining variants are only found in patients with uncomplicated diarrhea or might be not toxic to humans, as for instance Stx2e (Scheutz et al., 2012b).

The potency of the Stx subtypes is due to the interaction between the toxin and Stx-sensitive cell types, many of them present in the kidney, leading to renal cell death and kidney failure (Obata & Obrig, 2015). Fuller *et al*. (2011) studied the *in-vivo* toxicity of Stx in Vero cells (kidney epithelial cell from monkey) and human renal cells finding that Stx2a and Stx2d were 40 to 400 times more potent than Stx2b and Stx2c, while Stx2b and Stx2c showed similar toxicity as Stx1.

1.3.2. Locus for enterocyte effacement (LEE)

The locus for enterocyte effacement (LEE) is a pathogenicity island (PAI) of approximately 35 kb, present in the chromosome of both EPEC and LEE^{+} (LEE-positive) STEC (McDaniel et al., 1995). LEE encodes proteins responsible for the formation of the characteristic attaching and effacing lesion (A/E) required for colonization of the host intestinal mucosa

LEE contains the *eae* gene that encodes the adhesin intimin, the genes encoding for the type III secretion system (T3SS) responsible for pedestal formation, as well as regulators and effector proteins. LEE^{+} *E.coli* uses T3SS to inject effector proteins into intestinal epithelial

cells. The effector proteins transform the target cell surface and rearrange it to a pedestal (Stevens & Frankel, 2015). Figure 1.2 shows the pedestal formation accomplished by LEE$^+$-STEC and some effector proteins involved.

The presence of LEE in STEC gives them an advantage for colonization of the intestinal epithelium and consequently *eae* positive (*eae*$^+$)-STEC are normally associated with severe disease (Blanco et al., 2004).

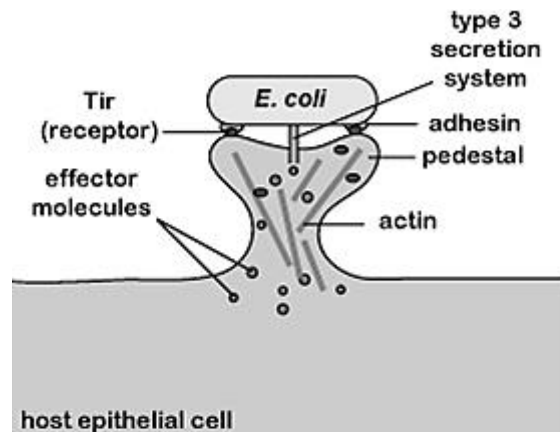

Figure 1.2. Pedestal formation: LEE$^+$ -*E.coli* attaches to microvilli, effaces its normal structure, adheres to the epithelial cell surfaces by receptor-adhesin junction and finally forms the characteristic pedestal formation. (Figure reprinted with permission of the author Copyright © Gary E. Kaiser).

1.3.3. Entero-haemolysin

Entero-haemolysin (EhxA), also known as Hly, is a toxin encoded in a 60-MDa plasmid that belongs to the RTX family, a large family of toxins that share common features. Its function in STEC pathogenesis is to lyse red blood cells and release Iron, which is important for the colonization and pathogenesis of STEC (Mellies & Lorenzen, 2014). The toxin contributes to pore-formation in cell membranes and it has been found to be cytotoxic to endothelial cells and may contribute to the development of HUS (Croxen et al., 2013).

1.3.4 Virulence gene regulation

Although the virulence genes described above are encoded in different genetic elements within the bacterial genome, coordination among them is decisive for disease outcome.

Stx expression is LEE -independent and believed to be under the control of a phage promotor. Nevertheless, for LEE$^+$ -STEC, an unspecified synergism between intimin and s*tx2* has been

suggested to exist (Boerlin et al., 1999). Toxin production is repressed while the phage exists as a lysogen in the STEC and is expressed first after phage induction. The lytic cycle of Stx-phages and thereafter toxin release is triggered by environmental factors such as nutritional stress, oxidative stress, UV radiation, antibiotics, heat shock, quorum sensing among others (Scheutz, 2014).

Non-LEE effector EhxA, seems also to act in a coordinated manner with *eae* in LEE^{+}, *ehxA*$^{+}$ STEC. The expression of *ehxA* is controlled by regulator proteins encoded in LEE (Bielaszewska et al., 2014). LEE expresses T3SS based on environmental signaling and quorum sensing. At the same time, expression of *ehxA* is positively regulated by the same regulator proteins encoded in LEE (Mellies & Lorenzen, 2015).

1.4. STEC: reservoir, transmission and foodborne disease

The natural reservoir of STEC is ruminants; including cattle, which normally are healthy carriers, but shed STEC in their feces (Caprioli et al., 2005; Persad & LeJeune, 2015).

STEC are considered zoonotic microorganisms that can be transferred, through fecal contamination, from its wild and farm animal reservoir to meat and other products from animal origin, as well as to water, and further to fresh produce (Croxen et al., 2013, Feng 2015). STEC outbreaks have been traced back to game and bovine meat, minced meat, milk, and dairy products (from unpasteurized milk), fresh fruit and vegetables, juices, shellfish, mollusks and products thereof (EFSA-BIOHAZ, 2013:18), as well as to seed sprouts. STEC has also caused numerous outbreaks associated with recreational and municipal drinking water, person-to-person transmission and petting zoo and farm visits (Kaper et al., 2004). The Figure 1.3 shows an overview of reservoir and transmission. The figure is adapted from Croxen *et al*. (2013).

STEC infections are facilitated by an extremely low infectious dose, which is estimated to be <100 cells (Croxen et al., 2013).

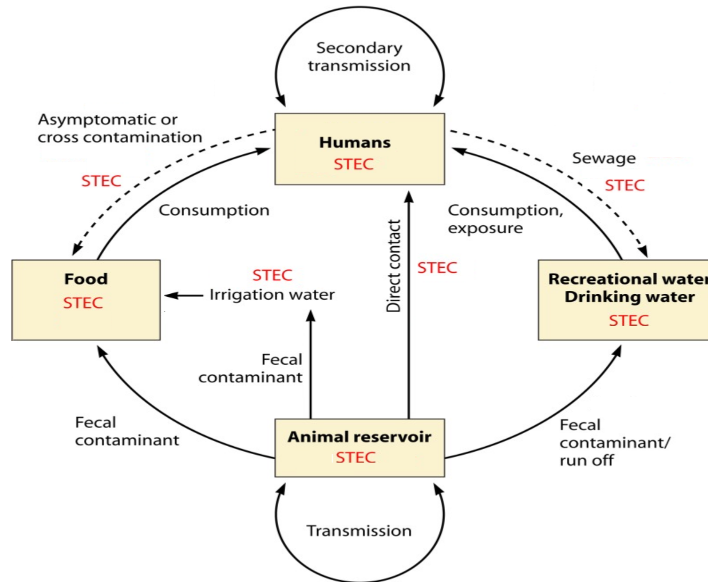

Figure 1.3. STEC: Potential reservoirs and modes of transmission. STEC can spread out from and among various animal reservoirs. Fecal matter can contaminate food, irrigation water, or recreational/drinking water. Humans can become exposed by contaminated food or water or through direct contact with colonized animals. Secondary transmission can occur between humans. Food can become contaminated through poor cooking practice. Additionally, symptomatic or asymptomatic food handlers can contaminate food through inadequate hygiene. Contamination of recreational or drinking water can occur through exposure of human sewage (Adapted with permission from Croxen *et al*., 2013).

1.5. Incidence of STEC human infections

The global incidence of STEC is unknown, but cases are reported from 21 countries belonging to14 World Health Organization (WHO) sub-regions (Majowicz et al., 2014).

In its annual report from 2012, the Center for Disease Control and Prevention (CDC) in the USA, reported 4654 laboratory-confirmed cases of STEC in 2012 (2460 STEC-O157 and 2194 non-O157).

The incidence in Europe has been calculated using "disease multipliers" to include the many unreported cases. From an average of reported notification per year of 3386 cases (average 2007-2011), the real incidence has been estimated to be of around 446 101 cases per year (EFSA, 2013:19-20). In 2013, EFSA reported 6043 confirmed human cases in Europa (EFSA, 2015).

In Norway, between 0 and 20 cases were reported annually from 1994 to 2005. In 2006, an outbreak involving 17 children where 10 developed HUS and one person died occurred (Schimmer et al., 2008). Moreover, in 2009, seven smaller outbreaks involved 111 patients,

from which nine children developed HUS and one died (Brandal et al., 2015a). After these outbreaks, many diagnostic laboratories for human clinical samples started detecting *stx* genes to cover every possible STEC serogroup in suspicious samples. The number of cases reported in 2013 was 103 and, in 2014, it increased to 151 (Brandal et al., 2015a).

1.6. STEC food-borne outbreaks

STEC outbreaks associated with food and water as the transmitting vector are continuously reported worldwide. The first reported big *E. coli* O157:H7 outbreak started in January 1993 and was associated with consumption of undercooked hamburgers. In total, 501 cases were reported, 477 were culture-confirmed, including 151 hospitalizations (31%), 45 cases of HUS (9%), and three deaths. The confirmed *E. coli* O157:H7 was characterized as stx^+, eae^+ and $exhA^+$ (O'Brien et al., 1993, Bell et al., 1994).

In 1999, one thousand New York habitants were infected with STEC O157:H7 after drinking water contaminated with cattle manure. In the last decade, the CDC have reported STEC-outbreaks traced back to beef, frozen pizza containing contaminated peperoni, cookie dough, cheese, lettuce, poultry, hazel nuts, organic spinach, sprouts, ready to eat salads, among other foods.

In Europe, several outbreaks involving different types of food have been reported. In 2007, Ireland registered an outbreak traced back to a contaminated water-well where STEC O157 was the contaminating agent. The same year, Sweden registered an outbreak with STEC O76 in cheese. In Germany, in 2008 raw milk was implicated in an O157:H7 outbreak. Table 1.1 shows a selection of outbreaks occurred in the past 10 years and associated with meat consumption (EFSA Panel on Biological Hazards, 2013).

Table 1.1 Selected STEC outbreaks related to meat and meat products in the past 10 years.

Country	Year	Vehicle	Number of patients (deaths)	Serotype	Reference
Denmark	2012	Ground beef	9	O157	Soborg *et al*. 2012
Japan	2011	Raw beef dish	181 (5)	O111	National Institute of Infection diseases, Japan (2012)
USA	2010	Blade tenderized steak	21	O157	CDC (2010)
Belgium	2008	Raw minced beef	6	Not-reported	EFSA (2013)
USA	2007	Frozen minced beef	40	O157	CDC (2007)
Denmark	2007	Organic fermented beef sausage	20	O26:H11	EFSA (2013)
Norway	2006	Cured mutton sausages	18 (1)	O103:H25	Schimmer *et al*. 2008

1.7. Prevalence of STEC in Norwegian cattle, sheep and food

According to the Zoonosis report (Heier et al., 2014) the prevalence of STEC is very low in Norway. In 1995 and 1999 examination of cattle herds was focused on *E. coli* O157, indicating a very low prevalence. In 2000 the investigations included serogroups O26, O103, O111, O145 and O157. The same year no O157-positive herds were found, but a low prevalence of *E. coli* O103 was detected. In 2003, 137 dairy farms were examined, finding high prevalence of *E. coli* O103 and several herds positive for O26, O145 and two herds positive for O111. Additional testing for intimin-*eae* demonstrated its presence in nine bacterial isolates from nine different herds.

In 2006, the Food Safety Authority initiated a two year project to examine at the incidence of STEC in sheep. The results show low prevalence of STEC O157, O26 and O103 of 0.9%, 0.8% and 0.7% respectively (Heier et al., 2014).

No routine monitoring for detection of STEC in food is carried out in Norway. However, between 1996 and 2004, thousands of different foods related to other monitoring programs and projects were examined for STEC O157. Only eight carcasses from cattle and two from sheep resulted positive for STEC O157:H7 (L'Abée-Lund & Wasteson, 2015).

In 2014, a surveillance program for pathogenic *E.coli* in cattle was initiated by the Norwegian Food Safety Authority. The aim was to examine the prevalence of the "top five" STEC serogroups in Norwegian cattle. The results indicated a prevalence of STEC O26, O103, O111, O145 and O157 of 5.6%, 2.2%, 0%, 0.6% and 2.2%, respectively (Sekse et al., 2015).

1.8. Detection and Isolation of STEC

Because of the widespread variety of food that can be contaminated with STEC, the development of methods to detect STEC in complex matrices has become essential. The main challenge is the need for rapid and sensitive methods applicable to different matrices and that can also scope the diversity of STEC strains. The problem is complicated by the absence of common phenotypical markers in STEC that could serve to differentiate them from other *E. coli*.

After the first large outbreaks with *E. coli* O157:H7, standard methods were developed to detect this serotype. STEC O157:H7 can be phenotypically identified in media due to its

unique biochemical characteristics such us the inability to metabolize sorbitol. Suspicious colonies are easily distinguishable on MacConkey agar in which lactose is substituted by Sorbitol (SMAC) as colorless colonies. Further, the selected colonies should be confirmed by agglutination assays. The method was effective and extensively used. However, soon it became clear that STEC from serogroups other than O157 were responsible of many outbreaks and severe disease and other O157:H^- do possess the capacity to ferment sorbitol and could not be detected on SMAC (Croxen et al., 2013).

The increasing diversity of STEC involved in human disease made it necessary to change the basis of identification from serotypes to the production of Shiga toxin or the presence of *stx* genes. Phenotypical assays such as Cytotoxicity assays and immunological assay were developed to detect the production of the toxins, while molecular methods based in PCR have been developed for detection of *stx* genes (Beutin & Fach, 2015).

In 2012 a new ISO method was released, this method starts with DNA extraction from the matrix to analyze, followed by detection of *stx*, *eae*, and serotypes O157, O26, O103, O111 and O145 by real-time PCR. Nevertheless, isolation of STEC strains is required to confirm that the positive PCR signals are generated from genes present in the same living bacterial cell (International Organization for Standardization, 2012). This culture-based isolation process is time- and labor-intensive and the outcome depend on many factors such as enrichment and isolation medium that favors STEC grow and dismiss background flora (Verhaegen, 2016).

1.9. *stx2a*$^+$ STEC and its association with HUS

Comparative analysis of STEC isolated from HUS patients has found the presence of the virulence genes *stx2a* and *eae* as common markers (Boerlin et al. 1999, Friedich et al. 2002, Jenkins et al. 2003, Ethelberg et al. 2004, Persson et al. 2007, Brandal *et al*. 2014a). In Norway, all the STEC strains isolated from HUS patients harbored *stx2a*, while strains harboring other *stx* subtypes with or in absence of *eae* have been more often connected with bloody diarrhea and gastroenteritis (Haugum et al., 2014b, Brandal 2015b). Additionally, the toxic effect of *Stx2a* over endothelial uremic cells has been also confirmed (Fuller et al. 2011, Betzen et al. 2015).

1.10. Aim of the study

As described in this introduction, STEC has its main reservoir in cattle and the presence of *stx2a* in STEC strains is often associated with HUS. In this study, 178 fecal samples from Norwegian cattle herds from a nationwide study were included. The objectives of this thesis are:

i. To examine the PCR prevalence of *stx1, stx2 and stx2a* in the fecal samples.
ii. To isolate *stx2a*$^{+}$ STEC from the PCR -positive fecal samples.
iii. To evaluate the pathogenic potential of *stx2a*$^{+}$ *E. coli* strains isolated from Norwegian cattle by characterization of typical virulence factors and comparison with virulence profiles from human isolates.

2. Materials and Methods

2.1 Materials

In this study, a total of 178 fecal samples from cattle, collected from herds in different areas of Norway, were used. The fecal samples were collected for the Surveillance program for pathogenic *E.coli* in cattle by the Norwegian Food Safety Authority between August and October of 2014. Each fecal sample contained cattle feces from 10 different points of one farm, representing feces from animals of different ages.

DNA extracted from 178 fecal samples (QIAamp® DNA stools Mini kit, QIAGEN, Hilden, Germany), and enriched fecal material from each sample were used in this study.

2.2 Methods

The figure 2.1 shows a flow diagram of the study. The different methods are described below.

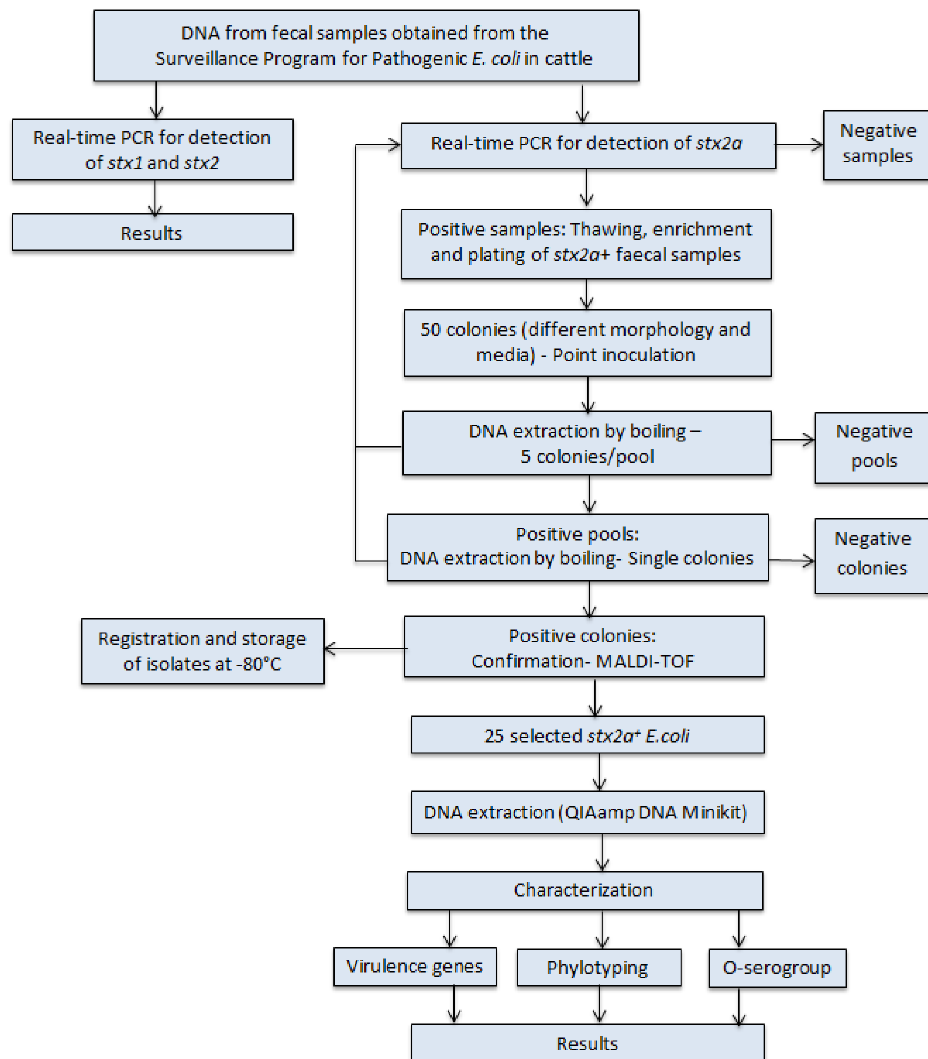

Figure 2.1. A flow chart of the present study

2.2.1. Real-Time PCR

Real-time PCR allows the detection of PCR product during the PCR reaction progresses. By including a fluorescent molecule (probe) in the reaction that binds to a specific DNA sequence (given by the target gene), DNA accumulation can be detected by reading the increase in fluorescence. The instrument provides a measure, the Ct-value, which is the cycle number at which the fluorescence is detectable. Using the Ct-value, quantification of the original amount of template can be calculated. However, real-time PCR was mainly used as a qualitative indicator to determine the presence or absence of a target gene.

Stratagene Mx3005P (Agilent Technologies, Germany) together with software MxPro-Mx3005P were used in all experiments during this study. The set of primers, probes and real-time PCR programs are shown in Tables 2.1 and 2.2. The master mixes used are described in the respective PCR methods.

Table 2.1. Primers and probes used for detection of virulence genes/O-serotyping

Target gene/ serogroup	Primer/probe	Sequence (5'-3')	Reference
stx1/*stx2*	stx-RT-fwd	TTTGTYACTGTSACAGCWGAAGCYTTACG	ISO/TS 13136:2012 (E). Annex E
	stx-RT-rev	CCCCAGTTCARWGTRAGRTCMACRTC	
stx1	stx1-RT-probe	6FAM-CTGGATGATCTCAGTGGGCGTTCTTATGTAA-BHQ1	
stx2	stx2-RT-probe	HEX-TCGTCAGGCACTGTCTGAAACTGCTCC-BHQ1	
stx2a	VT2a-QfLNA1	GGCGG+TTTT+ATT+TGCATTA+G	Pers. communication, Tomas Jinnerot. Statens Veterinärmedicinska anstalt, Sweden (SVA)
	VT2a-QrLNA2	CG+TC+AAC+CTT+CACTGT+A	
	VT2a-Qp	HEX-CRCAATCCGCCGCCATTGCATTAACAGAA-BHQ1	
eae	stx-RT-fwd	CATTGATCAGATTTTTCTGGTGATA	ISO/TS 13136:2012 (E). Annex E
	stx-RT-rev	CTCAGCGGAAATAGCCGTTA	
	eae-RT-probe	6FAM-ATAGTCTCGCCAGTATTCGCCACCAATACC-BHQ1	
ehxA	stx-RT-fwd	GTGTCAGTAGGGAAGCGAACA	Bugarel *et al.*(2010)
	stx-RT-rev	ATCATGTTTTCCGCCAATG	
	ehxA-RT-probe	FAM-CGTGATTTTGAATTCAGARCCGGTGG-BHQ	
O26-specic *wzx*	wzx-O26-fxd	CGCGACGGCAGAGAAAATT	ISO/TS 13136:2012 (E). Annex E
	wzx-O26-rev	AGCAGGCTTTTATATTCTCCACTTT	
	wzx-O26-probe	HEX-CCCCGTTAAATCAATACTATTTCACGAGGTTGA-BHQ1	
O91-specific *wzy*	wzyO91-F	CGA TTT TCT GGA ATG CTT GAT G	Perelle *et al.*, (2004)
	wzyO91-R	CAA TAC ATA GTT TGA TTT GTG TTT AAA GTT TAA T	
	wzyO91.P	FAM- CCT GGG TTG TTA GGA ACA ATT TCA GCA CTT C-BHQ1	
O103-specific *wzx*	wzx-O103-fxd	CAAGGTGATTACGAAAATGCATGT	ISO/TS 13136:2012 (E). Annex E
	wzx-O103-rev	GAAAAAAGCACCCCCGTACTTAT	
	wzx-O103-probe	6FAM-CATAGCCTGCCTGTTGTTTTAT-MGBNFQ	
O111-specific *wbdl*	wbdl-O111-fwr	CGAGGCAACACATTATATATGCTTT	ISO/TS 13136:2012 (E). Annex E
	wbdl-O111-rev	TTTTTGAATAGTTATGAACATCTTGTTTAGC	
	wbdl-O111-probe	6FAM-TTGAATCTCCCAGATGATCAACATCGTGAA-BHQ1	
O121 *wzx*	wzxO121-F	TGGTCTCTTAGACTTAGGGC	Bugarel *et al.* (2010)
	wzxO121-R	TTAGCAATTTTCTGTAGTCCAGC	
	wzxO121-P	FAM- TCC AAC AAT TGG TCG TGA AAC AGC TCG-BHQ1	

Target gene/ serogroup	Primer/probe	Sequence (5'-3')	Reference
O145 *wyz2*	O145wyz2-F	ATATTGGCTGCCACTGATTGGGAT	Fratamico *et al.*, 2009
	O145wyz2-R	TATGGCGTACAATGCACCGCAAAC	
	O145wyz2-P	6FAM-AGCAGTGGTTCGCGCACAGCATGGT-BHQ1	
O157 *rfbE*	rfbE-O157-fwd	TTTCACACTTATTGGATGGTCTCAA	ISO/TS 13136:2012 (E). Annex E
	rfbE-O157-rev	CGATGAGTTTATCTGCAAGGTGAT	
	rfbE-O157-probe	6FAM-AGGACCGCAAGAGGAAAGAGAGGAATTAAGG-BHQ1	

Table 2.2. Real-time PCR programs

Target Gene/ serogroup	Real-time PCR program		
		Time (seconds)	Temperature (°C)
(*stx1*/+*stx2*), *eae,* O26, O103, O91, O111, O121, O145, O157	Pre-PCR: De-contamination	120	50
	Pre-PCR: Polymerase activation and template denaturation	600	95
	PCR 45 cycles:		
	Step 1: Denaturation	15	95
	Step 2: Annealing and DNA synthesis	60	60
stx2a	Pre-PCR: Polymerase activation and template denaturation	180	95
	PCR 45 cycles		
	Step 1: Denaturation	3	95
	Step 2: Annealing and DNA synthesis	30	60
ehxA	Pre-PCR: polymerase activation and template denaturation	180	95
	PCR 45 cycles		
	Step 1: Denaturation	10	95
	Step 2: Annealing and DNA synthesis	30	60

2.2.2. Conventional PCR

PCR primers use sequences complementary to the target DNA segment desired to amplify, and DNA Taq polymerase that elongates the primers using dNTPs available in solution. The PCR program first increases the temperature to 95°C to separate double stranded DNA, and thereafter the temperature is decreased to the optimal for annealing of primers to the DNA template. For elongation of the primers by addition of dNTPs with the DNA Taq polymerase, the temperature is adjusted to 72°C. The process is repeated in several cycles to increase DNA concentration of the target fragment. The PCR products are detected by gel electrophoresis.

A Bio-rad T100 Thermal cycler (Bio-rad, Singapore) was used during this study. The set of primers and PCR programs used for amplification of *stx1* and *stx2* subtypes, as well as for the phylogenetic analysis of *stx2a*$^{+}$ *E. coli* isolates are shown in Table 2.3 and 2.4. The master mixes are defined in the respective PCR description.

Table 2.3 Primers used for subtyping of *stx1*/*stx*2 and phylotyping

Target gene(s)	Primer	Sequence	Amplicon size	Reference
Multiplex: *stx1a*, *stx1c*, *stx1d*	vtx1a-F1	CCTTTCCAGGTACAACAGCGGTT		Scheutz F. (2012a) WHO Collaboration Centre for Reference and Research on *Escherichia* and *Klebsiella*, SSI
	vtx1a-R2	GGAAACTCATCAGATGCCATTCTGG	478 bp	
	vtx1c-F1	CCTTTCCTGGTACAACTGCGGTT		
	vtx1c-R1	CAAGTGTTGTACGAAATCCCCTCTGA	252 bp	
	vtx1d-F1	CAGTTAATGCGATTGCTAAGGAGTTTACC		
	vtx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA	203 bp	
stx2a	vtx2a-F2	GCGATACTGRGBACTGTGGCC		
	vtx2a-R3	CCGKCAACCTTCACTGTAAATGTG	349 bp	
	vtx2a-R2	GGCCACCTTCACTGTGAATGTG	347 bp	
stx2b	vtx2b-F1	AAA-TAT-GAA-GAA-GAT-ATT-TGT-AGC-GGC		
	vtx2b-R1	CAG-CAA-ATC-CTG-AAC-CTG-ACG	251 bp	
stx2c	vtx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTA		
	vtx2c-R2	CCGGCCACYTTTACTGTGAATGTA	177 bp	
stx2d	vtx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG		
	vtx2d-R1	TTYCCGGCCACTTTTACTGTG	179 bp	
	vtx2d-R2	GCCTGATGCACAGGTACTGGAC	280 bp	
stx2e	vtx2e-F1	CGG-AGT-ATC-GGG-GAG-AGG-C		
	vtx2e-R2	CTT-CCT-GAC-ACC-TTC-ACA-GTA-AAG-GT	411bp	
stx2f	vtx2f-F1	TGG-GCG-TCA-TTC-ACT-GGT-TG		
	vtx2f-R1	TAA-TGG-CCG-CCC-TGT-CTC-C	424 bp	
stx2g	vtx2g-F1	CAC-CGG-GTA-GTT-ATA-TTT-CTG-TGG-ATA-TC		
	vtx2g-R1	GAT-GGC-AAT-TCA-GAA-TAA-CCG-CT	573 bp	
Multiplex: *gadA, chuA, yjaA, TSPE4.C2*	gadA.F	GATGAAATGGCGTTGGCGCAAG		Doumith *et al.* (2012). Clermont *et al.*8 2000).
	gadA.R	GGCGGAAGTCCCAGACGATATCC	373 bp	
	chuA.F	ATGATCATCGCGGCGTGCTG		
	chuA.R	AAACGCGCTCGCGCCTAAT	281 bp	
	yjaA.F	TGTTCGCGATCTTGAAAGCAAACGT		
	yjaA.R	ACCTGTGACAAACCGCCCTCA	216 bp	
	TSPE4.C2 F	GCGGGTGAGACAGAAACGCG		
	TSPE4.C2 R	TTGTCGTGAGTTGCGAACCCG	152 bp	

Table 2.4. PCR programs for subtyping of *stx1*/*stx2* and phylogenetic analysis

Target Genes	Conventional PCR program			
			Time (seconds)	Temperature (°C)
Multiplex: *stx1a*, *stx1c*, *stx1d*. Subtyping *stx2a*, *stx2b*, *sttx2c*, *stx2d*, *stx2e*, *stx2e*, *stx2f*, *stx2g*	Step 1		900	95
	PCR 35 cycles:	Step 2	50	95
		Step 3	40	64/66*
		Step 4	60	72
	Step 5		180	72
	Step 6		∞	4
Multiplex: *gadA*, *chuA*, *yjaA*, *TSPE4.C2*	Step 1		900	95
	PCR 45 cycles	Step 2	30	95
		Step 3	30	60
		Step 4	30	72
	Step 5		300	72
	Step 6		∞	8

*The annealing temperature used for multiplex *stx1* PCR and for *stx2a, stx2b, stx2e, stx2e, stx2f, stx2g* PCR program was 64°C, and 66°C for *stx2c* and *stx2d* to avoid cross-reactions seen in such subtyping studies (Scheutz et al., 2012a)

2.2.3 Reference strains

All experiments carried out in this study included a positive control. The reference strains carrying the target gene/belonging to the serogroup tested are described in Table 2.5. When no other is specified, a blank negative control without any template was used.

Table 2.5. Reference strains used in this study

Reference to the target gene/ serogroup	Reference Strain	Serotype	Reference	Method
Stx1 , stx2, stx2a, eae, ehxA	EDL933(D2653)	O157:H7	O'Brien *et al*. 1984	Real-time PCR
O26	G08	O26	European Union Reference Laboratory VTEC (EURL VTEC)	
O91	F08	O91:H14		
O103	E08	O103		
O111	C08	O111:H-		
O121	B08	O121:H19		
O145	A08	O145:H28		
O157	D08	O157:H7		
stx1a	EDL933(D2653)	O157:H7	O'Brien *et al*. (1984)	Conventional PCR
stx1c	DG131/3(D3602)	O174:H8	Scheutz *et al*. (2012a)	
stx1, stx1c, stx1d	Ecoli vtx1 &vtx2 subtyping PCR kit (SSI, Hillerød, Denmark)			
stx2b	EH250	O118:H12	Piérard *et al*. (1998)	
stx2c	O31(D2587)	O174:H21	Paton *et al*. (1992)	
stx2d	C165-02(D3435)	O73:H18	Persson et al. (2007)	
stx2e	S1191(D3648)	O139:k12:H1	Weinstein *et al*. (1988)	
stx2f	T4/97(D3546)	O128ac:[H2]	Schmidt *et al*. (2000)	
stx2g	7v(D3509)	O2:H25	Leung *et al*. (2003)	
gadA, chuA, yjaA, TSPE4.C2	BÆ14	-	Internal reference	

2.2.4. Validation of master mix´s efficiency

Three different master mixes were tested to assess the amplification efficiency and optimize the reaction. Using serial dilutions of a template, it is possible to determine whether the amplification efficiency is the same for different template copy numbers. The efficiency assay was carried out for the target genes *stx1* and *stx2,* using DNA dilutions from reference strain *E. coli* EDL-933. Undiluted DNA was assigned an initial template quantity, and subsequently template quantities of the dilutions were assigned based on the dilution factor.

Each reaction was composed by a mix of the following reagents (Sigma-Aldrich, USA):12 µl of the different master mixes (2X), 0,5 µl 50 µM forward primer, 0,5 µl 50 µM reverse primer, 1,0 µl 5µ M probe Stx1 (FAM), 1,0 µl 5 µM stx2 probe (HEX), 4,5 µl Nuclease free water, and 5 µl of DNA template. The real-time PCR program is described in Table 2.2.

Using the Mx3000P software attached to the instrument Stratagene Mx3005P, standard curves were created by plotting the initial template quantity against the Ct-value obtained during amplification of each dilution (Mx3000P software manual, pg.227-262). An optimized quantitative real-time PCR is characterized by a R^2 >0.980 and an amplification efficiency of 90-100% (Bio-rad, 2006, pg.4)

Reaction efficiency was assessed for three master mixes: TaqMan® Universal (2X) (Applied Biosystems, Foster City, USA), TaqMan® Environmental Master Mix 2.0 (2X) (Applied Biosystems, Foster City, USA) and Brilliant III Ultra-fast QPCR Master Mix (2X) (Agilent Technologies, USA). The experiment included two parallels of each dilution: 1/1, 1/8, 1/32, 1/64, 1/128.

2.2.5. Screening of fecal samples for the presence of *stx1*, *stx2* and *stx2a* by real-time PCR.

For screening of *stx1* and *stx2*, the combination of reagents used and PCR program was as the described in section 2.2.4. The master mix giving the best result in 2.2.4, 2X Brilliant III Ultra-Fast QPCR Master Mix was used.

For screening of *stx2a*, a mix comprising 10 µl 2X Brilliant III Ultra-Fast QPCR Master Mix , 0,66 µl 10 µM forward primer, 0,66 µl 10 µM reverse primer (Exiqon, Vedbaek, Denmark), 0,2 µl 10 µM probe (Sigma-Aldrich, USA), 3,48 µl Nuclease free water, and 5 µl of DNA template were used for each reaction. The oligonucleotides used as primers to amplify *stx2a* are Locked Nucleic Acids (LNA™), which have the ribose ring “locked” in the ideal structural conformation (Watson-Crick binding), this give them high affinity and higher thermal stability when hybridized to a complementary DNA strand.

Primers and probes are described in Table 2.1. Two concentrations of DNA were used in the assay: undiluted and 1/10 dilution of DNA in milliQ water. The real-time PCR program is described in Table 2.2.

2.2.6. Isolation of *stx2a*$^+$ isolates from PCR -positive fecal samples.

The *stx2a* PCR -positive fecal samples were thawed in water bath at 50°C for 1 minute until the sample was defrosted. The tubes were incubated at room temperature for one hour and then transferred to 9 ml fresh BPV-ISO (Buffered Peptone Water [BioRad]) pre-warmed to 37°C. The tubes were incubated for 2-3 hours at 37°±1°C. After incubation, the cultures were mixed using a Vortexer (IKA®M53 basic, USA) and serial dilutions from 10^0 to 10^{-4} were prepared. Aliquots of 100 µl from 10^{-3} and 10^{-4} dilutions were plated onto three agar media plates, spread with a sterile L-shaped spreader and incubated overnight at 37±1°C.

The three agar media used were: CHROMagar™ O157 (CHROMagar Microbiology, Paris, France), Sorbitol MacConkey agar (SMAC) (Media production, NVI) and MacConkey agar (Media production, NVI).

Subsequently, ISO/TS 13136:2012 for isolation of STEC strains was followed with modifications. A total of 50 colonies with typical or suspicious *E. coli* morphology from the 6 agar plates available for each sample (2 different concentrations x 3 agar media) were selected. The colonies were point inoculated in blood agar plate (BA) (Media production, NVI) and incubated overnight at 37±1°C. Next, five and five colonies were pooled, giving a total of 10 pool from each sample, and DNA was extracted by boiling (described in section 2.2.7.1), and used as template for detection of *stx2a* by real-time PCR (as described in 2.2.5). When PCR -positive pools were identified, DNA from single colonies was obtained in the same manner and tested for the presence of *stx2a*. When no PCR -positive pools were obtained from the collection of 50 colonies, the result was reported as PCR -positive without STEC isolation.

Stx2a$^+$ isolates were tested on MALDI-TOF (description on 2.2.8) for species identification. Pure cultures of confirmed *stx2a*$^+$ -*E. coli* were stored in Kryo-tubes containing 25% (vol/vol) glycerol (Media production, NVI) at −80°C for later use.

2.2.7. DNA extraction

2.2.7.1. DNA Extraction by boiling

Bacterial material was suspended in 100 µl of milliQ water in 1,5 ml Eppendorf tube and heated at 100°C for 10 minutes in a heating block (Grant Instruments, England), followed by

centrifugation at 10000 rpm for 5 minutes in an Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was immediately used as template for real-time PCR reactions.

2.2.7.2. DNA extraction using QIAamp DNA Mini kit®

DNA extraction was carried out using the QIAamp DNA Mini Kit® (Qiagen, Bruz, France) according to the manufacturer's instructions. For the detailed procedure for DNA extraction from Gram-negative bacteria see www.qiagen.com.

Nano Drop 2000-Spectrophotometer (Thermo-Scientific, USA) was used to measure DNA concentration after DNA extraction with QIAamp Mini Kit®.

The DNA was used as template for real-time and conventional PCR reactions.

2.2.8. Confirmation of *E. coli* by MALDI-TOF

Matrix assisted desorption/ionization time-of-flight mass spectrometry, MALDI-TOF (Microflex, Maldi Biotyper, USA) is an established method for identification of bacterial isolates. The method is based on the bacterial mass spectra given by a representative number of microbial proteins that give peaks with a determinable mass to charge (m/z) ratio. The results are given by comparison of these peaks with known spectra from reference microorganisms. For MALDI-TOF analysis, a fresh single colony was applied to a metal plate with a toothpick and left to dry. Then, 1 µl of MALDI-TOF matrix (Bruker, Bremen, Germany) was applied to the dried bacterial material and left to dry. The metal plate was placed in the instrument and the MALDI Biotyper software (Bruker, Bremen, Germany) gave the most probable species to which the bacterium belongs to.

2.2.9. Characterization of *stx2a*$^{+}$ *E. coli* isolates

The characterization of *stx2a*$^{+}$ *E. coli* isolates was carried out using both phenotypical methods (serotyping) and molecular methods (Conventional PCR and real-time PCR) for detection of virulence genes.

2.2.9.1 Determination of virulence genes and O-group typing by real-time PCR

The characterization of *stx2a*$^{+}$-STEC was first carried out by real-time PCR for determining the presence of additional virulence genes as *eae, ehxA* and *stx1*, as well as for seven of most common serogroups O26, O91, O103, O111, O121, O145 and O157. For primer and probes see Table 2.1, for PCR programs see Table 2.2, and for positive controls see Table 2.5.

Further serotyping with antisera was performed for s*tx2a*$^{+}$ *E. coli* that were negative for the first seven serogroups tested.

Table 2.6. Master mix composition for specific serogroups O26, O91, O103, O111, O121, O145 and O157 gene amplification by real-time PCR

	Final concentration of components in the master mix					
Characterization: gene/serogroup	*eae*	*ehxA*	O26, O103, O145, O157	O111	O91	O121
Component						
x2 Brilliant III Ultra-Fast QPCR Master Mix	1x	2x	2x	2x	1x	1x
Primer F, (10µM)	0,6 µM	0,8 µM	0,5 µM	1 µM	0,5 µM	0,33 µM
Primer R, (10µM)	0,6 µM	0,8 µM	0,5µM	1µM	0,5 µM	0,33 µM
Probe (10µM)	0,2 µM	0.8 µM	0,2 µM	0,2 µM	0,2 µM	0,1 µM
Nuclease free water	-	-	-	-	-	-
Total master mix vlume	15 µl	19 µl	18 µl	18 µl	20µl	5 µl
DNA volume	5 µl	1 µl	2 µl	2 µl	5 µl	20 µl
Total volume per reaction	25 µl	20 µl	20 µl	20 µl	25µl	25 µl

2.2.9.2. O-group typing by antisera assay

Agglutination of boiled culture with O-antisera was carried out for the O-serogroups: O45, O55, O104, O113 and O146. O-antisera kit (Statens Serum Institut, Copenhagen, Denmark) was used according to the manufacturer's instructions.

2.2.9.3. Subtyping of *stx1* genes by multiplex PCR assay and Bioanalyzer

Conventional multiplex PCR was used to amplify the three subtypes of *stx1*-gene. The amplification was carried out using BioRad T100 Thermal cycler (BioRad, Singapore). The set of primers used (Sigma-Aldrich, USA), are described in Table 2.3 and the PCR program is listed in Table 2.4. The reagents and 1µl of PCR products were applied to a miniaturized microcapillary electrophoresis chip (Agilent Technologies Inc., Germany) following the manufacturer's instructions are the results were read using 2100 Bioanalyzer chip reader (Agilent Technologies Inc., Germany).

2.2.9.4. Subtyping of *stx2* genes by conventional PCR and gel electrophoresis

Conventional PCR was carried out for each of the seven *stx2* subtypes. The instrument used for amplification was Bio-rad T100 Thermal cycler (Bio-Rad, Singapore City, Singapore). The set of primers used are listed in Table 2.3 and the PCR programs are defined in Table 2.4. Primers from amplification of *stx2a, stx2c* and *stx2d* were purchased from Sigma-Alorich, (St Quentin Fallavier, France), primers for amplification of *stx2b, stx2e, stx2f* and *stx2g* were purchased from Eurogentec (Liège, Belgium).

For each reaction 12,5 µl HotStar Taq® Master mix, 0,75 µl of each primer, a water volume necessary complete a total of 23 µl, and 2 µl of template DNA were used.

6x Orange loading dye (5 µl/25 µl PCR product) (Thermo Fisher Scientific, Canada) was added to the PCR products. 10 µl of PCR products, 100 bp ladder (Gene Ruler™, Fermentas, Life Technologies Corporation, Van Allen Way Carlsbad, CA, USA), positive and negative controls were applied to agarose gel (2% agarose (Prolano, Belgium) in 100ml TAE buffer,10µl GelRed™ Nucleic acid gel staining (Biotium, Hayward, CA, USA) and ran at 90 volt for 60 minutes. The bands were visualized under UV light using the Molecular Imager® ChemiDoc XRS System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Images were taken using Image Lab™ Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Different sized PCR products travels at different velocities. Their final position of the product was determined comparing to the molecular markers given by the 100 bp DNA ladder and the position of the amplicon in the positive control.

2.2.9.5. Phylotyping of *E. coli* strains by multiplex PCR and gel electrophoresis

Conventional Multiplex PCR was used for grouping of *E. coli* strains into the phylogenetic groups: A, B1, B2 and D. The sequence-based method targets housekeeping genes and is used to determine genetic relatedness among isolates (Scheutz, 2014). The method was based on Clermont *et al*. (2000) and Doumith *et al*. (2012).

For the PCR reaction, a mix of 12,5 µl 2x Qiagen Multiplex PCR master mix (QIAGEN®, Germany), 0,5 µl primer mix (Table 2.3) (0,2 µM)(Invitrogen, Gaithersburg, Maryland) and 10 µl milliQ was prepared. A volume of 2 µl template DNA was added. The primer mix

composition is shown in Table 2.3 and the PCR program is described in Table 2.4. Agarose gel electrophoresis was run as described in 2.2.9.4. The interpretation of the results is based on Table 2.7.

Table 2.7. Interpretation of results for phylotyping of *E. coli*. (+) indicate the presence of band and (-) the absence of band.

Phylogenetic group	*gadA* 373 bp	*chuA* 216bp	*yjaA* 216 bp	*TSPE4.C2* 152 bp
A	+	-	-/+	-
B1	+	-	-	+
B2	+	+	+	-/+
D	+	+	-	-/+

3. Results

3.1. Validation of master mixes

Three different master mixes were assessed for efficiency in detecting the genes *stx1* and *stx2*. Figure 1 shows the amplification curves and standard curve generated by amplification of a serial dilution of DNA from strain EDL-933 using Brilliant III Ultra-fast QPCR master mix® (left) and TaqMan® Universal PCR Master mix. The results obtained for the three master mixes tested are shown in Table 3.1.

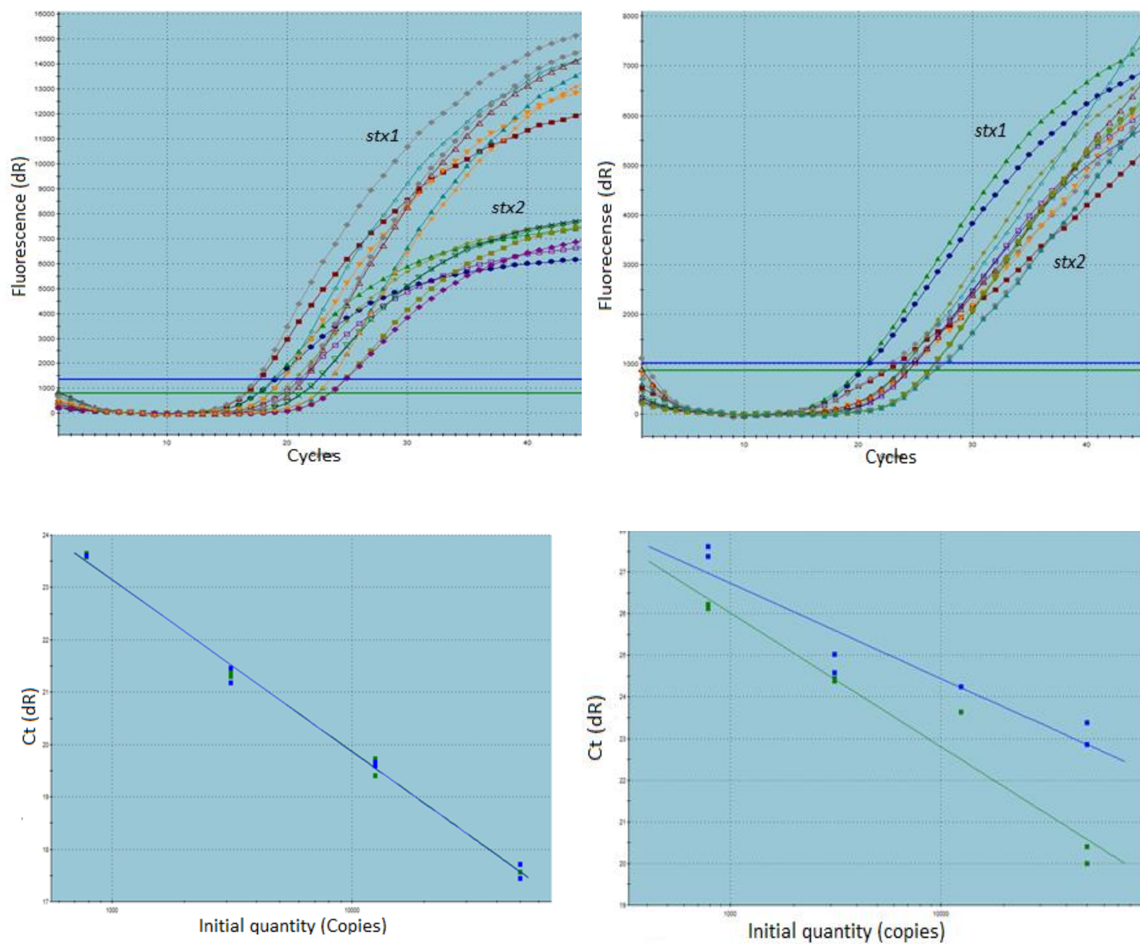

Figure 3.1. Amplification plots and standard curves resulted from the validation of master mixes. Upper images: Amplification curves for the target genes *stx1* and *stx2* obtained by serial dilutions of DNA template from strain EDL-933. Down: Standard curves obtained by plotting the initial template quantities against the Ct-value obtained during amplification of each dilution. Left: Results using Brilliant III Ultrafast QPCR Master Mix®. Right: Results obtained using TaqMan® Universal PCR Master mix. Results obtained using TaqMan® Environmental Master Mix 2.0 are not shown.

Table 3.1. **Amplification efficiency**. R^2 and amplification efficiency estimated for *stx1* and *stx2* using DNA template from reference strain EDL-933.

TaqMan® Universal PCR Master mix		TaqMan® Environmental Master Mix 2.0		Brilliant III Ultra-fast QPCR Master Mix®	
HEX[*] standards	R^2 = 0,954	HEX standards	R^2 = 1,000	HEX standards	R^2 = 0,995
	Eff =105.4%		Eff =97.94%		Eff =102.0%
FAM [**] standards	R^2 = 0,892	FAM Standards	R^2 = 0,853	FAM Standards	R^2 = 0,995
	Eff =175.1%		Eff =141.5%		Eff =101.6%

[*]Reporting dye for detection of *stx2*
[**]Reporting dye for detection of *stx1*

Brilliant III Ultra-fast Master Mix® resulted in the best combined efficiency for *stx1* and *stx2* detection, while TaqMan® Universal showed the lowest efficiency for *stx1*and *stx2* detection. TaqMan® Environmental showed the best efficiency for detection of *stx2* alone, together with the lowest efficiency for detection of *stx1* gene. As a result, Brilliant III Ultra-fast Master Mix® was selected for use in the following work.

3.2. Screening of DNA extracted from fecal samples for virulence genes: *stx1*, *stx2* and *stx2a*

From a total of 178 DNA samples from an equal number of dairy herds tested by real-time PCR, 141 herds (79.2%) were *stx1* positive and 166 herds (93.3%) were *stx2* positive (Ct-values 24.5-44.6). 100 herds (70.9%) containing genes coding for both Shiga toxins, and just seven herds were *stx*-free. 30 herds (16.8%) were positive for the presence of *stx2a* gene (Ct-values 37.1-44.5). The Appendix 6.1 shows the complete results obtained by real-time PCR. Ct -values from *stx1* and *stx2* screening are not shown.

3.3. Isolation of *stx2a*-positive *E. coli*

A total of 30 dairy herds returned positive real-time PCR results for the presence of *stx2a*-genes. Isolation of STEC was then attempted from PCR positive fecal samples. Bacteria containing *stx2a* was successfully isolated from 15 of the 30 PCR -positive fecal samples. A total of 86 *stx2a*$^+$ isolates were obtained from 15 different herds and 76 of them were further confirmed as *E. coli* by MALDI-TOF test. Table 3.2 shows information about the isolates found, such as sample number (herd number), colony morphology, and MALDI-TOF test results.

The number of *stx2a*-positive isolates found in positive samples was variable, from one in fifty to twenty in fifty. The positive colonies were isolated from three different agar media and showed variable morphology.

Table 3.2. Characteristics of the 86 *stx2a*⁺ isolates obtained. The colony number denotes the number of the positive colony on blood agar. The number of positive isolates recovered from each fecal sample, colony morphology on agar media and results on MALDI-TOF are also annotated. To the right, the selected 25 *E. coli* isolates are numerated.

#	Sample number (PJS-nr*)	Colony #	Number of *stx2a+* colonies/sample	Colony morphology	MALDI-TOF	Selected for characterization
1	2014-22-143	34		Small, pink on Chromagar	*E. coli*	1
2	2014-22-143	32	2 of 50	Small, pink on Chromagar	*E. coli*	2
3	2014-22-137	2		Small, pink on Chromagar	*E. coli*	
4	2014-22-137	3		Small, pink on Chromagar	*E. coli*	
5	2014-22-137	4		Small, pink on Chromagar	*E. coli*	
6	2014-22-137	5		Small, pink on Chromagar	*E. coli*	
7	2014-22-137	7		Turquoise on Chromagar	*E. coli*	
8	2014-22-137	8		Turquoise on Chromagar	*E. coli*	3
9	2014-22-137	16		Big, pale pink on Smac agar	*E. coli*	4
10	2014-22-137	19		Big, pale pink on Smac agar	*E. coli*	
11	2014-22-137	20		Big, pale pink on Smac agar	*Citobacter amalonaticus*	
12	2014-22-137	21		Dark pink on MacConkey agar	*E. coli*	
13	2014-22-137	22		Dark pink on MacConkey agar	*E. coli*	
14	2014-22-137	23		Dark pink on MacConkey agar	*E. coli*	
15	2014-22-137	24		Dark pink on MacConkey agar	*E. coli*	
16	2014-22-137	31		Small, pink on Chromagar	*E. coli*	
17	2014-22-137	32		Small, pink on Chromagar	*E. coli*	
18	2014-22-137	34		Small, pink on Chromagar	*E. coli*	
19	2014-22-137	35		Small, pink on Chromagar	*E. coli*	
20	2014-22-137	40		Pink on Smac agar	*E. coli*	
21	2014-22-137	46		Pink on MacConkey agar	*E. coli*	
22	2014-22-137	48	20 of 50	Pink on MacConkey agar	*E. coli*	
23	2014-22-162	9		Blue on Chromagar	No result on MALDI	
24	2014-22-162	26		Pink on Smac agar	*Citrobacter farmeri*	
25	2014-22-162	27		Pink on Smac agar	*E. coli*	5
26	2014-22-162	28	4 of 50	Pink on Smac agar	*E. coli*	6
27	2014-22-164	39	1 of 50	Dry, pink on MacConkey agar	*E. coli*	7
28	2014-22-219	11		Purple/Blue on Chromagar	*E. coli*	
29	2014-22-219	14		Purple/Blue on Chromagar	No result on MALDI	
30	2014-22-219	15		Purple/Blue on Chromagar	*E. coli*	
31	2014-22-219	16		Purple/Blue on Chromagar	*E. coli*	
32	2014-22-219	18		Purple/Blue on Chromagar	*E. coli*	8
33	2014-22-219	38		Pink on MacConkey agar	*E. coli*	
34	2014-22-219	39		Pink on MacConkey agar	*E. coli*	9
35	2014-22-219	40		Pink on MacConkey agar	*E. coli*	
36	2014-22-219	46	9 of 50	Pink on MacConkey agar	*E. coli*	
37	2014-22-232	1		Blue on Chromagar	No result on MALDI	
38	2014-22-232	3		Blue on Chromagar	*E. coli*	
39	2014-22-232	4		Blue on Chromagar	*E. coli*	
40	2014-22-232	5		Blue on Chromagar	*E. coli*	10
41	2014-22-232	25		Big, pale pink on Smac agar	*E. coli*	11
42	2014-22-232	37		Pink on MacConkey agar	*E. coli*	
43	2014-22-232	38	7 of 50	Pink on MacConkey agar	*Citrobacter amalonaticus*	
44	2014-22-288	5	1 of 50	Pink on Chromagar	*E. coli*	12
45	2014-22-293	6		Blue on Chromagar	*E. coli*	
46	2014-22-293	8		Blue on Chromagar	*E. coli*	
47	2014-22-293	9		Blue on Chromagar	*E. coli*	
48	2014-22-293	14		Blue on Chromagar	*E. coli*	13
49	2014-22-293	31		Pink on Smac agar	*E. coli*	14
50	2014-22-293	35		Pink on Smac agar	*E. coli*	

#	Sample number (PJS-nr*)	Colony #	Number of *stx2a+* colonies/sample	Colony morphology	MALDI-TOF	Selected for characterization
51	2014-22-293	36	7 of 50	Pink on Smac agar	*E. coli*	
52	2014-22-159	4	1 of 50	Small, pink on Chromagar	*E. coli*	15
53	2014-22-188	2	1 of 50	Pink on Chromagar	*E. coli*	16
54	2014-22-241	1		Small, pink on Chromagar	*E. coli*	
55	2014-22-241	2		Small, pink on Chromagar	*E. coli*	
56	2014-22-241	3		Small, pink on Chromagar	*E. coli*	
57	2014-22-241	4		Small, pink on Chromagar	*Citrobacter amalonaticus*	
58	2014-22-241	5		Small, pink on Chromagar	*E. coli*	17
59	2014-22-241	29		Pink on Smac agar	*E. coli*	18
60	2014-22-241	32		Pink on Smac agar	*Citrobacter amalonaticus*	
61	2014-22-241	35	8 of 50	Pink on Smac agar	*E. coli*	
62	2014-22-253	23	1 of 50	Pink on Smac agar	*E. coli*	19
63	2014-22-184	1		Small, pink on Chromagar	*Proteus vulgaris*	
64	2014-22-184	2		Small, pink on Chromagar	*E. coli*	
65	2014-22-184	3		Small, pink on Chromagar	*E. coli*	
66	2014-22-184	4		Small, pink on Chromagar	*E. coli*	
67	2014-22-184	5		Small, pink on Chromagar	*E. coli*	
68	2014-22-184	24		Big, pale pink on Smac agar	*E. coli*	
69	2014-22-184	33		Red on Smac agar	*E. coli*	20
70	2014-22-184	48	8 of 50	Pink on MacConkey agar	*E. coli*	21
71	2014-22-255	1		Small, pink on Chromagar	*Citrobacter amalonaticus*	
72	2014-22-255	18		Medium, turquoise on Chromagar	*E .coli*	22
73	2014-22-255	35		Pale pink on MacConkey agar	*E. coli*	
74	2014-22-255	43		Pink on Smac agar	*E. coli*	23
75	2014-22-255	44		Pink on Smac agar	*E. coli*	
76	2014-22-255	46		Pink on Smac agar	*E. coli*	
77	2014-22-255	47	7 of 50	Pink on Smac agar	*E. coli*	
78	2014-22-158	2		Small, pink on Chromagar	*E. coli*	
79	2014-22-158	4		Small, pink on Chromagar	*E. coli*	
80	2014-22-158	26		Red w/transparent edges on Smac	*Proteus vulgaris*	
81	2014-22-158	27		Red w/transparent edges on Smac	*E. coli*	
82	2014-22-158	28		Red w/ transparent edges on Smac	*E. coli*	
83	2014-22-158	29		Red w/ transparent edges on Smac	*E. coli*	24
84	2014-22-158	31		Pink/grey on Smac agar	*E. coli*	25
85	2014-22-158	32		Pink/grey on Smac agar	*E. coli*	
86	2014-22-158	44	9 of 50	Small, pink in MacConkey agar	*E. coli*	

*Identification number used by the NVI

3.4. Characterization of *stx2a*$^+$ *E. coli*

A total of 25 *stx2a*$^+$ *E. coli* isolates were selected for characterization: one from each positive herd and one additional isolate from herds with more than one positive isolate. When two isolates per herd were included, they presented different morphology on agar media or were isolated from different agar plates.

3.4.1. Virulence genes: *stx1*, *eae* and *ehxA*

The 25 *stx2a*$^+$ *E. coli* isolates were screened for additional virulence genes: *stx1, eae* and *ehxA* by real-time PCR.

The results indicated that four isolates were *stx1* positive, 23 isolates gave signals of *ehxA* gene amplification, showing Ct -values ranging from 14, 2 to 32,53; and 24 of 25 isolates gave positive signals for the *eae* gene (Ct from 15,75 to 36,67). A more detailed revision of the amplification curves led us to dismiss all isolates with Ct-values>30.

Finally, 21 *E.coli* isolates were determined as *ehxA* positive and seven isolates were determined as *eae* positives. An overview of results is shown in Table 3.3.

3.4.2. *stx1* and *stx2* subtyping.

Subtyping of *stx1* was carried out for the four isolates harboring *stx1*. All the isolates harbored *stx1a*. Figure 6.2 in the Appendix shows the image obtained by Bioanalyzer chip reader.

The results for subtyping of *stx2* genes showed that in addition to *stx2a*, two isolates (14 and 19) harbored *stx2b* gene, while isolates 5, 15, 17, 20 (marked with +/- in Table 3.3) showed bands with the corresponding amplicon size in agarose gel, but were dismissed due to the low intensity of the band compared with the intensity showed by the positive control and other clear positive results (see appendix 6.3). Three isolates (3, 4 and 8) harbored the *stx2c* in addition to *stx2a*. No isolates carried *stx2* subtypes d, e, f or g. The results are summarized in Table 3.3, and gel images are showed in appendix 6.3.

Table 3.3. Characterization of the selected *stx2a*⁺ *E. coli:* Virulence genes. The presence of virulence genes *eae, ehxA, stx1, stx2* and subtypes were examined by real-time PCR. Isolate number consist in the last three digits of the cattle herd NVI intern number and number of the positive colony on agar media. The positive results are colored. For *eae* and *ehxA*, values under Ct=30 were dismissed. Subtypes not showed in the table were not detected in any of the isolates.

#	Isolate number	Virulence genes						
						Stx2 subtype		
		eae	*ehxA*	*stx1*	*stx1a*	*stx2a*	*stx2b*	*stx2c*
1	143-34	36,25	21,77	-	-	+	-	-
2	143-33	33,54	21,33	-	-	+	-	-
3	137-8	16,76	17,64	-	-	+	-	+
4	137-16	17,19	19,27	-	-	+	-	+
5	162-27	17,36	17,31	-	-	+	+/-	-
6	162-28	16,1	17,19	-	-	+	-	-
7	164-39	34,24	20,16	-	-	+	-	-
8	219-18	34,82	17,63	-	-	+	-	+
9	219-39	36,45	-	16,45	+	+	-	-
10	232-5	36,23	14,2	-	-	+	-	-
11	232-25	34,95	16,85	-	-	+	-	-
12	288-5	34,99	21,54	-	-	+	-	-
13	293-14	36,67	-	-	-	+	-	-
14	293-31	31,01	31,45	-	-	+	+	-
15	159-4	15,75	16,71	15,92	+	+	+/-	-
16	188-2	-	20,25	-	-	+	-	-
17	241-5	34,93	20,44	-	-	+	+/-	-
18	241-29	35,76	19,23	-	-	+	-	-
19	253-23	32,52	32,53	-	-	+	+	-
20	184-33	31,61	20,13	-	-	+	+/-	-
21	184-48	33,74	19,96	-	-	+	-	-
22	255-18	26,54	16,39	-	-	+	-	-
23	255-43	26,48	16,57	-	-	+	-	-
24	158-29	17,77	17,58	15,75	+	+	-	-
25	158-31	16,43	17,6	16,03	+	+	-	-
	PC*	15,73	19,85	14,74	+	+	+	+

*Positive control

3.4.3. O-Serogroup typing by real-time PCR

Seven O-groups were tested by real-time PCR. The O-groups tested were the most common associated with human disease: O26, O91, O103, O121, O111, O145 and O157. The isolates were negative for O91, O103, O121, O111, O145 and O157. However, most isolates (23 of 25) returned Ct-values (16,3 – 39,1) when testing for the presence of O26-spesific gene. More detailed analysis of the curves showed a clear difference between the groups that showed a signal similar to the positive Control (Ct = 17,38) and the ones that gave late signals (after 30 cycles). To clarify the results, slide agglutination with O-antisera was used. It confirmed that the two isolates (5 and 6), both from the same herd and which showed curves similar to the

positive control (Ct_5 =16,29; Ct_6 =16,74) were O26 positive, while no agglutination was observed for the isolates with higher Ct-values. The results for all isolates are shown in Table 3.4.

3.4.4. O-agglutination of boiled culture with O-antisera.

Stx2a$^+$*E. coli* isolates were tested for five O-groups also associated with human disease: O45, O55, O104, O113, O128 and O146 by agglutination with O-antisera. The results are shown in Table 3.4: two isolates (10 and 11) belonging to the same herd tested positive for O113, while the remaining 21 isolates could not be serotyped with the antisera that was available for use in this study. Isolates #1 and #2 react with all the antisera tested and also with physiological salt water and were thus serogrouped as O-rough.

Table 3.4. Characterization of *stx2a*$^+$ *E. coli*: O-serogroups. The table shows the results obtained by real-time PCR for detection of seven O-groups and results from agglutination with O-antisera for detection of six serogroups.

		O-serotype												
	Isolate	Real-time PCR							Agglutination with O-antisera					
#	number	O26	O91	O103	O111	O121	O145	O157	O45	O55	O104	O113	O128	O146
1	143-34	No Ct	-	-	-	-	-	-				O-rough		
2	143-33	31,16	-	-	-	-	-	-				O-rough		
3	137-8	34,34	-	-	-	-	-	-	-	-	-	-	-	-
4	137-16	38,3	-	-	-	-	-	-	-	-	-	-	-	-
5	162-27	16,29	-	-	-	-	-	-	-	-	-	-	-	-
6	162-28	16,74	-	-	-	-	-	-	-	-	-	-	-	-
7	164-39	25,99	-	-	-	-	-	-	-	-	-	-	-	-
8	219-18	35,34	-	-	-	-	-	-	-	-	-	-	-	-
9	219-39	38,23	-	-	-	-	-	-	-	-	-	+	-	-
10	232-5	37,71	-	-	-	-	-	-	-	-	-	+	-	-
11	232-25	37,77	-	-	-	-	-	-	-	-	-	-	-	-
12	288-5	34,72	-	-	-	-	-	-	-	-	-	-	-	-
13	293-14	39,06	-	-	-	-	-	-	-	-	-	-	-	-
14	293-31	32,83	-	-	-	-	-	-	-	-	-	-	-	-
15	159-4	33,67	-	-	-	-	-	-	-	-	-	-	-	-
16	188-2	32,91	-	-	-	-	-	-	-	-	-	-	-	-
17	241-5	36,08	-	-	-	-	-	-	-	-	-	-	-	-
18	241-29	37,35	-	-	-	-	-	-	-	-	-	-	-	-
19	253-23	No Ct	-	-	-	-	-	-	-	-	-	-	-	-
20	184-33	32,13	-	-	-	-	-	-	-	-	-	-	-	-
21	184-48	37,54	-	-	-	-	-	-	-	-	-	-	-	-
22	255-18	34,93	-	-	-	-	-	-	-	-	-	-	-	-
23	255-43	32,67	-	-	-	-	-	-	-	-	-	-	-	-
24	158-29	32,59	-	-	-	-	-	-	-	-	-	-	-	-
25	158-31	32,59	-	-	-	-	-	-	-	-	-	-	-	-
PC*		17,38	15,81	18,14	18,94	17,17	18,38	19,28						
NC**		-	-	-	-	-	-	-	-	-	-	-	-	-

*Positive control
**Negative controls

3.4.5. Phylogenetic analysis

Multiplex PCR and agarose gel electrophoresis were used for phylogenetic analysis of *stx2a*⁺ *E. coli*. All isolates belonged to two phylogenetic groups. A total of 13 *E. coli* isolates fell into the A-group and the remaining 12 isolates fell into the B1-group. The results are shown in table 3.5 and images of agarose gel are shown in appendix 6.4.

Table 3.5. Phylogenetic analysis of 25 *stx2a*⁺*E. coli* isolates. The presence or absence of four genes housekeeping genes determine the phylogenetic group of *E. coli* isolates.

#	Isolate number	*gadA* 373 bp	*chuA* 216 bp	*yjaA* 216 bp	*TSPE4.C2* 152 bp	Phylogenetic group
1	143-34	+	-	+	-	A
2	143-33	+	-	+	-	A
3	137-8	+	-	-	-	A
4	137-16	+	-	-	-	A
5	162-27	+	-	-	+	B1
6	162-28	+	-	-	+	B1
7	164-39	+	-	+	-	A
8	219-18	+	-	-	+	B1
9	219-39	+	-	-	+	B1
10	232-5	+	-	-	+	B1
11	232-25	+	-	-	+	B1
12	288-5	+	-	+	-	A
13	293-14	+	-	-	+	B1
14	293-31	+	-	-	+	B1
15	159-4	+	-	-	+	B1
16	188-2	+	-	+	-	A
17	241-5	+	-	+	-	A
18	241-29	+	-	+	-	A
19	253-23	+	-	-	+	B1
20	184-33	+	-	+	-	A
21	184-48	+	-	+	-	A
22	255-18	+	-	+	-	A
23	255-43	+	-	+	-	A
24	158-29	+	-	-	+	B1
25	158-31	+	-	-	+	B1

3.5. Summary: Characterization of 25 *stx2a*$^{+}$ *E. coli* isolates from Norwegian cattle

The table 3.6 summaries the results from characterization assays carried out for the 25 selected *stx2a*$^{+}$ *E. coli* isolates. The characterized isolates belong to two phylogroups: 52% fall into the A-group and 48% fall into B1-group. Four isolates were serogrouped as O26 and/or O113. Seven isolates were *eae* positives, 21 were *ehxA* positives, and four were *stx1a* positive. In addition to *stx2a*, subtypes *stx2b* were present in 2 isolates and *stx2c* was present in three isolates. Different virulence profiles are shown, but generally, isolates from the same herd present the same profile. Virulence profiles are more uniform within phylogroup A, while more diverse profiles were distributed among phylogroup B1.

Table 3.6. Characterization of selected *stx2a*$^{+}$ *E.coli* isolated from Norwegian cattle.

#	Isolate number	Morphology	Phylogenetic group	O-Serogroup	Virulence genes					
								Stx2 subtype		
					eae	*ehxA*	*stx1a*	*stx2a*	*stx2b*	*stx2c*
1	143-34	Pink on CHROMagar	A	Rough	-	+	-	+	-	-
2	143-33	Pink on CHROMagar	A	Rough	-	+	-	+	-	-
3	137-8	Turquoise on CHROMagar r	A	NF*	+	+	-	+	-	+
4	137-16	Pale pink on SMAC	A	NF	+	+	-	+	-	+
7	164-39	Dry colony on MacConkey	A	NF	-	+	-	+	-	-
12	288-5	Pink on CHROMagar	A	NF	-	+	-	+	-	-
16	188-2	Pink on CHROMagar	A	NF	-	+	-	+	-	-
17	241-5	Pink on CHROMagar	A	NF	-	+	-	+	+/-	-
18	241-29	Pink on SMAC	A	NF	-	+	-	+	-	-
20	184-33	Red on SMAC	A	NF	-	+	-	+	+/-	-
21	184-48	Pink on MacConkey	A	NF	-	+	-	+	-	-
22	255-18	Turquoise on CHROMagar	A	NF	-	+	-	+	-	-
23	255-43	Pink on SMAC	A	NF	-	+	-	+	-	-
5	162-27	Pink on SMAC	B1	O26	+	+	-	+	+/-	-
6	162-28	Pink on SMAC	B1	O26	+	+	-	+	-	-
8	219-18	Purple/blue on CHROMagar	B1	NF	-	+	-	+	-	+
9	219-39	Pink on MacConkey	B1	NF	-	-	+	+	-	-
10	232-5	Blue on CHROMagar	B1	O113	-	+	-	+	-	-
11	232-25	Pink on SMAC	B1	O113	-	+	-	+	-	-
13	293-14	Blue on CHROMagar	B1	NF	-	-	-	+	-	-
14	293-31	Pink on SMAC	B1	NF	-	-	-	+	+	-
15	159-4	Pink on CHROMagar	B1	NF	+	+	+	+	+/-	-
19	253-23	Pink on SMAC	B1	NF	-	-	-	+	+	-
24	158-29	Red w/transparent edges on SMAC	B1	NF	+	+	+	+	-	-
25	158-31	Pink/grey on SMAC	B1	NF	+	+	+	+	-	-

*Not found

4. Discussion

4.1 Prevalence

In this study, the prevalence of *stx2a*$^{+}$ STEC in Norwegian cattle herds was examined. Our results indicated a surprisingly high PCR -occurrence of *stx2a* genes (16.9%) in fecal samples. However, to the best of our knowledge, this is the first study of its type, and no comparable data is available, neither in Norway nor in other European countries.

Knowledge on prevalence of Shiga toxin genes in Norwegian cattle was also limited. The PCR screenings carried out in the present study indicate wide distribution of *stx* genes in fecal samples. A total of 93.3% of the herds were PCR -positive for *stx2,* while 79.2% were positive for *stx1*. The high rates of *stx* genes detected suggest that future studies should rather focus on detection of *stx* subtypes. By doing so, efforts can be concentrated in detection of strains most frequently associated with human disease and at the same time, reduce the number of samples to analyze to manageable numbers.

Although the prevalence of STEC on its animal reservoirs and especially in cattle has been studied in different countries in the past three decades, most of the studies have concentrated on detection and isolation of the “top five” human pathogenic serogroups: O157, O26, O111, O103 and O145. The last survey of potentially zoonotic *E. coli* in Norway investigated the prevalence precisely of these serogroups in the same fecal samples examined in this study. The results indicated a low prevalence of STEC of these serogroups. However, STEC from serogroups O26, O103 and O157 were found in 5.6%, 2.2% and 2.2% of cattle herds, respectively (Sekse et al., 2015).

Yet few studies have examined the prevalence of STEC using *stx* genes as pathogenic marker: In Spain, Blanco *et al*. (1997) found STEC in 95% of the examined cattle farms and found *stx2* more often than *stx1* in the isolates. The prevalence rates of STEC in asymptomatic cows and calves were estimated to be 35% and 37%, respectively. In Germany, Geue *et al*. (2002) carried out a three years’ study where the prevalence of STEC varied widely between the farms and animal groups included. In average, 45% of the samples harbored *stx*-sequences simultaneously. Moreover, STEC were detected at least once in fecal samples from every animal examined. Sequences of *stx2* were more often reported than *stx1* within the isolated strains. In Switzerland, Zweifel *et al*. (2005) analyzed cattle carcasses at the slaughterhouses. They detected STEC harboring *stx1* more frequently than *stx2* among the obtained isolates. In

Japan, Mekata *et al*. (2014) reported *stx* genes in 64.2% of the analyzed cattle feces and STEC strains were isolated from 22% of the PCR -positive samples.

4.2. *stx2a*$^{+}$ isolates

A total of 86 s*tx2a*$^{+}$ isolates were recovered from 50% of the PCR -positive samples (15 of 30). A selection of 25 *stx2a*$^{+}$ *E. coli* representing the 15 cattle herds was characterized. The characterized isolates belong to two phylogenetical groups with 52% in the A-group and 48% in the B1-group. Within phylogroup A, the isolates presented very similar virulence profiles, while more diverse profiles were distributed among phylogroup B1 (Table 3.5). Isolates from the same herd fell in the same phylogroup, and the majority of isolate pairs isolated from the same herd, also showed identical virulence profiles.

To assess the pathogenicity of STEC isolated from cattle, their virulence profiles were compared with genetic information from 95 non-O157 STEC strains isolated from Norwegian patients (Haugum et al., 2014). Additional information from that study is shown in appendix 6.5. The human strains were not tested for *ehxA* genes, thus comparison will be based on phylogroups, serotypes, *stx* subtypes and *eae*.

E. coli strains fall into determined phylogenic groups A, B1, B2, D, E and F. Generally, commensal strains fall into groups A and B1, whilst intestinal pathogenic strains fall in phylogroups A, B1 and D (Carlos et al., 2010). In Norway phylogroup B1 is the most common among STEC isolated from human patients, nonetheless phylogroups A, E and B2 are also represented. Moreover, all strains associated with HUS in the study from Haugum *et al*. (2014) fell in phylogroup B1.

The selected *stx2*$^{+}$ isolates analyzed in this study belonged to two phylogroups: A and B1. Within the 13 cattle isolates in phylogroup A, 11 isolates presented identical virulence profile: s*tx2a*$^{+}$, *ehxA*$^{+}$ and *eae*$^{-}$. These isolates did not belong to any of the tested serogroups. Similar virulence profile can be observed in five STEC isolated from Norwegian patients (Appendix 6.5, Table 6.2). None of these strains were associated with HUS. Four of them fell into phylogroup B1 and one fell into phylogroup A. Two isolates with unknown serotype (FHI89 and FHI 99) caused gastroenteritis, one of them FHI99 belong to phylogroup A. Other two strains (from serogroups O104 and O111) were associated with bloody diarrhoea. Although

these 11 cattle-isolates lack *eae*, there is not possible to discard the presence of other adhesion factors not tested in this study, thus, the pathogenicity of these isolates cannot be excluded.

Two STEC in the A phylogroup isolated from cattle were *stx2a*$^{+}$, *eae*$^{+}$, *ehxA*$^{+}$ and *stx2c*$^{+}$. None of the human STEC presented a similar profile in Haugum *et al*. (2014). However Brandal *et al*. 2015b describes one HUS patient from which STEC harboring both *stx2a* and *stx2c* was isolated, yet no information on *eae* and *ehxA* is describe for that strain. The virulence combination *eae*$^{+}$, *stx2a* and *stx2c* has been reported in strains O157:H7, O177 and O-rough isolated from German patients (Beutin et al. 2004) and from eight Danish patients with HUS (Persson et al., 2007), and the combination of *eae* and *stx2c*, in absence of *stx2a*, have also been associated with HUS (Friedrich et al., 2002).

The cattle isolates in phylogroup B1 presented several different virulence profiles. Two cattle isolates were O26, *eae*$^{+}$, *ehxA*$^{+}$, *stx2a*$^{+}$. In Haugum's study, seven human isolates presented the same virulence profile (FHI3, FHI4, FHI24, FHI79, FHI36 and FHI2); all of them fell into phylogroup B1 and are grouped into the HUS-group 2, and three of them were associated with HUS. Other two cattle-isolates were O113, *eae*$^{-}$, *ehxA*$^{+}$ and *stx2a*$^{+}$. The same serogroup has been isolated from Norwegian patients (two isolates) and minced meat (one isolate), however, these isolates harbored other subtypes of *stx2* (*stx2d* or *stx2b*), two belong to phylogroup A, one to the phylogroup B1 and none of them caused disease. In Australia in 1998, a STEC O113:H21 strain lacking *eae* and positive for *stx2* and *ehxA* was responsible for three cases of HUS (Paton et al. 1999).

Another cluster in the phylogroup B1 is formed by three STEC with unknown serotype and virulence profile: *stx2a*$^{+}$, *ehxA*$^{+}$, *eae*$^{+}$ and *stx1a*$^{+}$. None of the STEC analyzed in Haugum and colleagues' study presented similar profile. In general, the human isolates harboring both *stx1* and *stx2* had different subtype combinations. Moreover, according to Brandal *et al*. 2014b, the presence of *stx1* was negatively associated with HUS in their study. However, the presence of the same virulence factors lacking *stx2a* has been observed in many human isolates from Norwegian patients. Some of them belong to known pathogenic serogroups, while five isolates had unknown serotype. Among them, just one FHI64 was associated with bloody diarrhea, the outcomes of the rest of the cases was unknown. One STEC O111 (*stx2a*$^{+}$, *eae*$^{+}$ and *stx1a*$^{+}$) was associated with HUS. The rest of s*tx1a*$^{+}$ STEC isolated from humans were *eae*$^{+}$ but *stx2a*$^{-}$, and belonged to known O-groups: O26, O103, O11 and O145 and were

associated with gastroenteritis and HC. STEC from other O-groups harboring *stx1* and *stx2* have been isolated from human patients, O77:H1 and O118:H16 (Beutin et al. 2004).

The last cluster in phylogroup B1included isolates of unknown serogroup that harbored *stx2a* and *stx2b*. As named before, no STEC isolated from the Norwegian patients were characterized with more than one *stx2* subtype (Haugum et al. 2014b). *stx2b* is often associated with less virulence, not only because the toxin is less potent, but because the strains carrying this subtype normally do not have *eae* (Haugum et al. 2014b, Friedrich et al, 2002, Persson et al. 2007). However, in Denmark, some unusual cases of HUS caused by strains harboring *stx2b* in absence of *eae* have been reported (Scheutz, 2014).

Referring to the phylogroups in general, groups A and B1 are the most closely related among the six phylogroups in which *E. coli* strains are clustered (Carlos et al. 2010). Groups A and B1 are often described as commensal *E. coli,* but some intestinal pathogen such as STEC fall also into these groups. *E. coli* belonging to the B1-group is more often present in cattle, but also found in sheep and goats, while group A is most commonly isolated from humans and less common in cattle (Herzer et al. 1990, Carlos et al. 2010). According to Lecointre *et al*. (1998), phylogroup B2 strains are highly pathogenic harboring several virulence factors and often associated with extra-intestinal infection in healthy humans. Group D is associated with lower virulence than B2, and is present in humans and primates. Still, it is important to point out that the phylotyping method used in this study do not included phylogroups E and F, and that some known STEC pathogens belong to these groups, such as *E. coli* O157:H7 which often falls in the E phylogroup.

Summarizing these remarks, human STEC isolates associated with HUS were *eae*$^{+}$ and belong to the B1 phylogroup. Furthermore, all of them harbor *stx2a* (Haugum et al., 2014b), except for the serotype O103:H25 from the 2006 outbreak where the strain had presumable lost the *stx2* phage (Andersen et al. 2013). Many of the cattle isolates contain enough virulence genes to be considered as potential human pathogens. However, a more in-depth examination of the strains will be done by analyzing their whole genomes. The selected 25 STEC isolates will therefore be sent to whole-genome sequencing.

4.3 Methodological considerations

Real-time PCR was extensively used in this study, first to screen the fecal samples, and thereafter to detect positive pools and colonies in the isolation process.

The consecutive methods used for detection and isolation of *stx2a*$^+$ STEC have not been tested in other studies before. Detection and isolation was successfully achieved through these methods, and some of the advantages and disadvantages are described below.

Validation of the reaction efficiency

An assessment of three master mixes was made to optimize the PCR reaction for detection of *stx* genes. There are several master mixes available to detect genetic markers in samples from complex matrices. The principal challenge is to detect the target gene in the presence of high levels of inhibitors. An optimized quantitative real-time PCR is characterized by a R^2 >0.980 and an amplification efficiency of 90-100% (Bio-Rad, 2006:3-6).

Comparison of the standard curves obtained, indicated that the master mix Brilliant III Ultra-Fast was the most efficient in the quantitative detection of target genes in DNA samples from feces. The R^2 and Efficiency values reported were within the optimal values for detection of both target genes (Table 3.1).

PCR as screening method

The DNA samples used in the screening of *stx* were extracted with QIAamp® DNA stools Mini kit (Qiagen). Detection of relative low quantities of STEC in fecal samples depends on the quality and quantity of the DNA extracts. This DNA extraction method combines enzymatic extraction and removal of PCR inhibitors, increasing the accuracy and sensitivity of the PCR –reactions.

Additionally, 10X DNA dilutions were included in the *stx* screenings to dilute potential inhibitors present in the DNA samples. Including the dilutions had the disadvantage of doubling the number of samples to examine.

Ct-values obtained in the screening of *stx* genes ranged between 24.5 and 44.6, and generally, the undiluted sample showed lower Ct-value, moreover, some diluted DNA templates showed

no Ct-value. However, during the screening of *stx2a*, the Ct-values were higher, between 37.1 and 44.5, and signals from some samples (5 of 30 positives) were only detected in the diluted samples. Nevertheless, after the isolation attempts, it was clear that Ct-value and the quantity of *stx* harboring bacteria in the sample were correlated. That is, except from one sample, 2014-22-164, with Ct>44 for the diluted DNA, and no-Ct for undiluted DNA, the rest of isolates were recovered from samples with the lowest Ct-values.

For characterization of the selected isolates by real-time PCR, DNA extracted with QIAamp DNA Mini Kit was used. When detecting the *eae* gene, 24 of 25 isolates gave positive signals (Ct from 15,75 to 36,67). A more detailed revision of the amplification curves leaded us to dismiss all isolates with Ct-values>30. This decision was based on the fact that real-time PCR experiments using DNA template from pure cultures should show lower Ct-values because of the absence of potential inhibition factors. In the other hand, unspecific bind of the primers to the template can be a source of late fluorescence signals. Based on the same argument, two isolates were dismissed when detecting *ehxA* and 21 isolates were considered negative when testing for O26 serogroup.

Isolation method

The isolation of STEC isolates is required to confirm that the previous positive PCR signals were generated from genes present in living bacterial-cells (International Organization for Standardization, 2012). In this study, the isolation method used permitted the recovery of *stx2a*$^{+}$ isolates from 50% of the PCR -positive fecal samples. This rate is acceptable because of the difficulty to isolate STEC from complex matrices. Particularly non-O157 STEC are difficult to isolates because no common biochemical markers exists in order to differentiate them from other *E.coli* (EFSA, 2013).

Different variants of *E. coli* may harbor Shiga toxin genes, therefore different agar media were included to broaden the selection of potential STEC to diverse phenotypes.

Recovery of *stx2a*$^{+}$-STEC was more often achieved from CHROMagar^{TM}, in concordance with other experiments isolating STEC from complex matrices (Mekata et al., 2014; Verhaegen et al., 2016). Isolates obtained from CHROMagar^{TM} media represented 44 of 86 (51%) of the total isolates, 27 of 86 isolates (31.4%) were obtained from SMAC agar and 16 (18.6%) were obtained from MacConkey agar. The advantage of using three agar media is

that *E. coli* colonies grow showing different colors, making it easier to select different colonies.

Low STEC recovery rates from samples positive for *stx* genes have occurred in previous studies (Hoang Minh et al., 2015). This might be explained by the presence of free phages in the positive samples (EFSA, 2013) and the small numbers of the bacteria of interest compared with the background micro-flora in the samples (Hoang Minh et al., 2015). Congruently, in the present study, isolation was more often achieved from samples that gave lower Ct -values in the initial real-time PCR for the detection of *stx2a*. Moreover, the number of isolates obtained from PCR -positive samples with lowest Ct-values was considerable higher, suggesting a higher initial quantity of STEC present in the fecal sample. These results indicated high sensitivity of real-time PCR for detection of STEC in DNA extracted from fecal samples.

DNA extraction by boiling was used in the isolation process. Pools of five colonies were boiled and examined by PCR. The Ct-values obtained range from 23 to 30. Similar Ct-values were obtained in the confirmation of single colonies. The DNA extraction method was simple and effective.

Phenotype of *stx2a*$^{+}$ isolates

The *stx2a*$^{+}$ isolates presented different morphologies in agar media. Positive colonies were purple, blue, and turquoise or pink in CHROMagar^{TM}, both big and small, pale pink, intense pink, grey, pink/grey or red with transparent edges on SMAC agar, and pink in MacConkey agar (see Figure 6.11 in Appendix 6.6).

The phenotypical variety showed by the positive isolates indicates that *stx2a*$^{+}$ STEC cannot be differentiated from other *E. coli*. Furthermore, when isolating *stx2a*$^{+}$ STEC, all possible morphologies should be considered as suspicious and consequently be included in the investigation.

Confirmation (Other bacteria carrying *stx2a*)

MALDI-TOF was used to identify *E. coli*. Confirmation is particularly important because *stx2* subtypes can be present in other *Enterobacteriaceae*, such as *Acinobacter haemoliticus*

(*stx2a*), *Enterobacter cloaceae* (*stx2a*), *Citrobacter freundii* (*stx2d*) and *Escherichia albertii* (*stx2d*) (Scheutz et al., 2012b). Identification of cattle-isolates as *C*. *amalonaticus* and *C*. *farmeri* might suggest contamination of the agar plates. These particular isolates were not further tested for *stx2a,* therefore is not possible to conclude that they gave the positive PCR signals.

Particularly the presence of *Proteus* species in the agar plates was problematic in the isolation process. The use of BA containing chloral was intended to resolve the problem, although this was just partially achieved.

4.4. Conclusion and prospective studies

In the present study, PCR screening of the fecal samples revealed a surprisingly high prevalence of *stx2a* in Norwegian cattle. This is the first study of prevalence of STEC harboring *stx2a* as virulence marker in Norway, and to the best of our knowledge, in Europe. Thus, no comparable data is available yet.

Shiga toxin genes were detected in almost all the herds included in the study. These results suggest that a change of approach may be beneficial when examining prevalence of pathogenic STEC in its animal reservoir. Screening and isolation based on *stx* subtypes more often associated with human disease might broaden the detection to all serotypes and, at the same time, reduce the number of samples to more manageable numbers.

stx2a$^+$ STEC were isolated from Norwegian cattle and characterized. The methods used for detection and isolation of s*tx2a* performed rather well with a 50% rate of recovery from PCR positive samples. However, the isolation method was work and time consuming.

The most distributed virulence gene, in addition to the selected virulence marker *stx2a*, was *ehxA*. The intimin encoding gene *eae* was also present in some isolates. Comparison of virulence profiles indicate both similarities and differences between the strains isolated from human patients and from cattle. Nevertheless, prediction of the pathogenicity of the cattle isolates becomes difficult in the absence of suitable models. It is possible that many of these isolates are not pathogenic to humans, but based on their virulence profiles, it cannot be discarded. Thus, more information about the STEC isolated from cattle is needed in order to make wider comparative analysis. Consequently, the selected 25 *stx2a*$^+$ STEC will be sent to whole genome sequencing. As mentioned, analysis of their whole-genomes can provide valuable information not obtained in this study.

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6. Appendices

6.1. Results from real-time PCR for detection of virulence genes: *stx1, stx2* and *stx2a*

A complete overview of the real-time PCR results for detection of virulence genes *stx1, stx2* and *stx2a* is shown in Table 6.1.

Table 6.1. Virulence genes: *stx1, stx2* and *stx2a* detected by real-time PCR. The results obtained for *stx1* and *stx2* are shown as "+" when the DNA sample gave a signal of gene amplification, and as "-" when no signal was detected. The result was considered positive when either of the two dilutions tested gave a signal. For *stx2a* positive samples, the Ct-values obtained for the two tested DNA dilutions are included.

	STEC genotype					STEC genotype		
Sample name	*Stx1*	*Stx2*	*Stx2a* (Ct-value*)		Sample name	*Stx1*	*Stx2*	*Stx2a*(Ct-value)
2014-22-90	+	+	-		2014-22-180	-	-	-
2014-22-91	+	+	43,18/44,54		2014-22-181	+	+	-
2014-22-92	+	+	41,41/42,23		2014-22-182	+	+	-
2014-22-93	+	+	-		2014-22-183	+	+	43,63/No Ct
2014-22-94	+	+	-		2014-22-184	+	+	42,58/42,13
2014-22-95	+	+	-		2014-22-185	+	+	-
2014-22-96	+	+	-		2014-22-186	+	+	-
2014-22-97	-	+	-		2014-22-187	+	+	-
2014-22-98	+	+	-		2014-22-188	+	+	44,94/No Ct
2014-22-99	+	+	No Ct/43,94		2014-22-189	+	+	-
2014-22-100	+	+	No Ct/42,34		2014-22-218	+	+	-
2014-22-101	+	+	-		2014-22-219	+	+	39,34/37,49
2014-22-102	+	+	-		2014-22-220	+	+	-
2014-22-103	+	+	-		2014-22-221	+	+	No Ct/43,16
2014-22-104	+	+	-		2014-22-222	+	+	-
2014-22-105	+	+	-		2014-22-223	+	+	-
2014-22-106	+	+	-		2014-22-224	+	+	-
2014-22-107	+	+	-		2014-22-225	+	+	-
2014-22-108	+	+	-		2014-22-226	+	+	-
2014-22-109	+	+	-		2014-22-227	+	+	-
2014-22-110	-	+	-		2014-22-228	+	+	-
2014-22-111	+	+	-		2014-22-229	+	+	-
2014-22-112	+	-	-		2014-22-230	+	+	-
2014-22-113	+	+	-		2014-22-231	+	+	-
2014-22-114	+	+	-		2014-22-232	-	+	40,84/42,32
2014-22-115	+	+	-		2014-22-233	+	+	-
2014-22-116	+	+	-		2014-22-234	-	+	-
2014-22-117	+	+	-		2014-22-235	+	+	-
2014-22-118	-	+	-		2014-22-236	+	+	-
2014-22-119	+	+	-		2014-22-237	+	+	-
2014-22-120	-	+	-		2014-22-238	-	-	-
2014-22-121	-	+	-		2014-22-239	-	+	-
2014-22-122	+	+	42,44/42,69		2014-22-240	-	+	-
2014-22-123	+	+	-		2014-22-241	+	+	42,46/43,28
2014-22-124	-	-	-		2014-22-242	+	+	-
2014-22-125	+	-	-		2014-22-243	+	+	-
2014-22-126	+	+	-		2014-22-244	-	+	-
2014-22-127	+	+	44,32/No Ct		2014-22-245	-	+	-
2014-22-128	+	+	42/40.9		2014-22-246	+	+	-
2014-22-129	+	+	-		2014-22-247	+	+	-
2014-22-131	+	+	-		2014-22-248	+	+	-
2014-22-132	+	+	-		2014-22-249	+	+	-
2014-22-133	+	+	-		2014-22-250	+	+	-
2014-22-134	+	+	-		2014-22-251	+	+	-
2014-22-135	+	+	-		2014-22-252	+	+	-
2014-22-136	-	-	-		2014-22-253	+	+	41,2/No Ct

2014-22-137	+	+	37,13/37,32		2014-22-254	+	+	41,53/No Ct
2014-22-138	+	+	44,13/No Ct		2014-22-255	-	+	37,44/39,56
2014-22-139	-	+	-		2014-22-256	+	+	42,53/44,38
2014-22-140	+	+	-		2014-22-257	-	+	-
2014-22-141	+	-	-		2014-22-258	+	+	-
2014-22-142	+	+	-		2014-22-259	+	+	-
2014-22-143	+	+	41,84/43,08		2014-22-260	-	+	-
2014-22-144	-	+	-		2014-22-261	+	+	-
2014-22-145	+	+	-		2014-22-262	+	+	-
2014-22-146	+	+	-		2014-22-263	+	+	-
2014-22-147	-	+	-		2014-22-264	-	+	-
2014-22-148	+	+	-		2014-22-265	-	-	-
2014-22-149	+	+	-		2014-22-266	-	+	-
2014-22-150	+	+	-		2014-22-267	+	+	-
2014-22-151	-	+	-		2014-22-268	+	+	-
2014-22-152	+	+	43,7/No Ct		2014-22-269	+	+	-
2014-22-153	+	+	-		2014-22-270	-	-	-
2014-22-154	+	+	-		2014-22-271	-	+	-
2014-22-155	+	+	-		2014-22-272	-	+	-
2014-22-156	-	+	-		2014-22-273	+	-	-
2014-22-157	-	-	-		2014-22-274	+	+	-
2014-22-158	+	+	34,54/38,17		2014-22-275	-	+	-
2014-22-159	+	+	43,21/43,71		2014-22-276	-	+	-
2014-22-160	+	+	-		2014-22-277	-	+	-
2014-22-161	+	+	-		2014-22-278	+	+	-
2014-22-162	+	+	37,79/39,03		2014-22-279	+	+	-
2014-22-163	-	+	-		2014-22-280	+	+	-
2014-22-164	+	+	No Ct/44,47		2014-22-281	+	+	41,47/42,58
2014-22-165	+	+	-		2014-22-282	-	+	-
2014-22-166	+	+	-		2014-22-283	+	+	-
2014-22-167	-	+	-		2014-22-284	+	+	-
2014-22-168	+	+	-		2014-22-285	+	+	-
2014-22-169	+	+	-		2014-22-286	+	+	-
2014-22-170	+	+	-		2014-22-287	+	+	-
2014-22-171	+	+	-		2014-22-288	+	+	43,88/41,88
2014-22-172	+	+	-		2014-22-289	+	+	-
2014-22-173	+	+	-		2014-22-290	+	+	-
2014-22-174	+	+	-		2014-22-291	+	+	-
2014-22-175	-	+	-		2014-22-292	+	+	No Ct/41,96
2014-22-176	+	+	-		2014-22-293	+	+	42,64/39,53
2014-22-177	+	+	-		2014-22-294	+	+	-
2014-22-178	+	+	-		2014-22-295	+	+	-
2014-22-179	+	+	-		2014-22-296	+	+	-

6.2. Bioanalyzer results for *stx1* subtyping

Figure 6.1 shows an image of the microcapillary electrophoresis chip taken under running of PCR products in the Bioanalyzer.

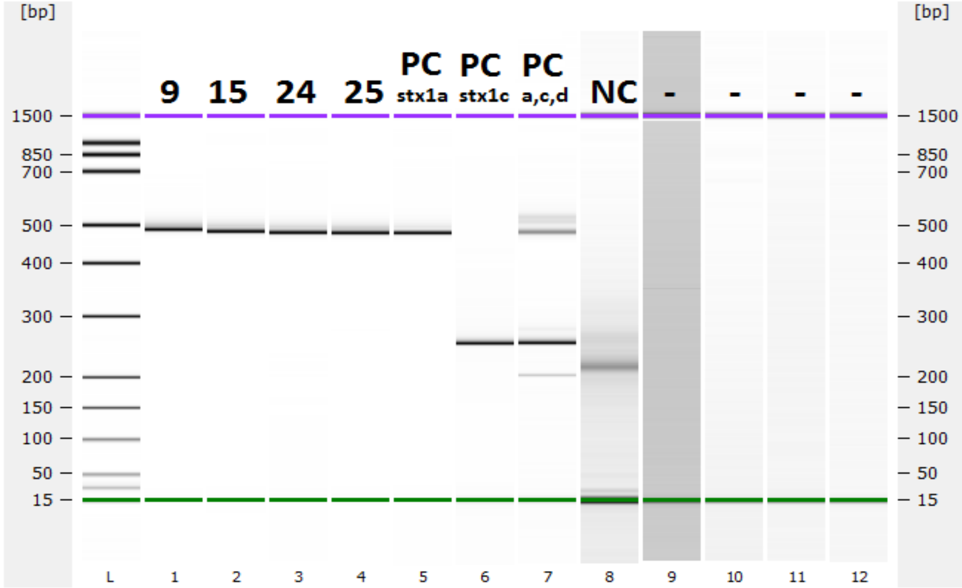


Figure 6.1. Image of the miniaturized microcapillary electrophoresis chip taken by Bioanalyzer chip reader. The image shows that the four *stx1*-positive *E. coli* isolates harbor the *stx1a* variant of the gene.

6.3. Agarose gel electrophoresis for *stx2* subtyping

The following figures show the pictures of agarose gel taken under *stx2* subtyping of the 25 *stx2a*$^{+}$ *E.coli* isolates.

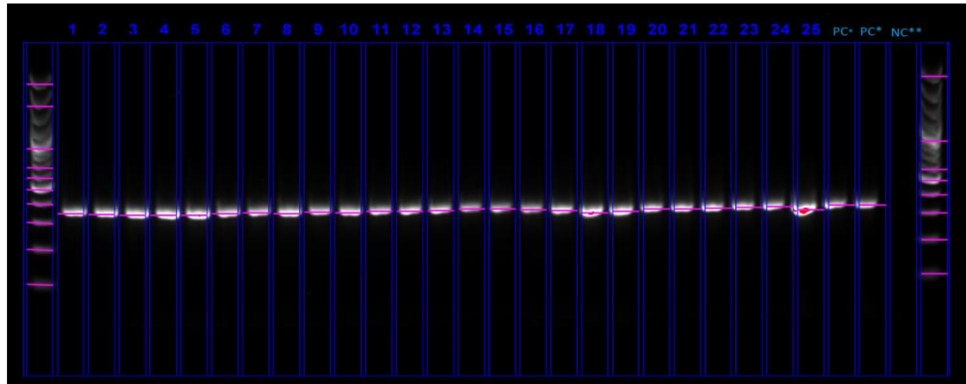


Figure 6.2. *stx2a*: All 25 *E. coli* isolates showed a band with a size of 347/349 bp. Two positive controls and one negative control were included.

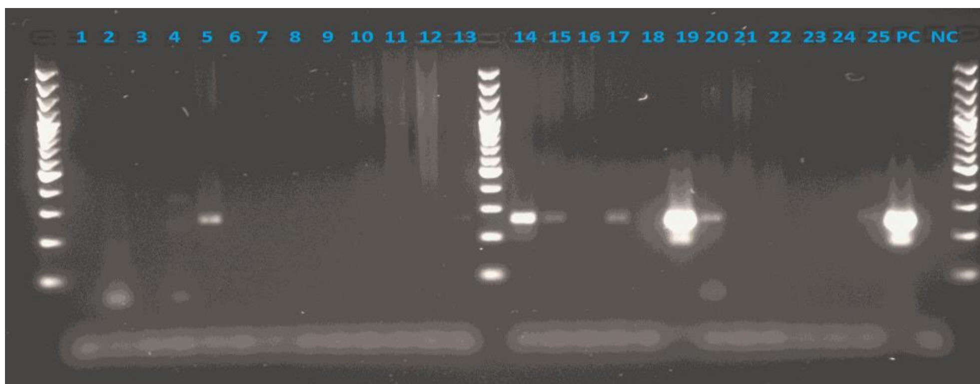


Figure 6.3. *stx2b*: Isolate #19 showed an intense band with the amplicon size of around 251 bp, likewise the Positive. Isolate #14 was also considered positive, whereas isolates # 5, 15 and 17 were considered negatives because of the weakness of their bands.

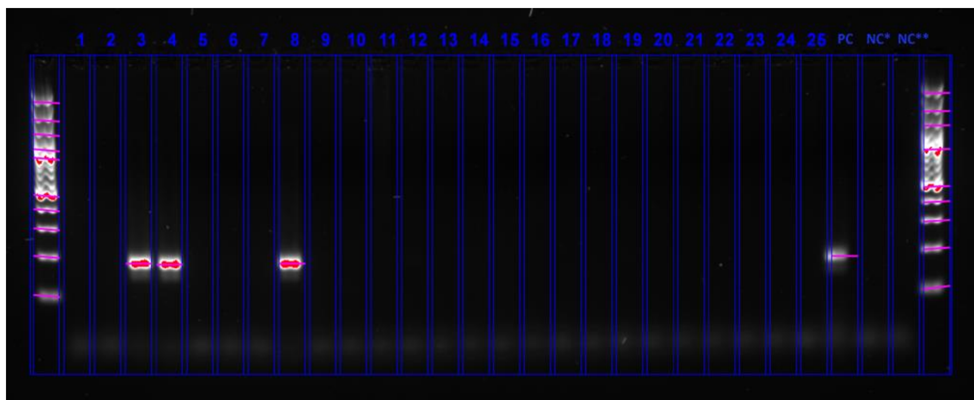


Figure 6.4. *stx2c*: Isolates 3, 4 and 8 showed bands with the respective amplification size of 177 bp corresponding to *stx2c* amplicon. Two Negative Controls were included.

Figure 6.5. ***stx2d***: Non isolates showed bands, except for the Positive Control that illustrated the band with the amplification size of 179/235/280 bp corresponding to stx2c amplicon. Two negative controls were included.

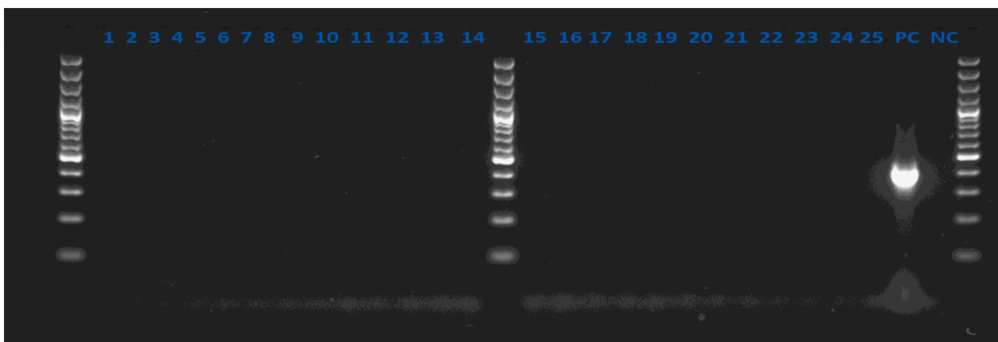


Figure 6.6. ***stx2e*****:** Non isolates showed bands, except for the Positive Control that illustrated the band with the amplification size of 592bp corresponding to *stx2e* amplicon. Two negative controls were included.

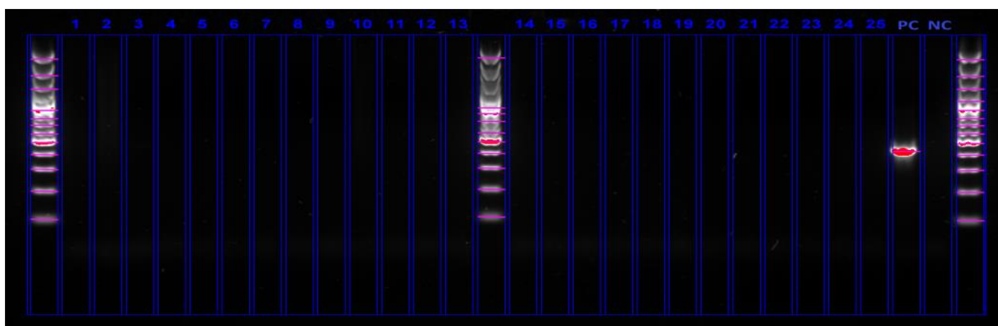


Figure 6.7. ***stx2f***: Non isolates showed bands, except for the Positive Control that illustrated the band with the amplification size of 465 bp corresponding to *stx2f* amplicon.

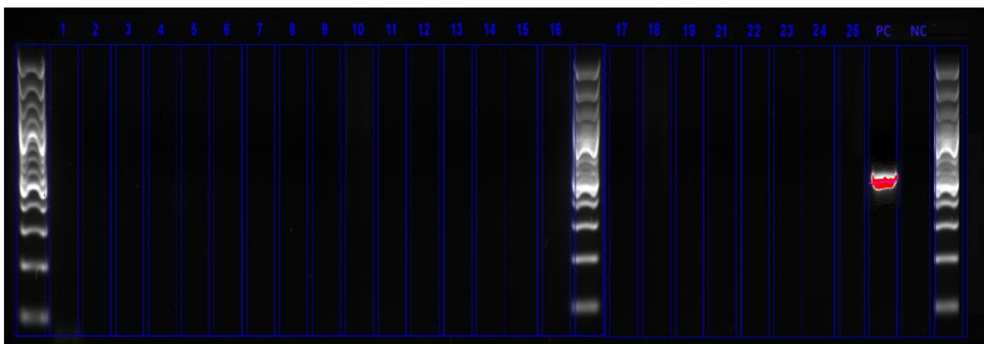


Figure 6.8. ***stx2g*****:** Non isolates showed bands, except for the Positive Control that illustrated the band with the amplification size of 573 bp corresponding to *stx2f* amplicon.

6.4. Gel image taken for phylogenetic analysis

Figure 6.9. The image shows the 25 *stx2a*$^{+}$ *E. coli* isolates in agarose gel. The figure was used to group isolates into Phylogenetic groups.

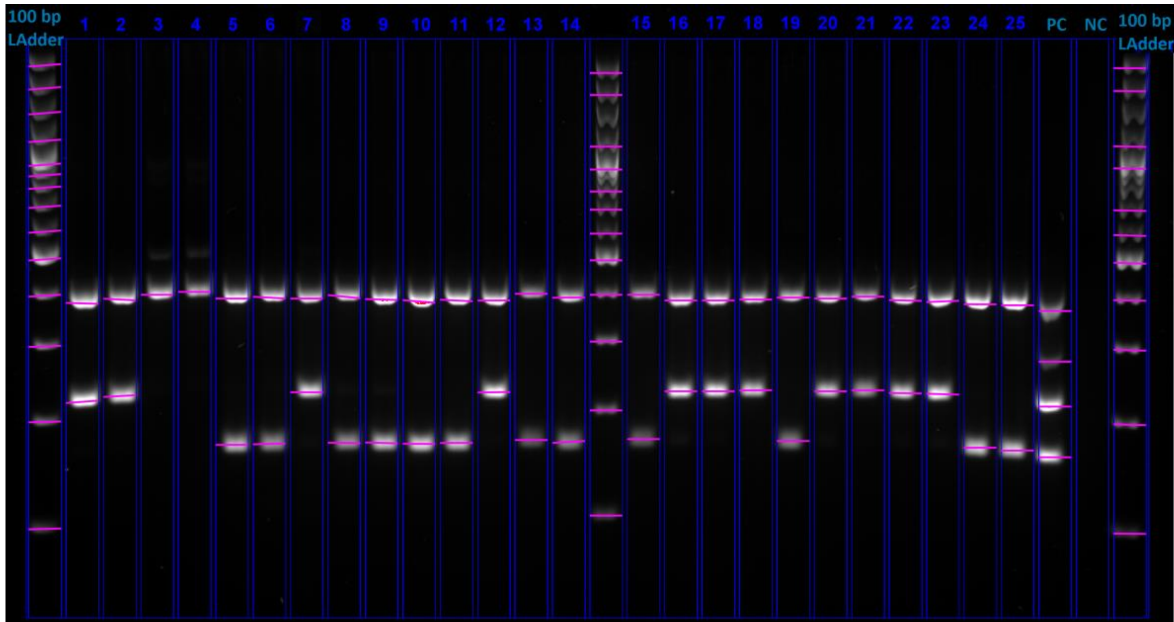


Figure 6.9. Agarose gel picture of the 25 *stx2a* positive *E. coli* isolated. The bands produced were used to group the isolated into phylogenetic groups.

6.5. Supplementary information on 95 Norwegian non-O157 STEC isolated from human patient.

The figure used for comparison between STEC isolated from humans and STEC isolated from Norwegian cattle.

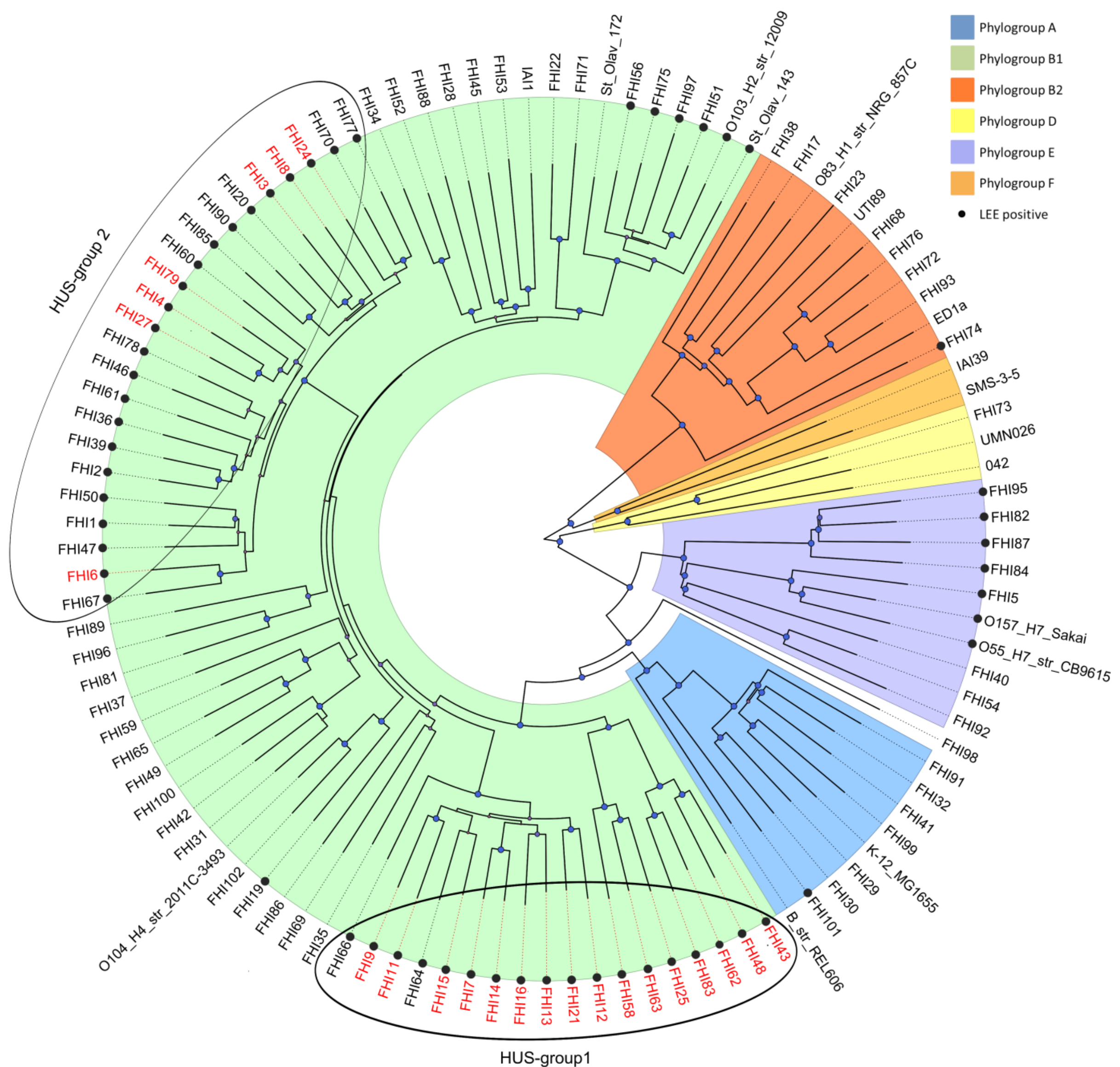


Figure 6.10. Core gene phylogeny of 95 non-O157 STEC isolated from human patients in Norway and 14 *E. coli* reference genomes. The *E. coli* phylogroups are marked with the colors blue (A), green (B1), orange (B2), yellow (D), ochre (F) and indigo (E). LEE positive STEC were marked with •, while all HUS and HUS-associated STEC included in the study were indicated with red letters (Haugum et al. 20014).

Table 6.2. Supplementary information on 95 Norwegian non-O157 STEC genomes isolated from Norwegian patients (Haugum et al. 2014) and used in this study for comparison with cattle-isolates.

Group	Strain ID	O-type[1]	Lab_stx1[2]	Lab_stx2	Lab_eae	Pred_stx1[3]	Pred_stx2	Pred_eae	stx1 subtype	stx2 subtype	MLVA Allele	Source	Clinic	HUS-associated	Outbreak	Sex	Age	Accession No.	Sequencing method	Contigs
LEE positive STEC n=54; LEE positive and stx2 positive STEC n=33; HUS and HUS-associated STEC n=23	FHI3	26	0	1	1	0	1	1		stx2a	6-1-0-8-3-5-1-6-11-15	Human faeces	Unknown	1	1	F	31	ERS480135	Illumina PE[9]	130
	FHI4	26	0	1	1	0	1	1		stx2a	6-1-0-8-3-5-1-6-11-15	Human faeces	HUS	1	1	F	4	ERS480136	Illumina PE, MP[10]	35
	FHI24	26	0	1	1	0	1	1		stx2a	6-1-0-8-3-4-1	Human faeces	HUS	1	0	F	2	ERS480154	Illumina PE, MP	42
	FHI27	26	1	1	1	0	1	1		stx2a	6-1-0-8-3-4-1	Human faeces	HUS	1	0	M	1	ERS480156	Illumina PE, MP	27
	FHI79	26	0	1	1	0	1	1		stx2a	6-0-0-8-3-4-1-6-13-12	Human faeces	HUS	1	0	M	0	ERS480204	Illumina PE, MP	28
	FHI8	86	0	1	1	0	1	1		stx2a	6-3-0-8-3-7-1	Human	HUS	1	0	F	1	ERS480140	Illumina PE, MP	33
	FHI7	103	0	1	1	0	1	1		stx2a	7-3-0-5-0-8-1-16-9-11	Human faeces	HUS	1	0	F	1	ERS480139	Illumina PE, MP	30
	FHI9	103	0	1	1	0	1	1		stx2a	7-3-0-5-0-3-1-16-9-11	Human faeces	HUS	1	0	F	0	ERS480141	Illumina PE, MP	34
	FHI10	103	0	0	1	0	0	1			7-3-0-5-0-7-1-16-9-12	Human faeces	HUS	1	2	F	4	-	Illumina PE	ND
	FHI11	103	0	0	1	0	0	1			7-3-0-5-0-7-1-16-9-12	Human faeces	HUS	1	2	F	2	ERS480142	Illumina PE, MP	25
	FHI12	103	0	1	1	0	1	1		stx2a	7-3-0-5-0-7-1-16-9-12	Human faeces	HUS	1	2	F	4	ERS480143	Illumina PE, MP	31
	FHI13	103	0	0	1	0	0	1			7-3-0-5-0-7-1	Human faeces	HUS	1	2	F	1	ERS480144	Illumina PE	120
	FHI14	103	0	1	1	0	0	1			7-3-0-5-0-7-1	Human faeces	HUS	1	2	F	2	ERS480145	Illumina PE, MP	29
	FHI15	103	0	0	1	0	0	1			7-3-0-5-0-7-1	Human faeces	HUS	1	2	F	1	ERS480146	Illumina PE, MP	42
	FHI16	103	0	0	1	0	0	1			7-3-0-5-0-7-1-16-9-11	Fermented sausage	NA	1	2	NA	NA	ERS480147	Illumina PE	129
	FHI21	103	0	0	1	0	0	1			7-3-0-5-0-5-1	Human faeces	HUS	1	2	M	1	ERS480151	Illumina PE, MP	32
	FHI6	111	1	1	1	1	1	1	stx1a	stx2a	6-3-0-5-3-10-1	Human faeces	HUS	1	0	F	1	ERS480138	Illumina PE, MP	33
	FHI43	121	0	1	1	0	1	1		stx2a	6-3-0-5-3-6-1-6-11-13	Human faeces	Gastroenteritis	1	3	M	1	ERS480171	Illumina PE, MP	17
	FHI48	121	0	1	1	0	1	1		stx2a	6-3-0-5-3-6-1-6-11-16	Human faeces	HUS	1	3	F	1	ERS480175	Illumina PE, MP	34
	FHI62	121	0	1	1	0	1	1		stx2a	6-3-0-5-3-5-1-6-11-15	Human faeces	Gastroenteritis	1	3	F	2	ERS480187	Illumina PE, MP	23
	FHI83	121	0	1	1	0	1	1		stx2a	8-0-0-6-3-6-1-6-11-0	Human faeces	HUS	1	0	F	1	ERS480207	Illumina PE, MP	27
	FHI25	145	0	1	1	0	1	1		stx2a	5-3-0-8-4-1-1-16-8-12	Human faeces	HUS	1	0	M	2	ERS480155	Illumina PE, MP	27
	FHI58	145	0	1	1	0	1	1		stx2a	5-3-0-8-4-1-1-16-9	Human faeces	HUS	1	4	M	1	ERS480183	Illumina PE, MP	28
	FHI63	145	0	1	1	0	1	1		stx2a	5-3-0-8-4-1-1-16-9	Human faeces	HUS	1	4	M	1	ERS480188	Illumina PE, MP	43
	St. Olav104	145	0	1	1	0	1	1		stx2a	5-3-0-8-4-1-1-16-9	Human faeces	Asymptomatic	1	4	F	1	ERS480228	Pacific Biosciences	78
LEE positive STEC n=54; LEE positive and stx2 positive STEC n=33	FHI5	0	1	0	0	1	1	1	stx1a	stx2c	6-10-3-5-4-7-2-6-9	Human faeces	Gastroenteritis	0	0	M	4	ERS480137	Illumina PE	89
	FHI66	0	1	1	1	1	1	1	stx1c	stx2a	7-3-0-5-4-7-1-16-11	Human faeces	Asymptomatic	0	0	M	5	ERS480191	Illumina PE, MP	32
	FHI85	0	1	1	1	1	1	1	stx1a	stx2c	5-3-0-8-3-4-1-6-20-0	Human faeces	ND	0	0	F	69	ERS480209	Illumina PE, MP	33
	FHI101	0	0	1	1	0	1	1		stx2d	5-1-0-8-3-9-1-64-0-13	Human faeces	Gastroenteritis	0	0	F	1	ERS480224	Illumina PE	152
	FHI36	26	0	1	1	0	1	1		stx2a	6-1-0-8-3-5-1-6-16	Human faeces	Bloody diarrhoea	0	0	F	1	ERS480164	Illumina PE	137
	FHI39	26	0	1	1	0	1	1		stx2a	6-1-0-8-3-5-1-6-15	Sheep faeces	NA	0	0	NA	NA	ERS480167	Illumina PE	133
	FHI2	26	0	1	1	0	0	1			6-0-0-8-3-4-1-6-15-10	Human faeces	ND	0	0	F	36	ERS480134	Illumina PE	157
	FHI19	104	0	1	0	0	1	1		stx2a	6-3-10-8-3-6-1-6-6-0	Human faeces	Gastroenteritis	0	0	M	74	ERS480149	Illumina PE	283
	FHI82	145	0	1	1	0	1	1		stx2a	7-0-0-8-3-2-1-35-0-0	Human faeces	Bloody diarrhoea	0	0	M	21	ERS480206	Illumina PE, MP	29
	FHI95	145	0	1	1	0	1	1		stx2a	7-3-0-8-3-2-1-35-0-0	Human faeces	ND	0	0	F	2	ERS480218	Illumina PE	100
LEE positive STEC n=54	FHI51	0	1	0	1	1	0	1	stx1a		6-3-0-8-3-7-1-6-7	Human faeces	ND	0	0	F	3	ERS480178	Illumina PE	189
	FHI64	0	1	0	1	1	0	1	stx1a		5-3-0-5-4-5-1-6-10	Human faeces	Bloody diarrhoea	0	0	F	2	ERS480189	Illumina PE	111
	FHI74	0	1	0	1	1	0	1	stx1a		6-3-0-8-3-5-1-6-0-13	Human faeces	ND	0	0	M	3	ERS480199	Illumina PE, MP	23
	FHI90	0	1	0	1	1	0	1	stx1a		6-0-0-8-3-6-1-6-10-11	Human faeces	ND	0	0	F	1	ERS480214	Illumina PE, MP	31
	FHI1	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-5-1-6-23	Human faeces	ND	0	0	M	2	ERS480133	Illumina PE	151
	FHI20	26	1	0	1	1	0	1	stx1a		6-1-0-8-3-5-1-6-23	Human faeces	Gastroenteritis	0	0	F	1	ERS480150	Illumina PE, MP	26
	FHI46	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-6-1-6-40-13	Human faeces	Bloody diarrhoea	0	0	F	22	ERS480173	Illumina PE	162
	FHI47	26	1	0	1	1	0	1	stx1a		6-1-0-5-3-9-1-6-21	Human faeces	Bloody diarrhoea	0	0	F	24	ERS480174	Illumina PE	211
	FHI50	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-6-1-6-40	Human faeces	Bloody diarrhoea	0	0	F	42	ERS480177	Illumina PE	194
	FHI60	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-6-1-6-22	Human faeces	Bloody diarrhoea	0	0	M	32	ERS480185	Illumina PE	252
	FHI61	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-4-1-6-20	Human faeces	Gastroenteritis	0	0	F	1	ERS480186	Illumina PE	447
	FHI70	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-15-1-0-39-16	Human faeces	Annet	0	0	M	0	ERS480195	Illumina PE, MP	40
	FHI77	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-6-1-6-0-17	Human faeces	Bloody diarrhoea	0	0	M	9	ERS480202	Illumina PE	246
	FHI78	26	1	0	1	1	0	1	stx1a		6-0-0-8-3-7-1-6-21-15	Human faeces	Bloody diarrhoea	0	0	M	0	ERS480203	Illumina PE	243
	FHI56	103	1	0	1	1	0	1	stx1a		6-3-0-8-3-5-7-6-7	Human faeces	Gastroenteritis	0	0	M	0	ERS480182	Illumina PE	197
	FHI75	103	1	0	1	1	0	1	stx1a		6-3-0-8-3-5-7-6-7-0	Human faeces	ND	0	0	M	6	ERS480200	Illumina PE, MP	26
	FHI97	103	1	0	1	1	0	1	stx1a		6-3-0-8-3-6-1-6-10-15	Human faeces	Bloody diarrhoea	0	0	F	1	ERS480220	Illumina PE, MP	46
	St. Olav143	103	1	0	1	1	0	1	stx1a		6-3-0-8-3-4-7-6-7-0	Human faeces	Asymptomatic	0	0	F	1	ERS480226	Illumina PE	102
	FHI67	111	1	0	1	1	0	1	stx1a		6-3-0-5-3-5-1-95-19	Human faeces	Gastroenteritis	0	0	M	0	ERS480192	Illumina PE	129
	FHI84	145	1	0	1	1	0	1	stx1a		1-0-0-8-3-7-1-35-0-0	Human faeces	Bloody diarrhoea	0	0	F	65	ERS480208	Illumina PE	150
	FHI87	145	1	0	1	1	0	1	stx1a		1-3-0-8-3-6-1-35-0-0	Human faeces	Asymptomatic	0	0	M	1	ERS480211	Illumina PE, MP	25

LEE negative STEC n=41			FHI31	0	0	1	0	0	1	0		stx2b	6-3-0-8-3-6-1-6-6	Human faeces	ND	0	0	F	56	ERS480160	Illumina PE	87
			FHI38	0	0	1	0	0	0	0			7-0-0-8-3-7-1-0-0	Sheep faeces	NA	0	0	NA	NA	ERS480166	Illumina PE	47
			FHI53	0	0	1	0	0	1	0		stx2a	6-3-0-8-3-2-1-6-14	Human faeces	ND	0	0	M	5	ERS480180	Illumina PE	54
			FHI89	0	0	1	0	0	1	0		stx2a	6-3-0-8-3-11-1-6-9-0	Human faeces	Gastroenteritis	0	0	M	10	ERS480213	Illumina PE, MP	22
			FHI99	0	0	1	0	0	1	0		stx2a	5-3-0-8-3-14-1-6-0-0	Human faeces	Gastroenteritis	0	0	F	90	ERS480222	Illumina PE, MP	32
			FHI100	0	0	1	0	0	1	0		stx2b	6-3-0-8-3-10-1-6-6-0	Human faeces	ND	0	0	M	58	ERS480223	Illumina PE, MP	32
			FHI32	0	1	1	0	1	1	0	stx1c	stx2b	5-0-4-8-4-4-1-6-0	Human faeces	ND	0	0	F	51	ERS480161	Illumina PE	75
			FHI37	0	1	1	0	1	1	0	stx1c	stx2b	12-3-0-8-3-8-1-6-8	Sheep faeces	NA	0	0	NA	NA	ERS480165	Illumina PE	103
			FHI92	0	1	1	0	1	1	0	stx1c	stx2b	6-14-0-8-3-3-1-16-6-0	Human faeces	Bloody diarrhoea	0	0	M	72	ERS480216	Illumina PE, MP	24
			FHI98	2	0	1	0	0	1	0		stx2b	5-0-0-8-4-2-1-16-0-0	Human faeces	Gastroenteritis	0	0	F	84	ERS480221	Illumina PE, MP	31
			FHI28	8	0	1	0	0	1	0		stx2c	6-3-0-8-3-8-1-6-11	Human faeces	Gastroenteritis	0	0	F	68	ERS480157	Illumina PE, MP	21
			FHI86	8	0	1	0	0	1	0		stx2e	6-3-0-8-3-4-1-6-0-0	Human faeces	Gastroenteritis	0	0	F	20	ERS480210	Illumina PE	46
			FHI42	84	0	1	0	0	1	0		stx2b	6-3-0-8-3-7-1-6-6	Human faeces	ND	0	0	F	4	ERS480170	Illumina PE, MP	33
			FHI59	91	0	1	0	0	1	0		stx2b	6-3-0-8-3-4-1-6-7	Human faeces	ND	0	ND	ND	ND	ERS480184	Illumina PE, MP	22
			FHI81	91	1	1	0	1	1	0	stx1a	stx2b	7-3-0-8-1-6-1-6-7-0	Human faeces	Gastroenteritis	0	0	F	1	ERS480205	Illumina PE	143
			FHI102[5]	104	0	1	0	0	1	0		stx2a	6-3-0-8-3-10-1-6-6-0	Human faeces	Bloody diarrhoea	0	0	M	40	ERS480225	Illumina PE, MP	31
			FHI88	111	0	1	0	0	1	0		stx2a	6-3-0-10-3-6-1-6-6-0	Human faeces	Bloody diarrhoea	0	0	F	1	ERS480212	Illumina PE	129
			FHI35	113	0	1	0	0	1	0		stx2d	6-3-0-8-3-8-1-6-6	Minced meat	NA	0	0	NA	NA	ERS480163	Illumina PE	49
			FHI41	113	0	1	0	0	1	0		stx2d	5-0-8-8-3-6-1-6-0	Human faeces	ND	0	0	F	71	ERS480169	Illumina PE	98
			FHI30	113	1	1	0	1	1	0	stx1c	stx2b	5-0-5-8-3-6-1-6-0	Human faeces	ND	0	0	F	37	ERS480159	Illumina PE, MP	28
			FHI71	128	0	1	0	0	1	0		stx2b	2-3-0-1-3-6-1-6-13-0	Human faeces	ND	0	0	F	0	ERS480196	Illumina PE, MP	27
			FHI49	146	1	1	0	1	1	0	stx1c	stx2b	6-3-0-8-3-5-7-0-7	Human faeces	Asymptomatic	0	0	F	4	ERS480176	Illumina PE	102
			FHI22	146	1	0	0	1	1	0	stx1c	stx2b	6-3-0-8-3-2-1-0-7	Human faeces	Asymptomatic	0	0	F	31	ERS480152	Illumina PE	101
			FHI65	146	1	1	0	1	1	0	stx1a	stx2b	6-3-0-8-3-6-1-6-7-0	Human faeces	Bloody diarrhoea	0	0	F	55	ERS480190	Illumina PE, MP	26
			FHI40	0	1	0	0	1	0	0	stx1d		6-15-0-8-3-1-1-6-3	Human faeces	Gastroenteritis	0	0	M	2	ERS480168	Illumina PE, MP	32
			FHI45	0	1	0	0	1	0	0	stx1a		6-3-0-8-3-13-1-6-7	Human faeces	Gastroenteritis	0	0	M	2	ERS480172	Illumina PE	71
			FHI54	0	1	0	0	1	0	0	stx1c		6-13-0-8-3-3-1-16-6	Human faeces	Gastroenteritis	0	0	M	3	ERS480181	Illumina PE	70
			FHI69	0	1	0	0	1	0	0	stx1c		8-3-0-8-3-5-1-6-0-0	Human faeces	ND	0	0	M	0	ERS480194	Illumina PE	79
			FHI73	0	1	0	0	0	0	0			7-16-0-8-3-2-1-55-3-0	Human faeces	ND	0	0	F	1	ERS480198	Illumina PE	62
			FHI91	0	1	0	0	0	0	0			5-3-7-8-3-3-1-0-7-0	Human faeces	Gastroenteritis	0	0	F	45	ERS480215	Illumina PE	95
			FHI96	0	1	0	0	1	0	0	stx1a		7-3-0-8-3-10-1-6-9-0	Human faeces	Gastroenteritis	0	0	F	68	ERS480219	Illumina PE	123
			FHI17	26	1	0	1	0	0	0			6-0-0-8-3-7-1-0-6-0	Human faeces	ND	0	0	F	1	ERS480148	Illumina PE	65
			FHI23	76	1	0	0	1	0	0	stx1c		6-0-0-8-3-9-1-6-0	Human faeces	Gastroenteritis	0	0	F	2	ERS480153	Illumina PE, MP	33
			St. Olav172	103	1	0	0	1	0	0	stx1a		6-3-0-8-3-4-7-6-7-0	Human faeces	ND	0	0	F	0	ERS480227	Illumina PE	101
			FHI34	104	1	0	0	1	0	0	stx1c		6-3-0-8-3-5-1-6-7	Human faeces	Bloody diarrhoea	0	0	M	0	ERS480162	Illumina PE, MP	26
			FHI52	104	1	0	0	1	0	0	stx1c		6-3-0-8-3-4-1-6-7	Human faeces	Gastroenteritis	0	0	F	11	ERS480179	Illumina PE	171
			FHI68	117	1	0	0	1	0	0	stx1a		5-0-0-8-3-5-1-0-37-0	Human faeces	Gastroenteritis	0	0	M	44	ERS480193	Illumina PE	171
			FHI72	117	1	0	0	1	0	0	stx1a		5-0-0-8-3-6-1-0-36-0	Human faeces	Bloody diarrhoea	0	0	F	22	ERS480197	Illumina PE, MP	34
			FHI76	117	1	0	0	1	0	0	stx1a		5-0-0-8-3-5-1-6-36-15	Human faeces	Gastroenteritis	0	0	F	32	ERS480201	Illumina PE	178
			FHI93	117	1	0	0	1	0	0	stx1a		5-0-0-8-3-6-1-0-37-0	Human faeces	Gastroenteritis	0	0	F	45	ERS480217	Illumina PE	162
			FHI29	118	1	0	0	1	0	0	stx1a		5-1-0-8-4-4-1-6-7-0	Human faeces	Gastroenteritis	0	0	M	1	ERS480158	Illumina PE, MP	24

[1] Strains that did not belong to the tested serotype
[2] Laboratory results of *stx1*, *stx2* and *eae*.
[3] *stx1*, *stx2* and *eae* predicted by sequence analysis.

6.6. *E. coli* phenotypes included in the *stx2a*+ STEC isolation process

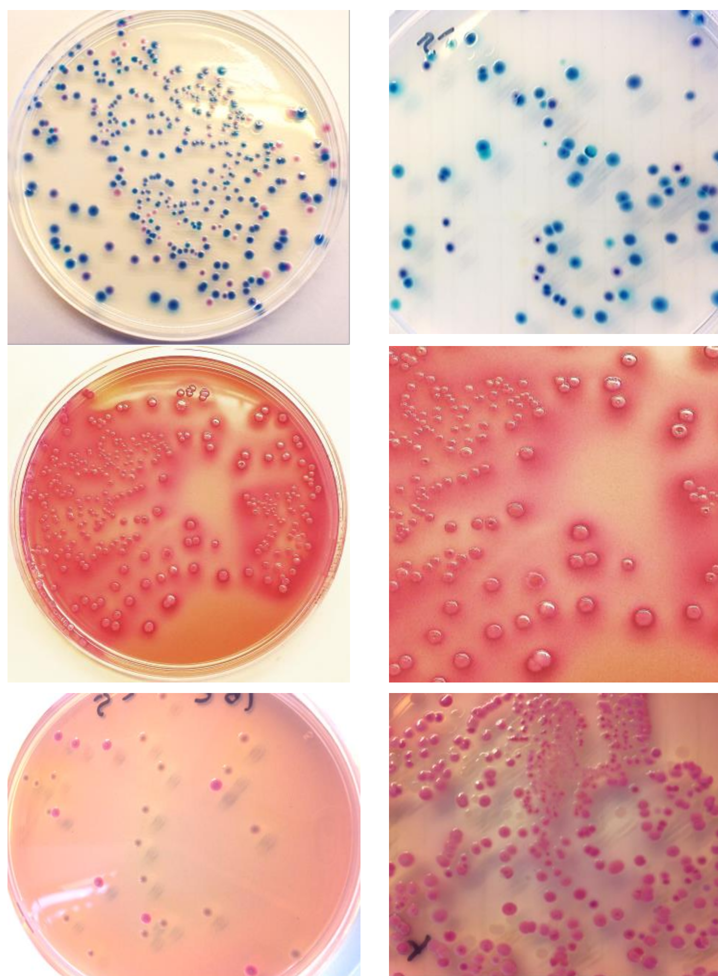
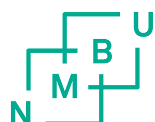


Figure 6.11. *E. coli* phenotypes on agar plates. Presumptive STEC were tested for *stx2a*+ in the isolation process. All the phenotypes on three different agar media were included. Upper images: CROMagar. Central images: MacConkey agar. Down: SMAC agar



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