Edinburgh Napier UNIVERSITY

Scottish wild deer as a potential source of human pathogenic non-O157 Shiga toxin producing *Escherichia coli* (STEC)

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Declaration

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification, and that it is the result of my own independent work.

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Abstract

Shiga-toxin producing *E. coli* (STEC) are zoonotic pathogens, which release phage-encoded Shiga toxins (Stx). Stx subtypes *stx2a* and *stx2d* are associated with severe human disease. STEC O157 is the most common serotype in human disease although other pathogenic serotypes exist. Cattle, sheep and deer can carry STEC. A recent study found STEC O157 prevalence of 0.34 % (95 % CI = 0.02 – 6.30) in Scottish wild deer; however 69.5 % of faecal samples were *stx* positive suggesting presence of pathogenic non-O157 STEC serotypes.

The aims of this project were: (i) to investigate prevalence and factors associated with carriage of *stx2a* genes in Scottish wild deer; (ii) to determine pathogenic potential of non-O157 STEC strains isolated from Scottish wild deer using whole genome sequencing (WGS).

PCR testing of faecal samples found 12 % of *stx* positive samples were subtype *stx2a*. In an 'all deer species' model, roe deer and sheep density had significant positive associations with *stx2a*. In a 'roe deer only' model, South of Scotland, % semi-natural grassland and rain-days in month had significant positive association with *stx2a*. WGS of 56 non-O157 STEC strains isolated from deer faeces identified five strains genetically similar to individual Scottish human clinical non-O157 STEC isolates. Of these, two deer isolates had identical *stx* and virulence gene profiles to the closest human isolates, and three strains differed only by one or two virulence genes, including a *stx2d* positive strain. The majority of isolates (47/56) had low pathogenic subtypes *stx2b* or *stx2b*:*stx1c*. Although *stx2a* was found in three isolates, none were genetically similar to human clinical strains.

In conclusion, this study found roe deer were more likely to carry pathogenic *stx2a* and presence of sheep and environmental factors may influence this. Non-O157 STEC strains from deer are likely to cause diarrhoea but not severe human disease.

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Chapter 1: Introduction

1.1 Shiga toxin producing *Escherichia coli*

Shiga toxin producing *Escherichia coli* (STEC) are zoonotic bacterial pathogens that are carried in the gastro-intestinal tract of ruminants. Historically, the term Verotoxigenic *Escherichia coli* (VTEC) has been used and it is interchangeable with the term STEC. STEC are defined by production of bacteriophage (phage) encoded Shiga toxins (Stx) which are the main mediators of their pathogenicity. Ruminants including cattle, sheep, goats and deer appear to be the main hosts. However, STEC has been isolated from a wide range of other wild and domestic animals including birds, cats, dogs, horses and pigs (Persad and LeJeune, 2014; Espinosa *et al.*, 2018). Humans become infected by consuming contaminated food or water, or by the faecal-oral route after contact with animals or infected humans. STEC are shed in the faeces of an infected animal which can result in contamination of meat, milk, food crops and water. Human STEC infections have been associated with diverse sources including fruit, vegetables, dairy products and meats (WHO-FAO, 2018). Analysis of food products in European countries in 2018 found 3.4 % of 1,992 samples of fresh beef contained STEC and 10.9 % of 695 sheep meat samples (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2019). Adequate cooking of meat products will destroy STEC present (Food Standards Scotland, 2020a). However, there is an infection risk from undercooked meat and improper storage or handling of raw meat allowing cross contamination with ready to eat foods. Studies of outbreaks of STEC O157, the most common serotype, which have estimated the number of bacteria consumed in contaminated foods, suggest a low dose of infection ranging from 2 to 216 colony forming units per gram (cfu/g) (Hara-Kudo and Takatori, 2011). Dose response modelling using data from outbreaks suggests the risk of becoming infected after ingesting a single bacterium is probably 1 – 10 % (Teunis *et al.*, 2008). Due to the low number of bacteria required to cause infection, secondary person to person spread can contribute to cases during an outbreak (Locking *et al.*, 2011).

1.2 Impact on human health

Human cases of STEC infection are found in most countries worldwide and were the third most reported zoonosis in the EU in 2018 with 8,161 reported cases and 11 deaths (EFSA

and ECDC, 2019). In the UK, there were 1,840 cases in 2018 (EFSA and ECDC, 2019). Infection causes diarrhoea which is frequently bloody, with symptoms of fever, abdominal pain and vomiting less often reported; gastrointestinal symptoms usually resolve within a week. Asymptomatic carriage is also possible (Byrne *et al.*, 2015). A third of infections, including adults and children, result in hospital admission and 14.8 % of cases admitted to hospital in England and Wales between 2009 – 2012 were reported to result in the serious complication of Haemolytic Uraemic Syndrome (HUS) (Byrne *et al.*, 2015).

In the UK and other countries, the majority of STEC infections occur in children less than 5 years old (Brandal *et al.*, 2015; Byrne *et al.*, 2015; De Rauw *et al.*, 2018). Young age groups also have the highest rates of HUS, which is rare in adults (Byrne *et al.*, 2015; Hamilton and Cullinan, 2019). Of note, HUS is a leading cause of acute renal failure in children in Scotland (Locking *et al.*, 2011). In 20 to 40 % of HUS cases, chronic health problems can persist. Although these are usually relatively mild, for example treatable hypertension and decreased renal glomerular filtration rate, the long term health impacts are largely unknown (Spinale *et al.*, 2013). HUS usually affects the kidneys, but may also result in neurological and cardiac disease, and is fatal in 1- 4 % of cases (Spinale *et al.*, 2013). The pathology of HUS is mediated by a combination of the cytolethal effect of Stx, platelet and complement activation and destruction of red blood cells (Obrig and Karpman, 2012; Lee and Tesh, 2019). There is no widely accepted specific treatment for STEC infection or HUS other than symptomatic treatment (Rahal *et al.*, 2012). There is debate over the use of antibiotics to treat STEC infection and antibiotic use has been linked to increased risk of HUS (Wong, 2000; Smith *et al.*, 2012). Stx is encoded by a prophage (lysogenic bacteriophage genome integrated in bacterial chromosome). When exposed to concentrations of antibiotic that are sub-lethal for STEC bacteria, induction of the bacteriophage lytic cycle can occur resulting in increased Stx production compared to untreated bacteria (McGannon *et al.*, 2010). In particular, sub-inhibitory concentrations of antibiotics targeting DNA synthesis (ciprofloxacin and sulfomethoxazole) have been shown to increase levels of Stx production *in vitro* (McGannon *et al.*, 2010). As an alternative approach, the monoclonal antibody drug Eculumizab, which inhibits complement activation, has been trialled as a treatment for HUS but has not shown significant benefit (Walsh and Johnson, 2019).

Infections are classified as an outbreak where more than one household (or multiple residents of an institution) is affected or as sporadic where only one household is affected

(Gastrointestinal and Zoonoses Team and Scottish *E. coli* O157/STEC Reference Laboratory, 2019). Worldwide, the majority of STEC cases are thought to be sporadic (Kintz *et al.*, 2017). In Europe 95 % of cases are estimated to be sporadic (Koutsoumanis *et al.*, 2020) and in the UK 62 % to 80 % of infections with STEC O157 are reported to be sporadic (Locking *et al.*, 2011; Public Health England, 2018). Identifying the source of infection in outbreaks is possible by determining common exposure among cases, whereas in sporadic infections, large case control studies are required to identify possible risk factors (Food Safety Authority of Ireland, 2019). Nonetheless, in approximately 50 % of food borne outbreaks the source is not identified (Ebel *et al.*, 2016; Pires *et al.*, 2019). A global study with the aim of attributing different food sources to STEC infections was carried out based on foodborne outbreak data (WHO-FAO, 2018). It estimated that beef is the most common food implicated in outbreaks of STEC in Europe and North and South America, followed closely by produce (fruit and vegetables). In contrast, in the Western Pacific Region, produce was estimated to cause the majority of STEC infections. In Europe, it is estimated that 60 % of STEC infections are food borne and 11 % of STEC infections are associated with contact with animals (Hald *et al.*, 2016).

Incidence for all serotypes of STEC have been reported as 6.3 per 100,000 people for the USA, 8.9 for New Zealand and rates in Europe ranging from an incidence of zero in Bulgaria, Cyprus and Lithuania to up to 20/100,000 population in Ireland (Surveillance Atlas of Infectious Diseases, 2018; Browne *et al*., 2018; Tack *et al*., 2020). Scotland has the highest rates of STEC infection in the UK with data from 2017 showing an average of 4.1 cases /100,000 population compared to 1.5 cases /100,000 for the UK as a whole (Food Standards Scotland, 2020b). There have been several possible reasons proposed for increased incidence in Scotland compared to the rest of the UK, including higher numbers of cattle per human population, a greater rural population, higher rainfall and circulation of specific STEC strains (Halliday *et al.*, 2006; Money *et al.*, 2010). Of note, strain types of STEC O157 which are associated with high levels of cattle shedding and with human infection are more common in cattle in Scotland than in other parts of the UK (Chase-topping *et al.*, 2007; Pearce *et al.*, 2009; Matthews *et al.*, 2013; Dallman *et al.*, 2015).

1.3 Classification of STEC by serotyping

STEC can be classified on the basis of cell wall lipopolysaccharide O antigens and protein flagellar H antigens, both of which were originally determined by testing for agglutination

with specific anti-sera against a known O or H type (Chattaway *et al.*, 2017). The serogroup provides information on the O antigen type and is often used for comparison of strains, while the serotype includes details of O and H type. Globally, STEC O157 is most frequently detected in cases of human infection and is the top serogroup detected in the USA, UK and most countries in Europe (EFSA and ECDC, 2019; Gastrointestinal and Zoonoses Team and Scottish *E. coli* O157/STEC Reference Laboratory, 2019; González-Escalona and Kase, 2019). STEC O157 implicated in human disease is predominantly H7, although non-motile strains which lack flagellin have been implicated in human disease (Rosser *et al.*, 2008). In 2018, 59 % of infections in Scotland were identified as O157 with the remaining cases caused by a range of other serogroups (Gastrointestinal and Zoonoses Team and Scottish *E. coli* O157/STEC Reference Laboratory, 2019). Of the non-O157 serogroups causing human infection O26, O103, O91, O146, O145 and O128 are the most commonly found in Europe including the UK (EFSA and ECDC, 2019). In the USA serogroups O26, O45, O103, O111, O121 and O145 are most common cause of human infections after O157 (USDA, 2019). However, many other serogroups are isolated from human clinical cases, including strains which are of previously unknown O-types.

Detection of non-O157 STEC infections have increased in recent years including in the USA, UK and Europe and in Ireland STEC of serogroup O26 are now the most commonly reported in association with human disease (Food Safety Authority of Ireland, 2019). While serological testing for common O-groups has provided a rapid method of identifying STEC O157 and other serogroups known to cause human disease, a disadvantage is that identifying an isolate by serogroup does not provide virulence gene information (Food Standards Scotland, 2020b). Furthermore, it is not possible to identify serogroups for which serological tests are not available. Additionally, false positive results are possible as some strains of bacteria auto-agglutinate and will bind anti-sera non-specifically (Food Standards Scotland, 2020b). For these reasons, there is now a shift to using PCR testing for *stx* genes to identify STEC infections and to isolating STEC from positive samples by testing individual colonies for *stx* genes.

1.4 Detection of STEC by culture, serological and molecular methods

Most strains of STEC O157 have biochemical properties which distinguish them from other serogroups and commensal *E. coli,* including the lack of ability to ferment sorbitol and lack of glucuronidase activity, thereby simplifying isolation using selective and differential agar plates. Sorbitol MacConkey (SMAC) Agar is frequently used to isolate STEC O157 as this grows as colourless colonies in comparison to commensal *E. coli* (and other serogroups) which grow as pink colonies due to their ability to ferment sorbitol (March and Ratnam, 1986; Feng *et al.*, 1998). Addition of chromogenic substrate to the growth medium to detect glucuronidase activity also allows differentiation of STEC O157 which is generally lacks glucuronidase activity in comparison to other *E. coli* which usually have glucuronidase activity (Gouali *et al.*, 2013). Addition of cefixime and tellurite to Sorbitol MacConkey agar (CT-SMAC) also increases selectivity for STEC O157 (Chapman *et al.*, 1991; Zadik *et al.*, 1993). Non-O157 STEC, along with commensal *E. coli,* tend to have sorbitol fermenting ability and glucuronidase activity and a proportion will be sensitive to cefixime and tellurite (Verhaegen *et al.*, 2015).

Amplification of STEC O157 by culture in nutrient broth and subsequent immunomagnetic separation (IMS) can also be used to increase sensitivity of detection by using magnetic beads linked to O157 antibodies to capture bacteria from faecal, water or food samples (Cubbon *et al.*, 1996). IMS has been reported to have a 100 fold greater sensitivity than isolating bacteria by direct culture, with a lower limit of detection of 100 STEC O157 organisms per g of faeces (Chapman *et al.*, 1994; Omisakin *et al.*, 2003). While IMS can be used for other serogroups of bacteria, only one serogroup can be identified at a time. Since only the most common STEC serogroups tend to be tested for using IMS, rarer serotypes will not be detected. Latex agglutination testing uses agglutination of latex beads coated with O-antigen specific antibodies to confirm the identity of particular *E. coli* serogroup. Although, this is a convenient method, as with IMS, only one serogroup can be identified at a time and testing is biased towards the expected most common serogroup. Furthermore, a positive latex agglutination test does not confirm that an isolate is STEC and further testing for *stx* genes is required.

Prior to the implementation of PCR testing, non-O157 infections may have been less likely to be reported as isolation was focussed on serological detection of O157. The adoption of PCR based screening for *stx* genes has made it easier to identify the presence of STEC regardless of serogroup. Since 2013, laboratories in England and Republic of Ireland have implemented PCR testing for *stx* genes to identify STEC (Jenkins *et al.*,

2020). While Scottish Regional Diagnostic laboratories do not undertake PCR screening, since 2014 all suspected STEC positive samples are submitted to the Scottish *E. coli* Reference Laboratory for PCR screening (Food Standards Scotland, 2020b). Diagnostic laboratories in Europe are adopting a molecular approach to test for the presence of *stx* genes, followed by isolation of bacteria from *stx*-positive samples by culturing samples and then screening individual colonies for *stx* genes (Koutsoumanis *et al.*, 2020). The United States has recently incorporated testing for *stx* and *eae* genes as an initial screening of meat samples. However, classification of foods being unsafe for consumption is still based on serological testing for O157 and six other STEC serogroups commonly implicated in human disease which include O26, O45, O103, O111, O121 and O145 (USDA, 2019).

1.5 Shiga toxin structure and mechanism of action

Shiga toxin (Stx) is a subunit protein comprised of one A subunit, which is responsible for biological effects on the target cell, and five B subunits, which mediate binding to target cells (Melton-Celsa, 2014). Stx can be classified as either Stx1 or Stx2 based on amino acid sequence. Further subtypes are recognised within Stx1 and Stx2 classifications based on differences in amino acid sequence. Within Stx1, subtypes 1a, 1c and 1d are recognised (Scheutz *et al.*, 2012). Within Stx2, seven different subtypes have been identified: 2a, 2b, 2c, 2d, 2e, 2f and 2g (Scheutz *et al.*, 2012). Antibodies raised against Stx1 do not cross react with Stx2. Stx1 and Stx2 have approximately 56 % similarity at the amino acid level (Jackson *et al.*, 1987). Subtypes are associated with varying clinical outcomes. In general, subtypes of Stx1 are associated with less severe disease than Stx2. Stx2a and Stx2c are most frequently associated with HUS, along with Stx2d which shows increased cytotoxicity when activated by elastase in host intestinal mucus (Naseer *et al.*, 2017; Sánchez *et al.*, 2017; De Rauw *et al.*, 2018). Stx2f and Stx2g are less frequently associated with human disease. However, severity is also dependent on host factors and most subtypes of Stx have the potential for serious disease depending on susceptibility of the host (Koutsoumanis *et al.*, 2020).

Stx is encoded by a bacteriophage which is integrated into the host bacteria DNA as a prophage (Kruger and Lucchesi, 2015). In its lysogenic state, the prophage DNA is replicated as the bacteria divide. The integrated phage produces its own repressor which during lysogeny prevents transcription of phage proteins (Chakraborty *et al.*, 2018). Induction of the lytic state leads to transcription of phage proteins including Stx and

eventual lysis of the host bacteria with release of bacteriophage (Wagner, Neely, *et al.*, 2001). Infection with more than one *stx* encoding phage is possible - STEC positive for 2 or more phage encoded Shiga toxins are frequently identified and there is evidence that additional *stx* genes result in increased Stx production (Fogg *et al.*, 2012). A switch to the lytic cycle known as phage induction is triggered by damage to bacterial DNA which triggers the bacterial SOS response, a ubiquitous bacterial response which results in pausing of bacterial cell division and transcription of proteins for DNA repair (Shinagawa, 1996). RecA is activated by damaged DNA resulting in cleavage of the SOS repressor LexA and also the phage repressor resulting in production of proteins for assembly of infectious phage and Stx (Shimizu *et al.*, 2009; Kruger and Lucchesi, 2015).

Phage induction also leads to transcription of proteins that mediate lysis of the bacterial cell, leading to release of phage and Stx (Wagner, Neely, *et al.*, 2001). While SOS-mediated induction is the main mechanism of Stx production and release, Stx production can also be induced in response to low iron levels particularly for Stx1 production (Calderwood and Mekalanos, 1987; Shimizu *et al.*, 2009). Stx 1 production is repressed by high iron concentration mediated by the regulatory protein Fur, whereas Stx 2 production is closely linked to the phage lytic cycle (Calderwood and Mekalanos, 1987). Triggers for switching to the lytic cycle include antibiotic exposure as described in section 1.2 but also endogenous factors within the host gastrointestinal tract. For example, microcins released by other bacteria and reactive oxygen species released by immune cells have been shown to induce Stx production (Wagner, Acheson, *et al.*, 2001; Nawrocki *et al.*, 2020). Stx1 is generally expressed at lower levels than Stx2 which may contribute to it being less pathogenic in human infection (Shimizu *et al.*, 2009).

Although lysis and death of the host bacterial cell represents the main route for Stx release, it is suggested that only a proportion of the bacterial population undergoes a switch to the lytic cycle, with toxin production conferring a benefit on the surviving bacteria (Loś *et al.*, 2013). There is evidence for various mechanisms by which lysis of a proportion of bacteria and release of Stx may benefit the surviving bacteria of the same clone including protection from predation from protozoa, immune suppression and increasing colonisation success in the ruminant host (Menge *et al.*, 1999; Koudelka *et al.*, 2018; Fitzgerald *et al.*, 2019). Free bacteriophage may survive stresses that the bacterial cell would not, so the lytic cycle also represents a survival strategy for the phage (Martínez-Castillo and Muniesa, 2014).

The major receptor for Stx1 and Stx2 on human cells is globotriaosylceramide (Gb3) (Ling *et al.,* 1998). An additional receptor globotetraosylceramide (Gb4) is also able to bind Stx at lower affinity, with the exception of the subtype stx2e which binds Gb4 with greater affinity than Gb3 (Melton-Celsa, 2014). In humans, Gb3 is expressed on vascular endothelial cells which line the small blood vessels in the gut, kidney and brain (Legros *et al.*, 2018). Human gut epithelial cells have been reported to lack Gb3 expression based on lack of anti-Gb3 antibody reactivity (Schüller *et al.*, 2004). However, Gb3 synthase mRNA has been detected in human colonic epithelial cells using quantitative PCR (qPCR) and anti Gb3 antibodies have been show to bind to Paneth cells in the epithelium (Schüller *et al.*, 2007; Zumbrun *et al.*, 2010). It has been suggested that Gb4, which has been detected on human colonic epithelial cells, may provide an alternative receptor for Stx (Zumbrun *et al.*, 2010). Stx is capable of binding and causing apoptosis and necrosis in human epithelial cells and can also be transported from apical to basal surfaces in an *in vitro* model, although it is unclear what cellular receptor(s) are involved in these processes (Schüller *et al.*, 2004; Pradhan *et al.*, 2020).

Since STEC colonise apical epithelium and do not invade underlying tissue, translocation of Stx from gut lumen to blood vessels and dissemination via the circulation is key to the pathology seen in other organs including the brain and kidneys (Ståhl *et al.*, 2015). Epithelial cell damage, along with damage to underlying endothelial cells in the intestine, may explain characteristic STEC symptoms of bloody diarrhoea (Proulx *et al.*, 2001). Stx is able to be transported through gut epithelia via cells or through junctions between cells (paracellularly) to gain access to underlying endothelial cells which are positive for Gb3 and susceptible to the cytolethal effects of Stx (Hurley *et al.*, 1999). After translocation across the epithelial cell layer, binding of Stx occurs to host immune cells including neutrophils and also microvesicles derived from platelets, leukocytes and red blood cells (te Loo *et al.*, 2000; Ståhl *et al.*, 2015). Bacterial lipopolysaccharide (LPS) may also play a role in priming leukocytes to bind Stx. LPS-treated myeloid leukocytes were shown to transport Stx2 and cause HUS in a mouse model (Niu *et al.*, 2018). Cell bound or microvesicle bound Stx can be transported via the circulation to other tissues which are positive for Gb3 and susceptible to toxin induced cell death (te Loo *et al.*, 2000; Ståhl *et al.*, 2015).

Shiga toxins are type-2 ribosome inactivating proteins (Chan and Ng, 2016). Binding to Gb3 on the cell surface via the B subunit causes the toxin to be taken into the cell by endocytosis. This is mediated by the cell surface protein clathrin and by a clathrin

independent mechanism whereby Stx itself may induce endocytosis (Mukhopadhyay and Linstedt, 2013). Once inside the cell, the A subunit is cleaved to its active form by host cell furin as it undergoes retrograde trafficking to ribosomes, leading to inhibition of protein synthesis and cell death (Schüller *et al.*, 2004). Immune cells, including monocytes and neutrophils, are resistant to cell death through binding of Gb3 and may use TLR4 as an alternative receptor for Stx which induces inflammatory cytokine release leading to further tissue injury (Brigotti *et al.*, 2013; Menge, 2020). Resulting inflammation and complement activation contributes to the characteristic features of HUS - microangiopathic anaemia, thrombocytopenia and renal failure, due to accumulation of damaged red blood cells and platelets in kidney glomeruli (Obrig and Karpman, 2012; Rahal *et al.*, 2012).

The effect of Stx on cells is related to the presence of Gb3 and also the location of Gb3 in the cell membrane. Binding of Stx to Gb3 in lipid rafts leads to trafficking of toxin to the ribosome resulting in cell death (Higashi *et al.*, 2010). In humans, Stx is able to traverse the gut epithelia and gain access to vascular endothelial cells which have lipid raft associated Gb3 and are sensitive to Stx toxicity (Higashi *et al.*, 2010). As described above, there is also evidence that when Stx gains access to the circulation it can bind blood leukocytes and blood cell derived microvesicles and be transported to Gb3 positive cells. In contrast, on bovine intestinal epithelial cells, Stx has been shown to bind non-lipid raft associated Gb3 on epithelial crypt cells (Hoey *et al.*, 2003). Binding to non-lipid raft associated Gb3 appears to limit the toxic effects on the cells and the toxin is trafficked to endosomes for degradation (Hoey *et al.*, 2003). Although Stx binding to bovine epithelium does not cause cytolethal effects, it may inhibit intestinal stem cell regeneration, leading to reduced epithelial cell turnover and increased persistence of STEC at the apical epithelium (Fitzgerald *et al.*, 2019). Cattle do not appear to express Gb3 on vascular endothelial cells and have an additional isoform of Gb3 within the kidney compared to humans (Hoey *et al.*, 2002). The degradation of Stx in gut epithelial cells and absence of Gb3 expression on endothelial cells may explain why ruminants can carry STEC without being affected by the cytotoxic effects of Stx.

1.6 Additional virulence factors of relevance to STEC pathogenesis

STEC have additional virulence factors which can be broadly defined as factors mediating bacterial attachment to the host epithelium and factors which enhance survival of the bacteria once colonisation has occurred. A major virulence factor is the bacterial cell

surface protein intimin which is encoded by the *eae* gene. The *eae* gene is located within a sequence of genes termed the Locus for Enterocyte Effacement (LEE) due to its importance in facilitating attachment to cells in the intestine (Kaper *et al.*, 2004). The LEE encodes Type III secretion system proteins which assemble into a syringe-like structure, allowing the bacteria to secrete proteins directly into the host cell (Gaytán *et al.*, 2016). Initial attachment of the bacteria is via intimin binding to nucleolin on the host cell surface (Sinclair and O'Brien, 2004). The bacterial encoded receptor for intimin, Tir, is then translocated into the host cell via the Type III secretion system where it is integrated into the host cell membrane. Tir then binds to intimin with higher avidity than nucleolin allowing the bacteria to form secure attachments with host epithelial cells (Zaharik *et al.*, 2002; Sinclair and O'Brien, 2004; Mohawk and O'Brien, 2010). Subsequently, LEE encoded effectors cause cytoskeletal rearrangements in the host cell leading to a pedestal like formation on the host epithelium and effacement of microvilli. The mechanism of LEE mediated attachment of STEC is shown in Figure 1.1.

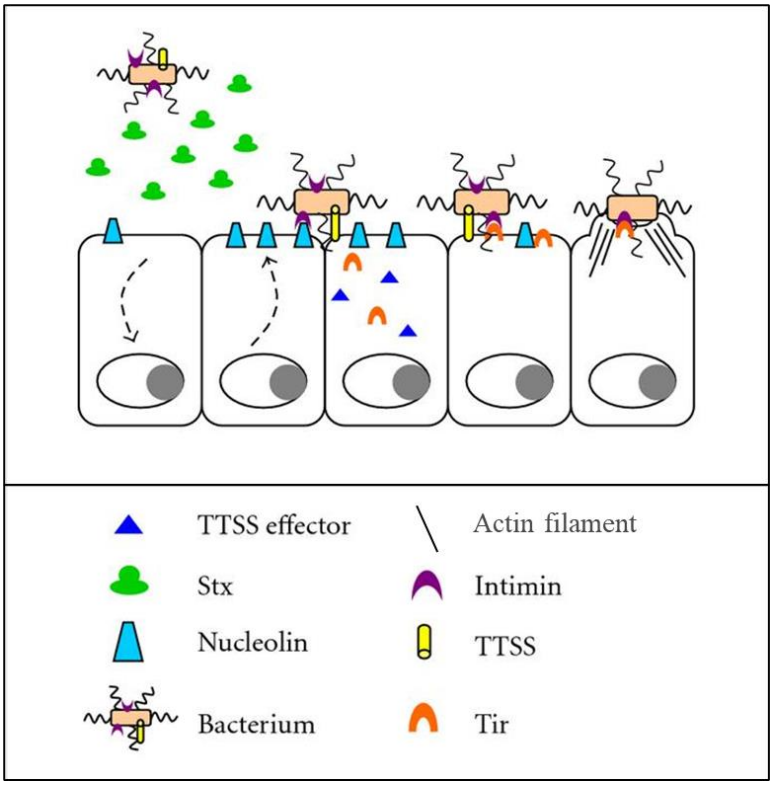

Figure 1.1 Mechanism of LEE- mediated attachment of STEC to host epithelial cells (adapted from O'Brien and Mohawk, 2019) Lysis of STEC releases Shiga toxin (Stx) which causes up-regulation of cell-surface associated nucleolin. Initial attachment occurs through binding of bacterial intimin to host cell nucleolin. Using the type III secretion system (TTSS), the bacterium injects various proteins including Tir into the host epithelial cell. Tir is incorporated into the host cell membrane where it binds with high avidity to intimin. Other TTSS effectors mediate host cell cytoskeleton re-organisation (represented by actin filaments) leading to characteristic pedestal formation and attaching / effacing lesion formation.

STEC strains positive for *eae* have been identified as an important risk factor for development of HUS (Brandal *et al.*, 2015). A review of human STEC infections caused by O157 and other serotypes in Norway from 1992 to 2012 showed that 73.9 % (246) of cases were caused by *eae* positive STEC and 100 % (25) of cases of HUS were linked to *eae* positive strains. (Brandal *et al.*, 2015). For 129 disease-causing non-O157 strains in the Netherlands collected from 2006 to 2010, 80.9 % were *eae* negative, and these were generally associated with less severe disease outcomes than *eae* positive STEC O157 infections (Franz *et al.*, 2015). Surveillance data for all STEC from the EU for 2012 to 2017 showed 71 % (517/726 cases) and 90 % (200/222 cases) of hospitalized cases and HUS cases respectively were positive for both *eae* and *stx2* (Koutsoumanis *et al.*, 2020). Although less often implicated in HUS than *eae* positive strains, STEC which are negative for *eae* have been associated with cases of HUS (Koutsoumanis *et al.*, 2020).

In addition to *eae,* over 100 other virulence genes have been identified in STEC including adhesins, siderophores, microcins, colicins and SPATEs (Serine Protease Autotransporters of *Enterobacteriaceae*) (González-Escalona and Kase, 2019). Adhesins include the fimbrial proteins FimH and LpfA and which allow the bacteria to make initial attachments to the gut epithelium so preventing the bacteria being excreted from the intestine (Tarr *et al.*, 2000; Farfan and Torres, 2012). In STEC which lack the adhesin gene *eae,* it is hypothesised that alternative bacterial adhesin proteins allow colonisation of the intestinal epithelium. The adhesin genes *aggR, iha, hra* and *saa* have also been proposed as providing alternative attachment mechanisms for *eae* negative strains (Paton *et al.*, 2001; Montero *et al.*, 2017; WHO-FAO, 2018).

Proteins which suppress host immune responses promote survival of STEC in the host. The host factor NFκB is key to initiating an inflammatory response and STEC proteins NleB and EspB have been shown to inhibit translocation of NFκB to the nucleus, thereby mediating immune suppression (Clements *et al.*, 2012). The gene *iss* allows STEC to avoid damage from host serum complement proteins associated with the innate immune response (Johnson *et al.*, 2008).

Siderophore genes (*ira*, *fyuA*) encode iron binding proteins which allow the bacteria to take up iron for growth. In the host, iron is usually sequestered by host protein, so iron binding proteins allow the bacteria to compete with the host for iron (Page, 2019). The haemolysin genes *ehxA* and *hlyD* may also play a role in nutrient acquisition by causing lysis of red blood cells and release of iron containing proteins which advantageous for bacterial growth (Law and Kelly, 1995).

STEC also face competition from commensal bacteria; microcins and colicins produced by STEC can inhibit growth of competing bacteria (Montero *et al.*, 2019). The gene *gad* confers acid resistance allowing the bacteria to survive transit through the stomach (Vanaja *et al.*, 2009). Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs) are proteases which were initially identified as secreted proteins from pathogenic *E. coli*. Their exact roles in virulence have not been fully determined but they may help STEC overcome host defences. Specific proteases have been shown to digest host mucin and clotting factor V (Dautin, 2010). In addition to Stx, STEC may also have additional toxins, for example the subtilase cytotoxin (Paton and Paton, 2010). In Scottish non-O157 STEC isolated from human clinical cases between 2002 and 2018, the most common virulence genes other than *stx* were *fimH, gad*, *iss, ehxA hlyD, lfpA* and *eae* (Food Standards Scotland, 2020b). The haemolysin gene *ehxA* and the adhesin gene *lpfA* have also been shown to be common in human STEC strains in the Netherlands, Norway and Belgium (Ferdous *et al.*, 2016; Naseer *et al.*, 2017; De Rauw *et al.*, 2018).

Genome size of STEC can vary widely between different strains due to the plasticity of the STEC genome. On average, STEC have a larger genome than non-pathogenic *E. coli* or Stx negative pathogenic *E. coli* (Van Hoek *et al.*, 2019). In addition, a study of bovine and human clinical isolates in the Netherlands found *eae* positive isolates had significantly more virulence genes than *eae* negative isolates (Franz *et al.*, 2014).

Associations exist between groups of virulence genes due to them being found within the same lineage of bacteria and also being co-located on the same plasmid or pathogenicity island. Significant association has been observed between *eae* positive isolates and the virulence genes *ureC* (urease), *toxB* (adhesin), *etpD* (secreted effector), *adfO* (adhesin) and *cfk* (toxin), due to being encoded within the Locus for Enterocyte Effacement (LEE) (Franz *et al.*, 2015). The presence of *eae* is also correlated with *tir* and genes for formation of the Type III secretion system (Kaper *et al.*, 2004). Conversely, *eae* negative isolates have been found to be significantly associated with virulence genes including adhesin genes *iha* and *saa*, microcin genes *mchB*, *mchC* and *mchF*, toxin genes *subA* and *senB*, and the siderophore *ireA* (Franz *et al.*, 2015; Ferdous *et al.*, 2016). There is also evidence that acquisition of virulence genes is dependent on the genetic background of the strain, so gene distribution may not be completely random (Escobar-Páramo *et al.*, 2004).

1.7 Prevalence and characteristics of STEC in deer

Studies to determine prevalence of STEC using PCR to test for *stx* genes without isolation of bacteria reveal variation in prevalence of *stx* genes in deer faeces, rectal swabs and carcass or meat samples. As shown in Table 1.1, this variation ranges from 32.6 % (Laaksonen *et al.*, 2017) to 83 % of deer faeces (Eggert *et al.*, 2013). Carcass samples and frozen venison meat were found to have an *stx* prevalence of 25.1 % and 45.8 %, respectively (Díaz-Sánchez *et al.*, 2012; Diaz-Sanchez *et al.*, 2013). The study by Obwegeser *et al.* (2012) detected *stx* in combination with *eae* in 20.3 % of samples; although it should be noted that the other studies detailed in Table 1.1 did not test for the *eae* gene. The approach of determining prevalence of STEC by PCR testing for *stx* genes in samples is regarded as presumptive as, without isolation and further testing, it is not possible to know if the virulence genes detected are present in one or more isolates of *E. coli* or possibly in other species of bacteria.

Table 1.1 Prevalence of STEC virulence genes in deer samples based on PCR of *stx* genes

Species	Sample type	No samples	Location	% *stx*	Reference
Red	Deer meat samples	48	Spain	45.8	Díaz-Sánchez *et al.*, 2012
Red	Faecal samples	264	Spain	35.2	Diaz-Sanchez *et al.*, 2013
Red	Carcass samples	271	Spain	25.1	Diaz-Sanchez *et al.*, 2013
Red, Roe	Faecal samples	60	Germany	83.3	Eggert *et al.*, 2013
Roe	Rectal swabs	77	Germany	74.60	Frank *et al.*, 2019
Reindeer	Faecal samples	470	Finland, Norway	32.6	Laaksonen *et al.*, 2017
Roe	Rectal swabs	179	Spain	67.0	Mora *et al.*, 2012
Red, Roe	Faecal samples	148	Switzerland	37.8	Obwegeser *et al.*, 2012

Based on published literature up to 2019.

1.7.1 Prevalence of STEC O157 in deer

Previous studies in Spain and Japan screening colonies for *stx* genes followed by determination of serotype indicated that STEC O157 could be isolated from 0.4 % (Diaz-Sanchez *et al.*, 2013) to 3 % (Kabeya *et al.*, 2017) of deer faecal samples as shown in Table 1.2. Although, prevalence of STEC O157 in deer is reported to be low, outbreaks associated with venison consumption or contact with deer faeces have been reported. An outbreak in Oregon, USA was associated with consumption of strawberries thought to be contaminated with deer faeces. In analysis of this outbreak, identical strains based on pulsed-field gel electrophoresis (PFGE) were isolated from human cases and environmental samples contaminated with deer faeces (Laidler *et al.*, 2013). In 2015, an outbreak of 12 cases occurred in Scotland which was associated with consumption of venison from Scottish wild deer (Smith-Palmer *et al.*, 2018). This resulted in a study being undertaken to determine the risk of STEC contamination in venison including determining the prevalence of STEC O157 in wild deer. In total, 1087 samples from individual animals were analysed, of which 3 samples contained STEC O157 (McNeilly *et al.*, 2020). Prevalence of STEC O157 was estimated at 0.34 % (Confidence Interval 0.02 – 6.30). PCR testing was undertaken for *stx* genes in samples which were negative for STEC O157 and 69.5 % of samples were positive for *stx1*, *stx2* or a combination of both genes suggesting that non-O157 STEC are present in a high proportion of deer.

Table 1.2 Distribution of O157 STEC isolated from deer

Species	Sample Type	No of animals sampled	Country	No. individual non-O157 *stx* positive strains isolated*	No. of STEC O157 isolated	% of samples positive for STEC O157	*stx* subtype	*eae* positive	Reference
Red	Faecal samples	264	Spain	89	1	0.4	*stx1, 2*	yes	Diaz-Sanchez *et al.*, 2013
Roe	Rectal swabs	179	Spain	103	1	0.6	*stx2c*	yes	Mora. *et al.*, 2012
Sika	Faecal samples	30	Japan	19	1	3.3	*stx2c*	yes	Kabeya *et al.*, 2017
Red Roe Fallow	Rectal swabs	252	Poland	51	5	2.0	*stx2c* (1) *stx2b* (1) *stx2g* (2) *stx2g* (1)	yes yes yes no	Szczerba-Turek *et al.*, 2020
Red Roe	Faecal samples	148	Switzerland	34	1	0.7	*stx2c*	yes	Hofer *et al.*, 2012

*This includes isolates from wild deer only

1.7.2 Non-O157 STEC isolated from deer

A wide variety of STEC serotypes have been isolated from deer, some of which appear to be typical of deer. O types of non-O157 STEC isolated from deer include O21, O146, O128, O113 and O22 (Miko *et al.*, 2009; Martin and Beutin, 2011). However, little is known about the serotypes and potential for causing human disease for non-O157 STEC from Scottish wild deer.

Stx and *eae* profiles of non-O157 STEC which were isolated from deer are shown in Table 1.3. These studies use the approach for estimating non-O157 STEC prevalence by culturing STEC from samples followed by PCR screening of individual colonies for the presence of *stx* genes. While this gives a clearer picture of *stx* and *eae* virulence gene profile, a limitation of this approach is that it may underestimate the overall prevalence due to difficulty in isolating STEC from a background of enteric bacteria. Overall, *stx2* alone or in combination with *stx1* was more common than *stx1* alone in non-O157 STEC isolated from deer. Only 5 of 11 studies identified non-O157 STEC which were positive for *eae,* ranging from 5.4 % to 58.8 % of isolates (Mora *et al.,* 2012; Obwegeser *et al.*, 2012; Carrillo-Del Valle *et al.*, 2016; Kabeya *et al.*, 2017; Szczerba-Turek *et al.*, 2020).

Studies that subtyped *stx* genes in non-O157 STEC from deer by faecal sampling found that *stx2b* alone or in combination with *stx1c* was the most common *stx* subtype (Hofer *et al.*, 2012; Mora *et al.*, 2012; Eggert *et al.*, 2013; Dias *et al.*, 2019; Frank *et al.*, 2019; Szczerba-Turek *et al.*, 2020), accounting for between 22 % (11/51) (Szczerba-Turek *et al.*, 2020) and 97 % (31/32) of isolates (Eggert *et al.*, 2013). While considered to be less pathogenic than the *stx2a* subtype, *stx1c* and *stx2b* positive strains still have the potential to cause severe disease in humans (Koutsoumanis *et al.*, 2020). The subtype *stx2a* was identified in non-O157 isolates in 2 studies at a rate of 4 % (4/103) and 12 % (6/51) of isolates (Mora *et al.*, 2012; Szczerba-Turek *et al.*, 2020).

Table 1.3 Distribution of *stx* and *eae* genes in non-O157 STEC isolated from deer

Species	Source	Location	No. non-O157 STEC isolates tested	% *stx1* + alone	% *stx2* + alone	% *stx1*+/*stx2*+	% *stx1*+/ *eae*+	% *stx2*+/ *eae*+	% *stx1*+/*stx2*+/ *eae*+	Reference
Red, Roe	Faecal sample	Belgium	16	25.0	68.8	6.3	0.0	0.0	0.0	Bardiau *et al*., 2010
Red	Rectal swab	Mexico	19	26.3	10.5	42.1	10.5	0.0	10.5	Carrillo-Del Valle *et al*., 2016
Red	Meat	Spain	5	0.0	100.0	0.0	0.0	0.0	0.0	Díaz-Sánchez *et al.*, 2012
Red	Faecal sample	Spain	89	4.7	90.7	4.7	0.0	0.0	0.0	Diaz-Sanchez *et al.*, 2013
Red	Carcass swab	Spain	19	10.5	84.2	5.3	0.0	0.0	0.0	
Red, Roe	Faecal sample/Tonsil /Lymph node	Germany	32	0.0	93.8	6.3	0.0	0.0	0.0	Eggert *et al.*, 2013
Roe	Faecal sample/ Rectal swab	Germany	143	14.7	85.3	0.0	0.0	0.0	0.0	Frank *et al*., 2019
Sika	Faecal sample	Japan	15	6.7	80.0	6.7	6.7	0.0	0.0	Kabeya *et al*., 2017
Roe	Rectal swab	Spain	103	11.7	61.2	21.4	3.9	1.0	1.0	Mora *et al.*, 2012
Red, Roe	Faecal sample	Switzerland	37	29.7	59.5	5.4	2.7	2.7	0.0	Obwegeser *et al.*, 2012
Red, Roe, Fallow	Rectal swab	Poland	51	9.8	27.5	3.9	15.7	39.2	3.9	Szczerba-Turek *et al.*, 2020

1.8 STEC in other ruminants

1.8.1 Prevalence of STEC in sheep and goats

Similar to deer and cattle, overall levels of STEC carriage in sheep and goats are relatively high. A survey of 1300 lambs from different flocks in Spain isolated STEC strains from 36 % of lambs (Blanco *et al.*, 2003). In a separate study in Spain based on PCR testing of pooled samples, 56.5 % of 115 sheep flocks sampled had animals carrying STEC (Oporto *et al.*, 2019). In a study of dairy goats in Spain, STEC isolates were obtained for 47.7 % of animals (Cortés *et al.*, 2005). PCR testing of faecal samples showed an animal level prevalence of 87.6 % in sheep on farms in Norway (Urdahl *et al.*, 2003).

Prevalence of STEC O157 in sheep and goats appears to be lower than is seen in cattle. Two studies which sampled 129 sheep in Norway and 222 goats in Spain did not isolate STEC O157 (Urdahl *et al.*, 2003; Cortés *et al.*, 2005). In a longitudinal study of two herds of dairy goats, STEC O157:H7 was only isolated from three kids on one occasion (Orden *et al.*, 2008). A study of sheep in Scotland found 3.4 % prevalence of STEC O157:H7 in sheep at slaughter (Evans *et al.*, 2011). In Ireland, O157 STEC was isolated from 5.8 % of rectal swabs collected from sheep at slaughter (Prendergast *et al.*, 2011). An investigation of STEC O157 in Scottish sheep flocks found 40 % flock prevalence, with an animal prevalence of 6.5 % (Ogden *et al.*, 2005). A study of sheep flocks in Spain found 20 % of flocks had at least one animal carrying STEC O157 (Oporto *et al.*, 2019).

A range of non-O157 serogroups have been identified in sheep, with 64 different serogroups identified in 384 isolates from sheep in Spain, O128 and O91 were the most common serogroups (Blanco *et al.*, 2003). In Scottish sheep, 5.2 % and 2.3 % of samples contained isolates of O26 and O103, respectively, however testing was limited to O157, O26, O103, O111 and O145 serogroups (Evans *et al.*, 2011). The most common serotype isolated from sheep in Norway was O128:H2 (Urdahl *et al.*, 2003). Modelling of data from human and animal isolates collected over a 4 year period in the Netherlands was used to estimate the relative contribution of different animal species to human STEC infection. It was estimated that 24 % - 26 % of human STEC cases in the Netherlands could be attributed to small ruminants including sheep and goats and 71 – 77 % of cases of STEC O146 were attributed to small ruminants (Mughini-Gras *et al.*, 2018).

In a survey of goats (28) and sheep (20) on city farms in Southern Germany, STEC was isolated from 100 % of sheep samples and 25 of 28 goat samples (Schilling *et al.*, 2012).

The most common *stx* profile was *stx1c* followed by *stx1c*:*stx2b* and *stx2b* alone. Only one goat sample was positive for *stx1a* and 2 sheep were positive for *stx1a:stx2b* isolates. The subtypes *stx1c* and *stx2b* have been shown to be significantly associated with isolates from sheep and goats, and from their meat and milk (Martin and Beutin, 2011). No STEC strains positive for *eae* were isolated from 129 sheep in a Norwegian study and only 6 % of isolates in a survey of Spanish sheep farms were *eae* positive (Blanco *et al.*, 2003; Urdahl *et al.*, 2003).

1.8.2 Prevalence of STEC in cattle

Studies which have used PCR testing for *stx* genes in faecal samples to determine the overall prevalence of STEC suggest that a high proportion of cattle carry STEC strains. A study in Norway detected *stx* genes in 64.6 % of cattle and 90 % of cattle were positive for *stx* genes in a study from China (Urdahl *et al.*, 2003; Fan *et al.*, 2019). Studies in Scotland and Ireland found 20 % and 40 % respectively of beef cattle positive for *stx* genes (Jenkins *et al.*, 2002; Monaghan *et al.*, 2011).

Estimated prevalence of STEC O157 from faecal sampling in Scottish cattle close to date of slaughter was 23.6 % of herds and 10.6 % of individual cattle. The same survey found a 21.3 % herd prevalence and 6.9 % pat level prevalence on farms in England and Wales (Henry *et al.*, 2017). STEC O157 is found in cattle worldwide and studies in South Korea, Argentina, Spain and USA which detected STEC O157 using immunomagnetic separation and culture have found animal prevalence from zero at certain times of the year up to 21.5 % (Cabal *et al.*, 2016; Dong *et al.*, 2017; Schneider *et al.*, 2018; Rhades *et al.*, 2019). A study looking at beef cattle at slaughter in Ireland which quantified STEC O157 rectal swabs found an animal prevalence of 4.2 % for STEC O157 (McCabe *et al.*, 2019).

Non-O157 STEC isolates from cattle comprise a wide range of serotypes. The most common serotypes identified in beef cattle in Ireland included O113:H4 and O26:H11, with17 different serotypes identified in total (Monaghan *et al.*, 2011). In a study of a Scottish beef farm, serotypes O26:H11, O113:H21 and O128:H8 were identified most frequently over an eight month period (Jenkins *et al.*, 2002). The serotypes O113:H4 and O113:H21 were the most common strains isolated from cattle in a study of Norwegian farms (Urdahl *et al.*, 2003). *Stx* subtypes *stx1a*, *stx2a*, *stx2c* and *stx2d* have commonly been detected in non-O157 STEC isolates from cattle (Monaghan *et al.*, 2011; Shridhar *et al.*, 2017; Fan *et al.*, 2019).

1.8.3 STEC in wild ruminants other than deer

Wild ruminants which have been shown to carry STEC include ibex and chamois. Faecal sampling of hunted wild ruminants in Spain found 2/117 ibex samples positive for STEC O157 (Navarro-Gonzalez *et al.*, 2015). In a study of wild ruminants in Switzerland, 12/64 chamois samples and 6/27 ibex were STEC positive (Hofer *et al.*, 2012). From these, the Stx profiles were identified as either *stx1c* or *stx2b* or a combination, with *stx1a*:*stx2b* being found in 1 ibex sample, no isolates were *eae* positive. STEC has also been detected in elk, wild sheep, bison, antelope, buffalo, wild goat, moose, and yak (Espinosa *et al.*, 2018).

1.8.4 Faecal shedding and transmission of STEC in ruminants

The average duration of shedding in calves experimentally infected with STEC O157 is thought to be around 30 days, with some animals shedding bacteria for 20 weeks (Cray and Moon, 1995; Sanderson *et al.*, 1999). Longitudinal studies suggest that a high proportion of naturally infected cattle will shed at some point - 61.9 % of cattle in a year-long study in Argentina were positive at least at one point during the year and all 23 cattle in a Australian herd shed at some point during a 9 month period (Jones *et al.*, 2017; Rhades *et al.*, 2019). Naturally infected sheep kept in pens have been observed to shed STEC O157 for up to 4 weeks (McPherson *et al.*, 2015). In a longitudinal study of two dairy goat herds, most animals sampled over a year were positive for STEC at least at one sampling point of the year, with 11/33 animals shedding over several months (Orden *et al.*, 2008). Repeat sampling in a study of wild roe deer suggests that deer may shed the same strain over long time periods of up to 778 days, with a mean shedding duration of 42 days (Frank *et al.*, 2019).

The term super shedding has been used to describe animals which shed STEC at high levels and disproportionately contribute to the overall levels of STEC shed into the environment, and therefore contribute most to animal-to-animal transmission (Chase-Topping *et al.*, 2008; Matthews *et al.*, 2013). A level of greater than 10^4 cfu/g of STEC in faeces is generally considered as the definition of super shedding although 10^3 cfu/g or higher has also been considered as super shedding (Chase-Topping *et al.*, 2008). Both cattle and sheep have found to be super shedders of O157 STEC with studies enumerating $>10^4$ cfu/g of bacteria in faeces (Ogden *et al.*, 2005; Matthews *et al.*, 2013). Other serogroup strains which also have potential to be shed at high levels include O26

(McCabe *et al.*, 2019). There is evidence that wild deer can become super shedders of high levels of STEC O157 (>10^4 cfu/g faeces) meaning there is potential for high levels of contamination of meat at slaughter and processing (Matthews *et al.*, 2013; McNeilly *et al.*, 2020).

Animals may be re-infected and while it has been shown that cattle experimentally infected with STEC O157 shed lower levels of the same strain on re-infection, suggesting some immunity, this is not complete (Naylor *et al.*, 2007). Sheep experimentally infected with a Stx negative O157 strain generated humoral and cellular responses to bacterial antigen but were not protected from reinfection with the same strain (Vande Walle *et al.*, 2011). There is evidence that STEC produces factors including Stx which suppress the host immune response, thereby enabling it to persist in the host. Stx has been shown to inhibit *in vitro* bovine lymphocyte proliferation in response to mitogen stimulation (Menge *et al.*, 1999). Lymphocytes from calves inoculated with a Stx negative strain of O157 showed lymphoproliferation when challenged with heat killed O157 STEC compared to lymphocytes from calves inoculated with Stx positive O157 which did not proliferate (Hoffman *et al.*, 2006). There is also evidence that the LEE encoded protein EspB plays a role in suppressing cytokine responses in STEC colonised epithelial cell (Hauf and Chakraborty, 2003).

1.8.5 Risk factors for STEC carriage in ruminants

Various risk factors for STEC carriage and level of shedding have been investigated with studies most frequently involving cattle. While seasonal variations on prevalence and shedding levels have been identified in cattle, it is difficult to draw specific conclusions. For example, studies from Italy, Ireland and USA suggest higher prevalence of STEC in warmer months (Bonardi *et al.*, 1999; McEvoy *et al.*, 2003; Cobbold *et al.*, 2004), while, a study in Scotland and an experimental study in the USA found higher prevalence in winter (Ogden *et al.*, 2004; Sheng *et al.*, 2016). It is possible that observed differences in shedding or prevalence are due to management factors and/or environmental factors, both of which are linked to seasonality.

Management factors which may have an impact on STEC carriage include housing of animals, herd size, addition of animals to the herd, pasture management and diet. Larger herds have been observed to have greater prevalence of STEC (Herbert *et al.*, 2014; Widgren *et al.*, 2015; Henry *et al.*, 2019). This may be due to higher stocking density compared to smaller herds or more sources of potential infection. However, other factors

may confound this, farms with less than 100 animals were found to have higher risk of STEC infection possibly due to having less staff to carry out cleaning and hygiene measures (Cho *et al.*, 2013). In Scotland, farms producing beef cattle which also bought livestock other than cattle had lower incidence of STEC O157. It is possible that this indicates more extensive farms with lower stocking density which may reduce STEC transmission (Henry *et al.*, 2019). Changes in management have been correlated with increases in STEC O157 prevalence, for example, change of feed and weaning of calves (Lammers *et al.*, 2015). Movement of new animals into a herd has been identified as a potential risk factor that may increase STEC prevalence through exposure of uninfected animals to shedding animals and also through increased stress to animals (Chase-topping *et al.*, 2007). In contrast, moving cattle to new pasture without addition of new animals resulted in a decrease in STEC shedding (Lammers *et al.*, 2015).

Surveys and experimental studies have tested a range of dietary additions for association with STEC carriage. However, there is lack of agreement on dietary influences. It has been suggested that STEC may be more likely to persist in some animal food stuffs thereby increasing risk of infection (Herriott *et al.*, 1998). Diet may also have an effect by altering the gut microbiome. Lower gut microbial diversity has been linked to increased risk of colonisation with STEC O157 (Mir *et al.*, 2016). Dietary supplements such as monensin, seaweed and citrus oils in the form of orange pulp/peel may exert an effect on STEC carriage through directly inhibiting STEC growth or inhibition of growth of competing commensal bacteria (Jacob *et al.*, 2009).

Various environmental variables have been studied for their association with STEC shedding. Increasing rainfall has been found to be positively associated with shedding along with higher environmental temperature (Lammers *et al.*, 2015; Williams *et al.*, 2015; Dong *et al.*, 2017). Effects may be due to changes in animal behaviour increasing their likelihood of being infected or increased environmental persistence of STEC. Environmental conditions may impact on prevalence of STEC carriage by allowing STEC to persist in the environment thereby increasing exposure of animals to infection. STEC O157 has been shown to persist in soil and water for several months and strains re-isolated from water troughs were capable of re-infecting calves (LeJeune *et al.*, 2001; Franz *et al.*, 2011).

1.9 Control measures to prevent human STEC infections

As there are no specific treatments for STEC infections in humans, an effective control strategy is to prevent contamination of foodstuffs from the ruminant reservoir of infection. Processing of food in the UK is regulated by legislation to ensure the risks of microbiological hazards including STEC are minimised. The 2006 Food Hygiene (Scotland) Regulation implements the following EU regulations which cover hygiene of food stuffs and specific rules for food of animal origin:

Regulation (EC) No. 178/2002 – laying down the general principles and requirements of food law and procedures in matters of food safety, including establishing traceability of food producing animals.
Regulation (EC) No. 852/2004 – the hygiene of foodstuffs.
Regulation (EC) No. 853/2004 – specific hygiene rules for food of animal origin
Regulation (EC) No. 854/2004 – specific rules for the organisation of official controls on products of animal origin intended for human consumption

Specific rules cover food of animal origin and include identification of critical control points in processing where controls are necessary to prevent, eliminate or reduce hazards to an acceptable level (HACCP guidance). Examples include rejection of carcasses with faecal contamination and ensuring meat is chilled during processing, transport and storage (Food Standards Agency, 2019a). There is no specific routine testing for STEC serotypes in animal-derived foodstuffs in the UK or Europe although, in the US, testing of meat products for STEC of O-type O157, O26, O45, O103, O111, O121 and O145 is required (USDA, 2019). However, in the UK there are guidelines for acceptable generic *Escherichia coli* counts in meat and products from farmed food producing animals (EC Regulation 2073/2005). Generic *E. coli* counts indicate levels of faecal contamination present and therefore the potential for STEC contamination (Food Standards Agency, 2019b).

Producers are advised by Food Standards Scotland that if their products are likely to contain STEC this must be considered in a Food Safety Management System (FSMS) or Hazard Analysis and Critical Control Point (HACCP) plan. This includes animal and plant derived foodstuffs. Specific testing for STEC in foodstuffs may take place to validate FSMS or HACCP plans to demonstrate they are effective and working properly (Food Standards Scotland). Testing for STEC in food stuffs may also take place in the case of an outbreak where there is evidence that a particular foodstuff may be

contaminated. Scottish Government policy for testing of STEC in foodstuffs and actions to be taken if STEC is identified are outlined in the document 'Protecting consumers from infection with Shiga toxin-producing *E. coli* (STEC)'. If STEC presence in ready to eat food is confirmed by culture, the food must be withdrawn from sale or be further processed in a way that would inactivate STEC present before it can be sold. Food that is intended to be further processed by consumers in a way that that would render STEC non-infectious is permitted to be sold providing guidance is provided on safe handling and cooking.

Venison production is included in legislation requiring hygienic handling from point of cull to reaching consumers (The 2006 Food Hygiene (Scotland) Regulation). However, in contrast to meat from farmed livestock, there is no requirement for carcass bacterial counts. Venison is required to be processed by Approved Game Handling Establishments (AGHE) which comply with hygiene and traceability legislation (FSA, 2015). Exemptions from the requirement for AGHE processing are possible in cases where venison is for private consumption by the hunter, and when carcasses are supplied directly to the final consumer or to local retailer supplying final consumer. Additionally, hunters or estates supplying small quantities of processed venison to final consumers are exempt. In comparison to farmed animals for food production, initial processing of deer carcasses is relatively uncontrolled with culling and removal of the gastrointestinal tract undertaken outdoors, followed by transport of the carcass at ambient temperature, before the hide can be removed and chilling takes place (Best Practice Guidance on the Management of Wild Deer in Scotland). Good practice guidelines however recommend practice to follow to minimise risk - carcasses should be rejected for human consumption if there is sign of contamination of the meat, for example, by gut contents or if the animal is showing signs of disease (Best Practice Guidance on the Management of Wild Deer in Scotland).

Adequate cooking of meat products can kill any STEC present. Guidelines to consumers recommend cooking minced meat products to reach an internal temperature of 75ºC; however, steaks can be eaten rare as long as the external surface has been cooked (Food Standards Scotland, 2020a). Foods which are ready to eat and are not cooked prior to consumption (for example, salad crops, sprouted seeds and fruits) may present a higher risk of STEC infection if they become contaminated with animal faeces. Subsequent to an outbreak in Germany in 2011 of STEC O104:H4 which was associated with sprouted seeds, the EU implemented a requirement for testing sprouted seeds for O157, O26, O103, O111, O145, and O104:H4 (Buchholz *et al.*, 2011; European Food Safety Authority,

2011). Currently, this is the only legal microbiology criterion relating to STEC in food in the EU (Food Safety Authority of Ireland, 2019). An outbreak of STEC O157 associated with strawberries has been linked to deer faecal contamination (Laidler *et al.*, 2013). A recent study has shown that STEC can be internalized in plant tissue making it resistant to removal by washing (Merget *et al.*, 2019). The risk of contamination can be reduced by following best practice when using organic fertiliser on food crops - for example, treating animal waste used as fertiliser and avoiding the application on growing crops (Food Safety Authority of Ireland, 2016). Due to the ability of *E. coli* to persist in the environment, the possibility of contamination by wildlife such as by deer faeces remains.

1.10 Aims and objectives of project

Scotland has a relatively high rate of human STEC infections. In many cases, the source of infection is unknown and a wildlife reservoir such as deer could be a possible source. The aim of this project is to determine the potential contribution of Scottish wild deer to human STEC infections. From the recent study The Risk Of STEC Contamination In Wild Venison which investigated prevalence of STEC O157 in Scottish wild deer, it is known that 69.5 % of deer may be carrying STEC strains, as determined by *stx* PCR of faecal samples, which are of serogroups other than O157 (McNeilly *et al.*, 2020). Information on the serogroups of these strains, their virulence genes and also the subtypes of Stx present would enable assessment of their potential risk to human health and the dynamics of spread between deer and farmed livestock. Since Stx produced by STEC can be classified in subtypes of varying pathogenic potential, this study will test specifically for the presence of the subtype *stx2a* which is the *stx* subtype most often linked to severe human disease. Analysis will be undertaken within all *stx* positive faecal samples to identify associations between *stx2a* presence and factors such as deer age, species, sex, condition score, time of cull, environmental conditions and proximity of farmed livestock including cattle and sheep. This will allow a better understanding of the factors which drive *stx2a* selection within wild Scottish deer populations. This will inform possible control measures for prevention of more severe human STEC infections arising from wild deer, as well as providing information on whether *stx2a* positive strains are potentially circulating between deer and farmed livestock.

In parallel, this study will characterise STEC strains isolated from deer by whole genome sequencing to provide information on virulence factors, serotype and core genome MLST of the STEC isolates. This will allow a detailed comparison of Scottish wild deer STEC

isolates with Scottish human clinical isolates with the aim of determining if human non-O157 STEC isolates are genetically similar to strains found in deer, and by extension whether STEC strains found in wild Scottish deer pose a risk of causing disease in humans.

Objectives:

1. To determine prevalence and identify risk factors associated with the presence of highly pathogenic *stx2a* subtype of Stx in wild deer in Scotland. Factors tested will include deer age, species, sex, time of cull, environmental factors and proximity to cattle and sheep. The outcome of this will be to determine if transmission of *stx2a* positive strains may occur between farmed livestock and wild deer and to determine if there any factors which are associated with risk of *stx2a* presence.
2. To determine if deer non-O157 STEC strains represent a risk to human health by comparison of non-O157 STEC isolates from Scottish wild deer with Scottish non-O157 STEC human clinical strains.

Chapter 2: Risk factors associated with the presence of the *stx2a* gene in faecal samples from wild deer

2.1 Introduction

Ruminants are considered to be the main carriers of STEC and are capable of shedding high levels of pathogenic bacteria that either directly or indirectly can cause serious illness in humans. As a result many studies have focused on estimating the prevalence of STEC in cattle and finding risk factors associated with cattle carriage, particularly STEC O157. Many potential risk factors have been identified, however there is a lack of agreement across studies as to the main risk factors.

For STEC O157, seasonality in shedding has been observed in cattle. In a study of beef cattle herds in Scotland, more farms had animals shedding in autumn compared to summer (Synge *et al.*, 2003). A study of dairy farms in Scotland revealed a greater number of herds were positive for STEC O157 in autumn compared to spring, but there was not a significant seasonal effect for farms sampled in England and Wales (Henry *et al.*, 2019). Conversely, cattle in New Zealand had lower prevalence of STEC O157 in autumn compared to spring (Jaros *et al.*, 2016). Environmental temperature may play a role in seasonality, with increased overall prevalence of STEC observed in warm months (Fernández *et al.*, 2009). In addition to an effect on overall shedding of STEC, variations in *stx* gene subtypes in cattle have also been linked to temperature, with warmer temperatures associated with higher prevalence of *stx1* and *stx2* in combination compared to *stx1* alone (Fernández *et al.*, 2009).

Modelling cattle behaviour in warmer temperatures suggests there may be increased transmission due to cattle congregating in shade and increased grooming (Dawson *et al.*, 2018). Increased environmental temperature has been shown to increase numbers of *E. coli* in faecal pats, which may also influence growth of STEC, leading to higher contamination of pasture and increased likelihood of infection of grazing animals (Oliver and Page, 2016). Although specific STEC strains were not analysed, detectable levels of *E. coli* have been shown to persist in cow faecal pats for up to 98 days (Oliver and Page, 2016). STEC O157 is able to persist in experimentally infected water troughs for up to 6 months and was able to infect calves (LeJeune *et al.*, 2001). STEC O157 strains from cattle and humans have been shown to persist in soil for between 47 and 266 days (Franz *et al.*, 2011). STEC of the same serotype and virulence gene profile were isolated from

faecal samples of cattle and soil samples in their grazing area, demonstrating contamination of soil by grazing animals (Monaghan *et al.*, 2011). Additionally rainfall may cause leaching of O157:H7 from faeces onto pasture land, potentially increasing the risk of grazing animals becoming infected (Williams *et al.*, 2008). Increased rainfall has been identified as a risk factor for higher shedding of STEC O157 in cattle in Australia (Lammers *et al.*, 2015; Williams *et al.*, 2015).

Seasonal effects may be linked to management practices such as housing cattle during winter, a practice which has been associated with increased prevalence of STEC O157 (Synge *et al.*, 2003; Gunn *et al.*, 2007; Henry *et al.*, 2019). A study of beef cattle in Scotland found a higher prevalence of STEC O157 in winter when cattle are often housed (Ogden *et al.*, 2004). A study that compared two groups of STEC O157 naturally infected calves found that calves turned out to pasture cleared infection whereas penned animals remained infected (Jonsson *et al.*, 2001). Housing may lead to animals being in closer proximity and exposure to wet and dirty bedding material when housed has also been identified as a risk factor for STEC O157 carriage in young cattle (Ellis-Iversen *et al.*, 2007).

Farms with a high number of cattle have been identified as a risk factor for STEC O157 infection in a study of British beef farms and of beef and dairy herds in Sweden (Widgren *et al.*, 2015; Henry *et al.*, 2017). In addition to the effect of herd size on STEC O157 infection, a study of Scottish farms also identified movement of animals onto farms as an additional risk factor (Herbert *et al.*, 2014; Henry *et al.*, 2019). This is in agreement with surveys in Sweden which have identified animal movement onto farms and increased herd size as risk factors for a farm being positive for STEC O157 (Widgren *et al.*, 2015).

In cattle, younger animals have been reported as having a higher prevalence of STEC O157 compared to adults (Widgren *et al.*, 2018). Levels of STEC shedding in beef cattle calves decreased with increasing age (Mir *et al.*, 2016). Prevalence of animals shedding STEC O157 and non-O157 STEC has been observed to be higher in beef herds compared to dairy herds (Widgren *et al.*, 2015; Oporto *et al.*, 2019). However, this may be due to differences in management of the herds rather than animal differences. Various associations with diet have been noted in the literature, although some are contradictory. Cattle fed distillers' grains were more likely to shed STEC O157 in a study in Scotland (Synge *et al.*, 2003). Feeding of silage was associated with increased likelihood of STEC O157 shedding in an Australian study (Lammers *et al.*, 2015). Feeding of root crops was

identified as a risk factor for STEC O157 infection in young cattle in the UK, whereas feeding silage, milk or grain was associated with lower infection rates (Smith *et al.*, 2016).

There is limited information available to date on the factors influencing the carriage of STEC in deer. Repeat sampling of a population of white tailed deer in the USA found no STEC in 73 deer sampled in March compared to seven STEC isolated from 74 deer sampled in June, indicating possible seasonal variation in STEC prevalence (Singh *et al.*, 2015). Red deer in Spain were found to have higher levels of Stx positive faecal samples in areas of higher deer density (Diaz-Sanchez *et al.*, 2013).

No age or sex related differences in prevalence of STEC in deer were observed in studies in Switzerland (Obwegeser *et al.*, 2012), Spain (Diaz-Sanchez *et al.*, 2013) and Belgium (Bardiau *et al.*, 2010). Studies which sampled both roe and red deer also found no species differences in overall STEC prevalence (Bardiau *et al.*, 2010; Obwegeser *et al.*, 2012; Eggert *et al.*, 2013).

Few studies have investigated possible STEC transmission between deer and domestic livestock. Red deer have been shown to be more likely to be infected with STEC in areas of Spain which included cattle, sheep and goat farms. However, STEC isolates from deer and livestock were not compared (Diaz-Sanchez *et al.*, 2013). In another study in Spain, a comparison of a roe deer isolate and cattle isolate from co-grazing animals found 90 % similarity in PFGE profile (Mora *et al.*, 2012). In a study of white tailed deer co-grazing with cattle, one deer isolate had the same MLST as three cattle isolates from the same area (Singh *et al.*, 2015). The direction of transmission in each case is not known. Farming activities may indirectly affect movement of deer, which may contribute to spread of STEC between deer populations. A study tracking red deer in different habitats suggested that deer movement on managed land is influenced by human activity and season, with deer ranging over a larger area in winter compared to summer (Náhlik *et al.*, 2009). Geographical area of sampling may also be important due to its' influence on other factors such as cattle density and environmental temperature (Widgren *et al.*, 2018).

Studies of deer in Germany, Spain and Switzerland have detected *stx* genes in 83.3 %, 37.8 % and 35.2 % of deer faecal samples respectively (Obwegeser *et al.*, 2012; Diaz-Sanchez *et al.*, 2013; Eggert *et al.*, 2013). A survey funded by Food Standards Scotland (FSS) to determine the Risk of STEC Contamination in Wild Venison (McNeilly *et al.*, 2020) analysed 1087 faecal samples collected between August 2017 to June 2018 for presence of STEC O157 using IMS of which three samples were positive. DNA was prepared from faecal samples and subject to multiplex PCR testing for *stx1*, *stx2* and *eae*

genes. Results are shown in Table 2.1. Samples which contained STEC O157 (n=3), samples which were initially thought to contain STEC O157 but which further testing determined to be *stx* negative (n=4) and, samples where DNA preparation failed (n=3) are not included in Table 2.1. In total 1077 faecal DNA samples were analysed by multiplex PCR, revealing 69.5 % of samples were positive for *stx* genes, with 60.1 % positive for *stx2* either with *eae* (162 samples) or without *eae* (485 samples), suggesting they could contain highly pathogenic strains of non-O157 STEC.

Table 2.1 Number of samples which were positive for *stx1*, *stx2* and *eae* by multiplex PCR

	stx1+	***stx2+***	***stx1+/stx2+***	***stx negative***	**Total**
eae +	29	92	70	69	260
eae -	72	325	160	260	817
					1077

There is evidence that the presence of *stx* of subtype *stx2a* along with the adhesin *eae* are major risk factors for development of more severe forms of human disease including HUS (Franz *et al.*, 2015; Naseer *et al.*, 2017; De Rauw *et al.*, 2018; WHO-FAO, 2018). However, the presence of *stx2* and *eae* in the same bacteria cannot be ascertained from PCR testing of samples. It should also be noted that there are reported cases of severe infection associated with *stx2a* positive strains which are *eae* negative (Buchholz *et al.*, 2011; Franz *et al.*, 2015; Koutsoumanis *et al.*, 2020, personal communication Lesley Allison, Scottish *E. coli* reference laboratory (SERL)). Looking specifically at *stx2a* prevalence in deer, four studies detected no *stx2a* (Hofer *et al.*, 2012; Eggert *et al.*, 2013; Dias *et al.*, 2019; Frank *et al.*, 2019), while studies from Spain and Poland revealed a prevalence of 3.9 % (4/103 non-O157 STEC isolates) and 11.8 % (6/51 non-O157 STEC isolates) respectively (Mora *et al.*, 2012; Szczerba-Turek *et al.*, 2020). However no studies have looked at the prevalence of *stx2a* in Scottish deer.

As such the aims of this chapter were to determine the prevalence of *stx2a* present in Scottish wild deer faecal samples and identify potential risk factors for the presence of *stx2a* over other *stx* subtypes. A second aim was to address the specific hypothesis that deer in close proximity of cattle, the proposed main reservoir of STEC, are more likely to be *stx2a* positive and therefore of greater concern for causing severe human illness. Determining risk factors associated with *stx2a* in deer may allow improved tracing of the origins of human disease outbreaks associated with this Stx subtype and assist in

identifying the control measures that would most impact on reducing the risk of severe human infections. Studying the distribution of *stx2a* positive samples may inform how *stx2a* positive strains spread within ruminant populations in Scotland.

2.2 Materials and Methods

2.2.1 Construction of database using questionnaire data gathered at point of culling of deer

Deer faecal samples were collected as part of a larger cross-sectional survey funded by Food Standards Scotland (FSS) to determine the Risk of STEC Contamination in Wild Venison (McNeilly *et al.*, 2020). Sample packs, instructions and questionnaires were distributed throughout Scotland to stalkers from Deer Management Groups (DMG) and Forestry and Land Scotland between August 2017 to June 2018. Sampling aimed to capture representative numbers of deer culled for the human food chain in Scotland. Proportions of the different species of deer culled are relatively stable from year to year and in the 2016 to 2017 hunting season this consisted of 55 % red deer and 37 % roe deer, with the remaining made up of sika and fallow (The Management of Wild Deer in Scotland: Deer Working Group report, 2020). The sampling strategy for deer was designed to sample similar proportions of wild deer species between 2017-2018 as those culled during the 2016-2017 hunting season.

Sampling packs contained sterile pots for faecal collection, gloves and instructions for collection method of faecal samples to avoid cross contamination. Faecal samples were collected directly from the rectum of deer after culling. Stalkers also completed a questionnaire form at the time of sampling recording the 6-figure grid reference of the cull site, date and time of cull, sex of deer, species of deer, condition score of deer, estimated age of deer, and details of co-grazing with other herbivores. Additionally, some stalkers provided information on carcass weight. A copy of the questionnaire is included in Appendix 1. Faecal samples and questionnaires were returned to the Moredun Research Institute - stalkers were asked to return samples on day of sampling or store them at 4^{o}C until they could be posted. On receipt at Moredun Research Institute samples were stored at 4^{o}C and the majority of samples were processed within 24 hours of being received.

Data collected from questionnaires were entered into an Excel spreadsheet. Location of the cull site obtained from the questionnaire was used to extract data relating to the nearest farm, defined as any farm holding regardless of type of livestock present, and the nearest

farm with cattle (both calculated as a straight line distance in metres). Data was also extracted for the species present on the nearest farm (i.e. number of cattle, sheep, pigs or poultry). Data relating to distance to nearest farm and species on farm was supplied by Paul Bessell (personal communication).

Sampling locations were mapped using QGIS (Quantum Geographical Information System) Version: QGIS Browser 2.18.26 with GRASS 7.4.2. Using the sampling location, further information was obtained including land cover, temperature, and cattle, sheep and deer density, and samples were assigned a categorical variable of geographical area of sampling. Information from the Land Cover Map 2015 was derived for the 1km square surrounding the cull site. Two variables were derived from the Land Cover Map - the categorical variable of overall dominant land cover type (Rowland *et al.*, 2017a) and also a continuous variable of percentage of each of the 6 land cover types in the 1km square (Rowland *et al.*, 2017b). The 6 land cover types used in this study and their description are shown in Table 2.2.

Table 2.2 Land cover variables adapted from LCM2015* dataset documentation

LCM2015 Cover Type	Description
Arable	Includes annual and perennial crops and ploughed land
Coniferous forest	Includes semi-natural stands and plantations
Broadleaf woodland	Vegetation dominated by trees including native and non-native broadleaved trees and yew
Improved grassland	Characterised by a few fast-growing grasses on fertile soils, typically managed as pasture or for silage production
Semi-natural grassland	Lower production than improved grassland, wider variety of plant species
Mountain, heath, bog	Inland rock, bog and heath

* Land Cover Map 2015, NERC

Environmental and temperature data was derived from the UK Met Office data and included Minimum, Maximum and Mean temperature, numbers of days of frost and rain, and hours of sunshine by region (North, West and East Scotland) and for whole country for month or season (UK Met Office, 2018). Cattle and sheep density as animals per 10km square area were obtained from Agriculture Census data for 2015 (EDINA at Edinburgh University Data Library and The Scottish Government). Densities of red and roe deer populations were determined by Massimino *et al* (2018) using data from the British Mammal Survey. Density maps supplied by Dario Massimino were used to derive deer densities at the cull site using QGIS. All the variables used in the risk factor analysis for presence of *stx2a* are listed in Appendix 2. Statistical analysis of the database was completed using R version 3.5.1 (2018-07-02) (R Core Team, 2013).

2.2.2 PCR to detect presence of *stx2a* gene in deer faecal samples

All *stx2* positive samples from the cross-sectional survey to determine the Risk of STEC Contamination in Wild Venison were tested in a *stx2a* specific PCR to determine the number of samples which were positive for this highly pathogenic subtype of *stx2*. Primer sequences were obtained from a method used by Wang *et al.* (2002). Primers sequences and product sizes are shown in Table 2.3. Primers were first tested with DNA from strains of known subtype to ensure specificity for *stx2a*. PCR reactions of 20 µl final volume contained 2 µl template DNA, GoTaq Colourless buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer and 0.5 units of GoTaq G2 DNA polymerase (Promega). The thermal cycling profile involved a 2 minute pre-incubation step of 95 ºC followed by 30 cycles consisting of denaturation at 95 ºC for 30 seconds, primer annealing at 50 ºC for 1 minute, extension at 72 ºC for 1 minute, and a final extension

step at 72 ºC for 5 minutes. PCR products were analysed by capillary electrophoresis using the Qiaxcel Advanced System (Qiagen).

Table 2.3 Details of primers used for *stx2a* PCR

***stx2a* PCR**	**Primer sequence (5' →3')**	**Product size (b.p)**
stx2a Forward	GCGGTTTTATTTGCATTAGC	115
stx2a Reverse	TCCCGTCAACCTTCACTGTA	

2.2.3 Analysis and selection of individual variables for inclusion in model

Determination of potential risk factors for the presence of *stx2a* was performed using a generalised linear model (glm) with a logistic link function (Brown and Prescott, 2006). Use of a glm with logistic link function allows the modelling of associations between categorical and continuous predictor variables with a binary response variable such as the presence/absence of *stx2a*. In addition, a glm can be constructed with continuous variables which are non-normally distributed. This is advantageous for inclusion of count data such as the number of animals on a farm (Bolker *et al.*, 2009). The output of interest from a glm model is an odds ratio. The odds ratio is the measure of association. It quantifies the relationship between an exposure (such as cattle density) and the presence of *stx2a*. The higher the odds ratio (along with significant p value < 0.5) the greater the association between the presence of *stx2a* and the exposure. An odds ratio of 1.0 (or close to 1.0) indicates that the exposure is not associated with *stx2a*. An odds ratio greater than 1.0 indicates that the odds of exposure might be a risk factor for the presence of *stx2a*. An odds ratio less than 1.0 indicates that the exposure might be a protective factor against the presence of *stx2a*.

All variables associated with the culled deer were initially analysed individually in a glm (univariable analysis). Shared range with other herbivores was not analysed as it was decided that this measure was subjective and open to different interpretation. Instead possible effects of shared range were investigated through variables related to cattle density, sheep density and distance to nearest farm. Cut-offs for categorising low and high cattle and sheep density were chosen based on mean density and are shown in Appendix 3. Variables with significance in a univariable glm of $p \leq 0.2$ were selected for inclusion in the multi-variable glm. The selected cut-off of $p \leq 0.2$ is arbitrary, although a similar approach using a cut-off value of 0.2 to 0.3 has been used in other risk factor studies relating to STEC (Halliday *et al.*, 2006; Herbert *et al.*, 2014). Variables were added to the model in order of decreasing significance (i.e. from low to high p value).

Prior to model construction, the correlation among all explanatory variables was determined. Categorical variables were checked for correlation using Cramer's V and continuous variables were checked for correlation using a Spearmann Rank correlation. Significance of dichotomous variables (categorical variable with only two categories) and continuous variables was checked using logistic regression. Significance with a categorical variable with > 2 levels was assessed using Kruskall Wallis test. For any pairs of variables with a correlation greater than 0.6 (Cramer's V or Spearman Rank) or a significant association ($p < 0.05$; logistic regression or Kruskall Wallis), only one variable was retained for addition to the model. This avoided collinearity in the final model, whereby more than one variable is explaining the same variation in the output variable. The variable chosen was the one with the lowest AIC (Akaike Information Criteria). A lower AIC indicates a more parsimonious model, relative to a model fit with a higher AIC.

A full model which included all deer species (red, roe and sika) was run initially. As roe deer had the highest number of *stx2a* positive samples (Table 2.4), a second model which only included roe deer, was run to determine non-species dependent effects. For the roe deer only model, sampling area categories were combined as described in Appendix 2 to create north and south due to low number of observations in some categories. For both multivariable models, variables were then added to the model by forward stepwise addition method, in order of increasing *p* - value. The model was assessed after each variable addition for a reduction in the AIC value. Variables that resulted in a reduction in AIC were retained in the model. The forward stepwise method of model construction favours a simple model with the minimal number of variables required to explain the observed results. Odds ratios (OR) and 95 % confidence intervals (CI) were calculated to determine the strength of association of each variable retained in the model with the presence of *stx2a*.

All models were run using R Version 3.5.1 (2018-07-02) using package MASS for model construction. A *p* value < 0.05 was considered significant. Assessment of the final model was made by Chi squared ($\chi 2$) test, comparing model prediction with actual values. A significant *p* value in this test indicates that prediction of samples being *stx2a* positive is significantly better than would be expected from chance.

2.3 Results

2.3.1 Results from *stx2a* PCR of *stx2* positive deer faecal samples

All faecal samples which were s*tx2* positive in multiplex PCR (Table 2.1), which represented 647 out of 1077 samples analysed by PCR in the previous STEC O157 prevalence study (McNeilly *et al.*, 2020), were tested for *stx2a* in a separate PCR with the aim of determining the prevalence of *stx2a* genes in deer faecal samples. The results for all samples which were *stx2a* positive are shown in Table 2.4. This table also included the three samples which were positive for STEC O157 in the previous study, as all three STEC O157 isolates were *stx2a* positive. In total, 92 samples were positive for *stx2a* and 657 samples were positive for *stx1* or a *stx2* subtype other than *stx2a*.

Table 2.4 Presence of *stx2a* in deer faecal samples shown for each deer species

***Stx* PCR results**	**Fallow**	**Red**	**Roe**	**Sika**
*Positive for *stx2a*	1	12	76	3
†Positive for *stx1* or a *stx2* subtype other than *Stx2a*	11	360	199	87
Total	**12**	**372**	**275**	**90**

*data obtained from this study
†data obtained from the STEC O157 prevalence study (McNeilly *et al.*, 2020)

2.3.2 Selection of samples for *stx2a* risk factor analysis

As the aim of this analysis was to determine factors associate with the presence of *stx2a* over all other *stx* subtypes, samples which were *stx* negative in the previous study (329), (McNeilly *et al.*, 2020) were excluded from glm analysis. Samples obtained from fallow deer were also excluded due to the small number of *stx2a* positive samples in this species (1/12) and two samples where species of deer was unknown were also excluded. This left a total of 737 of the 1087 total samples (68 %) from the previous study for statistical analysis. Of these 737 samples some were missing information on condition score of deer (31 samples), sex of deer (7 samples), estimated age of deer (12 samples), and carcass weight (412 samples), so these samples were excluded when these variables were analysed. Information on carcass weight was not specifically requested and therefore was not included on a large number of questionnaires.

2.3.3 Univariable analysis of risk factors for *stx2a* presence in samples from red, roe and sika deer

Categorical and continuous variables related to red, roe and sika deer were modelled using a glm with logistic link function in order to assess associations of individual variables with presence of *stx2a*. Univariable results for the categorical variables and continuous variables analysed for red deer, roe deer and sika deer are shown in Table 2.5 and Table 2.6, respectively. Variables which had a significant association in a glm as determined by a p value < 0.05 are shown in bold and variables with $0.2 \geq p > 0.05$ are shown in italics.

Variables with a significant positive association with *stx2a* were High sheep density, High cattle density, Species: roe, and Sampling area: 'South East' and 'South West' (Table 2.5). Of the continuous variables Sheep density, Cattle density, Number of sheep on nearest farm, Roe deer density, % Coniferous land cover and Condition score were significantly positively associated with *stx2a* (Table 2.6). Two variables related to land cover: Mountain, heath, bog and Semi-natural grassland had a significant negative association with *stx2a* (Table 2.5). Continuous variables Distance to nearest farm with cattle, Distance to nearest farm, Red deer density, Total no. of raindays in season, % Mountain, health, bog, Carcass weight and Age were significantly negatively associated with *stx2a* (Table 2.6).

Table 2.5 Univariable binomial regression analysis for categorical variables red, roe and sika deer

Variable*	no. *stx2a* negative	no. *stx2a* positive	Odds ratio	lower CI	upper CI	*p*-value
Livestock density						
Low sheep density (ref.)	535	39				
High sheep density	**111**	**52**	**6.43**	**4.05**	**10.21**	**< 0.001**
Low cattle density (ref.)	409	15				
High cattle density	**237**	**76**	**8.74**	**4.91**	**15.56**	**< 0.001**
Temperature						
Min temp region < 7 ˚C (ref.)	532	70				
Min temp region > 7 ˚C †	*114*	*21*	*1.40*	*0.83*	*2.37*	*0.212*
Max temp region < 7 ˚C (ref.)	334	41				
Max temp region > 7 ˚C	312	50	1.31	0.84	2.03	0.236
Mean temp region < 7 ˚C (ref.)	369	50				
Mean temp region > 7 ˚C	277	41	1.09	0.70	1.70	0.695
Cold months (ref.)	370	52				
Warm months	276	39	1.01	0.65	1.57	0.981
Species						
Red (ref.)	360	12				
Roe	**199**	**76**	**11.46**	**6.08**	**21.58**	**< 0.0001**
Sika	87	3	1.03	0.29	3.75	0.959
Sex						
Sex female (ref.)	323	50				
Sex male	316	41	0.84	0.54	1.30	0.433
Land cover						
Arable (ref.)	12	4				
Mountain, heath, bog	**97**	**3**	**0.09**	**0.02**	**0.39**	**0.0012**
Semi natural grassland	**89**	**7**	**0.24**	**0.06**	**0.93**	**0.0386**
Broadleaf	13	1	0.23	0.02	2.37	0.2169
Improved Grassland	41	7	0.51	0.13	2.05	0.3443
Coniferous	326	67	0.62	0.19	1.97	0.4146
Sampling area						
Central (ref.)	102	7				
SouthEast	59	29	**7.16**	**2.95**	**17.36**	**< 0.0001**
SouthWest	69	36	**7.60**	**3.20**	**18.06**	**< 0.0001**
NorthEast	*66*	*10*	*2.21*	*0.80*	*6.09*	*0.1259*
NorthWest	*350*	*9*	*0.37*	*0.14*	*1.03*	*0.0573*
Age category						
Calf (ref.)	64	9				
Yearling	95	16	1.20	0.50	2.88	0.687
Adult	477	64	0.95	0.45	2.01	0.902
Season						
Summer (ref.)	21	1				
Autumn	338	46	2.86	0.38	21.73	0.3105
Winter	223	34	3.20	0.42	24.59	0.2631
Spring	64	10	3.28	0.40	27.14	0.2706

*Variables are described in Appendix 2. Categories for cattle and sheep density are described in Appendix 3. Significant *p* values (<0.05) are highlighted in bold, non-significant variables with $0.2 \geq p > 0.05$ are shown in italics, ref. – reference variable. Odd Ratios relate to presence of *stx2a* over other *stx* subtypes. CI – confidence interval. † although this variable was above the cut-off value of 0.2 it was included in model construction because previous studies have shown an effect of temperature on STEC carriage; therefore it was biologically relevant to include it in this study.

Table 2.6 Univariable binomial regression analysis of continuous variables for red, roe and sika deer

	***stx2a* negative**		***stx2a* positive**					
Variable*	**Mean**	**SD**	**Mean**	**SD**	**Odds Ratio†**	**lower CI**	**upper CI**	**P-value**
Livestock density								
Sheep density	**4.49**	**0.99**	**5.38**	**1.12**	**2.1550**	**1.7473**	**2.6579**	**< 0.0001**
Cattle density	**3.98**	**1.91**	**5.63**	**1.60**	**1.5916**	**1.3977**	**1.8125**	**< 0.0001**
Distance to nearest farm with cattle (m)	**4075.27**	**3856.69**	**2670.42**	**2058.21**	**0.9998**	**0.9998**	**0.9999**	**0.0011**
Distance to nearest farm (m)	**2489.78**	**2143.30**	**1740.26**	**1377.14**	**0.9998**	**0.9996**	**0.9999**	**0.0016**
No. of cattle on nearest farm with cattle	*70.97*	*93.74*	*87.87*	*102.86*	*1.0017*	*0.9996*	*1.0037*	*0.1150*
No. of sheep on nearest farm with cattle	*508.10*	*754.10*	*639.93*	*864.95*	*1.0002*	*0.9999*	*1.0005*	*0.1280*
No. of sheep on nearest farm	**221.93**	**531.80**	**398.68**	**886.95**	**1.0004**	**1.0001**	**1.0007**	**0.0094**
No. of cattle on nearest farm	23.75	68.54	26.08	68.15	1.0005	0.9974	1.0035	0.7610
No. of pigs on nearest farm	0.37	3.70	0.75	5.66	1.0176	0.9757	1.0614	0.4160
No. of poultry on nearest farm	38.52	528.49	9.76	61.64	0.9996	0.9980	1.0012	0.6650
Deer density								
Red deer density	**3.13**	**1.64**	**1.44**	**1.01**	**0.4383**	**0.3533**	**0.5437**	**< 0.0001**
Roe deer density	**3.20**	**1.29**	**3.89**	**0.92**	**1.6504**	**1.3408**	**2.0315**	**< 0.0001**
Deer variables								
Carcass weight (kg)	**22.77**	**13.66**	**14.86**	**6.78**	**0.9182**	**0.8786**	**0.9595**	**0.0001**
Age (years)	**3.95**	**3.09**	**2.64**	**1.65**	**0.8181**	**0.7368**	**0.9083**	**0.0002**
Condition score	**3.57**	**1.11**	**3.93**	**1.06**	**1.3696**	**1.1035**	**1.6998**	**0.0043**

Table 2.6 continued

	***stx2a* negative**		***stx2a* positive**					
Variable*	**Mean**	**SD**	**Mean**	**SD**	**Odds Ratio†**	**lower CI**	**upper CI**	**P-value**
Temperature/environmental conditions								
Total no. of raindays in month	18.36	3.56	18.36	3.44	1.000	0.940	1.064	0.9974
Total no. of raindays in season	**55.61**	**11.81**	**51.14**	**9.78**	**0.9682**	**0.9502**	**0.9865**	**0.0007**
Hours of sunshine in month	*69.35*	*44.49*	*75.76*	*43.20*	*1.0030*	*0.9985*	*1.0075*	*0.1990*
Hours of sunshine in season	227.21	101.96	221.92	96.49	0.999	0.997	1.002	0.6410
Minimum monthly temperature (˚C)	3.09	3.56	3.28	3.58	1.0157	0.9551	1.0802	0.6200
Maximum monthly temperature (˚C)	8.82	4.08	9.23	4.06	1.0244	0.9712	1.0805	0.3750
Mean monthly temperature (˚C)	5.97	3.80	6.27	3.82	1.0210	0.9640	1.0814	0.4770
No. of days frost in month	7.66	6.74	7.66	6.90	1.0001	0.9681	1.0331	0.9960
Land cover								
% Arable	*1.72*	*9.21*	*3.40*	*12.55*	*1.0135*	*0.9959*	*1.0315*	*0.1340*
% Mountain, heath, bog	**27.04**	**34.84**	**7.81**	**17.60**	**0.9707**	**0.9581**	**0.9834**	**< 0.0001**
% Semi natural grassland	*15.30*	*27.69*	*10.56*	*20.01*	*0.9923*	*0.9827*	*1.0020*	*0.1190*
% Broadleaf	4.27	9.71	4.08	9.09	0.9979	0.9749	1.0214	0.8580
% Improved grassland	6.75	14.96	8.32	17.96	1.0061	0.9930	1.0194	0.3630
% Coniferous	**42.75**	**37.97**	**65.24**	**34.06**	**1.0164**	**1.0100**	**1.0229**	**< 0.0001**

*Variables are described in Appendix 2. Cattle and sheep density categories were assigned values from 1 (≤50 animals / 10km^2) to 7 (>20000 sheep / km^2 or >2000 cattle / km^2). Density categories 1 - 7 were analysed as a continuous variable. Further details of cattle and sheep density categories are described in Appendix 3. Red and roe deer density categories were assigned values from 1 (≤0.5 red deer / km^2 or ≤0.1 roe deer / km^2) to 5 (> 5 red deer / km^2 or >1 roe deer / km^2). Density categories 1 - 5 were analysed as a continuous variable. Further details of red and roe deer density categories are described in Appendix 4. Significant *p* values (<0.05) are highlighted in bold. variables with $0.2 \geq p > 0.05$ are shown in italics. † Odd Ratios relate to presence of *stx2a* over other *stx* subtypes. SD – standard deviation, CI – confidence interval.

2.3.4 Multivariable analysis of risk factors for presence of *stx2a* in samples from red, roe and sika deer

Variables which were chosen on the basis of likelihood ratio test with $p < 0.2$ which were included in the forward addition model construction were; Distance to nearest farm with cattle, Distance to nearest farm, Species, Cattle density, Sheep density, % Mountain, heath, bog, % Coniferous forest, Age (years), Total numbers of raindays in season, Condition score, Number of sheep on nearest farm, % Semi natural grass land, Number of cattle on nearest farm with cattle, Number of sheep on nearest farm with cattle, % Arable and Hours of sunshine in month. The variable: Minimum temperature greater than 7 ºC, which at $p = 0.212$ was above the cut-off of $p \leq 0.2$, was included as previous studies have found an effect of temperature on STEC carriage in other ruminants (Fernández *et al.*, 2009; Venegas-Vargas *et al.*, 2016; Dong *et al.*, 2017). High sheep density, High cattle density, Land cover, Sampling area, Red deer density, Roe deer density and Carcass weight were not used as they were correlated with other variables used in model construction.

After forward selection, variables which remained significant in the model were Species: roe and Sheep density (Table 2.7). Samples obtained from roe deer and increasing sheep density were both associated with a sample being positive for *stx2a*. Roe deer were almost eight times more likely to be positive for *stx2a* than red deer (odds ratio (OR) = 7.8, CI = 3.8-15.9). Increasing sheep density was associated with increasing likelihood of a sample being *stx2a* positive (OR = 1.3, CI = 1.0-1.7). The full model was statistically significant ($\chi 2 = 100.1$, DF = 3, $p = < 0.001$).

Table 2.7 Significant results from multivariable model of red, roe and sika deer *stx2a* positive samples *vs* other *stx* subtypes

Variable	Estimate	SE	P-value	OR (CI)
Intercept	-4.620	0.606	< 0.0001	
Species red	Baseline	-	-	1.000
Species roe	2.053	0.365	< 0.0001	7.788 (3.808 - 15.927)
Species sika	-0.096	0.662	0.8843	0.908 (0.248 – 3.322)
Sheep density	0.295	0.127	0.0199	1.343 (1.048 - 1.721)

Red deer are the reference category for Species

OR - Odds Ratio, CI – 95 % Confidence Interval

2.3.5 Univariable analysis of risk factors for presence of *stx2a* in roe deer samples.

The majority of *stx2a* positive samples (76 of a total of 92) were from roe deer (Table 2.4). To try to further elucidate environmental factors influencing presence of *stx2a,* roe deer were analysed separately from the combined data set of red, roe and sika deer samples. Categorical (Table 2.8) and continuous (Table 2.9) variables which had a significant association in a glm as determined by a p value < 0.05 are shown in bold. Categorical variables which were significantly associated with faecal samples being *stx2a* positive were High sheep density and Sampling area: South, no categorical variables were significantly negatively associated with *stx2a* (Table 2.8). Continuous variables which had significant association with *stx2a* positive samples were sheep density, Distance to nearest farm with cattle and % Semi natural grassland (Table 2.9). Red deer density and % Improved grassland were both significantly negatively associated with *stx2a* (Table 2.9).

Table 2.8 Univariable binomial regression analysis for categorical variables roe deer

Variable*	no. *stx2a* negative	no. *stx2a* positive	Odds ratio	lower CI	upper CI	*p*-value
Livestock density						
Low sheep density (ref.)	110	25				
High sheep density	**89**	**51**	**2.52**	**1.45**	**4.39**	**0.001**
Low cattle density (ref.)	38	9				
High cattle density	*161*	*67*	*1.76*	*0.81*	*3.83*	*0.157*
Temperature						
Min temp region < 7 ˚C (ref.)	171	62				
Min temp region > 7 ˚C	28	14	1.38	0.68	2.79	0.371
Max temp region < 7 ˚C (ref.)	117	38				
Max temp region > 7 ˚C	*82*	*38*	*1.43*	*0.84*	*2.43*	*0.189*
Mean temp region < 7 ˚C (ref.)	133	47				
Mean temp region > 7 ˚C	66	29	1.24	0.72	2.15	0.437
Cold months (ref.)	134	49				
Warm months	65	27	1.14	0.65	1.98	0.653
Sex						
Sex female (ref.)	117	46				
Sex male	80	30	0.95	0.56	1.64	0.864
Land cover						
Arable (ref.)	12	4				
Mountain, heath, bog	11	2	0.55	0.08	3.59	0.529
Semi natural grassland	*7*	*7*	*3.00*	*0.64*	*14.02*	*0.163*
Broadleaf	3	1	1.00	0.08	12.56	1.000
Improved grassland	21	4	0.57	0.12	2.71	0.481
Coniferous	144	58	1.21	0.37	3.90	0.752
Sampling area						
Sampling area North (ref.)	96	12				
Sampling area South	**103**	**64**	**4.97**	**2.53**	**9.78**	**< 0.001**
Age category						
Calf (ref.)	18	9				
Yearling	37	13	0.70	0.25	1.95	0.498
Adult	141	52	0.74	0.31	1.74	0.488
Season						
Summer (ref.)	9	1				
Autumn	*75*	*34*	*4.08*	*0.50*	*33.49*	*0.191*
Winter	78	31	3.58	0.43	29.43	0.236
Spring	37	10	2.43	0.27	21.53	0.424

*Variables are described in Appendix 2. Categories for cattle and sheep density are described in Appendix 3. Significant *p* values (<0.05) are highlighted in bold. Variables with $0.2 \geq p > 0.05$ are shown in italics. ref. – reference variable. Odd Ratios relate to presence of *stx2a* over other *stx* subtypes. CI – confidence interval

Table 2.9 Univariable binomial regression analysis of continuous variables for roe deer

	***stx2a* negative**		***stx2a* positive**					
Variable	**Mean**	**SD**	**Mean**	**SD**	**Odds Ratio†**	**lower CI**	**upper CI**	***p*-value**
Livestock density								
Sheep density	**5.28**	**1.09**	**5.61**	**1.05**	**1.3583**	**1.0382**	**1.7770**	**0.0256**
Cattle density	5.65	1.75	5.75	1.45	1.0379	0.8833	1.2197	0.6509
Distance to nearest farm with cattle (m)	**2089.80**	**1510.09**	**2521.22**	**1460.60**	**1.0002**	**1.0000**	**1.0004**	**0.0362**
Distance to nearest farm (m)	1467.04	1140.64	1641.93	1189.37	1.0001	0.9999	1.0003	0.2630
No. of cattle on nearest farm with cattle	102.60	114.13	87.22	96.65	0.9986	0.9960	1.0012	0.3000
No. of sheep on nearest farm with cattle	*586.69*	*813.72*	*746.71*	*908.59*	*1.0002*	*0.9999*	*1.0005*	*0.1610*
No. of sheep on nearest farm	*303.16*	*699.30*	*443.39*	*946.01*	*1.0002*	*0.9999*	*1.0005*	*0.1840*
No. of cattle on nearest farm	*37.68*	*90.11*	*22.13*	*58.18*	*0.9971*	*0.9930*	*1.0013*	*0.1740*
No. of pigs on nearest farm	0.93	6.50	0.89	6.19	0.9991	0.9583	1.0417	0.9680
No. of poultry on nearest farm	74.84	852.72	11.30	67.38	0.9993	0.9952	1.0033	0.7250
Deer density								
Red deer density	**1.60**	**1.11**	**1.22**	**0.70**	**0.6180**	**0.4297**	**0.8889**	**0.0095**
Roe deer density	4.04	0.86	3.89	0.86	0.8294	0.6124	1.1235	0.2270
Deer variables								
Carcass weight (kg)	13.43	2.58	13.52	2.80	1.0132	0.9017	1.1385	0.8260
Age (years)	2.56	1.45	2.38	1.27	0.9050	0.7414	1.1046	0.3261
Condition score	3.81	1.03	3.91	0.98	1.0998	0.8427	1.4353	0.4838

Table 2.9 continued

	***stx2a* negative**		***stx2a* positive**					
Variable*	**Mean**	**SD**	**Mean**	**SD**	**Odds Ratio†**	**lower CI**	**upper CI**	***p*-value**
Temperature/ environmental conditions								
Total no. of raindays in month	*17.30*	*4.12*	*18.18*	*3.63*	*1.0601*	*0.9881*	*1.1374*	*0.1038*
Total no. of raindays in season	48.98	11.19	49.84	9.47	1.0075	0.9829	1.0326	0.5530
Hours of sunshine in month	82.46	54.58	76.11	45.68	0.9975	0.9922	1.0029	0.3685
Hours of sunshine in season	*251.88*	*127.24*	*226.73*	*104.71*	*0.9982*	*0.9958*	*1.0005*	*0.1280*
Minimum monthly temperature (˚C)	2.35	3.33	2.79	3.42	1.0390	0.9610	1.1234	0.3360
Maximum monthly temperature (˚C)	8.42	4.23	8.73	3.95	1.0180	0.9556	1.0844	0.5807
Mean monthly temperature (˚C)	5.39	3.74	5.77	3.68	1.0276	0.9578	1.1026	0.4480
Frostdays region	9.21	6.68	8.49	6.76	0.9839	0.9457	1.0237	0.4227
Land cover								
% Arable	5.01	15.92	3.88	13.65	0.9949	0.9765	1.0135	0.5870
% Mountain heath bog	*8.42*	*18.51*	*5.00*	*13.20*	*0.9854*	*0.9658*	*1.0055*	*0.1530*
% Semi natural grassland	**6.48**	**13.88**	**12.37**	**21.40**	**1.0197**	**1.0044**	**1.0352**	**0.0115**
% Broadleaf	*5.37*	*10.87*	*3.28*	*8.49*	*0.9766*	*0.9466*	*1.0076*	*0.1380*
% Improved grassland	**11.35**	**17.54**	**6.55**	**15.53**	**0.9808**	**0.9626**	**0.9993**	**0.0419**
% Coniferous	*61.75*	*34.61*	*68.41*	*32.06*	*1.0060*	*0.9979*	*1.0142*	*0.1480*

*Variables are described in Appendix 2. Cattle and sheep density categories were assigned values from 1 (≤50 animals / 10km^2) to 7 (>20000 sheep / km^2 or >2000 cattle / km^2). Density categories 1 - 7 were analysed as a continuous variable. Further details of cattle and sheep density categories are described in Appendix 3. Red and roe deer density categories were assigned values from 1 (≤0.5 red deer / km^2 or ≤0.1 roe deer / km^2) to 5 (> 5 red deer / km^2 or >1 roe deer / km^2). Density categories 1 - 5 were analysed as a continuous variable. Further details of red and roe deer density categories are described in Appendix 4. Significant *p* values (<0.05) are highlighted in bold. variables with $0.2 \geq p > 0.05$ are shown in italics. † Odd Ratios relate to presence of *stx2a* over other *stx* subtypes. SD – standard deviation, CI – confidence interval

2.3.6 Multivariable analysis: Roe deer model

Variables which were selected to include in the Roe deer only model were: Distance to nearest farm with cattle, Sampling area South, High sheep density, Red deer density, % Semi natural Grassland, Sheep density, % Improved grassland, Total number of raindays in month, % Broadleaf, % Mountain, heath, bog, Hours of sunshine in season, % Coniferous forest, Number of sheep on nearest farm with cattle, Max temp region > 7 ºC and Number of sheep on nearest farm. High cattle density was excluded from model construction due to a low number of observations in one category and Number of cattle on nearest farm was excluded from model construction due to a high proportion of zero values. Categorical variables for Land Cover and Season were excluded due to correlation with other variables used in model construction.

Results for the roe deer only model are shown in Table 2.10. Sampling area South, % Semi natural grassland and Total no. of raindays in month had significant positive association with a sample being *stx2a* positive. Roe deer in the south were over 5 times more likely to be *stx2a* positive (OR = 5.49, CI = 2.73-11.04). Increasing % Semi-natural grassland was associated with increasing likelihood of a sample being *stx2a* positive as was increasing number of raindays in month of sampling. The full model was statistically significant ($\chi 2 = 37.35$, DF = 3, $p < 0.001$).

Table 2.10 Significant results from multivariable model of roe deer *stx2a* positive samples *vs* other *stx* subtypes

Variable	Estimate	SE	p-value	OR (CI)
Intercept	-3.717	0.793	< 0.0001	
Sampling area South	1.704	0.356	< 0.0001	5.49 (2.73 – 11.04)
% Semi natural grassland	0.020	0.008	0.016	1.02 (1.00 - 1.04)
Total no. of raindays in month	0.078	0.039	0.0424	1.08 (1.00 - 1.17)

2.4 Discussion

2.4.1 Prevalence of *stx2a*

The prevalence of *stx2a* in this study was found to be 12 % of all *stx*-positive samples from fallow, red, roe and sika deer. Previous studies of deer in Spain and Poland have found that the most common Stx subtypes identified in deer STEC isolates are *stx2b* and *stx1c* and that the prevalence of *stx2a* in deer is low (Mora *et al.*, 2012; Szczerba-Turek *et al.*, 2020). However, these studies were limited by recovery of low numbers of isolates and the challenges of isolating non-O157 STEC. As such, the incidence of *stx2a* positive

strains may have been underestimated. Four studies, one of which was based on only six isolates, found no *stx2a* positive isolates from deer (Dias *et al.*, 2019), with the other studies being based on 32, 33 and 96 isolates (Hofer *et al.*, 2012; Eggert *et al.*, 2013; Frank *et al.*, 2019). A study of roe deer in Spain found 3.9 % (4 out of 103 isolates) were *stx2a* positive (Mora *et al.*, 2012). In a study that sampled both red and roe deer in Poland, five isolates out of 33 isolates from roe deer were *stx2a* positive compared to one out of 21 isolates from red deer, suggesting there may be a species difference (Szczerba-Turek *et al.*, 2020). Although numbers were low in this study, these results are in agreement with the results from this study of Scottish wild deer where 27.6 % of roe deer (76/275) were positive for *stx2a* compared to 3.2 % (12/372) of red deer.

2.4.2 Risk factors for presence of *stx2a* determined from model incorporating samples from red, roe and sika deer species

Analysis of risk factors for presence of *stx2a* in deer was carried out using faecal samples collected from across Scotland as part of a larger survey on prevalence of STEC O157 in wild Scottish deer (McNeilly *et al.*, 2020), which included red, roe and sika species of deer. Samples from roe deer and increasing sheep density were identified as the main significant risk factors associated with the presence of *stx2a*, which is considered to pose the highest risk of causing severe human disease.

The association of *stx2a* positive samples with increasing sheep density suggest the possibility that *stx2a* positive strains circulate between sheep and deer. Information on subtypes of Stx present in sheep in Scotland is not known. However, in studies from Spain and Germany of non-O157 STEC from sheep, the most common subtypes were *stx1c* and *stx2b* (Schilling *et al.*, 2012; Otero *et al.*, 2017). STEC isolates from sheep meat and milk were associated with *stx1c* and *stx2b* (Martin and Beutin, 2011). Serotypes which are frequently *stx2a* positive include STEC O157:H7, which has a reported prevalence of 10.6 % of cattle and 3.4 % of sheep in Scotland (Evans *et al.*, 2011; Henry *et al.*, 2017). STEC of serotypes O26:H11 was detected in 5.2 % of sheep and 4.6 % of cattle samples in Scotland and this may also be a potential source of *stx2a* genes (Pearce *et al.*, 2006; Evans *et al.*, 2011). Although prevalence of STEC O157:H7 was found to be low in Scottish wild deer, it is possible that *stx2a* encoding phage cause lytic infection in serotypes that are more common in deer. STEC of serotype O146:H21 was one of the three most common serotypes found in deer samples in Spain and Germany (Martin and Beutin, 2011; Mora *et al.*, 2012). Although serotype O146:H21 is usually associated with

stx subtypes *2b* and *1c*, an isolate from deer has been reported as also being *stx2a* positive (Mora *et al.*, 2012).

There may be species differences that would make roe deer more likely to be infected with *stx2a* positive strains, for example behaviour, physiology and habitat differences to red and sika deer. In cattle and sheep, diet has been shown to have an effect on STEC shedding. It is therefore possible that differences in diet of different deer species may also contribute to differences in shedding of particular strains (Kudva *et al.*, 1997; Lammers *et al.*, 2015). Also, as roe deer are predominantly found in the South of Scotland and lowland areas, it is possible that there are environmental factors, for example higher temperature, which may make them more likely to be infected with *stx2a* positive strains.

2.4.3 Risk factors for presence of *stx2a* determined from roe deer only model

A model which included only samples from roe deer was constructed to determine variables within this population with an effect on *stx2a*. Variables significantly positively associated with samples being *stx2a* positive included samples obtained from South of Scotland, increasing % of semi-natural grassland and increasing number of days of rain in month of sample collection. Although sheep or cattle density did not remain significant in the model, the effect of semi natural grassland may be due to increased proximity with sheep or cattle. Also, higher densities of cattle and sheep are found in the South of Scotland, which has been reported as a risk factor for STEC infection in deer, and which may explain why *stx2a* was more prevalent in this region (Diaz-Sanchez *et al.*, 2013). It has been shown that cattle movement contributes to spread of particular strains, so it could be that *stx2a* positive strains are not yet as widespread in the North compared to the South (Widgren *et al.*, 2018). A study of STEC isolated from cattle at slaughter found different geographical associations dependent on serotype, so it is possible that *stx2a* positive serotypes are more prevalent in the South (Schneider *et al.*, 2018).

Studies of STEC O157 shedding in cattle have identified increasing rainfall as a positive risk factor (Williams *et al.*, 2015). Experimental inoculation of cattle and sheep faeces found that STEC O157 and generic *E. coli* are leached from sheep faeces in higher concentrations suggesting STEC from sheep may be more easily mobilised into water courses or distributed on pasture (Williams *et al.*, 2008). Super shedding of STEC has been linked to presence of *stx2a*. Although environmental factors may not be specific to *stx2a*, if *stx2a* isolates are present, they may be more likely to be present in higher

numbers than STEC with other Stx subtypes (Matthews *et al.*, 2013; Fitzgerald *et al.*, 2019).

2.4.4 Association of *stx2a* with cattle

It was hypothesised that proximity of deer to cattle or other livestock would increase the likelihood of deer faecal samples being positive for *stx2a*. While sheep density was significant in the multivariable model, significant positive associations were observed in univariable analysis of variables related to cattle density. Continuous variables which were negatively associated with increased probability of *stx2a* positive samples in the red, roe and sika deer univariable analysis were distance to nearest farm and distance to nearest farm with cattle, with *stx2a* positive samples collected on average closer to a farm than samples containing other *stx* subtypes. This supports the theory that contact with cattle may allow transmission of *stx2a* positive strains to deer.

2.4.5 Limitations of study

This study was limited in that *stx2a* PCR positive samples were only compared to other *stx* PCR positive samples and PCR negative samples were excluded from analysis. A positive PCR result does not necessarily show that viable STEC bacteria are present in a sample. Isolation of *stx2a* positive *E. coli* from samples would have increased the confidence that samples contained viable STEC capable of causing human disease. However, this was beyond what could be achieved in this project. Samples with STEC were focussed on to determine potential factors driving selection of *stx2a* over other *stx* subtypes. An assumption was made in this study that the location of cull was representative of normal habitat of the deer. This may limit the accuracy of analysis of effect of land cover as deer were not restricted to an area. It is therefore unknown how much time would be spent in different land cover areas. A study of red deer in managed and wild forest areas estimated home range for stags up to 5310 hectares or 53.1 km^2 although hinds in the same area had a smaller average home range of 25.8 km^2 (Náhlik *et al.*, 2009). Proximity to farms was also taken as a potential for deer to interact with livestock. However, whether livestock were grazing rather than being housed, and therefore more likely to have contact with deer, was not considered. An additional point of note is that cattle and sheep ranges overlap. Although sheep density was significant in the final model, cattle density was significant in a univariable analysis. Therefore a role for cattle as a source of *stx2a* positive strains cannot be discounted. Other livestock such

as pigs, chickens and horses are not considered to be major reservoirs of human pathogenic STEC (Persad and LeJeune, 2014). It would be informative to look at population density of pigs, chickens and horses where deer were sampled to assess if presence of large populations of pigs, chickens or horses could be protective for STEC infection in deer.

2.4.6 Future work

To determine how common transmission of strains between deer and farmed ruminants, a detailed comparison of non-O157 STEC *stx2a* strains isolated from deer, cattle and sheep, specifically targeting areas where species share grazing, could be performed. Isolation of similar or identical strains from different species would provide evidence for inter-species transmission. Also, targeted sampling of roe and red deer within the same areas would allow the determination of whether deer species or area of sampling has a greater influence on *stx2a* prevalence.

2.4.7 Conclusion

The prevalence of *stx2a* in this study was found to be 12 % of all *stx* positive samples from fallow, red, roe and sika deer. Modelling of factors associated with *stx2a* presence in deer faeces showed that samples from roe deer and high sheep density were positively associated with presence of *stx2a*. Proximity to cattle was hypothesised to be an important risk factor for presence of *stx2a* however this was not significant in either of the multivariable models. However, a role for cattle cannot be discounted because there is overlap in areas of high sheep and high cattle density.

Modelling of samples from roe deer only showed that samples collected from the South of Scotland, increasing rainfall and percentage of semi-natural grassland were associated with presence of *stx2a*. This is in agreement with previous studies which identified rainfall as influencing STEC transmission. However, further work is needed to determine mechanisms by which *stx2a* positive strains are found more commonly in roe deer compared to other species of deer.

Chapter 3: Determining human pathogenic potential of non-O157 STEC isolated from deer by comparison with human clinical strains using whole genome sequencing

3.1 Introduction

STEC O157 has historically been known as the most common cause of STEC infections in humans. However, STEC serotypes other than O157 (non-O157 STEC) have become increasingly recognised as a cause of human infection in recent years (Chattaway *et al.*, 2016; EFSA and ECDC, 2019; Food Safety Authority of Ireland, 2019). The use of immunomagnetic separation (IMS) to screen for STEC O157 followed by plating out onto selective media allows sensitive detection of this serotype (Cubbon *et al.*, 1996). On selective media, STEC O157 are identified by their characteristic failure to ferment sorbitol (March and Ratnam, 1986; Feng *et al.*, 1998). This is in contrast to non-O157 STEC which are more difficult to isolate as they are generally sorbitol fermenting (Byrne *et al.*, 2015; Fan *et al.*, 2018). Although IMS using antibodies specific for known common STEC serotypes is a sensitive method of detection, it is time consuming to screen samples individually and, for novel or less common serotypes, IMS is not possible (Noll *et al.*, 2016). Increased use of PCR based methods to screen for *stx* genes has increased the ability to detect non-O157 STEC infection, although numbers of human cases may still be underestimated due to difficult in isolating non-O157 STEC strains. Non-O157 STEC may also be indistinguishable in appearance to non STEC when grown on selective sorbitol containing media. Therefore isolation of non-O157 STEC involves testing of sufficient colonies from *E. coli* selective plates to identify those positive for *stx* genes.

In Scotland, regional laboratories identify O157 STEC by culture and latex agglutination testing, but not non-O157 STEC. Faeces from suspected cases of non-O157 STEC infections are sent to the central Scottish *E. coli* reference laboratory (SERL) for PCR testing and isolation of strains (Food Standards Scotland, 2020b). Non-O157 strains, which comprise a range of different serotypes, account for approximately 30 % of strains isolated from human clinical infections (Food Standards Scotland, 2020b). In Ireland, more than half of human infections are now attributed to non-O157 STEC and O26 strains have overtaken O157 strains as the most common cause of infection with 1135 culture confirmed cases of O26 compared to 920 cases of O157 in the period 2012 to 2016 (Food Safety Authority of Ireland, 2019). In 2018, data from the EU showed 65.5 % of STEC

cases were attributed to non-O157 strains (EFSA and ECDC, 2019). In a review of human STEC cases in the Netherlands, 70 % were due to non-O157 serogroups (Franz *et al.*, 2015).

Non-O157 STEC strains have been considered to be less pathogenic than STEC O157; however, particular serotypes such as O104:H4 can cause severe disease (Buchholz *et al.*, 2011). As with O157 STEC infections, *stx2a* and *eae* remain risk factors for development of more severe disease (Haugum *et al.*, 2014). In the UK, STEC O157 usually comprise some combination of *stx2a, stx2c* and *stx1a* (Dallman *et al.*, 2015; Holmes *et al.*, 2018). Given that non-O157 STEC are represented by many serotypes they have a wider range of toxin subtypes and virulence genes. All of the known *stx* subtypes of *stx1* and *stx2* have been found in non-O157 strains isolated from cases of human disease. The subtypes *stx1a*, *stx2a* and *stx2b* are the most common subtypes found in studies of non-O157 STEC in England and Belgium, with *stx2e* and *stx2g* rarely detected (Chattaway *et al.*, 2016; De Rauw *et al.*, 2018). In Scottish clinical isolates of non-O157 STEC, *stx1a* was the most common toxin profile seen followed by *stx2a* (Food Standards Scotland, 2020b).

STEC O157 and non-O157 strains have many virulence genes in common including those essential for survival in the host, such as *gad* conferring acid resistance and *iss* conferring increased survival in serum. The adhesin intimin is important for STEC O157 gut epithelial colonisation in human infection and in ruminant hosts. However, non-O157 STEC utilise both intimin and other adhesion molecules, including those encoded by *aggR, iha, hra* and *saa* (Paton *et al.*, 2001; Montero *et al.*, 2017; WHO-FAO, 2018). Non-O157 STEC may also carry additional toxin genes, including those encoding subtilase and enterotoxins (Sánchez *et al.*, 2012; Steyert *et al.*, 2012).

A study of STEC O157 prevalence in Scottish wild deer (McNeilly *et al.*, 2020) estimated a prevalence of 0.34 % (95 % Binomial Confidence Intervals = 0.02 – 6.30), which is low in comparison to the prevalence of cattle (10.6 %) and sheep (3.4 %) reservoirs of STEC O157 in Scotland (Evans *et al.*, 2011; Henry *et al.*, 2017). As described in Chapter 2 Table 2.1, out of a total of 1077 faecal samples from individual deer which were STEC O157 negative, 748 (69.5 %) were positive for *stx1* and/or *stx2*, suggesting the presence of non-O157 STEC. Of these, 101 (13.5 %) were *stx1* positive, 417 (55.7 %) were *stx2* positive and 230 (30.7 %) were positive for *stx1* and *stx2*. Furthermore, of the *stx2* positive samples, 162 of 647 (25.0 %) were also positive for *eae*.

As STEC strains positive for both *stx2* and *eae* are known to be associated with the most severe forms of human disease (Brandal *et al.*, 2015; De Rauw *et al.*, 2018; WHO-FAO,

2018), these results suggested that deer could be a source of highly pathogenic non-O157 STEC. However, presence of *stx* and *eae* genes in a faecal sample does not confirm that the genes are present in the same bacterial strain. Therefore isolation and characterisation of the STEC strains is important for determining the potential risk they pose to humans.

Whole genome sequencing (WGS) of STEC is becoming a valuable tool in health surveillance systems as it provides a sensitive and rapid way to determine the source and to track STEC human outbreak strains (Chattaway *et al.*, 2016). It provides information on a wide range of virulence genes and allows detailed analysis of relatedness of strains. The use of core genome multi locus sequence typing (cgMLST), which identifies alleles of genes in the core genome, allows a more detailed analysis of relatedness of strains than would be possible with PCR based multi-locus sequence typing (MLST) methods (Holmes *et al.*, 2015). Core SNP (Single Nucleotide Polymorphism) analysis of WGS data can also be used to assess relatedness of different strains (Rumore *et al.*, 2018). The use of cgMLST offers advantages as it is dependent on variation of several nucleotides rather than a point mutation, so is less susceptible to inaccuracies in sequencing which may introduce point mutations and is therefore more comparable between different laboratories (Pearce *et al.*, 2018). While core SNP analysis may provide a finer detail analysis of strains within an outbreak, cgMLST is a useful method for determining relatedness among a wide range of isolates (Gonzalez-Escalona *et al.*, 2016; Holmes *et al.*, 2018; Rumore *et al.*, 2018).

The aim of this chapter was to assess the potential of non-O157 STEC present in deer to cause human disease. Through the use of whole genome sequencing, a detailed comparison was made between deer isolates and existing available data from Scottish human clinical isolates.

3.2 Materials and Methods

3.2.1 Sample selection criteria

Samples were from a previous cross-sectional survey funded by Food Standards Scotland (FSS) to determine the Risk Of STEC Contamination In Wild Venison and as part of this study had been screened by IMS for STEC O157 (McNeilly *et al.*, 2020). The archived samples consisted of cryopreserved buffered peptone water enrichment (broth enrichment) of faecal samples. Faecal DNA was prepared from an aliquot of each broth enrichment sample using Instagene matrix (BioRad) according to the manufacturer's

instructions for bacterial DNA preparation. As detailed in Chapter 2 Table 2.1, faecal DNA samples were analysed by multiplex PCR for presence of *stx1, stx2* and *eae* genes (Bai *et al.*, 2010).

For this study, samples which were positive for *stx2* and *eae* genes, excluding samples which were known to contain STEC O157 (a total of 162 out of 1077 samples), were selected based on the rationale that these samples could contain more pathogenic *stx*2+/*eae*+ non-O157 STEC (WHO-FAO, 2018). Samples were further selected by quantitative PCR (qPCR) for *stx2* as described below to determine samples with highest levels of *stx2* DNA. This was in order to increase the likelihood that *stx2*+ STEC would be successfully isolated from the sample (Dr Anne Holmes, (SERL), personal communication).

3.2.2 qPCR assay for *stx2*

qPCR for *stx2* was carried out using a method from SERL. This assay was able to detect all subtypes of *stx2* with the exception of *stx2f* which is less likely to be associated with severe human disease (Holmes *et al.*, 2018). Primers for *rfb*, a gene specific for the O-antigen of STEC O157, were also included in the assay to confirm that the sample did not contain STEC O157. Briefly, each reaction contained 1 X QuantiTect PCR mix (Qiagen), 0.2 µM of each primer, 0.1 µM of each probe and 2 µl of DNA template in a final volume of 20 µl. Amplification conditions were: initial denaturation at 95 ºC for 15 minutes, 45 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. Details of primers and probes are provided in Table 3.1. A cycle threshold (CT) value of <30 for *stx2* was set as the cut-off for a sample to be considered for STEC isolation (personal communication Anne Holmes).

Table 3.1 Details of primers and probes for *stx2* quantitative PCR (qPCR) assay

Target	Forward primer	Reverse primer	Probe
stx2	TTTGTYACTGTSACAGCWGAAGCYTTACG	CCCCAGTTCARWGTRAGRTCMACRTC	FAM-TCGTCAGGCACTGTCTGAAACTGCTCC-BHQ1
rfb	TTTCACACTTATTGGATGGTCTCAA	CGATGAGTTTATCTGCAAGGTGAT	Texas Red-AGGACCGCAGAGGAAAGAGAGGAATTAAGG-BHQ2

BHQ: black hole quencher

3.2.3 Isolation of Shiga toxin positive bacteria for whole genome sequencing

For isolation of STEC strains, a loopful of cryopreserved broth enrichment prepared from faecal samples as described previously (Section 3.2.1) was inoculated into 20 ml Tryptone Soy Broth (TSB) and incubated for 18-20 hours statically at 37 ºC. Ten-fold dilutions of the broth (10^{-3}, 10^{-4}, 10^{-5}) were made and 25 µl of 10^{-3} and 50 µl of 10^{-4} and 10^{-5} dilutions were spread plated onto Sorbitol MacConkey Agar (SMAC) plates (E&O Laboratories, Bonnybridge, UK) which are selective for gram negative bacteria, including all *E. coli,* and ChromAgar STEC plates (E&O Laboratories, Bonnybridge, UK) which are selective for STEC. Selective agents in the ChromAgar STEC plates are intended to suppress growth of background commensal enteric bacteria making it easier to isolate STEC strains (Verhaegen *et al.*, 2015). However, as some STEC may also be sensitive to these selective agents (Jenkins *et al.*, 2020), culture of samples on SMAC plates was performed in parallel to capture STEC strains which would be unable to grow on ChromAgar STEC. After overnight incubation at 37 ºC, nine individual colony picks plus one sweep were sampled from each plate type and suspended in 100 µl sterile molecular biology grade water. Forty µl of bacterial suspension was heated to 99 ºC for 20 minutes and used as template for PCR detection of STEC virulence genes using a multiplex PCR for *stx1*, *stx2* and *eae* as detailed in Table 3.2. PCR reactions of 20 µl final volume contained 2 µl of template DNA, 10 µl of mastermix (iQ Multiplex Powermix, without additional supplement; Biorad) and 0.25 µM of each primer. The thermal cycling profile involved a 5 minute pre-incubation step of 94 ºC followed by 35 cycles each consisting of denaturation at 94 ºC for 30 seconds, primer annealing and extension at 67 ºC for 80 seconds and a final extension step at 72 ºC for 7 minutes. PCR products were analysed by capillary electrophoresis using the Qiaxcel Advanced System (Qiagen).

Table 3.2 Details of primers for multiplex PCR detection of *stx* and *eae* genes and singleplex PCR detection of *uidA*

Primer name *stx* / *eae* Multiplex PCR	**Primer sequence (5'→3')**	***Gene***	***Product size (bp)***
stx1-Forward	TGTCGCATAGTGGAACCTCA	*stx1*	655
stx1-Reverse	TGCGCACTGAGAAGAAGAGA		
stx2-Forward	CCATGACAACGGACAGCAGTT	*stx2*	477
stx2-Reverse	TGTCGCCAGTTATCTGACATTC		
eae-Forward	CATTATGGAACGGCAGAGGT	*eae*	375
eae-Reverse	ACGGATATCGAAGCCATTTG		
***uidA* PCR**			
*uidA_E. coli*_1 F	ATCACCGTGGTGACGCATGTCGC	*uidA*	486
*uidA_E. coli*_1 R	CACCACGATGCCATGTTCATCTGC		

If the bacterial sweep was positive for *stx1* or *stx2* but none of the individual colonies were positive for either *stx* gene, the sweep suspension was subsequently plated out to obtain individual colonies for further PCR testing. If this still did not identify individual *stx1* or *stx2* positive colonies, no further isolation was attempted. If a colony suspension was positive for either *stx1* or *stx2*, the residual 60 µl of suspension not used for PCR was plated on to the same agar plate used for the original isolation to obtain pure STEC isolates. A single STEC colony from the agar plate was inoculated into duplicate Tryptone Soy Broth (TSB) cultures and incubated for 18-22 hours at 37 ºC and shaking at 200 rpm. After overnight culture, 0.7 ml broth from one tube was mixed with 0.3 ml sterile 80 % glycerol in PBS and archived at -70 ºC. The second tube was used to prepare DNA for whole genome sequencing.

3.2.4 Whole Genome Sequencing of STEC isolates

The TSB culture of each STEC isolate was centrifuged for 4 minutes at 15,000 x g and DNA extracted from the bacterial pellet using a DNeasy® Blood and Tissue Kit (Qiagen) with RNAse A digestion. DNA was eluted in 100 µl EB buffer. The multiplex PCR described in section 3.2.3 was used to confirm the presence of *stx* genes prior to sequencing. Also, a separate PCR for the *uidA* gene was carried out to confirm that the isolate was *E. coli* (Juck *et al.*, 1996). Primers for *uidA* PCR are shown in Table 3.2. PCR

reactions of 20 µl final volume contained 1 µl template DNA, GoTaq Colourless 5X buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units *Taq* DNA polymerase and 0.5 µM of each primer. The thermal cycling profile involved a 5 minute pre-incubation step of 95 ºC followed by 30 cycles each consisting of denaturation at 95 ºC for 30 seconds, primer annealing at 50 ºC for 1 minute, extension at 72 ºC for 1 minute and a final extension step at 72 ºC for 5 minutes.

Quality assessment of DNA was made using Nanodrop® (Thermo Scientific) and Bioanalyser (Agilent®), and concentration was determined using Qubit® (Thermo Scientific). Genome sequencing was performed by MicrobesNG (http://www.microbesng.uk). The following protocol was used by MicrobesNG to prepare DNA for sequencing. DNA was quantified in triplicate using the Quant-iT™ dsDNA HS assay (Thermo Scientific) in an Eppendorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) according to the manufacturer's protocol with modification: 2 ng of DNA were used as input and PCR elongation time was 1 minute. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina HiSeq using a 250 bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger *et al.*, 2014). *De novo* assembly was performed on samples using SPAdes version 3.7, and contigs were annotated using Prokka 1.11 (Bankevich *et al.*, 2012; Seemann, 2014). The average read coverage was 89X ± 34. Each genome assembly had an average total length of 5,390,218 ± 224,005 bp, and an average N50 (>1,000 bp) of 162,391 ± 69,676 bp, with 50 % of the entire assembly contained in contigs equal to or larger than N50. The average number of contigs (>1,000 bp) within the assemblies was 197 ± 67.

3.2.5 Bioinformatic analysis of WGS data

Bioinformatic analysis was carried out by SERL. Whole genome sequencing data was analysed using two different pipelines. A pipeline originally developed at Public Health England was used to determine species ID, serotype, 7-gene MLST sequence type, presence of virulence genes *eae, bfpA, aggR, ipaH, aaiC, ltcA, sta1* and *stb*, and *stx* subtype. The 7-gene MLST derived from WGS data is based on the Achtman scheme which uses partial sequence information from the following housekeeping genes: *adk, fumC, gyrB, icd, mdh, purA* and *recA* (Wirth *et al.*, 2006). For this analysis, each gene

variation is assigned a number resulting in a 7 number allelic profile for an *E. coli* strain which corresponds to a particular sequence type. Each 7-gene MLST sequence type has a difference in at least one of the 7 gene sequences from other sequence types. The wgMLST and *E. coli* plug-in tools from BioNumerics v7.6 (Applied Maths) were used for the second pipeline analysis which identifies additional virulence genes including antibiotic resistance genes, in addition to species ID and serotype, and also calculates relatedness of isolates using core genome sequences from 2,513 core loci.

Strain sequences with novel sequence typing gene allelles were uploaded to Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) for further analysis and assignment of sequence type. Various open source software was used to process, quality control and analyse the raw sequence data. Trimmomatic was used to remove bases with a Phred score below 30 from the trailing edge (Bolger *et al.*, 2014). KmerID was used to confirm bacterial species as *E. coli* (Chattaway *et al.*, 2017). FASTQ reads were mapped to a panel of serotype and virulence genes using the GeneFinder tool and Bowtie 2 (Langmead *et al.*, 2009). The best match to each target was reported along with sequence coverage, depth, mixture and nucleotide similarity. The cut-off for acceptance of predictions was set as >80 % nucleotide identity and >80 % target gene length. MLST allelles of seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) were determined using Metric-Oriented Sequence typer (MOST) and sequence type was derived from this (Tewolde *et al.*, 2016). A combination of assembly free and assembly-based allelic detection was used to generate allelic profiles for each isolate. Assemblies were constructed using SPAdes integrated into the wgMLST plug-in.

Assembled genomes were analysed using the *E. coli* genotyping plug-in from the Centre for Genomic Epidemiology (DTU, Lyngby, Denmark) which contains reference databases for serotype, virulence and antibiotic resistance. Detection parameters were set to 90 % sequence identity (% of loci found belonging to the subset of core-loci) and 60 % sequence coverage. Only one STEC sequence was below the 90 % sequence identity cut-off, possibly due to being a rare *E. coli* type, but other QC parameters were within specification for this strain. All other strains were within specification in terms of sequence identity and coverage.

WGS data for Scottish non-O157 STEC human clinical isolates was obtained by SERL (Food Standards Scotland, 2020b). Sequences were obtained from all strains isolated or received by SERL between February 2002 and February 2018 and comprised of 522

strains. This included strains isolated from faecal samples submitted by all diagnostic laboratories in the different Scottish Health Board areas.

To compare deer isolates with human clinical isolates, a phylogenetic tree was constructed based on 2,513 core loci allel1es (Enterobase cgMLST) produced in BioNumerics v7.6 using categorical differences and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis. The distribution of WGS derived serotypes across four geographical regions was analysed by Fisher's Exact Test using R (R Core Team, 2018) Version 3.5.1. Regions were the same as the sampling areas described in Chapter 2 except south west and south east were combined as one region: south.

3.3 Results

3.3.1 Isolation and PCR analysis of non-O157 STEC from wild deer

The first objective of this study was to recover non-O157 isolates from STEC-positive deer faecal samples. Excluding those known to contain STEC O157, 162 samples which had been identified as positive for *stx2* and *eae* genes using multiplex PCR were further screened by qPCR for the *stx2* gene. Of these, 93 samples had a CT value < 30 for *stx2* and were selected for subsequent STEC isolation, starting with the samples which had the lowest CT value and therefore highest levels of *stx2* DNA. Only two strains were isolated from ChromAgar plates with the remaining isolates from SMAC agar plates, and 25 % of samples did not grow or had very limited growth on ChromAgar compared to growth on SMAC plates. From these 93 faecal samples, a total of 85 STEC strains were isolated from 72 different deer. In addition to strains isolated as described in Section 3.2.3, one non-O157 STEC strain which had been isolated during the previous study (McNeilly *et al.,* 2020) was also included in this analysis.

For 13 animals, two different isolates were recovered from each based on distinct colony morphology and/or *stx* PCR profile. Also, for eight faecal samples which were *stx1* and *stx2* positive, only a single isolate positive for either *stx1* or *stx2* was recovered, suggesting these animals might also have mixed STEC infections. Shiga toxin gene profiles for each isolate are summarised in Table 3.3. The majority of isolates contained *stx2* genes (77 isolates) alone or in combination with *stx1*, with nine isolates being *stx1* positive alone. All *stx* positive isolates were positive for *uidA* confirming that they were *E. coli*. None of the *stx* positive strains were *eae* positive. *E. coli* strains which were *eae* positive were also recovered (47 isolates in total); however, as these were *stx* negative

and therefore not STEC, these were excluded from further analysis.

Table 3.3 PCR profiles of non-O157 STEC strains isolated from deer samples

stx1+	*stx2*+	*stx1*+/*stx2*+	Total
9	53	24	86

3.3.2 Whole Genome Sequence analysis of non-O157 STEC deer faecal isolates.

Samples for isolation were processed in order of increasing *stx2* CT value. Due to time constraints, WGS data was obtained for the first 55 *stx2* positive isolates to be successfully cultured and confirmed as *E. coli* by use of *uidA* PCR. This included one non-O157 STEC strain isolated during the previous study to determine the risk of STEC contamination in wild venison. One *stx1* positive strain was included for sequencing as it was isolated alongside a *stx2* positive strain from the same deer. Two *stx2* positive isolates were obtained from an individual deer. Therefore, in total, 56 strains from 54 deer were sequenced. The source of the sequenced isolates by deer species and location of sampling are shown in Figure 3.1. The majority of the sequenced STEC isolates originated from the North West of Scotland, with higher proportions of STEC isolates in the North of Scotland originating from red deer, and higher proportions of STEC isolates in the South of Scotland originating from roe deer.

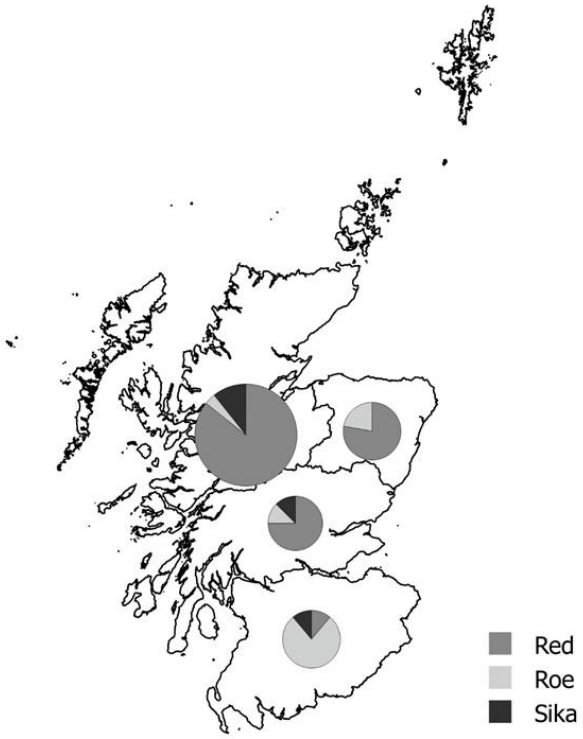

Figure 3.1 Source of non-O157 STEC isolates for WGS by sampling location and species of deer. STEC isolates originated from three deer species (red, roe, sika) from four designated regions in Scotland (North West, North East, Central and South). The pie charts indicate the proportion of deer species (red, roe, sika) from which the STEC isolates originated and the size of the pie chart is proportional to number of deer per region from which an STEC isolate was recovered. North West (n=28, including two isolates from one deer); North East (n=9); Central (n=8) and South (n=9, including two isolates from one deer). n = number of deer

QGIS Development Team (2019). QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org © Boundary Commission for Scotland, Local Government Boundary Commission for Scotland. Contains Ordnance Survey data © Crown copyright and database rights 2013.

The outputs of WGS analysis, including serotype, 7 gene Multi-Locus Sequence Type, and virulence genes, are presented in Table 3.4, together with the strain number, originating deer species and sampling location. Identical strains based on cgMLST, *stx* subtype, serotype and virulence genes were isolated from two deer that were culled at the same time and location (Strain 822 D1 and strain 827 G10 both of serotype O187:H28).

Two different STEC strains were isolated from the same deer on two occasions. Strain number 323 D1 and 323 D6 were isolated from the same roe deer. Strains 491 D6 and 491 D8 were isolated from the same sika deer. Isolates from the same animal had diverse serotype, MLST and *stx* subtypes. Isolates 323 D1 and 323 D6 had three virulence genes in common (*FimH, gad, iss*); however they differed in presence of 24 other virulence genes. Isolates 491 D6 and 491 D8 shared nine of the same virulence genes (*FimH, gad, subA, iss, ireA, iha, ehxa, hlyD* and *senB*); however they had differences in gene presence for 11 other virulence genes.

Table 3.4 Genetic characteristics of non-O157 STEC deer isolates

Strain number	Serotype	7 gene MLST Sequence Type	Virulence genes	Species	Sample location
152 B2	O128:H2	25	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA*	Red	NorthWest
582 D1	O128:H2	25	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA, TraT, cvi-cvaC*	Red	Central
588 E3	O128:H2	25	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA*	Red	NorthEast
694 B2	O128:H2	25	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, celb, PAI(malX), fyuA*	Red	NorthEast
68 C3	O128:H2	25	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, cba, cma, senB, PAI(malX), fyuA*	Red	NorthEast
69 B5	O128:H2	25	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA*	Red	NorthWest
134 E5	O128:H2	25	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, senB, PAI(malX), fyuA*	Red	NorthWest
730 F5	O128:H2	25	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA, cvi-cvaC*	Roe	NorthWest
643 B6[c]	O128:H2	10657	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA*	Roe	NorthEast
1070 G2[c]	O128:H2	10659	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, PAI(malX), fyuA, TraT, cvi-cvaC*	Red	NorthEast
737 F10	O22:H16	295	*stx2b, FimH, gad, lpfA, subA, iss, iha, astA, cba, cma, senB, espI, f17A, f17G*	Red	NorthWest
373 A4	O22:H16	295	*stx2b, FimH, lpfA, subA, iss, mchF, astA, celb, TraT, cvi-cvaC, epeA, f17A, f17G*	Red	NorthWest
304 E2	O22:H16	295	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, astA, cba, cma, espI*	Red	NorthWest
706 C3	O22:H16	295	*stx2b, FimH, lpfA, subA, iss, astA, cba, cma, f17A, f17G*	Red	NorthWest
1036 G5	O22:H16	295	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, astA, espI*	Red	NorthWest

Table 3.4 continued

Strain number	Serotype	7 gene MLST Sequence Type	Virulence genes	Species	Sample location
1083 G2	O22:H16	295	*stx2b, FimH, gad, lpfA, subA, iss, iha, astA, espI, f17A, f17G*	Red	NorthWest
103 C2	O22:H16	295	*stx2b, FimH, gad, lpfA, subA, iha, espI, f17A, f17G*	Red	NorthWest
552 G6	O146:H21	442	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, astA, cba, cma, celb, epeA*	Sika	Central
268 C2	O146:H21	442	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, celb*	Sika	NorthWest
428 D1	O146:H21	442	*stx2b, FimH, gad, lpfA, subA, iss, iha, ehxA, hlyD, mchF, mchB, mchC, cba, celb, mcmA, iroN, sfa/foc*	Roe	South
323 D1[a]	O146:H21	442	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, mchF, mchB, mchC, cba, cma, celb, espI, cvi-cvaC, epeA, mcmA, iroN, sfa/foc*	Roe	South
796 F2	O146:H21	442	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, astA, cba, cma, celb, cvi-cvaC, epeA, mcmA, iroN, sfa/foc*	Roe	South
432 E4	O146:H21	442	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, cba, cma, celb, TraT, epeA, mcmA, iroN, sfa/foc*	Roe	South
307 F2[c]	O146:H21	10658	*stx2b, FimH, gad, lpfA, subA, iss, ireA, iha, mchF, mchB, mchC, astA, cba, cma, celb, TraT, epeA*	Red	NorthWest
93 C6	O87:H16	2101	*stx2b, FimH, gad, lpfA, ireA, espI*	Red	NorthWest
764 D10	O87:H16	2101	*stx2b, FimH, gad, lpfA, ireA, espI*	Red	NorthWest
60 B2	O87:H16	2101	*stx2b, FimH, gad, lpfA, ireA, espI*	Red	NorthWest
76 B6	O87:H16	2101	*stx2b, FimH, gad, lpfA, ireA, espI*	Red	NorthWest
92 C5	O87:H16	2101	*stx2b, FimH, gad, lpfA, ireA, espI*	Red	Central
491 D6[b]	O174:H8	13	*stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, astA, cba, cma, celb, senB*	Sika	NorthWest

Table 3.4 continued

Strain number	Serotype	7 gene MLST Sequence Type	Virulence genes	Species	Sample location
127 D3	O174:H8	8630	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, mchF, mchB, mchC, astA, cba, cma, senB*	Red	Central
261 F4	O174:H8	8630	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, hlyD, mchF, mchB, mchC, astA, cba, cma, celb, senB*	Roe	Central
528 C1[c]	O174:H8	10655	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, astA, cba, cma, senB*	Red	Central
837 G3	O174:H8	13	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, astA, cba, cma, celb, senB*	Roe	NorthEast
696 F8	O113:H4	10	*stx2b, gad, subA, iss, ireA, iha, ehxA, hlyD, astA, cba, cma, senB*	Red	NorthEast
503 E1	O113:H4	10	*stx2b, FimH, gad, subA, ireA, iha, ehxA, hlyD, astA, cba, cma, senB*	Red	NorthWest
99 B4	O113:H4	10	*stx2b, stx1c, FimH, gad, subA, iss, ireA, iha, ehxA, hlyD, astA, cba, cma, celb, senB*	Red	Central
45 B2	O113:H4	10	*stx2b, stx1c, FimH, gad, subA, iss, ireA, iha, ehxA, hlyD, astA, cba, cma, celb, senB*	Red	NorthWest
221 C1	O166:H28	1819	*stx2b, FimH, gad, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, astA, senB, hra, eilA, air*	Red	NorthWest
491 D8[b]	O166:H28	1819	*stx2b, FimH, gad, subA, iss, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, senB, hra, eilA, air*	Sika	NorthWest
759 E10[c]	O166:H28	10656	*stx2b, FimH, gad, subA, ireA, iha, ehxA, hlyD, mchF, mchB, mchC, senB, hra, eilA, air*	Red	NorthWest
709 D4	O146:H28	738	*stx2b, FimH, lpfA, subA, iss, ireA, iha, mchF, mchB, mchC, PAI(malX), hra, usp*	Red	NorthEast
D0691	O146:H28	738	*stx2b, FimH, lpfA, subA, iss, ireA, iha, mchF, mchB, mchC, astA, PAI(malX), hra, usp*	Red	NorthEast
822 D1	O187:H28	200	*stx2g, FimH, gad, lpfA, ehxA, hlyD, astA, TraT, sta1, stb*	Red	NorthWest
827 G10	O187:H28	200	*stx2g, FimH, gad, lpfA, ehxA, hlyD, astA, TraT, sta1, stb*	Red	NorthWest

Table 3.4 continued

Strain number	Serotype	7 gene MLST Sequence Type	Virulence genes	Species	Sample location
97 D7	Ou:H7	5822	*stx2g, FimH, gad, lpfA, ehxA, hlyD, TraT, sta1, stb*	Sika	NorthWest
257 B6	Ou:H7	5822	*stx2g, FimH, gad, lpfA, ehxA, hlyD, TraT, sta1, stb*	Red	NorthWest
323 D6[a]	O11:H5	1104	*stx2a, FimH, gad, iss, ehxA, hlyD, astA, fyuA, eilA, air, stb*	Roe	South
793 G8	O113:H21	3695	*stx2d, FimH, gad, lpfA, subA, iss, ireA, iha, astA, cba, cma, celb*	Red	Central
652 F2	O117:H4	56	*stx2b, FimH, gad, lpfA, subA, iss, iha, ehxA, hlyD, mchF, mchB, mchC, astA, celb, espI, f17A, f17G, mcmA*	Roe	South
481 E3	O22:H8	446	*stx2a, FimH, gad, lpfA, iss, iha, ehxA, hlyD, celb, hra, espP, saa*	Sika	South
782 G3	O36:H14	1176	*stx2a, stx2g, FimH, gad, ehxA, hlyD, astA, TraT, hra, eilA, air, sta1, stb, usp*	Roe	South
355 G3	O38:H26	10	*stx2b, stx1c, FimH, gad, subA, iss, ireA, ehxA, hlyD, mchF, mchB, mchC, cba, cma, celb, senB*	Red	NorthWest
329 E1	O75:H8	13	*stx2b, stx1c, FimH, gad, lpfA, subA, iss, ireA, iha, ehxA, hlyD, mchF, celb, senB, espI, fyuA, cvi-cvaC, epeA*	Red	South
843 C10	Ou:H21	26	*stx2b, FimH, gad, lpfA, subA, iss, ireA, ehxA, hlyD, mchF, mchB, mchC, astA, cba, cma, celb, cvi-cvaC*	Roe	South
379 B3	Ou:H8	26	*stx2b, FimH, gad, lpfA, subA, iss, ireA, mchF, mchB, mchC, astA, cba, cma*	Red	NorthWest

Ou = O type unidentifiable; [a] denotes two isolates from the same animal; [b] denotes two isolates from the same animal; [c] previously unidentified sequence type.

3.3.3 Serotypes of non-O157 STEC strains from deer faecal samples determined using WGS data

Four STEC samples did not have an identifiable O type (Ou = O unidentifiable), but all had an identified H type. The most common serotype was O128:H2 with 10 isolates (17.9 %) followed by O22:H16 and O146:H21 each with 7 isolates (12.5 %). There were 5 isolates (8.9 %) of serotype O87:H16 and O174:H8 and four isolates (7.1 %) of serotype O113:H4. The remaining isolates (32.1 %) were serotypes O166:H28, O146:H28, O187:H28, Ou:H7, O11:H5, O113:H21, O117:H4, O22:H8, O36:H14, O38:H26, O75:H8, Ou:H21 and Ou:H8.

Eleven out of a total of nineteen different serotypes identified in the deer strains were also seen in Scottish human clinical isolates. Comparisons between serotypes of deer isolates and Scottish human clinical isolates is shown in Figures 3.2 and 3.3. The proportions of serotypes differed between deer and clinical isolates, with the three most common serotypes seen in human infections (O26:H11, O103:H2 and O145:H28) not identified in deer. The 4th and 5th most common human serotypes were the same as the top two serotypes in the deer isolates (O128:H2 and O146:H21). However, serotype O22:H16 was only seen in deer isolates.

For the seven most common STEC serotypes found in deer, there were significant differences between serotype and the area of sampling, (Fisher's Exact test, p value = 0.004) for 4 x 7 contingency table (analysis was of serotypes shown in Table 3.5 excluding 'Other' category). Serotype O22:H16 was only found in the North West and was the most frequent in this region, representing 7 of 29 isolates. Serotype O128:H2 was most frequent in the North East, representing 5 of 9 isolates. Serotype O174:H8 was most common in the Central area, representing 3 of 8 isolates. Serotype O146:H21 was most common in the South, representing 4 of 10 isolates.

Table 3.5 Association of serotype with area of sampling

	O128:H2	O22:H16	O146:H21	O87:H16	O174:H8	O113:H4	O166:H28	Other	Total
NorthWest	4	7	2	4	1	2	3	6	29
NorthEast	5	0	0	0	1	1	0	2	9
Central	1	0	1	1	3	1	0	1	8
South	0	0	4	0	0	0	0	6	10

Seven most common serotypes are shown only. 'Other' includes serotypes of two or less isolates including O146:H28, O187:H28, Ou:H7, O11:H5, O113:H21, O117:H4, O22:H8, O36:H14, O38:H26, O75:H8, Ou:H21 and Ou:H8.

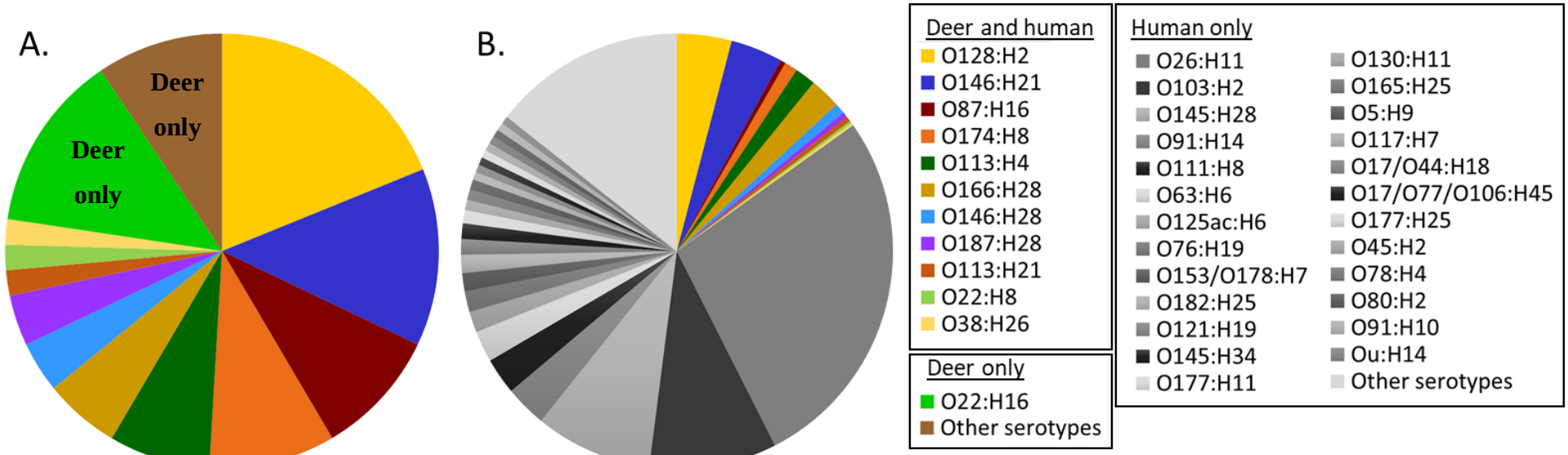

Figure 3.2 Comparison of serotypes present in Scottish wild deer and Scottish human clinical isolates. (A) Deer isolates (n = 53) **(B)** Human isolates (n = 518). Serotypes which are found in humans and/or deer are in colour; serotypes which are only seen in human isolates are shown in grey. Deer only 'Other serotypes' had fewer than two isolates per serotype and included O11:H5, O117:H4, O36:H14, O75:H8 and Ou:H21. Human only 'Other serotypes' include 60 different serotypes which were unique to humans and were represented by ≤ 2 isolates. For some serotypes, more than one O-type is shown as the sequences could not be assigned to a single specific O-type. Isolates of serotypes Ou:H7 and Ou:H8 were present in both human and deer isolates but were not included in the figure as the O-type was unidentified making it impossible to determine if the human and deer isolates were the same or different serotypes. Data shown for the human clinical isolates is adapted from Food Standards Scotland, 2020b.

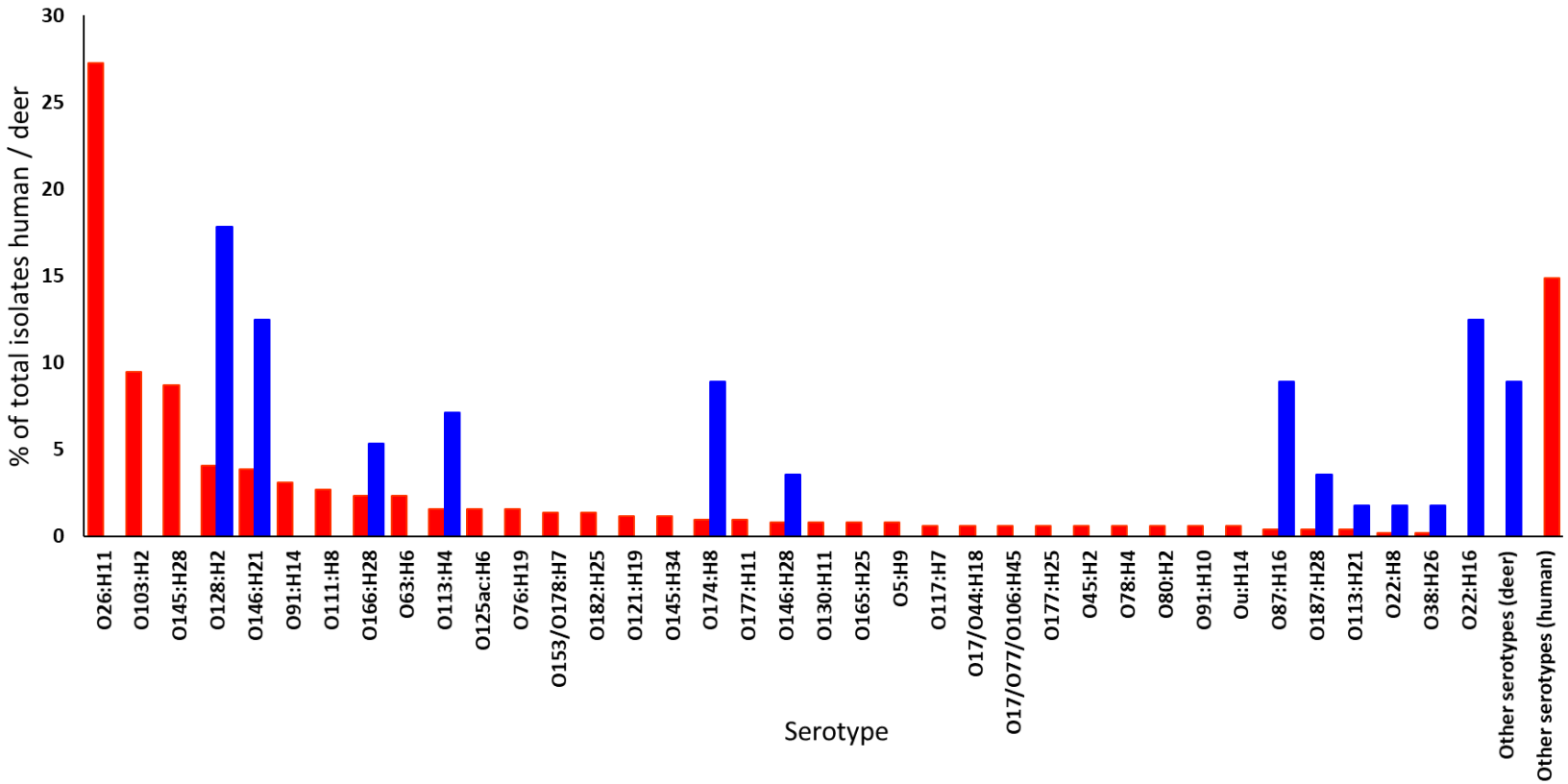

Figure 3.3 Frequency of non-O157 STEC serotypes within wild Scottish deer isolates and Scottish human clinical isolates. Red bars - Human clinical isolates. Blue bars – deer isolates. Bars represent the percentage of total number of isolates for either human clinical isolates (n=518) or deer isolates (n=53). For some serotypes more than one O-type is shown as the sequences could not be assigned to one specific O-type. Other serotypes (deer) include deer serotypes not identified in Scottish human clinical isolates and with fewer than two isolates including O11:H5, O117:H4, O36:H14, O75:H8 and Ou:H21. Other serotypes (human) included 60 different serotypes not identified in deer isolates and with two or less isolates for each serotype. Human and deer isolates of serotypes Ou:H7 and Ou:H8 were excluded from this analysis as the O-type was unidentified. Data shown for the human clinical isolates is adapted from Food Standards Scotland, 2020b

3.3.4 Sequence types of non-O157 STEC from deer determined by 7-gene MLST

As shown in Table 3.4, 22 sequence types were identified, of which five isolates (643 B6, 1070 G2, 307 F2, 528 C1 and 759 E10) were of a previously unidentified sequence type. Some serotypes were associated with sequence type: 8 out of 10 O128:H2 serotype isolates were sequence type 25, 6 out of 7 O146:H21 isolates were sequence type 442 and all O22:H16, O87:H16 and O113:H4 isolates were of the same sequence type (Table 3.4). Isolates of serotype O174:H8 were of three different sequence types.

3.3.5 Shiga toxin gene subtypes

Shiga toxin profiles for all isolates are summarised in Table 3.6. The *stx* subtype *stx2b* was the most common, found in 47 out of 56 isolates either alone or in combination with *stx1c*. Five isolates were *stx2g* positive, three were *stx2a* positive (one in combination with *stx2g*), one isolate was *stx2d* positive and one isolate was *stx1c* positive.

Table 3.6 Shiga toxin profiles of non-O157 STEC deer isolates

***Stx* profile**	**Total number of isolates**
stx2b	31
stx2b:stx1c	16
stx2g	4
stx2a	2
stx2a:stx2g	1
stx2d	1
stx1c	1

A comparison of *stx* profiles of deer isolates and human clinical isolates is shown in Figure 3.4. All deer and human non-O157 STEC *stx* subtypes profiles are compared in (A) and (B). Only subtypes of strains containing *stx2* are shown in (C) and (D), as *stx2* positive strains were selected for the deer strains leading to underrepresentation of *stx1* strains in the deer dataset. Overall *stx1a* (34 % of isolates) was the most common profile identified in human clinical isolates and this was not identified in the deer isolates. The most common profile in *stx2* positive human clinical isolates was *stx2a* (25 % of isolates). In *stx2* positive deer isolates, the most common *stx* profile was *stx2b* (56 % of isolates). In the *stx2* positive isolates, 85 % of deer isolates had the *stx2b* subtype alone or in combination with *1c* compared to 32 % of *stx2* positive human isolates, which were

positive for *stx2b* alone or in combination with other subtypes. In the *stx2* positive deer isolates, 5 % were positive for *stx2a* compared to 47 % of *stx2* positive human isolates.

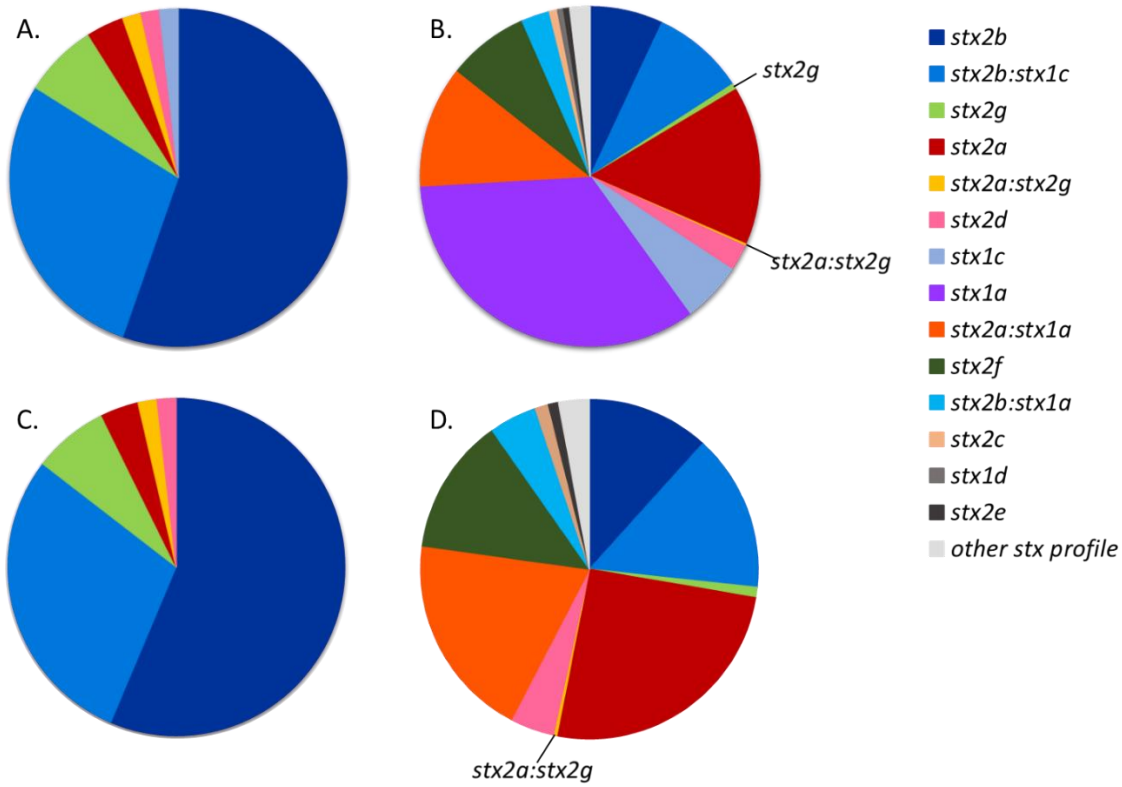

Figure 3.4 Shiga toxin gene profiles of non-O157 STEC isolates from Scottish wild deer and Scottish human clinical isolates (A) All deer isolates (n=56), **(B)** all human clinical isolates (n=517, *stx* subtype data was not available for five human strains), (C) *stx2* positive deer isolates (n=55) (D) *stx2* positive human clinical isolates (n=307). 'other' includes *stx* profiles : *stx2a*:*stx2c, stx2a*:*stx2c*:*stx1a, stx2c*:*stx1a, stx1a*:*stx1c, stx2b*:*stx1a*:*stx1c, stx2b*:*stx2c, stx2d*:*stx2b*; these were not seen in deer isolates and were identified in 2 or fewer human clinical isolates. Data shown for the human clinical isolates was adapted from Food Standards Scotland, 2020b.

3.3.6 Additional virulence genes

A total of 36 additional virulence genes (i. e. excluding *stx* genes) were identified and the percentage isolates positive for each virulence gene are shown in Figure 3.5. The most common virulence genes identified were *FimH*, (an adhesin), *gad* (glutamate decarboxylase) and *lfpA* (an adhesin). Only one single isolate was positive for *espP* and *saa*. The number of virulence genes per isolate ranged from 5 to 21 excluding *stx* genes. All the deer STEC isolates were negative for *eae*, *bfpA*, *aggR*, *ipaH*, *aaiC* and *ltcA*. Five and six isolates respectively were positive for *sta1* and *stb*. The virulence gene categories are provided in Appendix 5. Overall deer and human clinical isolates had very similar mean numbers of virulence genes. A comparison of the mean number of virulence genes

in deer and human clinical isolates for the seven most common serotypes in deer is shown in Table 3.7. The mean number of virulence genes for serotypes from human clinical isolates or deer isolates did not differ by more than two genes. Particular genes were observed to be associated with *eae* negative or *eae* positive Scottish human clinical strains (Food Standards Scotland, 2020b). In the deer strains 19/36 virulence genes detected were found to be associated with *eae* negative strains in Scottish human clinical isolates. In contrast 7/36 of the virulence genes observed in the deer strains were associated with *eae* positive isolates in the Scottish human clinical isolates.

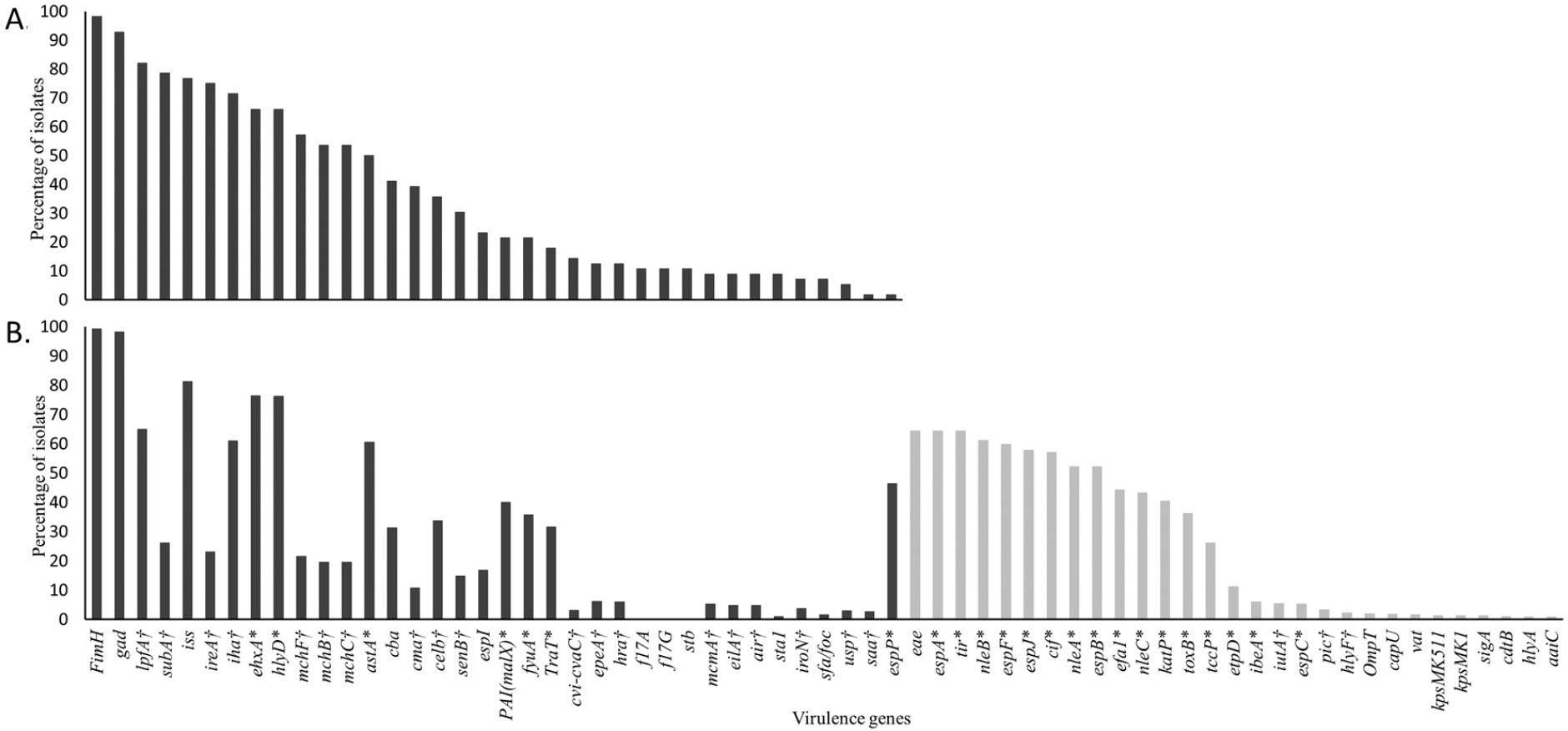

Figure 3.5 Virulence gene frequency in deer and human non-O157 STEC isolates. (A) Bars represent the percentage of deer isolates (n=56) positive for each virulence gene excluding Shiga toxin genes**. (B)** Bars represent the percentage of human isolates (n=522) positive for each virulence gene excluding Shiga toxin genes. * Genes with significantly higher prevalence in *eae* positive human clinical isolates. † Genes with significantly higher prevalence in *eae* negative human clinical isolates. Genes common to both deer and human isolates are shown as dark grey bars. Genes unique to human clinical isolates are shown as light grey bars. Data shown for the human clinical isolates and analysis of gene associations with presence of *eae* is adapted from Food Standards Scotland, 2020b.

Table 3.7 Mean number of virulence genes for most common deer non-O157 STEC serotypes compared to human clinical isolates

Serotype	Mean number of virulence genes in deer isolates ± standard deviation	Number of deer isolates	Mean number of virulence genes in human isolates*	Number of human isolates
O128:H2	15.0 ± 1.1	10	15	21
O22:H16	10.3 ± 1.8	7	na	na
O146:H21	17.4 ± 2.8	7	16	20
O87:H16	5.0 ± 0.0	5	6	2
O174:H8	15.6 ± 1.1	5	15	5
O113:H4	12.0 ± 1.2	4	11	8
O166:H28	15.0 ± 1.0	3	15	12

na = not available as there were no human clinical isolates of this serotype

* Data for human clinical isolates was adapted from Food Standards Scotland, 2020b and information on standard deviation was not available.

3.3.7 Antimicrobial resistance genes

Only one isolate, strain 1070 G2, carried acquired antimicrobial resistance genes for sulphonamide (*sul-2*), β-lactamase (*bla-TEM-1C*) and aminoglycosides (*aph(6), strB*). This isolate had a unique 7 gene MLST of 10659, although it was of the most common serotype O128:H2 and was obtained from a red deer in the NorthEast. Isolates 491 D8, 793 G8, 759 E10, 379 B3, 837 G3, 843 C10, 304 E2, D0691, 323 D6 and 796 F2 carried tellurite resistance genes. All isolates carried the gene *mdf(A),* a broad specificity transport protein which allows bacteria to transport a range of molecules including the antibiotics tetracycline and chloramphenicol (Edgar and Bibi, 1997).

3.3.8 Predicted human pathogenic potential of deer non-O157 STEC strains based on virulence gene profiles

A Joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO) Expert Meetings on Microbiological Risk Assessment (JEMRA) report classified risk to human health based on virulence gene presence (WHO-FAO, 2018). Based on this classification system, the majority of deer isolate serotypes have the potential to cause diarrhoea, with serotype O113:H21 having the potential to cause diarrhoea (D), bloody diarrhoea (BD) and haemolytic uraemic syndrome (HUS)

(Table 3.8). It should be noted that the potential to cause illness is also dependent on host susceptibility and other factors such as antibiotic treatment.

Table 3.8 JEMRA classification of STEC and potential to cause diarrhoea (D), bloody diarrhoea (BD) and haemolytic uraemic syndrome (HUS) based on Shiga toxin gene subtype and presence of *eae* or *aggR*

JEMRA Level (potential to cause illness)	Trait (Gene and subtype combination)	Deer isolate serotypes (no. of isolates)
1 (D/BD/HUS)	*stx2a* + *eae* or *aggR*	None
2 (D/BD/HUS)	*stx2d**	O113:H21 (1)
3 (D/BD)	*stx2c*, *eae*	None
4 (D/BD)	*stx1a*, *eae*	None
5 (D)	Other *stx* subtypes	O128:H2 (10), O22:H16 (7), O146:H21 (7), O87:H16 (5), O174:H8 (5), O113:H4 (4), O166:H28 (3), O146:H28 (2), O187:H28 (2), Ou:H7 (2), O11:H5 (1), O117:H4 (1), O22:H8 (1), O36:H14 (1), O38:H26 (1), O75:H8 (1), Ou:H21 (1), Ou:H8 (1)

* Potential to cause illness is dependent on strain background and *stx2d* variant. Adapted from (WHO-FAO, 2018).

3.3.9 Phylogenetic analysis of non-O157 STEC strains from deer and from Scottish human clinical isolates

To determine the genetic relationship between the non-O157 STEC strains isolated from deer and non-O157 STEC strains found in human clinical cases of STEC, a phylogenetic tree was constructed based on cgMLST for the 56 sequenced non-O157 STEC deer isolates and 105 human isolates from Scottish non-O157 STEC clinical cases isolated between 2002 and 2017 (Figure 3.6). The phylogenetic tree also included one sheep isolate, three isolates from venison meat, four *stx* negative/*eae* positive *E. coli* deer strains and three STEC reference strains (EQA) of known O type. The maximum difference between isolates that could be visualised through this analysis was 200 allellic differences.

In general, strains of the same serotype clustered together, although for the most common serotype, O128:H2 (n=10), deer and human strains formed separate branches, albeit two of the O128:H2 deer strains clustered with human clinical strains, and one human strain clustered within O128:H2 deer strains. The second most common serotype in deer was O22:H16 (n=7) and these strains formed a distinct cluster containing only deer isolates. The next most common deer serotypes, O146:H21 (n=7), O87:H16 (n=5), O174:H8 (n=5), O113:H4 (n=4) and O166:H28 (n=3), all clustered with human clinical isolates of the same serotype. Two deer strains of serotype O187:H28 were isolated from deer grazing in the same area and the strains were identical on the basis of cgMLST. Deer strains which did not cluster with other isolates and had more than 200 allellic differences from other isolates included strains of serotype O36:H14, O22:H8 and O11:H5.

Details of the most closely related deer and human clinical isolates are shown in Table 3.9. These included one isolate of each of the following serotypes: O166:H28 (strain 491 D8), O128:H2 (strain 134 E5), O113:H21 (strain 793 G8), O113:H4 (strain 99 B4) and O87:H16 (strain 764 D10). These deer strains had between 12 and 38 allelic differences with their corresponding human isolate; however the serotypes and MLST profile were the same for both deer and human isolates. In one serotype O166:H28 pair, the human clinical isolate had a *stx2b* and *stx1a* gene, while the corresponding deer sample was *stx2b* positive only. However, in each of the other pairs, the *stx* subtype profile was the same. In 3 of 5 pairs of deer and human strains, the additional virulence gene profile was identical and in the remaining two pairs virulence gene profiles in the human clinical and deer isolates differed by one or two genes. The five human isolates identified as being most similar to the deer isolates were isolated between 2003 and 2018. There was between

one year and 15 years between isolation of human strains and corresponding deer strains however there was no clear pattern between genetic relatedness and time between isolation. The pair of strains isolated one year apart were one of the least genetically similar.

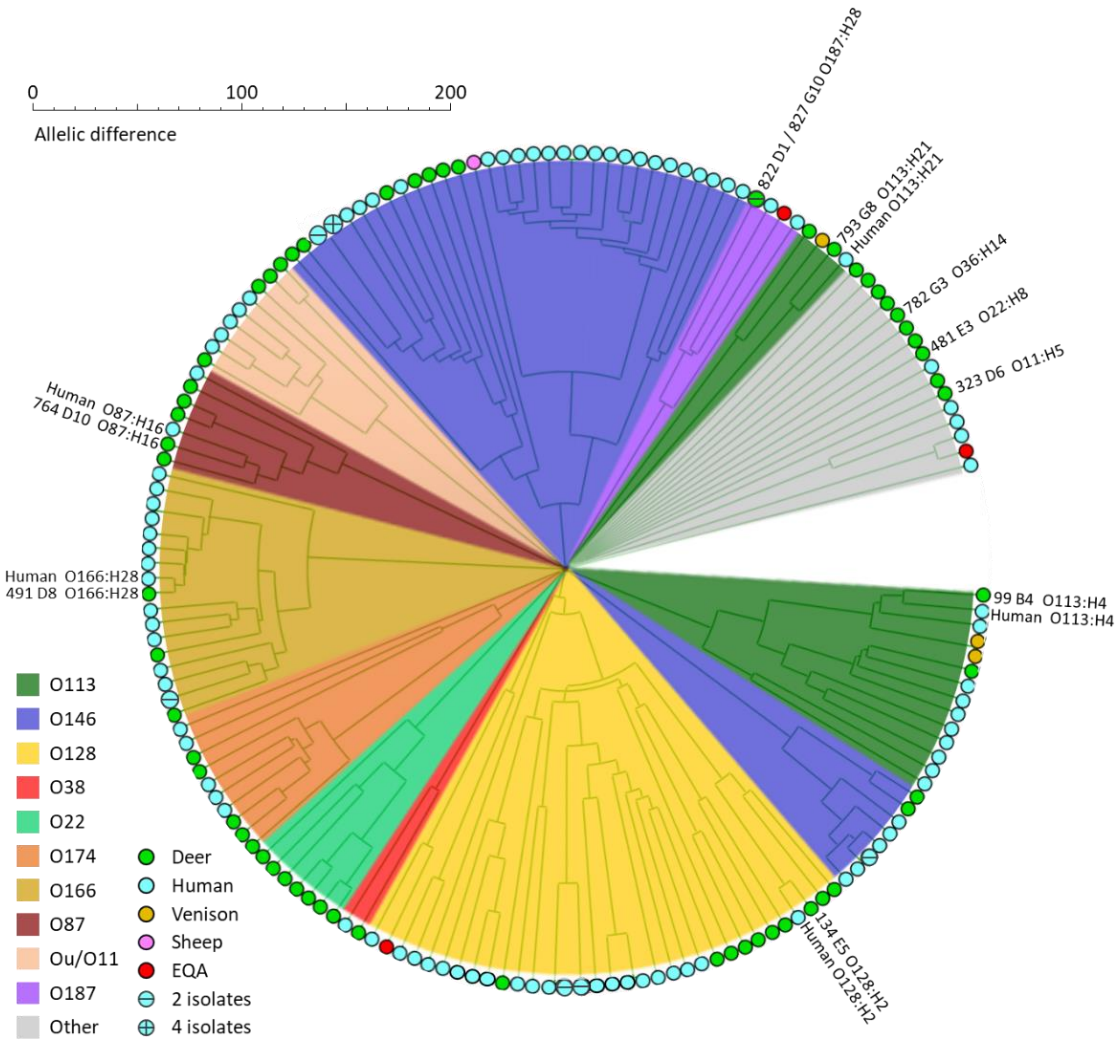

Figure 3.6 Phylogenetic analysis of non-O157 STEC isolates from Scottish human clinical cases and Scottish wild deer. The dendrogram is based on the allelic profile of 2,513 cgMLST target genes. Bionumerics v7.6 was used to produce the tree using the Advanced Cluster Analysis Tool and Topscore UPGMA (Unweighted Pair Group Method with Arithmetic Mean). Deer: strains isolated and sequenced in this study; Human: human clinical strains isolated from Scottish patients by SERL; Venison: isolates from venison meat obtained during investigation of STEC outbreak by SERL; Sheep: non-O157 STEC isolate from sheep from SERL strain archive; EQA: quality assurance reference strain of known O-types O187, O76 and O128. O groups of each cluster are highlighted in colour, 'Other' includes strains of O-group O117, O75, O36, O22, O11, O128, O76 and O113. Only O-groups are indicated for the majority of isolates. Individual isolates of interest are labelled with strain number and serogroup clockwise from top these are: Strains 822 D1/827 G10, identical based on cgMLST, isolated from two deer grazing the same area; deer strain 793 G8 most closely related to a human O113:H21 isolate; deer strains 782 G3, 481 E3 and 323 D6, all stx2a positive but phylogenetically distant from human clinical isolates and other deer strains; deer strain 99 B4 most closely related to a human O113:H4 isolate; deer strain 134 E5 most closely related to a human O128:H2 isolate, deer strain 491 D8 most closely related to a human O166:H28 isolate and deer strain 764 D10 most closely related to a human O87:H16 isolate. The scale bar corresponds to the branch length in the dendrogram and indicates number of allelic differences between isolates.

Table 3.9 Details of deer and human non-O157 STEC isolates most closely related based on cgMLST *

Strain ID	**Source**	**Serotype**	**7 gene MLST Sequence Type**	**Shiga toxin gene profile**	**No. of cgMLST allelic differences**	**Year isolated**	**Virulence genes**
491 D8	Deer	O166:H28	1819	*stx2b*	12	2017	*FimH,gad,subA,iss,ireA,iha,ehxA,hlyD,mchF,mchB,mchC,senB,hra,eilA,air*
Human O166:H28	Human	O166:H28	1819	***stx1a*** *stx2b*		2015	*FimH,gad,subA,iss,ireA,iha,ehxA,hlyD,mchF,mchB,mchC,senB,hra,eilA,air*
134 E5	Deer	O128:H2	25	*stx1c stx2b*	16	2017	*FimH,gad,lpfA,subA,iss,ireA,iha,ehxA,hlyD,mchF,mchB,mchC,senB,PAI(malX),fyuA*
Human O128:H2	Human	O128:H2	25	*stx1c stx2b*		2015	*FimH,gad,lpfA,subA,iss,ireA,iha,ehxA,hlyD,mchF,mchB,mchC,senB,PAI(malX),fyuA*
793 G8	Deer	O113:H21	3695	*stx2d*	33	2018	*FimH,gad,lpfA,subA,iss,ireA,iha,astA,**cba**,**cma**,celb*
Human O113:H21	Human	O113:H21	3695	*stx2d*		2003	*FimH,gad,lpfA,subA,iss,ireA,iha,astA,celb*
99 B4	Deer	O113:H4	10	*stx1c stx2b*	38	2017	*FimH,gad,subA,iss,ireA,iha,ehxA,hlyD,astA,cba,cma,celb,senB*
Human O113:H4	Human	O113:H4	10	*stx1c stx2b*		2018	*FimH,gad,subA,iss,ireA,iha,ehxA,hlyD,astA,cba,cma,celb,senB*
764 D10	Deer	O87:H16	2101	*stx2b*	38	2018	*FimH,gad,lpfA,ireA,espI*
Human O87:H16	Human	O87:H16	2101	*stx2b*		2005	*FimH,gad,lpfA,ireA,espI,**celb***

*Shiga toxin genes or virulence genes that differ between pairs of strains are underlined and in bold.

3.4 Discussion

From a total of 161 samples which were positive by PCR for *stx2* and *eae* and negative for O157, isolation was attempted for 93 samples which had a *stx2* CT value < 30 in qPCR. In total, 86 non-O157 STEC strains were isolated and 56 strains were subjected to WGS analysis. Samples for isolation were selected based on levels of *stx2* DNA, so were not truly randomly selected. However, the 56 strains included isolates from all deer species and from each main geographical area of Scotland, thereby providing information on the typical strains of non-O157 STEC circulating in Scottish wild deer.

3.4.1 Common deer non-O157 STEC serotypes

In this study the most common serotypes isolated from deer were O128:H2, O22:H16 and O146:H21, of which O128:H2 and O146:H21 are reported as the most common serotypes in studies of deer conducted in Spain and Germany. A study in Spain analysing roe deer rectal swabs found O146:H28, O146:H21 and O2:H6 STEC (Miko *et al.*, 2009) to be the three most common serotypes, whereas a study in Germany of red and roe deer meat found O21:H21, O146:H28 and O128:H2 to be the three most common serotypes (Mora *et al.*, 2012). A further study of isolates from deer meat in Germany found the three most common isolates to be O21:H21, O146:H28 and O146:H21 (Martin and Beutin, 2011). Although they were among the top three serotypes seen in Spain and Germany, neither O2:H6 or O21:H21 serotypes were observed in isolates from Scottish wild deer. These studies suggests that there are regional and national variation in STEC serotypes present in deer populations.

No studies to date have reported serotype O22:H16 in deer. It has been identified in sheep meat and beef in China, animal derived foods in Europe and in bovine isolates in North America (Hussein and Bollinger, 2005; Beutin *et al.*, 2007; Bai *et al.*, 2016). Other isolates identified in this study and which have not been reported previously in deer include O113:H4, O166:H28, O117:H4 and O38:H26. Each of these, with the exception of O117:H4, have previously been identified in sheep, cattle or wild boar, suggesting the possibility of circulation between deer and other wildlife and domestic livestock (Miko *et al.*, 2009; Martin and Beutin, 2011; Díaz-Sánchez *et al.*, 2012; Mora *et al.*, 2012). In addition to the above observations on serotype prevalence, five isolates were of novel sequence types (10655, 10656, 10657, 10658 and 10659) which have not been previously recorded in the 7 gene MLST database, suggesting that deer are a source of previously

unidentified STEC. Although cgMLST available from WGS data provides a much more detailed comparison of strains, 7 gene MLST is still useful for comparison with historical data on STEC strains as 7 gene MLST data exists from 2002, whereas cgMLST has only been employed in the past few years for routine characterisation of STEC (Zhou *et al.*, 2020).

3.4.2 Association of deer isolate serotypes and geographical area of sampling in Scotland

Associations between STEC serotype and geographical area of animal cull were observed in this study, although they should be interpreted with caution due to the low numbers of isolates in some categories. Serotype O22:H16 was predominant in the North West, serotype O128:H2 was predominant in the North East, serotype O174:H8 was predominant in Central Scotland and serotype O146:H21 was predominant in the South of Scotland. Studies of STEC O157 have shown that specific strains are linked to area (Herbert *et al.*, 2014; Widgren *et al.*, 2015). A cross-sectional study of dairy calves in New Zealand observed regional differences with STEC O26 more prevalent in the South Island and STEC O45 more prevalent in the North (Browne *et al.*, 2018). It is possible that certain serotypes may be specific to particular deer species, as samples from the South were mainly obtained from roe deer (7/9) and red deer make up the majority of samples from Central (6/8), North West (24/28) and North East (7/9). There is evidence that specific serotypes can be associated with either sheep or cattle (Urdahl *et al.*, 2003; Martin and Beutin, 2011). Given that the numbers of isolates sequenced from roe deer was low compared to those obtained from red deer, sequencing of additional STEC isolates from roe deer would be needed to determine whether there is an association of particular serotypes with species of deer.

3.4.3 Evidence of local transmission of non-O157 STEC strains between co-grazing deer

Two isolates, 822 D1 and 827 G10, sampled from two red deer culled at the same time and location were identical based on core genome MLST. Both strains had the *stx* subtype *stx2g* and had identical virulence gene profiles. Although there is a possibility of cross contamination during sampling, this result may indicate that strains spread between deer which were co-grazing. Alternatively, both deer may have acquired infection from a common source. Further characterisation of isolates from deer sampled from the same

area would provide information on circulation of strains between animals grazing in close proximity. Both samples were from female red deer (hinds) which have been observed to remain within a small home range along with other females so possibly grazing the same area (Best Practice Guidance on the Management of Wild Deer in Scotland-Red Deer; Froy *et al.*, 2018).

3.4.4 Evidence of mixed non-O157 STEC infections in wild deer

From two deer, two different STEC strains were isolated and sequenced. Within each deer, the strains were distinct, being of different serotype, sequence type and virulence gene profile. Looking at all 86 deer isolates, not just the 56 isolates sequenced, two different isolates were cultured for 13 deer. For an additional eight deer, only an isolate positive for either *stx1* or *stx2* was recovered, despite both genes being detected in the faecal sample by PCR. This suggests other STEC were present in these faecal samples, but were not isolated.

Previous studies in cattle, sheep, goats and deer have identified mixed STEC infections. However, as these studies did not specifically aim to determine the levels of mixed infection, the numbers of mixed infections may have been underestimated (Urdahl *et al.*, 2003; Schilling *et al.*, 2012; Dias *et al.*, 2019). This has implications for studying STEC strains in animal populations, as isolates may be missed when only one isolate per animal is chosen for sequencing. This may also affect attribution of STEC infection to a particular source or estimates of serotype prevalence. Screening and sequencing of additional isolates would reduce the chance of overlooking strains which may be important epidemiologically and would provide a better picture of the true incidence of mixed infections in animal populations.

3.4.5 Prevalence of antimicrobial resistance genes in STEC from Scottish wild deer

Only one isolate was positive for acquired antibiotic resistance genes for sulphonamide (*sul-2*), β-lactamase (*bla-TEM-1C*) and aminoglycosides (*aph(6), strB*) suggesting it had been exposed to antimicrobial selection. As deer are not treated with antimicrobials, it is possible that this strain had been acquired from livestock. No data is available for prevalence of antimicrobial resistance in STEC in cattle and sheep in Scotland. Studies from Ireland found antimicrobial resistance in 2/44 isolates of STEC O157 from cattle and sheep, although another study found 29 % of STEC isolated from beef cattle had

resistance to at least one antimicrobial (Prendergast *et al.*, 2011; Ennis *et al.*, 2012). Studies from Spain indicate a range from 2.8 % of cattle and sheep STEC isolates carrying resistance genes to 40 % of cattle STEC isolates (Mora *et al.*, 2005; Oporto *et al.*, 2019). A study in Belgium tested sensitivity of STEC isolates from deer against antibiotics widely used in bovine veterinary treatment (Bardiau *et al.*, 2010). Amoxycillin/clavulanic acid resistance was seen in STEC isolates from 13/16 deer but this was at an intermediate level, meaning the strains might not be fully resistant. Only one isolate from a red deer had full resistance to amoxicillin/clavulanic acid, and five animals had isolates resistant to the aminoglycoside spectinomycin. No resistance was found to 13 antibiotics tested in five STEC isolates from deer meat in Spain (Díaz-Sánchez *et al.*, 2012). In a separate study of faecal samples from deer in Portugal, resistance genes *ereB* and *mphA* genes, associated with macrolide resistance were identified in one isolate from a roe deer, while a second roe deer isolate had the *dfrA5* gene, conferring resistance to trimethoprim. All strains however were phenotypically sensitive to the antibiotics according to clinical cut-offs (Dias *et al.*, 2019).

The low level of antimicrobial resistance found in Scottish wild deer STEC in this study is similar to that found in other countries, although caution is needed due to the differing methods employed to characterise antimicrobial resistance – in the case of this study it was inferred through identification of antimicrobial resistance genes rather than phenotypic characterisation of resistance. In human non-O157 STEC isolates, 17.6 % of Scottish human clinical strains isolated between 2006 and 2018 had a least one antibiotic resistance gene and 27.3 % of isolates from England between 2014 and 2016 had at least one antibiotic resistance gene (Food Standards Scotland, 2020b; Gentle *et al.*, 2020). As antimicrobials are not generally used to treat STEC infections, there is not likely to be selection pressure for maintenance of antimicrobial resistance genes in STEC.

3.4.6 Isolation technique for non-O157 STEC in deer

In other studies determining STEC prevalence, IMS methods have been used to specifically isolate the top five serotypes considered to be most important in human disease (O26, O103, O145, O111 and O157) (Lillehaug *et al.*, 2005; Frank *et al.*, 2019). This study demonstrates the limitations of an IMS-based approach as none of the Scottish wild deer strains isolated are positive for these O groups, meaning that using IMS would have missed all of the non-O157 STEC the isolates recovered in this study. Similarly around half of the Scottish human clinical isolates used in this study were not of the top

five serotypes and would not have been detected using IMS methods for the most common serotypes.

The use of SMAC agar plates for the recovery of STEC was successful in that STEC were isolated from 77.7 % of PCR *stx2* positive samples. This is in agreement with another study which found STEC could be isolated from 70 % of PCR positive samples (De Rauw *et al.*, 2018). The use of Chromagar STEC plates did not improve ability to isolate STEC from deer and only two strains were isolated from these plates. A proportion of samples (25 %) did not grow any colonies or had very limited growth on Chromagar STEC plates, whereas growth was seen on SMAC plates for the same sample, suggesting that both commensal and STEC strains from deer are sensitive to the selective agents. The plates contain tellurite to which two studies have reported 74.3 % and 77.3 % of non-O157 STEC isolated from animals, food and humans as being sensitive (Orth *et al.*, 2007; Fan *et al.*, 2018). Based on the WGS analysis, only 10 out of the 56 deer isolates characterised in this study harbour tellurite resistance genes which may explain the poor growth of samples on the Chromagar STEC plates. Although only two isolates were originally isolated from Chromagar STEC, it would be useful to determine if there is a correlation between tellurite resistance genes and sensitivity of isolates to tellurite, to determine if this was the cause of lack of growth on Chromagar STEC plates. Chromagar STEC have been reported to be effective for isolating non-O157 from human cases of infection (Gouali *et al.*, 2013; Jenkins *et al.*, 2020). However, using Chromagar STEC plates alone to isolate STEC from other sources including food and animal sources may underestimate the full range of STEC present (Verhaegen *et al.*, 2015; Fan *et al.*, 2019).

3.4.7 Serotypes found in deer compared to human strains

Deer isolates were compared to 522 non-O157 STEC isolated by SERL from human clinical cases in Scotland over a 16 year period (Food Standards Scotland, 2020b). Around half of the serotypes (11 of 19) seen in the deer strains were represented in Scottish human clinical isolates and had the same *stx* subtype profiles. There was greater diversity in human isolates (98 different serotypes, including those with unidentifiable O-groups) compared to deer isolates (19 different serotypes, including unidentifiable O-groups), likely due to the greater number of human isolates included in this analysis. Isolates of serotype O22:H16 were only found in deer in this study. This serotype has been identified in one human infection in South Africa (Karama *et al.*, 2013), which was *stx2* positive although the subtype was not identified. The top three serotypes seen in

human clinical isolates (O26:H11, O103:H2, O145:H28), which were typically *eae* positive, were not identified in any of the deer isolates suggesting that, although the deer isolates were relatively few in number compared to the human isolates, they are from a different although possibly overlapping population of STEC.

3.4.8 Shiga toxin gene subtype profile and other virulence genes of non-O157 STEC from deer and humans

Amongst the deer isolates characterised in this study, *stx2* subtypes *a*, *b*, *d* and *g* and *stx1c* were observed. The subtypes found are in agreement with other studies of deer in Spain, Portugal, Germany, Poland and Switzerland which found *stx2b* to be the most common *stx2* subtype reported as 22 % (11/51) to 97 % (31/32) of non-O157 STEC isolates from deer followed by *stx2g* found in 3 % (3/103) to 24 % (8/33) of isolates (Hofer *et al.*, 2012; Mora *et al.*, 2012; Eggert *et al.*, 2013; Dias *et al.*, 2019; Frank *et al.*, 2019; Szczerba-Turek *et al.*, 2020). The most common *stx1* subtype reported was *stx1c*, frequently in combination with *stx2b*. The low prevalence of *stx2a* and *stx2d* positive strains observed in Scottish wild deer is similar to observations of non-O157 STEC from deer in other countries. A study in Spain found 4 % (4/103) of isolates to be *stx2a* positive and a study in Poland reported 12 % (6/51) of isolates were *stx2a* positive (Mora *et al.*, 2012; Szczerba-Turek *et al.*, 2020). Prevalence of *stx2d* in non-O157 isolates from deer has been reported as of 2 % (2/96) isolates, 3 % (3/103) isolates and 15 % (5/33) isolates in studies in Spain, Germany and Switzerland respectively (Hofer *et al.*, 2012; Mora *et al.*, 2012; Frank *et al.*, 2019).

None of the *stx* positive deer isolates were positive for *eae*, which encodes for the adhesin intimin and is associated with strains causing more severe forms of disease in humans including HUS (Brandal *et al.*, 2015; De Rauw *et al.*, 2018). This suggests that the deer non-O157 STEC strains isolated in this study would be unlikely to cause severe human disease. However, 186/517 (35.6 %) of the sequenced Scottish human non-O157 isolates also lacked *eae* (Food Standards Scotland, 2020b) which indicates that *eae* negative STEC strains can still cause clinical disease in humans. This is consistent with a study of 129 human clinical isolates from the Netherlands in which 80.9 % of non-O157 strains were *eae* negative (Franz *et al.*, 2015). Although *eae* negative STEC are isolated from cases where symptoms are consistent with STEC infection, this does not prove that the strains are causative of disease and further work is required to determine this. STEC can be isolated from asymptomatic individuals (Friedrich *et al.*, 2002; Brandal *et al.*, 2015).

Additionally STEC cases may have concurrent infections with parasites, other bacteria or viruses that may cause gastrointestinal symptoms (Ferdous *et al.*, 2016). Adhesins which may provide *eae* negative strains with an alternative means of attaching to host cells include the STEC autoagglutinating adhesion (*saa*) and the iron-regulated gene homo-log adhesion (*iha*) and the fimbriae *lpf* and *FimH* (Tarr *et al.*, 2000; Paton *et al.*, 2001; Clements *et al.*, 2012). In this study, *iha* was found in 71 % of the deer isolates although only one isolate carried *saa*. Additional adhesion encoding genes *FimH* and *lpfA* were common, being found in 98 % and 82 % of the sequenced deer isolates in this study respectively, and were also common in both the human *eae* positive and *eae* negative strains (Food Standards Scotland, 2020b). This suggests that while all the deer STEC strains isolated in this study were *eae* negative, many of the strains may still be able to colonise the intestinal epithelium via non-*eae* dependent mechanisms, although they would be expected to cause less severe human clinical disease. Virulence genes which were found to have significantly higher prevalence in *eae* negative Scottish human clinical isolates were also found in deer isolates with the exception of *iutA*, *pic* and *hlyF* which were seen in 5 %, 3 % and 2 % of human clinical isolates, respectively. The number of deer isolates analysed may have been too low to identify the presence of these lower frequency genes. Of the virulence genes detected in deer strains 19/36 genes detected were found to be associated with *eae* negative isolates in Scottish human clinical isolates. In contrast 7/36 of the virulence genes observed in the deer strains were associated with *eae* positive isolates in the Scottish human clinical isolates. All of the deer isolates were *eae* negative and it seems that additional virulence genes in deer STEC are also similar to those associated with human *eae* negative STEC strains.

The adhesins *f17A* and *f17G* were found in 5 out of 7 O22:H16 strains from this study, a serotype which has not been observed in deer before and was not seen in the human clinical isolates. These adhesins are associated with *E. coli* strains causing disease in cattle which are *stx* negative so may provide evidence that *E. coli* strains are transferred between cattle and deer or that acquisition of *stx* genes by non-STEC strains occurs within deer (Bertin *et al.*, 1996; Valat *et al.*, 2014). In addition to the adhesins, the most common virulence genes which were seen in both deer isolates and *eae* negative human clinical isolates were *gad*, *iss*, *subA* and *ireA*. The genes *gad*, *iss* and *ireA* contribute to survival of the bacteria in the host and their high frequency in deer and human isolates indicate that deer strains possess the necessary genes for causing human infection. The gene *gad* codes for glutamate decarboxylase which enables the bacteria to survive the acid environment of the digestive tract and probably contribute to the low infective dose of

STEC (Vanaja *et al.*, 2009). The gene *iss* codes for a protein which confers resistance to host complement (Johnson *et al.*, 2008). The gene *ireA* encodes a siderophore receptor which allows the bacteria to sequester iron necessary for growth in the host intestine (Page, 2019).

The toxin encoding gene *subA* was seen in 73.1 % of *eae* negative Scottish human clinical isolates but not in *eae* positive Scottish human clinical isolates. Strains positive for *subA* have been associated with HUS previously (Paton and Paton, 2010). Along with the co-transcribed gene *subB,* it forms subtilase (SubAB) toxin with serine protease activity which inactivates the chaperone protein BiP leading to apoptosis of cells (Paton and Paton, 2010; Seyahian *et al.*, 2017). The gene *subA* was present in 79 % of deer isolates. Four different variants of the subtilase (*subA*) gene have been reported, one plasmid associated variant and three chromosomal variants located in different genomic regions (Nüesch-Inderbinen *et al.*, 2015; Wyrsch *et al.*, 2020). The chromosomal variant of *subA* associated with the gene *tia* has been reported to be common in deer, sheep and isolates from human cases of diarrhoea (Sánchez *et al.*, 2012; Michelacci *et al.*, 2013; Nüesch-Inderbinen *et al.*, 2015). The plasmid associated variant which is present alongside the *saa* gene has been associated with cases of HUS (Paton *et al.*, 2001; Michelacci *et al.*, 2013). *In-vitro* plasmid encoded subtilase and chromosomal encoded subtilase have similar toxicity for Vero cells so it is possible that apparent differences in human disease outcome are related to co-transcribed genes (Tozzoli *et al.*, 2010; Michelacci *et al.*, 2013; Wyrsch *et al.*, 2020). Future work to determine the variant of *subA* present in deer isolates would provide more information on the potential to cause disease in humans.

3.4.9 Deer non-O157 STEC strains most closely related to human clinical isolates

The five deer strains most closely related to strains from the collection of Scottish human clinical isolates analysed by SERL had between 12 to 38 allelic differences in genes in the cgMLST scheme consisting of 2,513 genes. Isolates from the same outbreak are considered to have less than 10 allelic differences and typically 1 – 3 allele differences. Based on this criteria, these five deer strains were not closely related to the human STEC isolates (Holmes *et al.*, 2018; Rumore *et al.*, 2018; Jones *et al.*, 2019). The mutation rate in STEC O157 has been calculated as 2.6 mutation / genome/ year. However, rates of allelic changes in cgMLST are likely to be lower as these genes in the MLST scheme are selected for inclusion in the MLST scheme for their stability. Therefore they are less

likely to be mobile or subject to selection pressure than other genes within the genome (Dallman *et al.*, 2015).

Deer isolate 491 D8 of serotype O166:H28 was most closely related to a human clinical isolate of the same serotype, although the human isolate contained *stx1a* in addition to *stx2b*. This serotype was also documented in wild boar and sheep in Spain suggesting there are other animal reservoirs apart from deer (Blanco *et al.*, 2003; Diaz-Sanchez *et al.*, 2013). Deer isolate 134 E5 and human isolate of serotype O128:H2, the most common serotype found in deer and in *eae* negative Scottish human clinical isolates, differed by 16 alleles. Serotype O128:H2 has commonly been found in deer but also amongst STEC isolates from lamb meat and sheep faeces, suggesting possible sources other than deer (Urdahl *et al.*, 2003; Miko *et al.*, 2009; Martin and Beutin, 2011). In this study, a deer isolate 793 G8 of serotype O113:H21 was identified which was separated from a human clinical isolate of the same serotype by 33 allelic differences. This serotype has previously been identified in deer, wild boar and hares, and also in cattle in Scotland (Jenkins *et al.*, 2002; Miko *et al.*, 2009).

The O113:H21 deer isolate 793 G8 was positive for *stx2d*, which is associated with more severe forms of human disease (Bielaszewska *et al.*, 2006). However, there was only one deer isolate and only one human clinical isolate of this serotype and *stx* profile reported, suggesting that human infections with this serotype in Scotland are rare or of low pathogenicity. Cases of HUS associated with strains of serotype O113:H21, which were also positive for the adhesin *saa* have been reported in Australia and Canada (Feng *et al.*, 2014), The O113:H21 deer strain in this study and the human clinical strain that it was phylogenetically closest to were *saa* negative, suggesting they may not have the same potential for causing disease. The closely related deer isolate 99 B4 and a human isolate of serotype O113:H4 had a difference of 38 alleles. This serotype is commonly isolated from cases of human disease and is associated with uncomplicated diarrhoea or asymptomatic carriage (Friedrich *et al.*, 2002; Beutin *et al.*, 2004). Although not reported in deer previously, it has been reported as a common serotype in studies of cattle in Norway and Ireland (Urdahl *et al.*, 2003; Monaghan *et al.*, 2011). The closely related deer isolate 764 D10 and human isolate of serotype O87:H16 also had 38 allelic differences. Although four deer isolates were of this serotype, it was relatively rare (only 2 isolates) in Scottish human clinical isolates. Although not reported in deer previously, it has been isolated from sheep in Brazil and Switzerland (Vettorato *et al.*, 2003; Zweifel *et al.*, 2004).

Each of the five deer isolates which were clustered most closely with Scottish human clinical strains had the same *stx2* subtypes as the human isolates and three had identical additional virulence genes profiles. A further two differed only in the presence of colicin encoding genes which act to inhibit competing commensal bacteria so are unlikely to have major effect on pathogenicity of the isolate (Montero *et al.*, 2019). Although they are not closely related at the genetic level, the similarities between the *stx* and virulence gene profiles of the five deer isolates and their most closely related human clinical strains would suggest that these deer strains would be capable of causing human disease,

3.4.10 JEMRA assessment of potential to cause disease

A Joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organisation (WHO) Expert Meetings on Microbiological Risk Assessment (JEMRA) report classified risk to human health based on virulence genes present (WHO-FAO, 2018). All but one of the deer isolates in this study would be classified as having potential to cause diarrhoea in humans, having low risk of causing more serious disease such as bloody diarrhoea and HUS. However, the majority of deer strains were *stx2b* positive strains which has been associated with bloody diarrhoea (De Rauw *et al.*, 2018). This suggests that while deer strains are unlikely to cause HUS, they may potentially cause bloody diarrhoea and predictions have some limitations in determining disease severity.

3.4.11 Deer isolates with highly pathogenic *stx* subtype *stx2a*

In this study, only three *stx2a* positive strains were isolated and all were quite distant from human clinical samples with over 200 cgMLST allelic difference from any human clinical isolates. The first *stx2a* positive strain 323 D6 had the serotype O11:H5 which was not seen in any of the Scottish human clinical isolates and did not cluster with any other deer isolates, although it has been recorded in deer previously (Díaz-Sánchez *et al.*, 2012). A second *stx2a* positive isolate was strain 481 E3 which was serotype O22:H8 - this serotype was seen in a Scottish human clinical isolate, however with a different *stx* subtype of *stx2d*. Although phylogenetically distant from human clinical isolates, it was the only deer strain positive for *saa* which codes for STEC autoagglutination adhesin and has been suggested as an alternative adhesin in *eae* negative HUS associated strains (Paton *et al.*, 2001). Serotype O22:H8 has also been isolated from cattle and venison in other studies but not associated with *stx2a* (Miko *et al.*, 2009; Wang *et al.*, 2018).

The third strain 782 G3 was *stx2a*:*stx2g* positive and serotype O36:H14 which was not seen in the Scottish clinical isolates. Other studies have identified the same serotype in deer faeces and vegetables, although they were positive for *st2g* only (Mora *et al.*, 2012; González-Escalona and Kase, 2019). Strain 782 G3 was also positive for the heat stable enterotoxin *sta1*. This toxin is characteristic of non-STEC pathogenic *E. coli* meaning that this may represent a hybrid strain with characteristics of STEC and enterotoxigenic strains (Prager *et al.*, 2011). Hybrid strains are of interest as emerging pathogens as they combine virulence genes not commonly present in the same bacteria which may result in severe human disease (Bai *et al.*, 2019; Food Standards Scotland, 2020b).

Although *stx2b* positive strains appear to be more prevalent in deer and may be likely to cause human disease, *stx2a* positive strains may represent a source of emerging pathogens. As deer can carry more than one strain of STEC, they may be a source of *stx2a* which could transfer to other strains to generate highly pathogenic strains. Increasing *stx2a* prevalence has been observed in O26:H11 serotype strains and in STEC O157 (Bielaszewska *et al.*, 2013; Byrne *et al.*, 2018; Food Standards Scotland, 2020b). Presence of Stx2a has been shown to increase transmission of strains through increased colonisation success in cattle (Fitzgerald *et al.*, 2019). If Stx2a has a similar effect in deer, this may result in increasing prevalence of *stx2a* positive strains.

3.4.12 Study limitations

As this study focused on isolation of *stx2* positive strains from *stx2* PCR positive samples, *stx1* positive isolates which could also pose a risk of human disease may have been overlooked. Samples were also not chosen randomly, so the current observations may not be representative of the typical strains throughout the country and for all deer species present.

3.4.13 Future work

To investigate the source of non-O157 causing human disease in Scotland, it would be useful to carry out a similar investigation of non-O157 STEC present in other known reservoirs of STEC including cattle and sheep. Previous studies in cattle and sheep have focussed mainly on STEC O157 or on selected O types, which does not give a complete picture of the full range of STEC present. The use of WGS, which was not available in previous studies, would also allow more detailed comparison (Jenkins *et al.*, 2002; Pearce

et al., 2006; Evans *et al.*, 2011). The most frequent *stx* subtype found in human isolates both in Scotland and in other countries was *stx1a*. Future work should further characterise *stx1* isolates to determine the risk they pose to human health.

PCR analysis suggested that the *stx2a* gene is present in 27.6 % (76/275) of roe deer samples positive for *stx1* or *stx2* (see Chapter 2, Table 2.4). However, due to time limitations and the isolation approach taken, only two *stx2a* positive strains were analysed from roe deer. It would therefore be informative to isolate more strains from the other *stx2a* positive samples in order to test the hypothesis that they are similar to strains circulating in cattle or other livestock.

3.4.14 Conclusions

The main serotypes found in Scottish wild deer in this study were O128:H2, O22:H16 and O146:H21, and all of these isolates had a *stx* profile of *stx2b* or *stx2b*:*stx1c*. Both *stx1c* and *stx2b* subtypes are considered to be of low pathogenicity and none of the isolates were positive for the adhesion gene *eae* associated with serious human disease. Only three isolates were positive for the highly pathogenic subtype *stx2a*. These isolates included serotypes and *stx* profiles not seen in Scottish human clinical isolates and were phylogenetically distant from human clinical isolates, suggesting they do not pose a high risk of human disease. The only isolate that was classed as potential for causing severe disease according to JEMRA guidelines was an isolate with serotype O113:H21 which was positive for *stx2d*. The same serotype and virulence gene profile was only detected in one human isolate, suggesting that human infections of this serotype are rare and the risk of human infection with deer strains is low.

Chapter 4: General discussion and conclusions

The overall aim of this project was to evaluate the potential risk to human health of non-O157 STEC present in Scottish wild deer. Previously the prevalence of STEC O157 in Scottish wild deer in a nationwide survey has been found to be low (McNeilly *et al.*, 2020). However PCR testing for *stx* genes suggested a large proportion (69.5 %) of deer carry non-O157 STEC. The first aim of the project was determining the prevalence of the high pathogenic *stx* subtype *stx2a* in deer faecal samples and determine risk factors for the presence of *stx2a* compared to other *stx* subtypes. The hypothesis was tested that *stx2a* positive strains may be circulating between deer and livestock, in particular cattle, which are known to carry *stx2a* positive strains (Shridhar *et al.*, 2017; Fan *et al.*, 2019).

A model was constructed for all deer species (roe, red and sika) which showed that roe deer species was a significant risk factor along with increasing sheep density. Indeed, faecal samples from roe deer were found to be around 8 times more likely that red or sika deer to be *stx2a* positive. Although the effect of increasing sheep density had less impact, this study provides evidence to support the hypothesis that livestock may be a source of *stx2a* positive strains. A separate model, constructed using only data from roe deer highlighted South Scotland, increasing raindays in sampling month and % semi-natural grassland in area of cull site to be non-species related significant risk factors. Although cattle and sheep density were not present in the final roe deer only model, an association with livestock and presence of *stx2a* could not be discounted as South of Scotland, an area of high cattle and sheep density, was a significant factor in the roe deer only model. It is possible that livestock are a source of *stx2a* with environmental factors such as increased rainfall and type of land cover facilitating spread of *stx2a* positive strains. However, a physiological difference in roe deer compared to other species of deer, and/or the possibility of geographical distribution of specific STEC strains are also possible explanations for higher prevalence of *stx2a* in roe deer.

The second aim of the project was to assess the potential for non-O157 STEC strains from wild Scottish deer to cause human disease. A total of 56 deer strains were isolated from deer faeces and subject to WGS. The samples from which the STEC strains were isolated were not randomly selected, with only a subset of faecal samples positive for both *stx2* and *eae* chosen for isolation work. Despite this, the STEC isolates characterised in this study were from a range of deer species sampled across different geographical locations across Scotland and were considered partially representative of non-O157 STEC strains

in the Scottish wild deer population. These strains were compared to a collection of 522 human clinical isolates analysed by the Scottish *E.coli* Reference laboratory over a 16 year period from 2002 to 2018. The majority of strains isolated from deer carried genes for *stx* subtype *stx2b* alone or in combination with *stx1c*. These subtypes have been linked to mild human disease (WHO-FAO, 2018). In contrast the most common *stx* genes found in Scottish human clinical isolates were *stx1a* and *stx2a.* Only 15% of Scottish human clinical isolates were of a serotype seen in deer isolates and O22:H16, the second most common serotype in deer, was not identified in the human isolates.

Phylogenetic analysis based on cgMLST showed five deer STEC isolates clustered with five distinct human clinical isolates, although none of the deer and human isolates were closely related based on the number of allelic differences within the core genome. However, *stx* profiles and virulence genes were identical for two of the closely related deer – human isolate pairs which all had the *stx* profile *stx1c:stx2b*. A further related deer-human isolate pair had identical virulence genes except for the human strain also being positive for *stx1a*, whereas a further two pairs of deer – human isolates had identical *stx* profiles but differed in presence of one or two additional virulence genes. This suggests that the deer isolates most closely genetically related to the human clinical isolates would be capable of causing human disease. It should also be noted that one deer STEC was positive for *stx2d*, a subtype which has been associated with HUS.

The major difference between deer and human isolates was in the presence of *eae*, encoding the virulence factor intimin. The three most common serotypes in human isolates were *eae* positive, whereas no *eae* positive STEC isolates were identified in deer. A limitation of this study is that the isolation of the deer strains was biased towards *stx2* positive isolates, whereas the majority of non-O157 Scottish human clinical isolates were *stx1* positive. Therefore future work should focus on isolating *stx1* positive STEC strains from the deer samples to determine the similarity with human clinical isolates.

The apparent higher prevalence of *stx2a* genes in roe deer compared to other species may be concerning as *stx2a* is associated with severe human disease. However, the virulence genes, serotypes and genetic distance from Scottish clinical isolates of the three *stx2a* positive deer strains that were isolated suggest they are not likely to be a source of human disease. Future work to isolate and characterise additional *stx2a* positive strains from roe deer would increase confidence in this assessment.

The *stx2a* positive strains isolated from deer were relatively uncommon serotypes which did not appear to be associated with cattle or sheep in previous studies. However, little is

known about non-O157 strains present in cattle and sheep in Scotland. So at this point it is not possible to draw conclusions on whether the higher prevalence of *stx2a* in roe deer, which are predominantly found in areas of high cattle and sheep density, is linked to strains circulating between deer, cattle and sheep. Further work to identify non-O157 STEC strains present in cattle and sheep in Scotland would provide information on the circulation of strains between wild and domestic ruminants and cases of human infection. Isolation of *stx2a* positive strains from roe deer in areas of low and high livestock density would provide more evidence to prove or disprove the hypothesis that strains circulate between deer and domestic livestock.

Although this study shows that STEC strains with human pathogenic potential are present in Scottish wild deer faeces, the routes of transmission by which they could potentially infect humans is unknown. A future area of study would be to characterise STEC isolated from deer carcasses to determine if they are the same strains found in the faeces from the same animal, and whether faecal contamination of the carcass could represent a source of food borne infection. Another potential route of infection is through human contact with deer faeces in the environment, which may be of growing importance if deer and human interactions increase through encroachment of human development on deer habitats or deer adapting to urban areas.

A further future area of research would be to determine the population of STEC in farmed deer. In deer, increasing animal density has been linked to increased STEC prevalence (Diaz-Sanchez *et al.*, 2013) and so it could be expected that higher stocking density in farmed deer could have an impact on STEC prevalence. As part of the Ambition 2030 initiative (Beyond the Glen, 2018) which aims to increase food production in Scotland, the Scottish Government aims to increase the consumption of venison including by increasing numbers of farmed venison. If farmed deer have higher levels of STEC prevalence compared to wild deer, this initiative could lead to an increased risk of human STEC infection.

In conclusion, despite the relatively high incidence of the highly pathogenic *stx* subtype *stx2a* in wild deer faeces in Scotland, STEC strains containing both *stx2a* and *eae*, a virulence profile associated with the most severe forms of human disease (Naseer *et al.*, 2017; De Rauw *et al.*, 2018) were not found among non-O157 STEC from Scottish wild deer. This study has shown that there are serotypes, *stx* subtype and virulence gene profiles of STEC isolated from deer that are the same as a subset of human clinical isolates, suggesting that there may be some overlap in deer and human clinical strains.

However, unique serotypes and gene profiles are present in both deer and human clinical STEC isolates. The overall conclusion therefore is that deer do not appear to be a major source of human STEC infections. Despite this, the finding of potentially pathogenic strains in deer underlines the importance of following best practice guidelines for processing of venison. Even considering the relatively small sample size in this study, a wide variety of serotypes were isolated including many not previously seen in deer or identified rarely in human infection. This emphasises the diversity of STEC in wildlife sources. Based on the data from this study, it would seem that deer do not pose a high risk to human health, but they should be kept in consideration as a source of emerging novel pathogens.

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Appendix 1: Template for questionnaire returned with each deer faecal sample

Date	
Time	
OS Reference of cull site	OS Sheet:_____ 6-digit grid reference:_____
Larder Address	
Deer species	Red ☐ Roe ☐ Sika ☐ Other ☐ If other provide details:_____
Gender	Male ☐ Female ☐
Condition Score	1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 1 = very poor condition; 5 = very good condition
Estimated age	_____years
Shared range with other livestock/ wild herbivores	Cattle ☐ Sheep ☐ Wild herbivores ☐ If wild herbivores provide details below: _____
Other comments	

QUESTIONNAIRE

THANK-YOU FOR PARTICIPATING IN THIS SURVEY

Appendix 2: Details of variables

Variable	Description	Source
Min temp region > 7 ˚C	Min temp for region for month more than 7 ºC s	UK Met Office
Max temp region> 7 ˚C	Max temp for region for month more than 7 ºC	UK Met Office
Mean temp region > 7 ˚C	Mean temp for region for month more than 7ºC	UK Met Office
Warm months	Warm months (May to October) vs cold months (November to April)	UK Met Office
Species	Deer species roe/red/sika	Questionnaire (Appendix 1)
Sex	Male / Female	Questionnaire (Appendix 1)
Land cover	Dominant land cover for 1km square of cull site	Land Cover Map 2015 (1km dominant aggregate class, GB)
Sampling area	Geographical areas based on Animal Health administrative areas: South East, South West, Central, North East, North West	Ordnance Survey data © Crown copyright and database rights 2013
Sampling area for roe deer analysis	Consolidated categories for comparison of South (SouthWest and SouthEast), vs North (Central, NorthWest, NorthEast)	Ordnance Survey data © Crown copyright and database rights 2013
Age category	Age category calf, yearling, adult based on estimated age	Questionnaire
Season	Season culled; Summer : June - August, Autumn: September - November, Winter: December - February, Spring: March - May	UK Met Office
Distance to nearest farm with cattle (m)	Distance from cull site to nearest farm with cattle present in metres	supplied by Paul Bessell
Distance to nearest farm (m)	Distance from cull site to nearest farm in metres	supplied by Paul Bessell
No. of cattle on nearest farm with cattle	Number of cattle on nearest farm to cull site which has cattle present	supplied by Paul Bessell
No. of sheep on nearest farm with cattle	Number of sheep on nearest farm to cull site which has cattle present	supplied by Paul Bessell
No. sheep on nearest farm	Number of sheep on nearest farm to cull site	supplied by Paul Bessell
No. cattle on nearest farm	Number of cattle on nearest farm to cull site	supplied by Paul Bessell
No. pigs on nearest farm	Number of pigs on nearest farm to cull site	supplied by Paul Bessell

No. poultry on nearest farm	Number of poultry on nearest farm to cull site	supplied by Paul Bessell
Carcass weight (kg)	Weight of carcass	Questionnaire
Age (years)	Estimated age	Questionnaire
Condition score	Scale of 1 to 5, 1 = very poor condition 5 = very good condition	Questionnaire
Total no of raindays in month	Number of days of rain in month sample collected for Met Office region	UK Met Office
Total no. of raindays in season	Raindays in season; spring, summer, autumn or winter of sampling for Met Office region	UK Met Office
Hours of sunshine in month	Sunshine hours in month of sampling for Met Office region	UK Met Office
Hours of sunshine in season	Sunshine hours for season; spring, summer, autumn or winter of sampling average for whole country used	UK Met Office
Minimum monthly temperature (˚C)	Min temp for region for month	UK Met Office
Maximum monthly temperature (˚C)	Max temp for region for month	UK Met Office
Mean monthly temperature (˚C)	Mean temp for region for month	UK Met Office
Frostdays region	Frostdays for region for month	UK Met Office
% Arable	Percentage of Arable land use in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)
% Mountain, heath, bog	Percentage of Mountain/Heath/Bog land cover in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)
% Semi natural grassland	Percentage of Semi natural Grassland in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)
% Broadleaf	Percentage of Broadleaf Woodland in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)
% Improved grassland	Percentage of Improved Grassland in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)
% Coniferous	Percentage of Coniferous Woodland in 1km area of cull site	Land Cover Map 2015 (1km percentage aggregate class, GB)

Appendix 3: Details of sheep and cattle density information from Agricultural Census data for 2015

EDINA at Edinburgh University Data Library and The Scottish Government (formerly SEERAD)

Category (treated as continuous variable)	Sheep density (animals / 1000 hectares ($10km^2$)	Sheep Low / High	Cattle density (animals / 1000 hectares ($10km^2$)	Cattle Low / High
1	≤50	Low	≤50	Low
2	50.01 - 500		50.01 - 150	
3	500.01 - 1000		150.01 - 250	
4	1000.01 - 5000		250.01 - 500	
5	5000.01 - 10000		500.01 - 1000	High
6	10000.01 – 20000	High	1000.01 – 2000	
7	>20000		>2000	

Appendix 4: Details of Red and Roe deer density estimated from British Mammal Survey counts of abundance in 2011-2015

Category	**Red deer density (counts / 1 km square)**	**Roe deer density (counts / 1 km square)**
1	0 - 0.5	0 - 0.1
2	0.5 - 1	0.1 - 0.2
3	1.0 - 2.0	0.2 - 0.5
4	2. 0 - 5.0	0.5 - 1
5	> 5	>1

Appendix 5: Virulence genes descriptions

Gene	Name	Category
FimH	Type 1 fimbrial protein	Adhesin
gad	Glutamate decarboxylase	Acid resistance
lpfA	Long polar fimbriae	Adhesin
subA	Subtilase toxin subunit	Toxin
iss	Increased serum survival	Other
ireA	Siderophore receptor	Other
iha	Adherence protein	Adhesin
ehxA	Enterohaemolysin	Toxin
hlyD	Haemolysin D	Toxin
mchF	ABC transporter protein MchF	Microcin
mchB	Microcin H47 part of colicin H	Microcin
mchC	MchC protein	Microcin
astA	Heat-stable enterotoxin 1	Toxin
cba	Colicin B	Colicin
cma	Colicin M	Colicin
celb	Endonuclease colicin E2	Colicin
senB	Plasmid encoded enterotoxin	Toxin
espI	Serine protease	SPATE
PAI(malX)	Pathogenicity island	Other
fyuA	Ferric yersiniabactin receptor	Siderophore
TraT	Outer membrane lipoprotein	Other
cvi-cvaC	Transporter accessory protein - colicin V immunity protein	Other
epeA	Serine protease	SPATE
hra	Heat resistant agglutinin	Other
f17A	F17 fimbrial protein	Adhesin
f17G	F17 fimbrial protein	Adhesin
eilA	*Salmonella* HilA homolog	Other
mcmA	Microcin M part of colicin H	Microcin
air	Enteroaggregative immunoglobulin repeat protein	Other
sta1	Heat-stable enterotoxin 1a	Toxin
iroN	Enterobactin siderophore receptor protein	Other
sfa/foc	S and F1C fimbriae	Adhesin
usp	Uropathogenic-specific protein gene	Other
espP	Serine protease	SPATE
saa	Auto agglutinating adhesin	Adhesin
eae	Intimin	Adhesin
bfpA	Bundle forming pilus	Adhesin
aggR	AraC transcriptional activator Isoprenoid	Adhesin
ipaH	Invasion plasmid antigen	Other

aaiC	aggR-activated island C	Secretion system
ltcA	Heat labile enterotoxin	Toxin
stb	Heat stable enterotoxin	Toxin
aph(6)-Id	Aminoglycoside O-phosphotransferase	Antibiotic Resistance
strB	Streptomycin resistance protein	Antibiotic Resistance
Bla-TEM-1C	β-lactamase	Antibiotic Resistance
sul-2	Sulfonamide-resistant dihydropteroate synthase	Antibiotic Resistance
ereB	Erythromycin esterase type II	Antibiotic Resistance
mphA	macrolide 2'-phosphotransferase	Antibiotic Resistance
dfrA5	Dihydrofolate reductase	Antibiotic Resistance

SPATE – Serine Protease autotransporter of *Enterobacteriaceae*

Antibiotic resistance gene information from Uniprot (The UniProt Consortium, 2019)