Verotoxinogenic *Escherichia coli* O157:H7 in Swedish Cattle and Pigs

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Cover: Histopathological changes induced by *E. coli* O157:H7 colonization at the terminal rectum of cattle. A larger *E. coli* O157:H7 microcolony is shown on the lamina propria, following the shedding on the epithelial layer. Reproduced with the permission of Infection and Immunity, Nart *et al.*, 2008.

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Abstract

Verotoxinogenic *E. coli* O157:H7 (VTEC O157:H7) can cause severe disease in humans, with bloody diarrhea and complications such as haemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and even death. Animals carry VTEC O157:H7 asymptomatically. Ruminants, especially cattle, are considered to be the main reservoir, although the bacterium can occasionally be isolated from other species, such as pigs.

The main aim of this thesis was to increase our knowledge of VTEC O157:H7 in Swedish cattle and pigs and to assess the extent to which they could be a potential source of human infections. The studies have included prevalence investigations of VTEC O157:H7 in slaughtered Swedish cattle and pigs, with estimated prevalences of 1.2% and 0.1%, respectively. Moreover, a study was performed on 371 dairy herds, where VTEC O157:H7 was detected in 8.9% of the herds. Indentified risk factors for herds to prove positive were: median age of sampled animals, herd size, farms located in Halland and presence of pigs on a dairy farm. Studies were also performed on farms where pigs shed VTEC O157:H7. Direct or indirect contact with ruminants seemed to be of major importance for presence of the bacterium in pigs. Young pigs were monitored during rearing for slaughter and were found to rid themselves of the bacteria prior to slaughter. When VTEC O157:H7 isolates from the cattle prevalence studies (n=181) and farms linked to human cases (n=19) were subtyped, a specific variant, VTEC O157:H7 (PT4:vtx_{2} vtx_{2c}), predominated among the strains isolated from farms associated with disease in humans. By extended subtyping it was established that strains of this specific variant belonged to a group of putative hyper-virulent strains, clade 8, suspected of causing more severe disease in humans. Furthermore, different molecular subtyping techniques were evaluated regarding their ability to distinguish between VTEC O157:H7 strains isolated from Swedish cattle and pigs.

Keywords: Stx, VT, *E. coli*, O157; cattle, pig, PFGE, MLVA, VTEC, STEC

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Det är aldrig för sent att ge upp.

Ronny Eriksson standup comedian

List of Publications

This thesis is based on the work contained in the following papers, referred to by roman numerals in the text:

I Albihn, A., Eriksson, E., Wallen, C. & Aspán, A. (2003). Verotoxinogenic *Escherichia coli* (VTEC) O157:H7 a nationwide Swedish survey of bovine faeces. *Acta Veterinaria Scandinavica* 44, 43-52.

II Eriksson, E., Aspán, A., Gunnarsson, A. & Vågsholm, I. (2005). Prevalence of verotoxin-producing *Escherichia coli* (VTEC) O157 in Swedish dairy herds. *Epidemiology and Infection* 133, 349-358.

III Eriksson, E., Nerbrink, E., Borch, E., Aspán, A. & Gunnarsson, A. (2003). Verotoxin-producing *Escherichia co*li O157:H7 in the Swedish pig population. *Veterinary Record* 152, 712-717.

IV Aspán, A. & Eriksson, E. Verotoxinogenic *Escherichia coli* O157:H7 from Swedish cattle; isolates from prevalence studies versus strains linked to human infections – A retrospective study (submitted for publication).

V Eriksson, E., Söderlund, R., Boqvist, S. & Aspán, A. Genotypic characterization to identify markers associated with hyper-virulence in Swedish *Escherichia coli* O157:H7 cattle strains (in manuscript)

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Abbreviations

A/E lesions	Attaching and effacing lesions
CDC	Center for Disease Control and Prevention, Atlanta, USA
eaeA	Gene encoding intimin
HC	Haemolytic colitis
HUS	Haemolytic uraemic syndrome
kb	Kilo base pairs
kD	Kilodalton
LEE	Locus of enterocyte effacement
Mb	Mega base pairs
MLVA	Multi-locus variable number tandem repeat analysis
NVI	National Veterinary Institute (SVA)
IMS	Immuno magnetic separation
PT	Phage type
PCR	Polymeras chain reaction
PCR-RFLP	PCR-Restriction fragment length polymorphism
PFGE	Pulsed-Field Gel Electrophoresis
SMI	Swedish Institute for Infectious Disease Control
SNP	Single nucleotide polymorphism
Stx	Shiga toxin
STEC	Shiga toxin-producing *E. coli*
Tir	Translocated inimin receptor
TM	Terminal mucosa
TR	Terminal rectum
TTP	Thrombotic thrombocytopenic purpura
TTSS	Type III secretion system
VT	Verotoxin
VTEC	Verotoxin-producing *E. coli*
QS	Quorum sensing

1 Introduction

1.1 *Escherichia coli*

Escherichia coli was first described in 1885 by Theodor Escherich (Escherich, 1988). Escherich, a Bavarian paediatrician, had performed studies on the intestinal flora of infants and had discovered a normal microbial inhabitant in healthy individuals, which he named *Bacterium coli commune*. In 1919, the bacterium was renamed in his honour to *Escherichia coli* (Kaper, 2005)

The species *E. coli* comprises gram-negative, oxidase-negative straight cylindrical rods measuring 1.1-1.5 x 2.0-6.0 μm. They are aerobic and facultative anaerobic, rendered motile by peritrichous flagella, or non-motile (Scheutz & Strockbine, 2005).

The taxonomy of *E. coli* is summarized below

Phylum	*Proteobacteria*	
Class	*Gammaproteobacteria*	
Order	*Enterobacteriales*	
Family	*Enterobacteriaceae*	
Genus	*Escherichia*	
Species	*Escherichia coli*	(VetBakt, 2007)

The 16S rRNA based phylogenetic tree shown in Fig. 1 illustrates its relatedness with other representatives of genera within the *Enterobacteriaceae* family. Phylogenetic analysis has demonstrated a very close relation between *E. coli, Salmonella* spp. and *Citrobacter freundii*. With the exception of *Shigella boydii* serotype 13, the four species of *Shigella*, (*S. dysentieriae S. flexneri, S. boydi* and *S. sonnei*) show such a high degree of relatedness to *E. coli* that these five could be considered a single species. However, the distinction still prevails, for historical and medical reasons (reviewed by Scheutz & Strockbine, 2005).

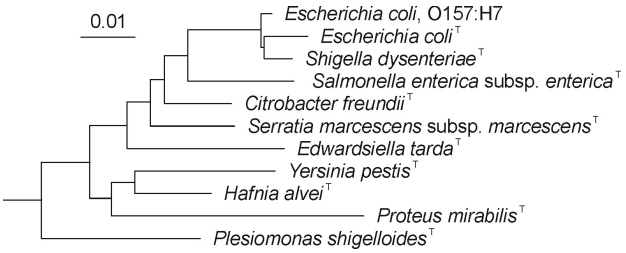

Figure 1. Evolutionary tree showing the phylogenetic relations of the *Enterobacteriaceae* family. Superscript T indicates type strains.

E. coli can be characterized by serotyping, a method based on differences in antigenic structure on the bacterial surface. The serotype is defined by the bacterium's O-antigen (Ohne), a polysaccharide domain in the bacterium's lipopolysaccharide (LPS) in the outer membrane, and the H-antigen (Hauch) consisting of flagella protein. Serotyping may also include the K-antigen (Kapsel) and the F-antigen (Fimbriae). There are many known O, H, K and F antigens and the existing number of different serotypes is known to be very high. Serotyping is an important tool which can be used in combination with other methods to distinguish pathogenic *E. coli* strains as specific pathogenicity attributes are often linked to certain serotypes. (Gyles, 2007; Kaper, 2005; Scheutz & Strockbine, 2005)

1.2 Pathogenic *E. coli*

Most *Escherichia coli* are harmless commensals which are part of the natural gastrointestinal flora in the lower intestine of warm-blooded animals. They are considered beneficial for maintaining a healthy intestinal ecosystem and have even been candidates for probiotic treatment to counteract a variety of enteric diseases. However, some subsets of *E. coli* have acquired specific virulence attributes that render them capable of causing a variety of illnesses in healthy humans and animals (Kaper *et al.*, 2004).

Interestingly, most acquired virulence factors that distinguish pathogenic *E. coli* from commensals are encoded by mobile genetic elements such as plasmids, bacteriophages and transposons. Genes coding for virulence factors are often located in the chromosome on pathogenicity islands (PAI), large genomic regions that cannot be found in commensals. These often include genetic elements that might once have been mobile but subsequently evolved to be locked into the genome (PEN, 2006b; Scheutz & Strockbine, 2005; Kaper *et al.*, 2004).The pathogenic *E. coli* are divided into different pathotypes according to the virulence factors they possess. In Tables 1 and 2 different pathotypes of *E. coli* in humans are described.

Table 1. *Intestinal pathogenic E. coli*

Pathotype, Mode of action	Main virulence factors
EPEC Enteropathogenic *E. coli* Adheres to small intestine enterocytes, destroys normal microvillar architecture, produces attaching and effacing lesions, an inflammatory response, increased intestinal permeability, active ion secretion. Watery to bloody diarrhea	Pathogenicity island LEE, -Type III secretion system, intimin, Tir, EspA, EspB, EspD, EspF EPEC adherence factor (EAF) plasmid - Bundle-forming pili (BPF) - Plasmid-encoded regulator (Per) No classic toxins produced (atypical EPEC lacks EAF plasmid)
ETEC Enterotoxigenic *E. coli* Adheres to small intestine enterocytes, Secretion of enterotoxins, cAMP, cGMP, stimulation of chloride secretion and inhibition of sodium absorption. Watery diarrhea	Colonization factor antigens (CFA) Heat-labile toxin (LT) Heat-stabile toxin (STa, STb)
EHEC Enterohaemorrhagic *E. coli* Adheres to large intestine enterocytes, destroys normal microvillar architecture, produces attaching and effacing lesions, causes apoptosis, cell death and inflammarory respons. Watery to bloody diarrhea. Systemic absorption of VT may lead to HUS, acute renal failure, TTP, neurological disorders.	Pathogenicity island LEE Type III secretion system, intimin, Tir, EspA, EspB, EspD, EspF Verotoxins VT1, VT2 pO157 Plasmid - Enterohaemolysin (EHEC-Hly) - Serine protease (EspP) - ToxB
EIEC Enteroinvasive *E. coli* Invades the colonic epithelial cell, lyses phagosomes, multiplies, moves through the cell, migrates into adjacent cells, causes inflammatory invasive colitis. Watery to bloody diarrhea	Invasion plasmid (pINV) - IpA, IpB, IpC, IpD - IscA
EAEC/ EAggEC Enteroaggregative *E. coli* Adheres to the small and large intestinal epithelia in a thick biofilm, causes increased mucus production, produces secretory enterotoxins and cytotoxins. Watery mucoid diarrhea, may be persistent	Aggregative adherence fimbrieae (AAFs) EAEC flagellin Toxins (Pic, ShET1, EAST, Pet)
DAEC Diffusely adherent *E. coli* Adheres and induces a cytopathic effect that makes small intestine enterocytes grow long, finger-like projections which wrap around the bacteria. Diarrhea	Dr adhesion family Fimbrial adhesin F1845

Table 2. *Extra-intestinal pathogenic E. coli (ExPEC)*

Pathotype, Mode of action	Main virulence factors
UPEC Uropathogenic *E. coli* Colonizes periurethral area, ascends the urethra to urine bladder, attaches and invades epithelial cells, may ascend to kidney. Cystitis and pyelonephritis	Adhesins (typ 1, F1C, S, M, Dr) P fimbriae (Pap) Cytotoxic necrotizing factor (CNF-1) Haemolysin (HlyA) Autotransported protease (Sat)
MNEC Meningitis/sepsis-associated *E. coli* Spreads haematogeneously, translocates from blod to CNS without damaging blood-brain barrier. Neonatal meningitis	Outer membrane proteins (OmpA, IbA, IbeB, IbeC, AsIA) Cytotoxic necrotizing factor (CNF-1) K1 kapsule

1.3 EHEC/VTEC/STEC

1.3.1 Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *E. coli* (EHEC) consists of a subset of *E. coli* strains that are known to be pathogenic to humans, i.e. they have the same clinical and pathogenic features associated with the EHEC prototype organism, *E. coli* O157:H7 (Levine *et al.*, 1987). In practice the definition EHEC is used to describe the subgroup of verotoxin producing *E. coli* that have the potential to cause haemorrhagic colitis (HC) in humans (reviewed by Scheutz & Strockbine, 2005).

1.3.2 Nomenclature VTEC/STEC

The cardinal virulence factor for EHEC is their ability to produce verotoxins (VT). These toxins are synonymously called shiga toxins (Stx), because of their similarity to those produced by *Shigella dysenteriae*. Consequently the *E. coli* bacteria that produce VT are called verotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC). These designations, VT/Stx and VTEC/STEC, are used interchangeably.

1.3.3 Seropathotypes

VTEC is considered a natural habitant in ruminants and VTEC is very frequently isolated from these animals. However, only a small subset of VTEC from ruminants should be considered as potential human pathogens, as VT alone is not sufficient to induce human disease (Law, 2000; Blanco *et al.*, 1996).

More than 400 different serotypes of VTEC have been isolated from humans but only few are associated with the majority of human EHEC cases (Scheutz & Strockbine, 2005). A classification system based on the concept of "seropathotypes" has been compiled by Karmali and colleagues. (Karmali *et al.*, 2003). This system classifies VTEC serotypes according to their ability to induce disease. The serotypes are ranked in five groups (A-E) ranging from the most pathogenic serotypes (A) to those that have never been associated with human disease (E). This classification is based on the reported occurrence of different serotypes in human outbreaks and by the frequency with which they are reported to have induced haemolytic uraemic syndrome (HUS) (See Table 3).

Table 3. *The classification of VTEC serotypes into seropathotypes (adapted from Karmali et al. 2003)*

Sero-pathotype	Relative incidence	Frequency of involvement in outbreaks	Association with severe disease[a]	Serotypes
A	High	Common	Yes	O157:H7, O157:H-
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111:NM, O121:H19, O145:NM
C	Low	Rare	Yes	O91:H21, O104:H21, O113:H21; others
D	Low	Rare	No	Multiple
E	Non-human only	NA[b]	NA[b]	Multiple

[a] HUS or hemorrhagic colitis
[b] NA, not applicable

The panel of the European Food Safety Authority (EFSA) concerned with Biological Hazards has recommended that animal and foodstuffs monitoring in the EU should initially be concentrated on VTEC O157, since this serotype is most frequently associated with severe human infections and HUS, but that monitoring should be extended to other serotypes such as O26, O103, O91, O145 and O111 that, after O157, are the serotypes most frequently causing human infections in Europe (Anonymous, 2007).
This thesis focuses on VTEC O157:H7 affecting cattle and pigs in Sweden and the importance of these species as sources of human EHEC infections. However, the importance of other serotypes should not be underestimated.

1.4 VTEC O157 (Seropathotype A)

VTEC O157:H7 is the prototype bacterium for EHEC and is the serotype most frequently isolated from outbreaks and severe human disease worldwide (Karmali *et al.*, 2003).

1.4.1 Non-sorbitol and sorbitol-fermenting VTEC O157

In contrast to other *E. coli* strains, VTEC O157:H7 strains cannot rapidly ferment sorbitol, do not produce ß-glucuronidase and are generally, resistant to the antimicrobiological agent tellurite. These are all features that can be used to indentify VTEC O157:H7 strains. Due to their inability to rapidly ferment sorbitol, VTEC O157:H7 are often referred to as non-sorbitol-fermenting (NSF) *E. coli* O157. However, some strains can ferment sorbitol rapidly within 24 h of incubation and these are referred to as sorbitol-fermenting (SF) *E. coli* O157 (Karch & Bielaszewska, 2001).

The SF VTEC O157:H- strains are non-motile due to a "12-p" deletion in the *fliC* gene (Monday *et al.*, 2004), They produce ß-glucuronidase and carry vtx_2 as their sole VT gene (Ammon *et al.*, 1999). Furthermore, they are not tellurite-resistant, as they lack the "tellurite adherence conferring island" (TAI) present in NSF VTEC O157:H7 (Karch & Bielaszewska, 2001; Tarr *et al.*, 2000). Moreover, SF VTEC O157:H- have acquired specific adhesion factors, novel pili, encoded by *spf* that distinguish them from other *E. coli*, another feature that can be used to identify SF VTEC O157:H- (Friedrich *et al.*, 2004).

1.4.2 The genome of VTEC O157:H7

Genome comparison studies have revealed a high degree of genomic diversity within the VTEC O157 population, which is attributed to many events of insertion/deletion and recombination of DNA. Most of these events seem to be prophage mediated or driven by other mechanisms of horizontal gene transfer (Ohnishi *et al.*, 2002).

Most VTEC O157:H7 strains carry a ~90 kb large plasmid, pO157 (often also referred to as the 60 MD plasmid). A similar but not identical plasmid, pSFO157, is found in SF VTEC O157:H- (Friedrich *et al.*, 2004; Karch *et al.*, 1993).

The first two strains of VTEC O157:H7 that were whole genome sequenced were "EDL 933" (Perna *et al.*, 2001) and "Sakai O157" (Hayashi *et al.*, 2001). When all sequence data were compiled they revealed that both strains had a genome of ~5.5 Mb which was ~0.9 Mb larger than the earlier sequenced genome of the non-pathogenic K12 laboratory strain, "MG 1655". Apart from a common, highly conserved backbone of 4.1 Mb

(shared by all three strains), the VTEC O157:H7 strains had about 1.34 million additional base pairs that not could be found in K12. On the other hand, about half a million of the base pairs from the K12 genome were missing in both sequenced VTEC O157:H7 strains. The additional "VTEC O157:H7-specific" 1.34 Mb were organized into several hundred "gene cassettes", O-islands or S-loops varying in size from 19 bp up to more than 15 kb. Prophage and prophage-like elements were abundant in these gene cassettes and about two-thirds of the Sakai O157 genome consisted of such elements (Ohnishi *et al.*, 2002).

1.4.3 Evolution of VTEC O157

In 1993 Whitham and colleagues suggested that *E. coli* O55:H7, an EPEC serotype known to cause infantile diarrhea, could be the ancestor of VTEC O157:H7 (Whittam *et al.*, 1993). Feng and colleagues elaborated on this hypothesis and in 1998 proposed a stepwise evolution model for VTEC O157:H7 with O55:H7 as its progenitor (Feng *et al.*, 1998). A recent study based on differences in single nucleotide polymorphisms (SNPs), located in the stable part of the genome, takes this evolution model even further (see Fig. 2) (Leopold *et al.*, 2009). In that study the authors even estimated the time spans in which the different subtypes evolved. For instance the SF O157:H- strain, commonly found among HUS cases in Germany (Subgroup B in Fig. 2) is believed to have emerged ~7000 years ago and Cluster 1 of Subgroup C strains, found among humans e.g. the American "spinach outbreak strain" (Manning *et al.*, 2008) might have emerged ~3,000 years ago.

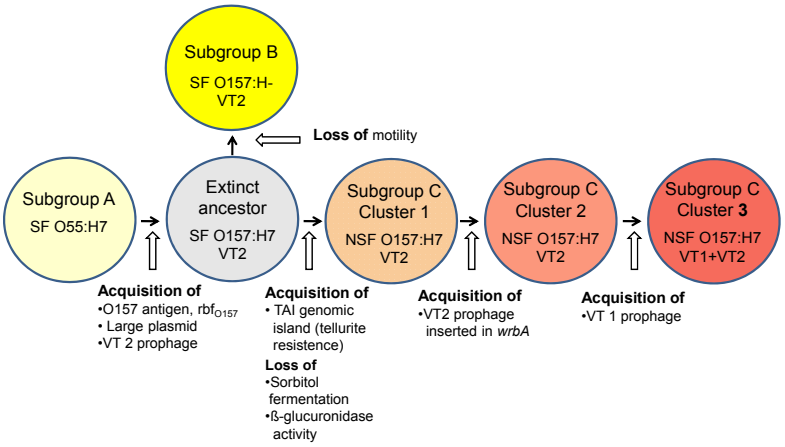

Figure 2. Proposed stepwise evolution model for VTEC O157:H7 from *E. coli* O55:H7. (Modified from Leopold *et al.*, 2009)

1.4.4 VTEC O157:H7 in a historical perspective

As early as from the 1960s, publications from Argentina have described HUS cases in children preceded by a period of diarrhea (Gianantonio *et al.*, 1964; Gianantonio *et al.*, 1962). In an article published in 1972, Gianantonio and colleagues reviewed 678 Argentinean HUS cases in children from 1957 to 1972 where no etiological agent could be identified (Gianantonio *et al.*, 1973).

VTEC was first described in Canada in the late 1970s by Konowalchuk and colleagues (Konowalchuk *et al.*, 1977). In 1982 it was first demonstrated that *E. coli* strains could produce a "shiga like toxin" (O'Brien *et al.*, 1982). A year later, O'Brien and Laveck managed to purify verotoxin from an *E. coli* strain, concluding that the toxin was both structurally and antigenically similar to the Shiga toxin produced by *Shigella dysenteriae* type 1, and therefore described the new toxin as a "shiga like toxin" (O'Brien & LaVeck, 1983; O'Brien *et al.*, 1983).

In 1982 Riley and colleagues were able to link a multistate food-born outbreak in USA (involving hamburger patties) to patients with bloody diarrhea (HC). A rare serotype "*E. coli* O157:H7" was isolated from stool samples and the strain was later shown to produce VT (Riley *et al.*, 1983). The same year, Karmali and colleagues established that HUS could be caused by VTEC O157:H7 and VTEC of other serotypes (Karmali *et al.*, 1983a; Karmali *et al.*, 1983b).

Following the observation that production of VT was linked to HUS, it could also be confirmed that HUS cases among children in Argentina were caused by VTEC (Novillo *et al.*, 1988).

1.5 Virulence factors of VTEC O157 in humans

1.5.1 Verotoxin (VT)

Verotoxins are the main virulence factor of EHEC. The name derives from their specific cytotoxicity to Vero cells (African green monkey kidney cells). VTs belong to a family of AB5 toxins, characterized by a single enzymatically active A subunit ~32kDa linked to a pentamer of five identical receptor-binding B subunits, each ~7.7kDa (see Fig. 3).

Based on toxin-neutralization and nucleotide sequence analyses the verotoxins are classified in two major groups, VT1 and VT2, showing approximately 60% nucleotide sequence identity. These two major groups can be further divided into several variants. VTEC strains may harbour a

single VT alone or possess several different (or similar) variants in combinations (reviewed by Müthing *et al.*, 2009).

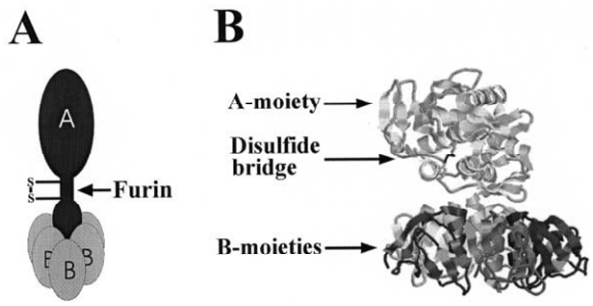

Figure 3. Schematic (A) and (B) crystallographic structure of verotoxin. The cleavage site for Furin is indicated with an arrow in (A). (Reproduced with the permission of Toxicon, Sandvig, 2001)

Generally, VT2 has been more closely associated with severe disease and HUS, than has VT1 (Jenkins *et al.*, 2003; Boerlin *et al.*, 1999; Ostroff *et al.*, 1989). VT2 has also proved to be 1000 times more potent as an agent toxic to human renal endothelial cells, than VT1 (Louise & Obrig, 1995).

VTs are encoded by temperate phages

VTs are encoded by a number of heterogeneous temperate lambdoid phages that follow the lysogenic pathway and are inserted as prophages at specific insertion sites in the bacterial genome (reviewed by Allison, 2007). VTEC O157:H7 can carry several different phages and at least five different insertions sites have been described, whereof *yehV* and *wrbA* are those most commonly occupied (Serra-Moreno *et al.*, 2007).

Events that provoke the bacterial SOS response, such as exposure to UV light or antibiotics, lead to induction of the phage's lytic cycle. The prophage is then excised and the bacterium starts to produce numerous phage particles and VT. This is followed by cell lysis whereby the phage particles and VT are released (Zhang *et al.*, 2000; Muhldorfer *et al.*, 1996). The phage particles released can also transduce other phage-sensitive bacteria in the gut, thus increasing the number of bacteria producing VT (Gamage *et al.*, 2003).

Furthermore, excision of integrated phages can take place without lysis of bacterial cells. For instance HUS patients can shed non-VTEC strains identical to the disease-causing EHEC strains, the only difference being absence of VT (Bielaszewska *et al.*, 2007). It has also been demonstrated that

identical VT-phages can be present at different insertion sites in strains from human outbreaks, indicating that the phages can move between different insertion sites (Bielaszewska *et al.*, 2006b).

Mode of action

The pentameric B unit of the VTs binds specifically to glycolipid receptors, Gb3 (globotriaosylceramide) or Gb4 (globotetraosylceramide), that are expressed on the cell surface by a variety of epithelial and endothelial cells (Gb4 is preferred by subgroup VT2e whereas all other VTs prefer Gb3; see below) (Lingwood *et al.*, 1998). After attachment the toxin is internalized into the cell by receptor-mediated endocytosis (Römer *et al.*, 2007). The toxin-receptorcomplex is transported via the trans-Golgi network to the endoplasmatic reticulum (ER) (Sandvig *et al.*, 1992). Near the nuclear membrane the ~32kDa Subunit A is cleaved by a protease, furin, into a catalytically active A1 fragment and an A2 fragment (Garred *et al.*, 1995). The A1 fragment is released into the cytosol where it exerts tRNA N-glycosidase activity that removes an adenine moiety from 28S rRNA in the eukaryotic 60S ribosomal subunit (Endo *et al.*, 1988). This modification inhibits protein synthesis and leads to cell death, as the acceptor site for aminoacyl-tRNA is blocked. There are also VT-resistant cells, in which VT is engulfed and rendered ineffective by lysosomes (Hoey *et al.*, 2003).

In addition to the cytotoxic effect, VT can induce apoptosis, programmed cell death, characterized by DNA fragmentation, cell shrinkage, membrane blebbing and condensation of nuclear chromatin. This is mediated via an independent pathway regulated by proteins from the BcL-2 family (Jones *et al.*, 2000; Suzuki *et al.*, 2000).

It has also been shown in renal carcinoma-derived cells that the specific binding of the B subunit of VT1 to Gb3 in itself produces intracellular signals which remodel cytoskeletal organizing proteins (Takenouchi *et al.*, 2004).

Furthermore, VTs stimulate epithelial cells and a variety of non-endothelial cells to secrete inflammatory mediators (cytokines, chemokines) that induce a pro-inflammatory response exaberating the detrimental effects of VTs on endothelial cells (reviewed by Naylor *et al.*, 2005a).

Variants of VT1 and VT2

The two major groups, VT1 and VT2, can be subdivided into variants. The nomenclature is not conclusive and new variants of VT are constantly being described. The VT1 group has three variants: VT1, VT1c and VT1d (reviewed by Müthing *et al.*, 2009). The VT2 group is comparatively more

heterogeneous and can be divided into seven variants: VT2, VT2b, VT2c, VT2d, VT2e, Vt2f and VT2g (reviewed by Persson *et al.*, 2007).
The VT variants found in VTEC O157:H7 are VT1, VT2 and VT2c; all have been implicated in HUS cases, although VT2 is the one most closely associated with severe disease, especially among children < 5 years old (Kawano *et al.*, 2008; Persson *et al.*, 2007; Friedrich *et al.*, 2002).

The other VT variants are found in non-O157 VTEC strains and are rarely implicated in severe human disease, with the exception of a particular variant of VT2d, $VT2d_{activatable}$ which has been associated with bloody diarrhea and HUS (Bielaszewska *et al.*, 2006a). Variant VT2e binds specifically to Gb4 and is associated with oedema disease in swine (Fan *et al.*, 2000; Bertschinger & Gyles, 1994) while the VT2f variant is associated with pigeons (Morabito *et al.*, 2001).

1.5.2 The LEE- mediated type III secretion system (TTSS)

EHEC and EPEC adhere to enterocytes by attaching and effacing lesions (A/E lesions). These are characterized by localized destruction of brush border microvilli and intimate attachment to the plasma membrane of host epithelial cells. The bacteria also induce actin polymerization and rearrangement of the cytoskeletal architecture in the host cells, a mechanism that anchors the bacterium on pedestals securely cupped by the host cell.
These events are mediated by a type III secretion system (TTSS). The major feature of TTSS is translocation of a variety of virulence factors from within the bacterium into the host cell via a filamentous needle complex (see Fig. 4). The bacterium translocates its own receptor Tir (transmembrane intimin receptor) which is inserted into the host cell's plasma membrane where it acts as an adhesion receptor for intimin, a bacterial outer membrane protein encoded by *eaeA* (see Fig. 5). The intimin–Tir interaction mediates intimate attachment for all bacteria that induce A/E lesions (reviewed by Garmendia *et al.*, 2005). Several different intimin types have been described and named after the Greek alphabet. Intimin-γ, the type associated with VTEC O157:H7, has a tissue specificity for follicle-associated epithelium overlaying Peyers´s patches (Phillips *et al.*, 2000).

In addition to Tir, a subset of Type III effector proteins e.g. Map, EspF, EspG, EspH, EspB and sepZ, translocate into the host cell where they elicit a variety of reactions resulting in diarrhea and transmigration of acute inflammatory cells to the infection site (reviewed by Garmendia *et al.*, 2005).

The structure and different components forming the filamentous needle complex (NC) are illustrated in Fig. 4. Beside its function as a syringe-like

"hollow needle", the mature EspA filament is also an important adhesion factor establishing a transient link between bacterium and host cell. After translocation of effector proteins, the whole needle complex is removed from the bacterial cell surface. This is necessary to make room for the intimate bacterial attachment between intimin and Tir that is essential for the A/E lesions (reviewed by Garmendia *et al.*, 2005)

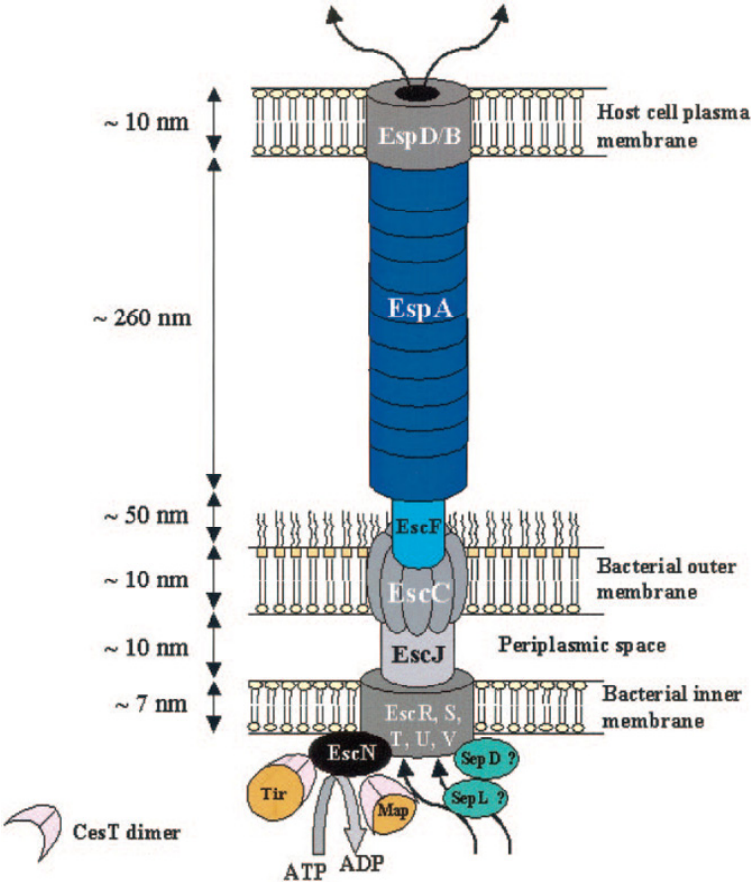

Figure 4. Schematic representation of the EPEC/EHEC type III secretion apparatus. The basal body of the TTSS is composed of the secretin EscC, the inner membrane proteins EscR, EscS, EscT, EscU, and EscV, and the EscJ lipoprotein which connects the inner and outer membrane ring structures. EscF constitutes the needle structure, whereas EspA subunits polymerize to form the EspA filament. EspB and EspD form the translocation pore in the host cell plasma membrane, connecting the bacteria with the eukaryotic cell via EspA filaments. The cytoplasmic ATPase EscN provides the system with energy by hydrolyzing ATP molecules into ADP. SepD and SepL have been represented as cytoplasmic components of the TTSS. (Reproduced with the permission of Infection and Immunity, Garmendia *et al.*, 2005)

The proteins involved in A/E lesions are encoded by a large pathogenicity island called locus of enterocyte effacement (LEE). LEE is organized in five operons which encode regulators, structural components, chaperones and effector proteins involved in the TTSS. Many effector proteins identified as EHEC virulence factors are also encoded outside LEE but still utilize the TTSS NC apparatus for translocation into the host cell (reviewed by Garmendia *et al.*, 2005).

LEE gene expression is regulated by complex mechanisms dependent on, among other things, cell contact and environmental conditions, e.g. levels of $NaHCO_3$. Expression is also regulated via quorum sensing (QS) which can be described as a communication system amongst bacteria via hormone-like compounds, auto inducers, that regulate bacterial gene expression (Pacheco & Sperandio, 2009; Sperandio *et al.*, 2003).

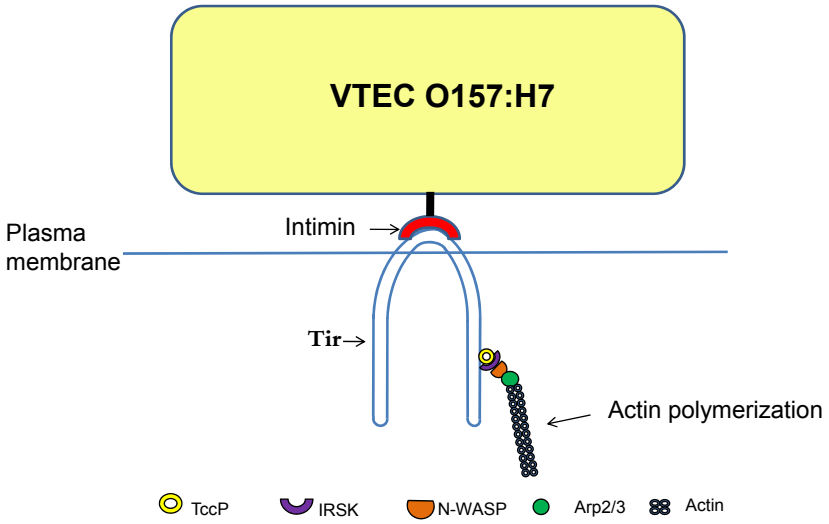

Figure 5. Attachment of intimin to the translocated receptor, Tir, which inserts into the host cell's plasma membrane. TccP, IRSK, N-WASP Arp2/3 and actin are different components involved in the actin polymerization process which "anchors" the bacteria to the host cell. (adapted from Frankel & Phillips, 2008)

1.5.3 Non-LEE associated virulence factors

Many putative virulence factors have been described for VTEC O157:H7 and new effector proteins are continually being identified. Selected virulence factors encoded outside the LEE pathogenicity island are listed in Table 4.

Table 4. *Virulence factors encoded by virulence plasmid pO157*

Effector	Functions	
EHEC-Haemolysin	RTX toxin, induces lysis of erythrocytes	[a]
KatP	Catalase peroxidase activity, can protect from oxidative stress exerted by host immune cells	[b]
EspP	Auto transported serine protease; proteolytic activity for coagulation factor V, expresses toxicity to vero cells, may promote mucosal haemorrhage and induces cell cytotoxicity	[c]
ToxB	Adherence factor, promotes adherence to epithelial cells and production/secretion of TTSS proteins (EspA, EspB and Tir), may also inhibit lymphocyte activation	[d]

[a](Schmidt *et al.*, 1995) [b](Brunder *et al.*, 1996) [c](Brunder *et al.*, 1997; Djafari *et al.*, 1997)
[d](Tatsuno *et al.*, 2001)

Table 5. *Non-LEE virulence factors encoded on the chromosome*

Effector	Functions	
Iha	irgA, adherence-conferring protein, membrane protein acts as an adherence factor to epithelial cells, found in VTEC O157:H7 but not in SF VTEC O157:H-.	[e]
Efa1	EHEC factor for adherence, adhesion factor; also inhibits proliferation of human lymphocytes, may modulate mucosal immune responses in cattle	[f]
CDT-V	Cytolethal distending toxin, disrupts cell cycle and blocks mitosis in G2/M phase, found in 6% of VTEC O157:H7 and in 87% SF VTEC O157:H-	[g]
TccP/EspFu	Tir-cytoskeleton coupling protein; associates with Tir, binds N-WASP and stimulates actin polymerization, seen in pedestal formation (see Fig 5)	[h]
Esp J	*E. coli* secreted protein, inhibits macrophage opsonophagocytosis	[g]

[e](Tarr *et al.*, 2000) [f](Stevens *et al.*, 2002) [g](Bielaszewska *et al.*, 2005) [h](Garmendia *et al.*, 2004)
[g](Marches *et al.*, 2008)

1.5.4 Variance in virulence among VTEC O157 strains

Based on genomic differences identified by octamer-based genome scanning (OBGS) (Kim *et al.*, 1999), lineage-specific polymorphism assay (LSPA) (Yang *et al.*, 2004) and micro array based comparative genomic hybridization (mCGH) (Zhang *et al.*, 2007), VTEC O157:H7 strains have been categorized in three lineages. Lineage I comprises strains commonly isolated from both cattle and humans, lineage II comprises strains isolated primarily from cattle whereas the third lineage, I/II, has not been strictly characterized regarding host distribution. The fact that lineage II strains are seldom

isolated from humans implies that these strains are less virulent for humans or ineffectively transmitted to humans from bovine sources (Kim *et al.*, 1999). Furthermore, it has been shown that strains from lineage I and lineage I/II produce significantly more VT2 than strains from lineage II and that this is due to genetic differences in the prophages carrying the VT2 genes from the different lineages (Zhang *et al.*, 2009).

A recent *in silico* study comprising six different DNA typing methods (Laing *et al.*, 2009) reported that lineage I/II strains belonged to a putative hyper-virulent clade of strains, called clade 8 (Manning *et al.*, 2008). By developing a single-nucleotide polymorphism (SNP) typing system, Manning and colleagues were able to distribute 519 VTEC O157:H7 strains into nine evolutionary clades. Using clinical data from isolated strains they found that patients infected with VTEC O157:H7 strains from one of the clades, clade 8, were seven times more likely to develop severe disease. Moreover, these patients were more likely to be younger (ages 0-18) than patients infected with strains from the other clades. Clinical outbreak data also revealed that clade 8 strains led to remarkably high rates of hospitalization (average 63%) and HUS (average 13%) compared with other outbreak strains.

A greater diversity of VT-encoding bacteriophage insertion sites has also been reported from VTEC O157:H7 strains isolated from cattle, than was found in isolates from humans (Besser *et al.*, 2007). Other studies have reported higher concentrations of secreted TTSS-proteins encoded by LEE in VTEC O157 strains associated with disease, than in strains shed from cattle (Roe *et al.*, 2004; Roe *et al.*, 2003).

SF VTEC O157:H- is also reportedly able to cause high rates of severe disease. In a Scottish outbreak in 2006, 10 (50%) of 20 cases with diarrhea progressed to HUS and in Germany the odds for developing HUS after SF VTEC O157:H- infection are estimated to 1:2 (reviewed by Rosser *et al.*, 2008). SF VTEC O157:H- is synonymous with subgroup B in Fig. 2.

1.6 VTEC O157 in humans

1.6.1 Disease in humans

Clinical manifestations of VTEC O157:H7 vary from no symptoms at all (asymptomatic carriers), to mild watery diarrhea, bloody diarrhea, to severe complications such as haemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and even death (reviewed by Mead & Griffin, 1998). Figure 6 shows alternative clinical pathways for a patient clinically infected with VTEC O157:H7.

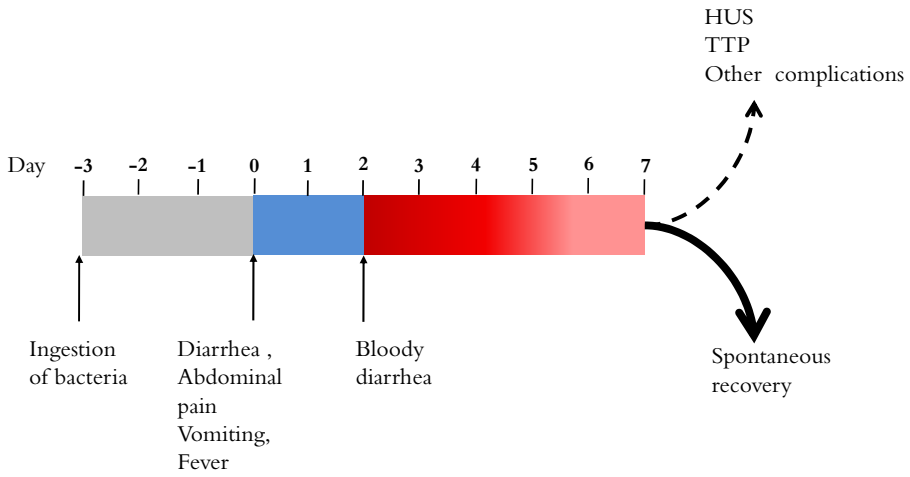

Figure 6. Illustrating how an infection with VTEC O157:H7 can develop along alternative clinical pathways. Note that indications of time are approximative. (Adapted from Tarr *et al.*, 2005)

The infectious dose of VTEC O157:H7 is very low, estimated to be <50 (Tilden *et al.*, 1996) or mere a few hundred bacteria (Bell *et al.*, 1994). After a mean incubation period of 3 days (can vary from 1 to 8 days) infected humans develop watery diarrhea, vomiting (30-60% of cases) and abdominal pain with cramps. About 30% of patients have mild fever, usually observed in the early stages of the disease (reviewed by Mead & Griffin, 1998). About 1-3 days after onset, over 70% of the patients develop bloody diarrhea (hemorrhagic colitis, HC) though lower frequencies have also been reported. The amount of blood in faeces varies from traces to almost entirely blood (reviewed by Tarr *et al.*, 2005; and Mead & Griffin, 1998).

Most patients recover spontaneously within a week of onset, whereas a small subset of cases progress to HUS or other complications. The proportion of patients who progress to severe bloody diarrhea and/or HUS varies for different strains of VTEC O157, as well as age and immunological status of the infected patient. In sporadic cases, 3-7% of cases progress into HUS whereas in specific outbreaks a HUS incidence of up to 20% has been reported (reviewed by Mead & Griffin, 1998). The risk that a child <10 years old with a diagnosed VTEC O157:H7 infection will develop HUS is estimated to be about 15% (reviewed by Tarr *et al.*, 2005).

HUS occurs 5-13 days after the onset of diarrhea and is characterized by haemolytic anaemia with fragmented erythrocytes, thrombocytopenia and acute renal failure (reviewed by Karch *et al.*, 2005). The syndrome can be

life threatening and patients need to be hospitalized and given intensive care including intravenous fluid perfusions. About half of all HUS patients require dialysis, 80% need erythrocyte transfusions, while 3-5% succumb (reviewed by Tarr *et al.*, 2005; and Mead & Griffin, 1998).

Elderly persons also run a greater risk of developing HUS – or a condition resembling HUS, called TTP. This is more common in adults and, in addition to acute renal failure, is also characterized by fever and neurological symptoms such as lethargy, severe headache, convulsions and coma (reviewed by Paton & Paton, 1998). Other acute complications reported after VTEC O157:H7 infections are cardiac dysfunction, pancreatitis, stroke, rectal prolapse and colonic perforation with peritonitis (reviewed by Karch *et al.*, 2005).

A small subset of patients develop chronic renal sequelae or other sequelae such as diabetes mellitus, neurological disorders, hypertension and colonic strictures (reviewed by Karch *et al.*, 2005).

1.6.2 Pathophysiology in humans

After ingestion, acid-resistance mechanisms of VTEC O157:H7 facilitates their survival through the low pH of the stomach (Palermo *et al.*, 2009). The bacteiria becomes attached by adhesins to the epithelium in the colon (reviewed by Spears *et al.*, 2006). Quorum sensing (QS) mechanisms probably up regulate expression of the bacterial genes needed for attachment, so that even small quantities of bacteria succeed in attaching (Pacheco & Sperandio, 2009). The bacteria colonize enterocytes via the TTSS pathway and A/E lesions are created (reviewed by Spears *et al.*, 2006). VT and endotoxin (LPS) are released and absorbed across the gut epithelium. VT, LPS and possibly other virulence factors lead to an increase in proinflammatory cytokines from host cells and subsequent release of chemokines from inflammatory cells. The susceptibility for VT in the intestine microvascular endothelial cells is exacerbated by this proinflammatory response (reviewed by Palermo *et al.*, 2009). VT and LPS activate thrombocytes, and the endothelial injuries, activation of the coagulation cascade and inhibition of fibrinolysis lead to formation of thrombi that occlude capillaries and small arterioles. The intestinal vascular injury leads to ischaemia and necrosis that, together with the inflammatory response, probably cause the bloody diarrhea (reviewed by Tarr *et al.*, 2005).

VT and LPS can also enter the bloodstream where, bound to polymorphonuclear leukocytes (VT) (Brigotti *et al.*, 2008; te Loo *et al.*, 2000) and thrombocytes (LPS), (Ståhl *et al.*, 2006) they can reach the kidneys. In

the kidneys, Gb3 receptors are expressed on endothelial glomerular cells, mesangial and tubular epithelial cells. The proinflammatory response and actions of VT elicit a pronounced endothelial swelling and the prothrombotic mechanism leads to occlusion of small blood vessels in the glomeruli. By acting on renal tubular cells, VT induces apoptosis. When acting in combination, these mechanisms lead to thromobcytopenia and acute renal failure (reviewed by Tarr *et al.*, 2005; and Proulx *et al.*, 2001). The anaemia with fragmented erythrocytes, as seen in HUS, is caused by mechanical breakdown when the erythrocytes pass partly occluded blood vessels, and/or by oxidative damage (reviewed by Proulx *et al.*, 2001).

Another organ containing endothelial cells with Gb3 receptors are the brain where the VTs can cause endothelial damage and thrombotic disorders leading to neurological symptoms (reviewed by Proulx *et al.*, 2001).

1.6.3 Routes of transmission to humans

In general, the different routes of transmission of VTEC O157:H7 to humans are via food or various items either directly or indirectly contaminated by ruminant faeces. As the infectious dose is very low the bacterium's excellent capacity to survive over time in different environments is of decisive importance.

Food-borne infections

Contaminated food of bovine or ovine origin such as unprocessed meat, undercooked hamburgers, coldfermented sausages, unpasteurized milk, yoghurt and cheese made from unpasteurized milk are common causes of VTEC O157:H7 infections. Vegetables and fruit products e.g. unpasteurized apple juice, and also vegetables and salad ingredients such as lettuce, spinach, alfa alfa and radish sprouts, have also been associated with several outbreaks. These food products may have been contaminated by direct spreading of cattle manure on growing crops or indirectly via contaminated irrigation or processing water (Anonymous, 2006; Hussein & Sakuma, 2005).

The largest known VTEC O157:H7 outbreak to date was caused by radish sprouts in Sakai City, Japan in 1996, where 6,000 mostly schoolchildren became ill and 1.2% of them developed HUS (Michino *et al.*, 1999). In 1996, 522 people in Scotland were infected and 22 died after eating contaminated meat from a butcher's shop (Cowden *et al.*, 2001).

Water in private wells contaminated by grazing cattle is a common infection route of VTEC O157:H7 in Scotland (Strachan *et al.*, 2006). In an

outbreak in Walkerton, Ontario, Canada in 2000, 2,300 people became ill, 28 developed HUS and 7 died after drinking municipal water contaminated with both *Campylobacter jejuni* and VTEC O157:H7 (Richards, 2005). For more details regarding foodstuffs as sources of infection, see publication from European comisson healt & consumer protection directorate- general (Anonymous, 2003).

In autumn 2002 there was a Swedish VTEC O157:H7 outbreak in the province of Skåne affecting 30 patients all of whom had consumed infected coldfermented sausage. Of these, 13 (43%) were hospitalized and 9 (30%) developed HUS (Sartz *et al.*, 2007). Another outbreak occurred in southwest Sweden in the summer of 2005 where 135 cases, including 11 (8%) HUS patients, proved culture-positive for VTEC O157:H7 after eating contaminated fresh lettuce (Söderström *et al.*, 2008).

Direct or indirect contact with animals

There are numerous reports of cases where people have contracted an EHEC infection after visiting a farm where ruminants have been found shedding VTEC O157:H7. Often these farms are so-called "visiting farms" where children are allowed to meet and pet animals (Strachan *et al.*, 2006; Crump *et al.*, 2002; Lahti *et al.*, 2002). Non-ruminant animals on these farms may also be transmitting VTEC O157:H7 infection after they have picked up the bacterium from ruminants (Pritchard *et al.*, 2001).

Exposure from environment

Ruminants – especially cattle – can excrete high levels of VTEC O157:H7 ($>10^5$ cfu/g) in faeces (Ogden *et al.*, 2004; Strachan *et al.*, 2001). The bacterium spreads to the environment via grazing ruminants or by direct spreading of infected manure on land. After heavy rainfall the bacterium can spread further in the environment by overland runoff.

VTEC O157:H7 has a great capacity to survive for lengthy periods in manure, soil and water. Survival in bovine faeces ranges from 46-126 days (Fukushima *et al.*, 1999) and up to 90 days in cattle slurry (McGee *et al.*, 2001). In water the bacterium can survive for 40 days at 21˚C and for > 70 days at 5˚C (reviewed by Fremaux *et al.*, 2008). In studies on manure amended soil, VTEC O157:H7 persisted for 25 to as long as 231 days, depending on experimental environmental conditions and inoculum levels (reviewed by Fremaux *et al.*, 2008).

Studies in Canada, Germany and Scotland have concluded that people living in rural areas with high cattle density run a higher risk of contracting VTEC O157 infections than people living in an urban environment (Frank *et al.*, 2008; Strachan *et al.*, 2006; Michel *et al.*, 1999). Swedish and Danish studies have shown that the risk for children ≤5 years old of contracting VTEC infection increases, the closer they live to a cattle farm (Ethelberg *et al.*, 2009; Rydevik *et al.*, 2008).

Person to person infections

The very low infectious dose facilitates spreading of VTEC O157:H7 infection from person to person via the faecal–oral route. Deficient hygiene after toilet visits enhances this transmission route which is common among family members, carers and in institutional settings such as day-care centres and homes for the elderly.

In outbreaks, several routes of transmission are often involved e.g. in food-borne outbreaks person to person spread is common between patients who have eaten infected food.

1.6.4 Epidemiology of infection in humans

VTEC infections show a seasonal variation, with an increase in human cases during summer and early autumn (PEN, 2006a). The incidence peaks in children ≤ 4 years, but falls rapidly with increasing age (PEN, 2006a). Severe complications are more commonly seen in children (HUS) and elderly patients (Both HUS and TTP) (Karmali *et al.*, 2009; PEN, 2006a) whereas asymptomatic carriers are more common in the age groups in-between. Most cases are sporadic, although large outbreaks do occur (reviewed by Strachan *et al.*, 2006; reviewed by Rangel *et al.*, 2005).

Reported incidences of human VTEC infections and VTEC serotypes vary between different parts of the world. VTEC O157:H7 is the predominant serotype in north America and Japan (Griffin *et al.*, 2009; Watanabe *et al.*, 2009; Anonymous, 1997a). Argentina has the highest global incidence rate of HUS in children ≤ 5 years old (15 per 100,000), the predominant etiological agent being VTEC O157:H7 (Rivas, 2009).

Although VTEC O157:H7 is not the predominant serotype in all European countries it is still the one most commonly associated with severe disease in Europe (Anonymous, 2009). Table 6 presents the countries having the highest incidence of VTEC in Europe in 2007.

Table 6. *Reported incidence rate of human VTEC cases in 2007 for the top 6 countries in the EU and proportions attributed to VTEC O157 among those cases where the serotype was established*

Country	Cases per /100,000 (all serotypes)	Proportion VTEC O157 in reported cases when serotype was established
Scotland [1]	4.9⋆	94%
Sweden[2]	2.9	ID ⋆⋆
Denmark[2]	2.9	16%
Ireland[2]	2.7	81%
United Kingdom (including Scotland)[2]	1.9	98%
Germany[2]	1.1	ID⋆⋆⋆

⋆ Scottish figures are for culture-positive VTEC strains only.
⋆⋆ ID, Insufficient data. Only 46% of reported cases serotyped.⋆⋆⋆ Only 34% of reported cases serotyped
[1] (*Personal communication Mary Locking, Health Protection, Scotland)* [2]*(Anonymous, 2009)*

In Sweden, infection by VTEC O157:H7 has been a notifiable disease under the Communicable Disease Act since 1st January 1996. The notification system was expanded in July 2004 to include all serotypes of VTEC. Both clinical and subclinical cases are reported by both the analysing laboratories and the treating physicians.

Before 1995, very few human cases of VTEC infection were reported, viz. between one and five cases annually, but after two consecutive VTEC O157:H7 outbreaks of probable food-borne origin in 1995-96 (Ziese *et al.*, 1996) there was an increase in reported cases. Fig. 7 illustrates the number of total and domestic reported VTEC cases in Sweden, 1997-2008. Note that the increased incidence seen since 2004 was due to all serotypes being included in the notification system (see above).

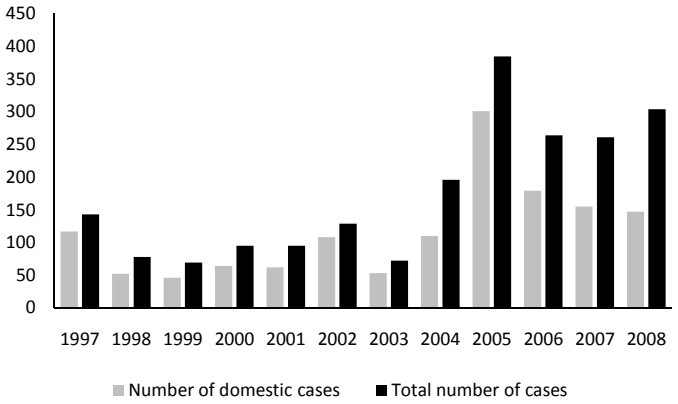

Figure 7. Reported VTEC cases, (total and domestic) in Sweden, 1997-2008 (Anonymous, 1997b)

There is an obvious geographical variation in incidence between regions (see map Fig. 8). The province of Halland, in the southwest, has consistently had the highest incidence of domestic VTEC cases (mean incidence, 1997–2008, 7.0 domestic cases /100,000 inhabitants) (Anonymous, 1997b)

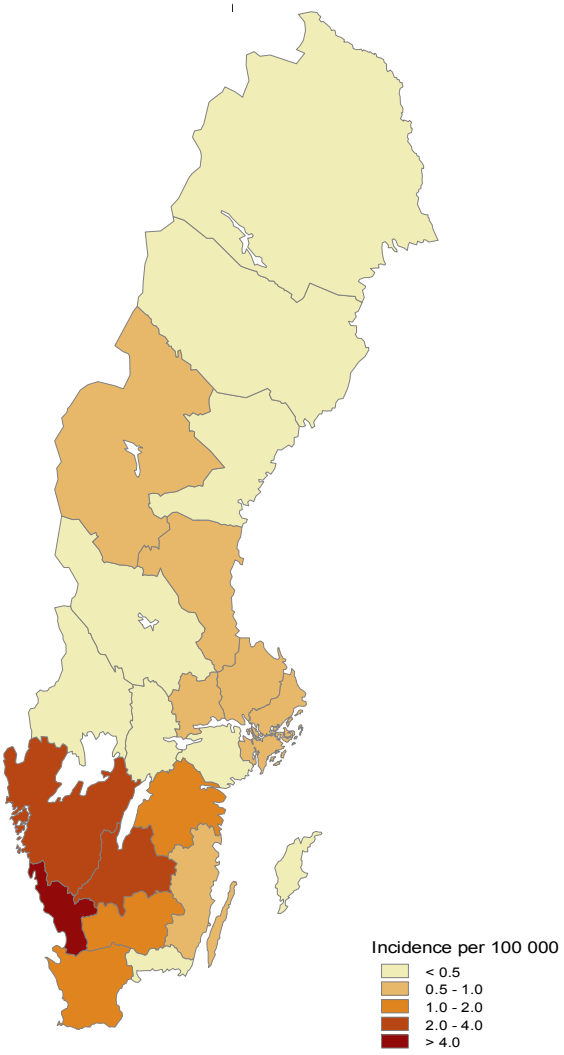

Figure 8. Map illustrating geographical variation in calculated main incidence rates of human VTEC cases in Sweden during 1997-2008. Map made by Martin Bergström, SVA (Source: Anonymous, 1997b)

Since 2004, all serotypes of VTEC are notifiable, but serotyping is not performed on all of the reported VTEC isolates. However, according to estimates by the Swedish Institute for Infectious Disease Control (SMI), VTEC O157:H7 accounts for approximately half of all reported cases of VTEC. Furthermore, there is a specific predominant variant among the VTEC O157:H7 strains isolated from humans, defined as phage type 4 and having two different verotoxin variants, VT2 and VT2c. More than two-thirds of the VTEC O157:H7 isolates from domestic cases during 2001-2007 belonged to this specific variant (personal communication, Sven Löfdahl, SMI; Löfdahl, 2008)

The most common serogroups in Sweden after O157 are O121, O26, and O103 (Löfdahl, 2008).

Deaths caused by VTEC infection are uncommon in Sweden, though three children have succumbed since 1996: one 5-year-old boy with VTEC O26 in 2006, in 2008 one 8-year-old boy infected by SF O157:H- and a 2-year-old girl infected with VTEC O157:H7 (PT4; Vtx_2, Vxt_{2c}) (Personal communication Sofie Ivarsson, SMI).

1.7 VTEC O157:H7 in cattle and pigs

1.7.1 Structure of the Swedish cattle population

In 2008 the total cattle population in Sweden was estimated to approx. 1,558,400 head (Anonymous, 2008) and registered herds numbered 25,847 (see Table 7). The maps in Fig. 9 demonstrate cattle density and cattle herd density within different postal areas in Sweden in 2008.

Table 7. *Registered number of cattle herds in Sweden in 2008*

Type of herd	No. of premises	Proportion of total no. of premises
Dairy	6,704	25.9%
Suckler	11,661	45.1%
Others	7,482	29.0%
Total	25,847	100.0%

(Source: Personal communication, Maria Nöremark, SVA)

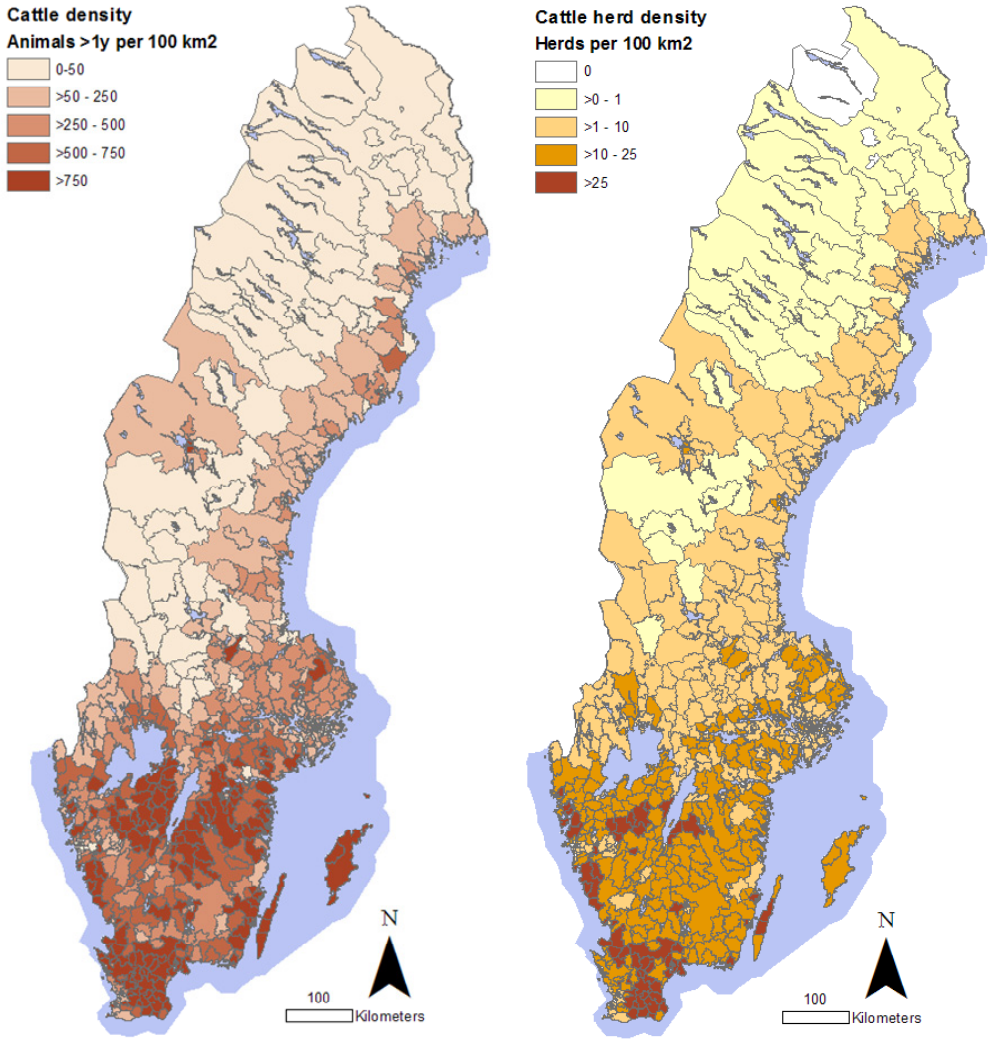

Figure 9. Differences in cattle density, cattle > 1 years old, (left) and cattle herd density (right) within different postal code areas in Sweden in 2008. Maps made by Jenny Frössling, SVA.

1.7.2 VTEC O157:H7 in animals

Cattle constitute the main reservoir for VTEC O157:H7, but sheep are also considered a significant source of infection in humans (reviewed by La Ragione *et al.*, 2009). Furthermore, VTEC O157:H7 has been isolated from goats (Pritchard *et al.*, 2000; Bielaszewska *et al.*, 1997) and water buffalos (Conedera *et al.*, 2004). Wild ruminants can also act as a potential reservoir; for instance VTEC O157:H7 has been isolated from wild deer (Renter *et al.*, 2001). Occasionally, the bacterium has been isolated from non-ruminant species e.g. horse, dog, rabbit, seagull, starling, wild boar and rat (Jay *et al.*, 2007; Wetzel & LeJeune, 2006; reviewed by Naylor *et al.*, 2005a; Cizek *et al.*, 1999). These animals are not considered as hosts, but rather as vectors transiently colonized by the bacterium following contact with ruminant faeces (reviewed by Caprioli *et al.*, 2005).

1.7.3 Prevalence of VTEC O157:H7 in cattle

Table 8 summarizes a subset of studies from different countries on the prevalence of VTEC O157 on cattle farms. It is difficult to compare results between the different countries due to differences in study design and methodology. However, in all the studies presented in Table 8 the faecal samples were analysed for VTEC O157:H7 with the sensitive immune magnetic separation (IMS) technique (see below under Considerations on Material and Methods).

Table 8. Prevalence of VTEC O157 on farms

Country	Cattle type	No. of farms	% positive farms	Reference
USA (Midwest)	Ranch and feedlot cattle	29	72.0%	(Elder *et al.*, 2000)
Canada (Saskatchewan)	Feedlot cattle	20	60.0%	(Vidovic & Korber, 2006)
USA (Ohio)	Dairy cattle	50	8.0%	(LeJeune *et al.*, 2006)
England&Wales	Dairy, suckler and fattener	75	38.7%	(Paiba *et al.*, 2003)
Scotland	Finishing/store cattle	481	18.9%	(Chase-Topping *et al.*, 2007)
Netherlands	Dairy cattle	678	7.2%	(Schouten *et al.*, 2004)
Spain	Dairy cattle	124	7.0%	(Oporto *et al.*, 2008)
	Beef cattle	82	1.6%	(Oporto *et al.*, 2008)
Denmark	Dairy cattle	60	16.7	(Nielsen *et al.*, 2002)
Sweden	Dairy cattle	371	8.9%	(Paper II)
Norway	Dairy cattle	50	0.0%	(LeJeune *et al.*, 2006)

In Sweden, a slaughterhouse prevalence survey of VTEC O157:H7 in slaughtered cattle was initiated in 1996. Between April 1996 and August

1997, 3,071 faecal samples were collected and analysed for VTEC O157:H7 (Paper I). These slaughterhouse investigations proceeded as annual prevalence studies, where approximately 2,000 faecal samples were collected yearly during 1998-2002. It was then decided that the studies should be performed every third year, i.e. two more studies have since been conducted; one in 2005-06 (Boqvist *et al.*, 2009) and one in 2008-09. In the latter two studies, in addition to the faecal samples, aural samples (piece of an ear, approx. 65 cm^2) were collected and analysed. The results from these studies are summarized in Table 9.

Table 9. *Results Swedish prevalence studies for VTEC O157:H7 in slaughtered cattle 1996-2009*

Year	No. Faecal samples	No. Positive feacal samples	No. Ear samples	No Positive ear samples
1996-1997	3071	37 (1.2%)[A]	NP	NP
1997-1998	2308	7 (0,3%)[B]	NP	NP
1999	2057	14 (0.7%)[C]	NP	NP
2000	2001	34 (1.7%)[A]	NP	NP
2001	1998	26 (1.3%)[A]	NP	NP
2002	2032	29 (1.4%)[A]	NP	NP
2005-2006	1758	60 (3.4%)[D]	446	54 (12.1%)
2008-2009	1993	65 (3.3%)[D]	500	41 (8.2%)

NP Not performed
[A] *10 g of faeces analysed. Analyses performed at SVA. Pre-enrichment broth BPW*
[B] *1 g of faeces analysed. Analyses performed at regional laboratory. Pre-enrichment broth BPW*
[C] *10 g of faeces analysed. Analyses performed at regional laboratory. Pre-enrichment broth BPW*
[D] *10 g of faeces analysed. Analyses performed at SVA. Pre-enrichment broth mTSB + novobiocin*

Table 10 summarizes a subset of slaughterhouse studies performed in different countries. All listed studies used IMS technology in their analyses.

Table 10. *Prevalence of VTEC O157 in slaughtered cattle in other countries*

Country	Year	No. faecal samples	% positive samples	Reference
USA (Midwest)	1999	327	27.8%	(Elder *et al.*, 2000)
Netherlands	1995-1996	937	6.3%	(Heuvelink *et al.*, 1998a)
Great Britain	2003	2736	4.7%	(Milnes *et al.*, 2008)
Belgium	1998-1999	1281	6.2%	(Tutenel *et al.*, 2002)
Poland	1999	551	0.7%	(Tutenel *et al.*, 2002)
Norway	1998-1999	1541	0.19%	(Johnsen *et al.*, 2001)
Finland	1997	1448	1.2%	(Lahti *et al.*, 2001)
Denmark	1999	227	3.0%	(Nielsen & Scheutz, 2002)

1.7.4 Cattle farms implicated in human VTEC O157:H7 cases in Sweden

In Sweden, in the period 1996 to 2008, 39 cattle farms (23 dairy farms, 15 beef producers and 1 "visitor farm") 1 sheep farm and 1 goat farm have been associated with human VTEC O157:H7 cases. All these farms have been suspected as sources of human infections and collected ruminant faecal samples proved positive for VTEC O157:H7. In most cases the infected humans have been children who had been visiting farms or had drunk unpasteurized milk from dairy farms. There have also been cases where people permanently living on the farms have become severely ill.

1.7.5 Epidemiology in cattle

VTEC O157:H7 colonization in cattle

Experimental infection with high doses of VTEC O157:H7 caused diarrhea, with A/E lesions in neonatal calves (Dean-Nystrom *et al.*, 1997) and transient watery diarrhea in weaned calves (Brown *et al.*, 1997). However, it is generally considered that cattle harbour the bacteria without expressing signs of disease (reviewed by Hancock *et al.*, 2001).

Gb3 receptors, the specific receptors for VT, are present in the proliferating crypt cells of bovine intestines but cannot be found in the vascular cells (Hoey *et al.*, 2002). In addition, when VT is internalized into the bovine cells it is engulfed and rendered ineffective by lysosomes. This could explain the evident resistance of cattle to the systemic effects of VT (Hoey *et al.*, 2003).

Cattle become infected via the faecal-oral route and, under controlled conditions, very low infectious doses <300 cfu VTEC O157:H7 have induced shedding in calves (Besser *et al.*, 2001). Sources of infection are considered to be direct contact with other cattle, contaminated water or feed, or exposure to the bacterium via the environment (reviewed by Chase-Topping *et al.*, 2008).

VTEC O157:H7 has been isolated throughout the whole bovine digestive tract from the oral cavity to the rectum, but it is more prevalent in the lower gastrointestinal tract (Grauke *et al.*, 2002). It has also been isolated from bovine bile bladders (Jeong *et al.*, 2007).

However, the primary site of VTEC O157:H7 colonization in cattle is considered to be in a defined region in the terminal rectum (TR), 5 cm proximal of the rectal anal junction (RAJ). This region, also referred to as

the terminal mucosa (TM), is characterized by high density of lymphoid follicles (Naylor *et al.*, 2003). In the TM the bacterium attaches and forms microcolonies (30-100 bacteria) and A/E lesions (Nart *et al.*, 2008). Successful colonization has also been established directly by rectal swabs inoculated with VTEC O157:H7 (Naylor *et al.*, 2007b; Sheng *et al.*, 2006b).

Following initial adhesion to bovine enterocytes by adhesins, such as fimbriae, outer membrane proteins and flagella, (Spears *et al.*, 2006), colonization of VTEC O157:H7 in the TM is dependent on the TTSS system (Naylor *et al.*, 2005b). It has been suggested that VTs potentiate VTEC colonization in cattle by modulating the bovine immune response, as VTs are able to block proliferation of bovine lymphocytes and are considered to have a significant effect on all main lymphocyte sub-populations. It is also speculated that VTs may act on local B cells in the gut to hinder antibody production or secretion (Menge *et al.*, 2003). Furthermore, it is reported that VT2 primes epithelial mouse cells for initial attachment by increasing expression of nucleolin on epithelial cell surfaces. Nucleolin, a multifunctional protein normally present in the nucleus, can act as an intimin receptor. VTEC may utilize this mechanism for initial attachment before the TTSS commences (Robinson *et al.*, 2006). It has also been suggested that VTs potentiate colonization by VTEC in cattle by specifically targeting bovine crypt cells, thereby lowering their metabolism and reducing cell turnover in the intestinal epithelium (Hoey *et al.*, 2002; Magnuson *et al.*, 2000).

Colonization in TM has been correlated to "high shedders" (see below) (Cobbold *et al.*, 2007; Low *et al.*, 2005) and to long-term shedding (up to 12 months) (Lim *et al.*, 2007).

Minor sites of VTEC O157:H7 carriage have also been identified in the bovine gastrointestinal system; the rumen, small intestine, proximal colon and in particular a region of lymphoid-rich tissue immediately distal to the ileocaecal valve (Nart *et al.*, 2008).

Hides from cattle can also be contaminated with VTEC O157:H7 and are considered to be a more important source of carcass contamination at slaughter than faecal excrement (Barkocy-Gallagher *et al.*, 2003).

VTEC O157:H7 shedding in cattle

There is a wide variation, in both magnitude and duration, in faecal shedding of VTEC O157:H7. Cattle that do not get colonized shed the bacterium transiently for less than 1 week but, if they do become colonized they typically shed for a month on average, but no longer than 2 months.

Very few animals may even shed for up to a year or longer (reviewed by Lim *et al.*, 2007; and Davis *et al.*, 2006).

Artificially infected calves shed VTEC O157:H7 in greater numbers and for longer periods of time than adult cattle (Cray & Moon, 1995). Calves from weaning up to 12 months old are those that that most often shed VTEC O157:H7 (Paiba *et al.*, 2003; Nielsen *et al.*, 2002; Heuvelink *et al.*, 1998b).

Shedding is often intermittent, and it has been reported that one and the same animal sampled just a few hours apart has shed in numbers varying from undetectable, up to ~10^3 cfu/g. However, there are also animals that can shed the bacterium both profusely and consistently for longer periods (Robinson *et al.*, 2004).

Most culture-positive animals (~75%) shed so few bacteria that VTEC O157:H7 can only be detected after IMS enrichment, viz. <100 cfu/g faeces, though a few may shed the bacteria more profusely, up to 1 x 10^7cfu/g (Chase-Topping *et al.*, 2007). Animals that shed VTEC O157:H7 in numbers ≥10^3 or ≥ 10^4/g faeces are referred to as "high-shedders" or "super-shedders" (reviewed by Chase-Topping *et al.*, 2008). These animals are responsible for most of VTEC O157:H7 spread by cattle. In one Scottish slaughterhouse study, high-shedders yielded 9% of the positive samples, yet it was calculated that these animals accounted for >96% of all VTEC O157:H7 shed (Omisakin *et al.*, 2003).

Epidemiology in cattle herds

VTEC O157:H7 positive faecal samples in sampled cattle herds are generally few, usually below 10% (reviewed by Hancock *et al.*, 1998). According to Scottish studies the distribution in prevalence varies between 0 and 100 %, where most herds test negative or have a very low prevalence, while a small proportion of herds have a very high prevalence (Gunn *et al.*, 2007; Matthews *et al.*, 2006b). Interestingly, if a high-shedder is present in a herd the likelihood of finding many low shedding animals in the same herd is high (Chase-Topping *et al.*, 2007). When cattle farms are sampled repeatedly over time the prevalence typically varies between the sampling occasions. A period with very low prevalence or no positive animals may be followed by a peak with many positive animals (Schouten *et al.*, 2005; Synge *et al.*, 2003; Hancock *et al.*, 1997). Thus, repeated sampling, i.e. longitudinal studies, gives higher and more accurate prevalence estimates than studies based on a single sampling (Synge *et al.*, 2003; Hancock *et al.*, 1997). Likewise, individual animals cannot be classified as non-shedders when

based on a single sampling occasion, as shedding may be low or intermittent (Robinson *et al.*, 2004; Garber *et al.*, 1995).

Statistic modelling based on data from Scottish field studies suggests that the observed variable distribution of prevalence at farm level (i.e. sporadic shedding by animals, occasional high prevalence and periods of apparent absence) is best explained by a model where a small proportion of animals with high excretion (high-shedders) infect the other cattle (Matthews *et al.*, 2006a; Matthews *et al.*, 2006b).

Greater numbers of cattle seem to shed VTEC O157:H7 during the warmer season than in wintertime and shedding seems to peak in late summer (Schouten *et al.*, 2005; Barkocy-Gallagher *et al.*, 2003; Paiba *et al.*, 2003). It is speculated that this phenomenon might be connected with day length and/or seasonal hormones (Edrington *et al.*, 2007). Studies have shown that housing of animals is a risk factor for shedding of VTEC O157:H7 (Gunn *et al.*, 2007; Synge *et al.*, 2003), while keeping animals on pasture seems to reduce the prevalence (Jonsson *et al.*, 2001).

The bacterium thrives in the farm environment (Randall *et al.*, 1999) and in one longitudinal study the same strain was shown to persist for more than 2 years on the farm (Shere *et al.*, 1998) where it could be isolated from birds, flies, from feed and drinking water. VTEC O157:H7 has also been isolated from rats (Cizek *et al.*, 1999), indicating that rodents can function as vectors of transmission.

In an American study, VTEC O157:H7 was found in 2.9% of house flies sampled from feedlot feed bunks (Alam & Zurek, 2004) and VTEC O157:H7 counts in house flies ranged from 3.0×10^{1} to $1.5\ 10^{5}$ cfu. Moreover, it has been shown that calves exposed to VTEC O157:H7 positive house flies start shedding the bacterium within a day after exposure (Ahmad *et al.*, 2007).

Contaminated water has under experimental conditions successfully infected cattle (Shere *et al.*, 2002) and the bacterium has been shown to survive for long periods (up to 245 days) in water-trough sediment. Thus, troughs can serve as a long-term reservoir for infection (LeJeune *et al.*, 2001). Furthermore, transmission of VTEC O157:H7 between herds of grazing cattle can occur if the cattle share water-courses e.g. rivers and lakes (LeJeune *et al.*, 2001; Jackson *et al.*, 1998).

A subset of reported risk-factors, other than the above-mentioned, for cattle shedding VTEC O157 on farms, is listed in Table 11.

Table 11. *Reported risk-factors for cattle shedding VTEC O157*

Risk-factor	Reference
Introduction of new cattle in herd	(Schouten *et al.*, 2004; Nielsen *et al.*, 2002; Wilson *et al.*, 1998)
Grouping of calves	(Cobbold & Desmarchelier, 2002; Garber *et al.*, 1995)
Shared buckets and bottles for calves without washing and rinsing	(Garber *et al.*, 1995)
High cattle density in feedlots	(Vidovic & Korber, 2006)
Number of animals in a group	(Ellis-Iversen *et al.*, 2007)
Group housed in pens with wet bedding	(Ellis-Iversen *et al.*, 2007)
Movement of calves/animals in herds	(Chase-Topping *et al.*, 2007; Rugbjerg *et al.*, 2003)
Stress (i.e. movement and weaning)	(Chase-Topping *et al.*, 2007)
Spreading of cattle slurry on pasture land	(Gunn *et al.*, 2007; Hancock *et al.*, 1994)
Presence of wild geese (if animals are grazing)	(Synge *et al.*, 2003)
Presence of pig at farm	(Paper II, Cernicchiaro *et al.*, 2009; Gunn *et al.*, 2007; Schouten *et al.*, 2004)
Use of corn silage during winter	(Cernicchiaro *et al.*, 2009)

1.7.6 Prevalence in pigs

Table 12 summarizes results from studies performed on slaughtered pigs.

Table 12. *Prevalence of VTEC O157:H7 in slaughtered pigs*

Country	Sampled material	No. of samples	% pos. samples	Reference
The Netherlands	Faecal samples	142	1.2%	(Heuvelink *et al.*, 1999)
Norway	Faecal samples	1996	0.1%	(Johnsen *et al.*, 2001)
Great Britain	Faecal samples	2060	0.3%	(Milnes *et al.*, 2008)
Sweden	Faecal samples	2446	0.1%	(Paper III)
Italy	Faecal samples	150	0,7%	(Bonardi *et al.*, 2003)
USA	Faecal samples	306	2.0%	(Feder *et al.*, 2003)
Japan	Rectal swabs	221	1.4%	(Nakazawa & Akiba, 1999)
Chile	Faecal samples	120	10.8%	(Borie *et al.*, 1997)

1.7.7 Pigs as a source for human VTEC O157:H7 cases

Pork salami (only meat from pigs) was reported as the source of an Italian family outbreak of VTEC O157:H7 in 1994 where two adults contracted bloody diarrhea (Conedera *et al.*, 2007). In a large American outbreak in 2006 caused by spinach, it was concluded that wild boars had contaminated

the spinach. Strains matching the outbreak strain were isolated in faecal samples from wild boars that had invaded the crop fields (Jay *et al.*, 2007).

In Sweden, four farms that kept both ruminants and pigs were linked to human cases and VTEC O157:H7 was isolated from pigs on two of these farms. On both farms, however, ruminants sampled at the same time also yielded positive samples and they were deemed to be the primary source of infection.

1.7.8 Epidemiology in pigs

E. coli strains of serotype O157 that are enterotoxin producing (ETEC O157) can be isolated from both healthy pigs and pigs with postweaning diarrhea (Hampson, 1994). These other pathogenic *E. coli* O157 are distantly related to VTEC O157, and have a separate evolutionary pathway (Whittam *et al.*, 1993).

Artificial infection of neonatal piglets with high doses of VTEC O157:H7 has induced severe disease and A/E lesions (Dean-Nystrom *et al.*, 2000). However, older pigs do not become clinically ill or exhibit any pathological changes following VTEC O157:H7 infection.

Experimental studies have shown that pigs have no innate resistance to colonization and can serve as a reservoir host, under suitable conditions. Three-month-old pigs have under experimental conditions been colonized after receiving an infectious dose of 10^{10} cfu and shed the bacterium for up to 2 months. (Booher *et al.*, 2002). Furthermore, it has been demonstrated that in contrast to ruminants, intimin is not necessary for persistent colonization in pigs (Jordan *et al.*, 2005). Pigs can transmit infection to other pigs if they share the same pen or if they have nose to nose contact and the infectious dose has under experimental conditions been estimated to approx. 6×10^3 cfu (Cornick & Vukhac, 2008; Cornick & Helgerson, 2004).

1.7.9 Control options for VTEC O157:H7 in animals

Management routines

Management routines that counteract risk factors mentioned in the section above, "epidemiology in cattle herds", may be of interest to reduce the risk of shedding in cattle. However, few scientific studies have evaluated such routines.

A British study on young cattle demonstrated that a combined intervention package of management routines, applied over 4½ months, significantly reduced the level of VTEC O157:H7 shedding. The package consisted of measures such as using designated boot-dips and overcoats,

keeping animals in the same group and ensuring dry bedding. The two measures of greatest importance were keeping young cattle in the same group throughout rearing, without introducing new animals, and ensuring constantly dry bedding. There was also weak evidence that not procuring new stock for the herd and avoiding direct contact with cattle from other farms were routines that reduced levels of shedding (Ellis-Iversen *et al.*, 2008).

Using sand instead of sawdust for bedding purposes has been found to lower the prevalence in dairy cows (Lejeune & Kauffman, 2005). Swedish field trials have indicated that addition of slaked lime to bedding materials can also reduce shedding of VTEC O157:H7 (unpublished data)

Calves aged 1-4 months have been reported to be less likely to become infected if they had suckled colostrum or stayed > 2 days with their mother (Rugbjerg *et al.*, 2003).

Feed control

Studies on feed composition and faecal shedding of VTEC O157:H7 in cattle have presented contradictory results. It has been suggested that a grain-rich diet could induce VTEC acid resistance in the rumen and that this would favour survival of VTEC O157:H7. This mechanism should lead to increased faecal shedding by cattle that are fed a grain diet compared with cattle fed a forage-based diet (Diez-Gonzalez *et al.*, 1998). Some studies support this hypothesis (Boukhors *et al.*, 2002; Tkalcic *et al.*, 2000), while others show that forage-fed cattle shed VTEC O157:H7 for longer periods than grain-fed animals (Midgley & Desmarchelier, 2001; Hovde *et al.*, 1999). There are yet other studies reporting no differences between the two feed categories (Grauke *et al.*, 2002; Hancock *et al.*, 1994).

Several studies have reported that feeding cattle with barley may increase shedding of VTEC O157:H7, compared with feeding a corn-based diet (Berg *et al.*, 2004; Boukhors *et al.*, 2002).

Several studies report that fasting increases the shedding of VTEC O157:H7 and makes calves more susceptible to colonization (Magnuson *et al.*, 2000; Cray *et al.*, 1998), while one study indicates that fasting has little effect on shedding (Harmon *et al.*, 1999).

It has been demonstrated under experimental conditions that VTEC O157:H7 can multiply in poorly fermented silage in numbers up to $>10^6$ cfu/g (Fenlon & Wilson, 2000) but does not survive a good silage fermentation process (Byrne *et al.*, 2002). Furthermore, inoculation of lactic acid-producing bacteria in ensilage hastens elimination of VTEC O157:H7 (Bach *et al.*, 2002).

Probiotics

Probiotics can be defined as "Live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbiological balance" (Fuller, 1989). Another term used in the USA for probiotics included in feed products for animals is direct-fed-microbials (DFD) (Elam *et al.*, 2003).

It has been proved experimentally that feeding cattle with specific *E. coli* strains that produce colicins (proteins that specifically target *E. coli*) can reduce shedding of VTEC O157:H7 (Schamberger *et al.*, 2004; Tkalcic *et al.*, 2003).

Numerous lactobacillus strains have also been tested regarding their potential to reduce shedding of VTEC O157:H7 in cattle. Their effectiveness varied widely between different lactobacillus strains, even within the same species (Wilderdyke *et al.*, 2004). The strain proven most effective to reduce shedding of VTEC O157:H7 in cattle is a specific *Lactobacillus acidophilus* strain called *Lactobacillus acidophilus* NP51. This strain has been proved in several American studies on feedlot cattle to reduce shedding of VTEC O157:H7 by up to 50%, where the maximum effect was achieved after the animals were fed the strain daily for 60 days (Younts-Dahl *et al.*, 2005; Brashears *et al.*, 2003; Elam *et al.*, 2003). It has also been shown that culture-positive animals fed *L. acidophilus* NP51 shed fewer VTEC O157:H7 than culture-positive animals not given this lacto-bacillus strain (Stephens *et al.*, 2007). There is a commercially available product containing *L. acidophilus* NP51 which is commonly used in American feedlots to reduce VTEC O157:H7 shedding by cattle prior to slaughter (reviewed by Callaway *et al.*, 2004a). This product also contains a *Propionibacterium freudenreichii* strain, included as a growth promoter (reviewed by Callaway *et al.*, 2009).

Phages

There are lytic bacteriophages (viruses) that specifically target surface receptors on VTEC O157:H7. After binding to the bacterium they inject their DNA into the cell and convert the bacterium's biosynthesis machinery to start producing new daughter phages. The latter are released when the host cell lyses and the released phages can infect new bacteria to repeat the process (reviewed by Johnson *et al.*, 2008).

Phages are plentiful in nature where they share the ecological niche with their bacterial host, which is why VTEC O157:H7 targeting phages can be isolated from cattle farms where VTEC O157:H7 is prevalent (Callaway *et*

al., 2006). Phages have also been detected in feedlot water troughs and it has been shown that the prevalence of VTEC O157:H7 in faecal samples collected in feedlots is lower when phages are present than if they are absent (Niu *et al.*, 2009).

Bacteriophages targeting VTEC O157:H7 can be included in animal feed to reduce faecal shedding of VTEC O157:H7. Administration can be oral or rectal, but the oral route seems to be more effective (reviewed by Johnson *et al.*, 2008). The bacteriophage preparations used in different studies often consist of a mixture of several different phages in order to prevent the development of resistance to the phages (Callaway *et al.*, 2008b). In one study, bacteriophages had successfully eliminated VTEC O157:H7 in weaned calves 8 days after administration (Waddel *et al.*, 2000) whereas other studies have reported only reduced shedding but not elimination in experimentally infected cattle (Rozema *et al.*, 2009; Sheng *et al.*, 2006a).

Other additives in feed and water

Orange peel and pulp, added at 2% of total volume, have in fermentation trials shown anti-VTEC O157:H7 activity, the efficacious component being inhibitory essential oils (Callaway *et al.*, 2008a; Fisher & Phillips, 2006). In a study on 200 cattle, feeding brown seaweed (*Ascophylum nodosum*) led to a reduction in prevalence of faecal shedding from 35% to 10% (Braden *et al.*, 2004).

Treating animals with the antibiotic neomycin sulphate has been demonstrated to reduce excretion of VTEC O157:H7 in cattle (Woerner *et al.*, 2006).

The chemical substance sodium chlorate is reduced to chlorite under anaerobic conditions by the enzyme nitrate reductase (Stewart, 1988) which is a bactericidal metabolite. When sodium chlorate was added to water or feed it was found to reduce VTEC O157:H7 populations in the intestines and faeces of cattle (Callaway *et al.*, 2002) and pigs (Anderson *et al.*, 2000). It has been suggested that sodium chlorate could be given as a supplement to cattle in the last meal as a pre-slaughter intervention and it has also been shown that the deleterious impact on ruminal fermentation is negligible (Anderson *et al.*, 2000).

Vaccines

It has been demonstrated that infection with VTEC O157:H7 can elicit an immune response in cattle. For instance Naylor and colleagues found a protective humoral response against LPS and H7 in calves after a challenge dose of VTEC O157:H7. The authors also showed that this induced

protective immunity resulted in lower shedding levels when the same calves were re-inoculated (Naylor *et al.*, 2007a).

Human convalescent patients have been found to develop an antibody response to Tir, intimin, Esp A and EspB, proteins that are secreted by the type III secretion system (TTSS) after VTEC O157:H7 infections (Li *et al.*, 2000). It was also demonstrated that intimin (Cornick *et al.*, 2002) and Tir (reviewed by Potter *et al.*, 2004) are necessary for successful colonization in adult cattle. Furthermore, it has been shown that the LEE4 operon, which encodes several proteins essential for A/E lesions, is needed for colonization in the terminal rectum of cattle (Naylor *et al.*, 2005b).

Intimin-based vaccines have proved efficient in experimental trials in piglets (Dean-Nystrom *et al.*, 2002) and mice (Judge *et al.*, 2004). In a Canadian study, cattle were treated with a vaccine prepared from culture supernatant material with type III secreted proteins. Vaccinated cattle developed high antibody titres against type III secreted proteins such as Tir, EspA, EspB and EspD (Potter *et al.*, 2004). This vaccine also significantly reduced the prevalence of VTEC O157:H7 in an experimental challenge model with 16 cattle and in a clinical trial including 192 steers (Potter *et al.*, 2004). However, when the same vaccine was tested in a large-scale study on nine Canadian feedlots it failed to produce any significant reduction in faecal shedding by vaccinated cattle (Van Donkersgoed *et al.*, 2005). This backlash might have been due to a change of adjuvant that altered the vaccine's immunogenicity. Nevertheless, with another adjuvant, used in subsequent feedlot studies with a two-dose regime, the vaccine reduced VTEC O157:H7 colonization in the terminal rectum by 92-98% in vaccinated feedlot cattle (Smith *et al.*, 2009b; Peterson *et al.*, 2007). Furthermore, in another feedlot study, vaccination significantly reduced shedding of VTEC O157:H7 among vaccinated cattle (4.8% positive samples) compared with unvaccinated cattle (11.5% positive), though, in this study no significant reduction in terminal rectum colonization could be proved in vaccinated animals (Smith *et al.*, 2009a). Since December 2006 the vaccine used in those studies "Bioniche *E. coli* O157:H7" has been an authorized vaccine for field use in cattle feedlots in Canada.

A Scottish study has revealed that systemic and mucosal immunization of cattle with purified H7 antigen leads to a humoral response and a delayed peak of VTEC O157:H7 shedding in calves. This indicates that H7 flagellin may be also a candidate to include in a systemic vaccine against VTEC O157:H7 (McNeilly *et al.*, 2008). Furthermore, in a recent study, the same research group demonstrated that an experimental vaccine consisting of a combination of purified recombinant proteins (EspA, Intimin-531 and Tir)

effectively reduced colonization and shedding of VTEC O157:H7 by cattle and that the protection may be enhanced by addtition of H7 flagellin to the vaccine preparation. (McNeilly *et al.*, 2009).

Other studies made on subunit vaccines for Efa1, intimin, and EspA have induced high titres of specific IgG and IgA antibodies, but these vaccines failed in experimental trials in calves to give any protection against VTEC O157:H7 intestinal colonization (Dziva *et al.*, 2007; van Diemen *et al.*, 2007).

Research is currently proceeding into new vaccines that will target epitopes of type III secreting proteins from several serotypes of VTEC that hopefully will reduce shedding in cattle of more EHEC serotypes than VTEC O157:H7 (Potter *et al.*, 2009).

Direct application of therapeutic agents to terminal recta

A Scottish study has evaluated local treatment with direct application of the antisepticum chlorhexidine to the terminal rectum of calves that have been experimentally infected with VTEC O157:H7. This treatment was found to eliminate "high shedding" and reduce low level shedding in the calves by killing VTEC O157:H7 that had colonized the terminal rectum (Naylor *et al.*, 2007b). When a group of 11 calves were sampled 3-4 weeks after treatment, there was no sign of re-colonizsation.

2 Aims of the thesis

The main aim of this study was to increase our knowledge of VTEC O157:H7 in Swedish cattle and pigs and to establish to what extent VTEC O157:H7 isolated from these animals can be held responsible for domestic human infections in Sweden.

The specific aims were:

- To estimate the prevalence of VTEC O157:H7 in slaughtered cattle in Sweden and establish the origin of identified "positive cattle".
- To estimate the prevalence of VTEC O157:H7 on Swedish dairy farms and to ascertain if there were any differences in farm prevalences between different regions in Sweden.
- To examine if there were any associations between VTEC O157:H7 status and farm size, age of sampled animals and presence of animals other than cattle on the dairy farms.
- To estimate the prevalence of VTEC O157:H7 in slaughtered pigs in Sweden and establish the origin of identified "positive pigs".
- To establish the period during which "positive pigs" on VTEC O157:H7 positive farms were shedding the bacteria and to ascertain if any specific factor influenced this period.
- To subtype VTEC O157:H7 strains isolated in prevalence studies on cattle and strains isolated from cattle farms linked to human cases, in order to establish if any specific VTEC O157:H7 variants were more closely associated with human VTEC O157:H7 infections.
- To evaluate the ability of different subtyping methods to distinguish between VTEC O157:H7 strains and their potential to identify a putative hyper virulent group of VTEC O157:H7 strains that predominate in Swedish humans cases.

3 Considerations on Material and Methods

3.1 Methods used in sampling of animals

Individual faecal samples in the cattle studies were collected directly from the rectum, using disposable plastic gloves. The samples were placed in plastic pots and sent to the laboratory by the regular postal service without a cooling device (except samples collected in the slaughterhouse studies). As VTEC O157:H7 can survive for lengthy periods in cattle faeces (46-126 days) (Fukushima *et al.*, 1999) and slurry (90 days) (McGee *et al.*, 2001) it was considered unnecessary to use a cooling device for transportation when analyses were initiated only 1-2 days after sampling.

An alternative sampling method vs. faecal sampling in the cattle studies could have been use of rectoanal mucosal swabs (RAMS), i.e. swabbing the specific colonization site in terminal rectum (Rice *et al.*, 2003). This method has been shown to yield more positive results compared with analysing faecal samples (Greenquist *et al.*, 2005). On the other hand analysis of faecal samples has proved more efficient than RAMS for detecting VTEC O157:H7 in transiently shedding cattle (shedding < 1 week) (Rice *et al.*, 2003).

In the farm studies, individual faecal samples were for practical reasons sometimes replaced by composite faecal pat samples from pens where faeces from up to 10 different pats were included. This method has been shown to reduce apparent pen prevalence, compared with collecting individual faecal samples (Smith *et al.*, 2004).

Two other alternative pen-sampling methods are placing "culturing rope devices" in the pens that cattle lick, chew or rub against (Smith *et al.*, 2004), and sampling faecal bedding material by walking around in the pens with absorbent overshoes (Cobbaut *et al.*, 2008).

3.2 Method for detection of VTEC O157:H7

All faecal samples were analysed by qualitative analysis involving immunomagnetic separation (IMS), a method that increases sensitivity up to seven-fold compared with conventional plating out from an enrichment broth (Chapman *et al.*, 1994).

The samples were diluted 1:10-1:20 in buffered peptone water (BPW) and pre-enriched at 37° ± 1°C for 6-8 h. IMS was then performed, where 1 ml of the enrichment broth was mixed with paramagnetic beads coated with antibodies against *E. coli* O157. Enriched *E. coli* O157 were allowed to bind to the beads, which were then subjected to several washes. After washing, the complex of bacteria and beads was spread on selective agar plates which were incubated overnight at 37°C. The plates were then screened for VTEC O157:H7.

IMS was performed either directly after 6-8 h incubation or after storing the enrichment broth overnight (16-20 h at 6-8°C). When evaluated this cold-storage procedure was not found to influence the analysis results negatively (Eriksson *et al.*, 1998).

Pooling of faecal samples together, five and five at analysis, was used to reduce costs in the dairy herd study and the slaughterhouse study on pigs. However, this procedure has been shown to impair the sensitivity of the analyses (Arnold *et al.*, 2008; Sanderson *et al.*, 2005; Eriksson *et al.*, 1998). The likelihood that pools would prove VTEC O157:H7 positive increases if more than one positive sample is included in the pool and/or if any of the included faecal samples contains high a level of VTEC O157:H7. One drawback of pooling is that background flora from several faecal samples accumulate, thereby interfering with the analysis. To compensate for this, faecal samples from animals of the same age and housed together can be pooled, as they are more likely to have largely the same gut flora. This strategy was utilized in the dairy herd study where the faecal samples were pooled according to an age-related sampling list. Also in this study, the amount of added faeces from each animal (5 g) exceeded the 1 g added faeces in the studies by Sanderson and colleagues and Arnold and colleagues. However, pooling probably reduced the apparent prevalence, especially in the pig studies where the analyses were performed during the period when only 1 g of faeces from individual samples was analysed (see below), while a pooled sample consisted of 5 g of faeces (1 g from each sample included).

IMS is considered as the official standard method for detection and isolation of E coli O157:H7 from food samples (Anonymous, 2001) but is also generally applied as the standard method in the analyses of animal faecal samples. In an evaluation study an IMS-based method was compared with

the performance of a PCR assay by testing series of animal faeces (from cattle and sheep) and meat samples artificially contaminated with VTEC O157:H7. In this study the IMS-method identified 20/21 (95%) of the faeces samples at the inoculation level 10^1 cfu/10 g faeces and 2/21 (10%) at inoculation levels 10^0 cfu/g faeces (Islam *et al.*, 2006).

The outcome of the VTEC O157:H7 IMS analyses of cattle faecal samples is very much dependent on background flora in the analysed faeces, amount of faeces included in the analysis, the individual laboratory´s performance and the enrichment procedure used. This is evident in the results from the Swedish cattle slaughterhouse studies, presented in Table 9, page 36. In the first study, 1996-97, 10 g of faeces were pre-enriched in 90 ml BPW giving an apparent prevalence of 1.2%. In 1997-98, when the analyses were conducted by another laboratory (not the National Veterinary Institute, NVI) the amount of faeces analysed was reduced to 1 g and the sample was enriched in 20 ml BPW (same enrichment protocol as used e.g. in Scotland). The apparent prevalence then fell to 0.3%. In 1999 the amount of faeces was again 10 g enriched in 90 ml BPW, which increased the apparent prevalence to 0.7%. Finally, when the analyses in 2000 were transferred back to NVI (10 g of faeces enriched in 90 ml BPW), the apparent prevalence increased further, to 1.7%. Moreover, in the slaughterhouse prevalence study performed in 2005-06, a new improved enrichment protocol was introduced, where 10 g of faeces was enriched in 90 ml modified Tryptic Soy Broth (mTSB) (with of 20 mg/l of novobiocin added), the enrichment period was prolonged to 18-24 h and the temperature was raised to 41.5°±0.5°C. With this new enrichment protocol the apparent prevalence increased to 3.4%. In the most recent slaughterhouse study, in 2008-09, the mTSB enrichment protocol was again used and the prevalence in faeces samples was almost identical (3.3%) with the results from 2005-06.

3.3 Detection of Verotoxins

VT secreted from VTEC can be detected by Vero cell cytotoxicity assay, enzyme immunoassay, latex agglutination assay and colony immunoblot, several tests of these being commercially available (reviewed by Karch *et al.*, 2005). With these methods the actual verotoxins are detected. With Vtx-ELISAs, one can also quantify and compare levels of VT production between different strains (Dowd & Williams, 2008; Ziebell *et al.*, 2008) which might have been interesting, e.g., for comparing VT2 production between VTEC O157:H7 strains in Paper V.

In the present studies it was not investigated if the bacteria produced VT; instead the genes encoding VTs by different PCR assays were identified. However, a positive PCR reaction does not confirm that the bacteria can produce VT, as the PCR reaction detects only a part of the VT gene. There may have been events such as mutations, insertions and deletions that rendered the gene unfunctional or unexpressed.

3.4 Phage typing

Phage typing, by published methods, was performed at the Laboratory of Enteric Pathogens (Central Public Health Laboratory, London, England) (Khakhria *et al.*, 1990; Ahmed *et al.*, 1987).

After the slaughterhouse survey in 1996-97 all the 37 VTEC O157:H7 isolates collected were sent for phage typing, six of them were typed as having a RDNC pattern (reacted but did not conform). When the next set of strains was submitted, in 2003, these six strains were sent again. This time three of those that at the previous typing proved RDNC were of phage type 8, a type that was also included in the typing scheme on the first typing occasion. These results illustrate that the correct phage type can sometimes be difficult to determine and that phage typing appears not to be completely reproducible. This also explains why the phage typing results of the survey performed in 1996-97 differ between Paper I and Paper IV.

3.5 Choice of molecular typing methods

For subtyping the VT2 genes, two different methods have been used, conventional PCR-RFLP (Pierard *et al.*, 1998) (Paper IV) and a method based on partial sequencing of the VT2 gene (Persson *et al.*, 2007) (Paper V). In the latter method the nucleotide sequence is defined from partial sequencing of the most variable part of the *vtx*AB_2 operon (Persson *et al.*, 2007). This method enabled division of the VT2 variants; vtx_2 and vtx_{2c}, into further variants where the new ones were defined by the 159 amino acids encoded by the sequenced DNA fragment. When several vtx_2 variants were present this could be detected as double peaks in each position where the two VT2 variants differed in nucleotide composition.

Pulsed-Field Gel Electrophoresis (PFGE) has been the basis for subtyping of all VTEC O157:H7 strains in these studies. By cutting the whole bacterial genome with specific macro-restriction enzymes, like *Xba*I, a varying number of DNA fragments of differing size are obtained. These fragments can be separated by PFGE according to a specific protocol.

Afterwards the fragments can be visualized as band patterns on agarose gels. In Paper V another typing method was evaluated; multi-locus variable number tandem repeat analysis (MLVA). MLVA does not analyse the whole genome, instead it focuses on specific loci and quantifies number of so-called tandem repeats in these loci. The resulting information is a number series of tandem repeats in the 8 different loci studied, which can be compared between different strains. Comparison of different band patterns by PFGE is more dependent on correct interpretation of gel images and is therefore more difficult to standardize. As PFGE and MLVA are based on different principles it was interesting to compare how they distinguish between the selected strains (Paper V).

A subtyping method based on single nucleotide polymorphism (SNP) was used in Paper V to detect single mutations localized in specific positions of four specific SNPs (Riordan *et al.*, 2008). By establishing which nucleotides were present in different SNPs, it was possible to establish if VTEC O157:H7 strains belonged to any of the evolutionary clades 1, 2, 3 or 8 described by Manning and colleagues (Manning *et al.*, 2008). To verify that clade 8 strains were correctly identified another SNP method was included. This one determined the nucleotide variants in four SNP in the *rhsA* gene by partial sequencing and the results could be used to specifically verify the clade 8 strains (Liu *et al.*, 2009).

3.6 Statistics

The statistics used in the studies are standard methods. In Paper II, however, the variable herd size was analysed in a novel way. It was assumed that the risk of a dairy herd being VTEC O157:H7 positive, depending on the variable herd size, was not linear. Rather, the incremental increase in risk would be higher from 20 to 100 cows than from 200-280 cows. Based on comparative considerations and using the approach in bacteriology for modeling infectious doses, the following formula was applied for the variable herd size:

$$1-\exp[-(\text{number of cows} \star \text{prevalence})]$$

where the prevalence among the animals was set to be 1.034% (based on the calculated individual prevalence from the observed prevalence in the pooled samples). When this formula was applied to the variable "herd size" the total risk was presented as a function that fitted the assumed hypothesis of increase in risk as a function of number of cows (see Fig. 10).

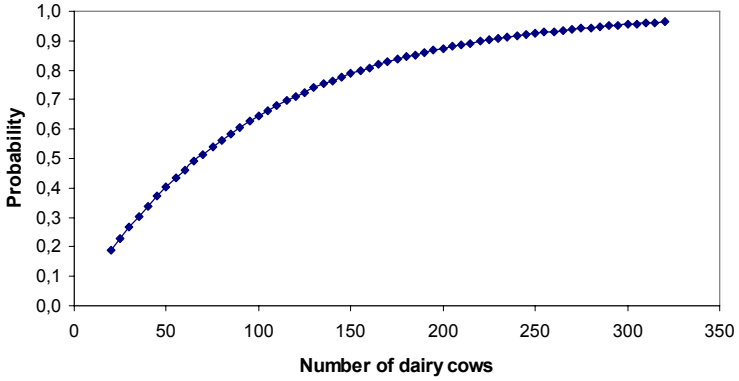

Figure 10. Relationship between the number of dairy cows and probability of a herd being deemed VTEC O157 positive if the variable herd size is presented as 1-exp [- (no.cows * prevalence)]. The total risk asymptotically approaches 1 for a infinite number of cows.

4 Results and Discussion

4.1 The prevalence studies on cattle

The first slaughterhouse study on cattle was launched as a survey 1996-97 and the sample size in this study was set to detect a very low prevalence of VTEC O157:H7 (prevalence of 0.1% with a 90% confidence level). The prevalence turned out to be 1.2% (CI_{95} 0.8-1.6) which was ten-fold higher that expected. This prevalence was in the lower range but nevertheless comparable to results from slaughterhouse studies in other countries in Europe (see Table 10, page 36). However, it is very difficult to compare results between different prevalence studies, as study design, number of samples, sampling strategy, amounts of faeces analysed, enrichment protocols etc, all vary in different studies. The effects of such factors are very explicitly demonstrated in the Swedish slaughterhouse studies, where relatively small changes in methodology led to considerable changes in apparent prevalence in different years (see above under "Method for detection of VTEC O157:H7").

The results from the survey and the subsequent slaughterhouse studies have confirmed the same relationships in Sweden as reported from other countries, i.e. that the prevalence is higher during the warmer season of the year (Barkocy-Gallagher *et al.*, 2003; Paiba *et al.*, 2003) and that the bacteria are more frequently found in younger than in older animals (Paiba *et al.*, 2003; Nielsen *et al.*, 2002; Heuvelink *et al.*, 1998b). Moreover, the results have demonstrated the importance of slaughter hygiene at Swedish slaughterhouses, especially after VTEC O157:H7 in the later studies was found in such a high prevalence in ear samples (Boqvist *et al.*, 2009) (see also Table 9, page 36).

With the exception of a few positive findings in 1999-2000, all VTEC O157:H7 positive animals have been geographically localized to the postal code of the farm of origin. Furthermore, the geographical postal codes of all

positive farms in the dairy herd study are known. The geographical distribution of the registered postal codes from the cattle prevalence studies performed during 1996-2002 is mapped in Paper IV. Generally, VTEC O157:H7 were detected in the areas in Sweden where cattle density is known to be high, see Fig. 9, page 34. No positive samples were detected in sampled animals from northern Sweden. These maps are consistent with the map in Paper II, where the prevalence of VTEC O157:H7 in dairy herds is shown.

The varying prevalences observed in the slaughterhouse prevalence studies among cattle originating from different geographical regions are consistent with observations in other countries. In a Finnish slaughterhouse study, no VTEC O157:H7 positive animals were found in northern Finland (Lahti *et al.*, 2001) and more positive samples were also found from slaughterhouses in eastern Great Britain compared to slaughterhouses from the north of Great Britain (Paiba *et al.*, 2002).

In the dairy herd study the livestock association (LA) "Halland Husdjur" situated on the Swedish southwest coast presented the highest prevalence (23.3%) of all LAs and LA "Halland Husdjur" was also included in the final fitted multivariate model as an increased risk for a herd being VTEC O157:H7 positive.

In Paper IV all the cattle farms that were associated with human cases during 1996-2002 are mapped. Most of these farms (61%) were located in Halland and at all those farms the causative agent was the variant VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) (see below). Most of the farms associated with human cases during this period were located in southwest Sweden, with only one farm in the east-central Sweden and one farm in Skåne province in the south. In latter years, more farms linked to human cases of VTEC O157:H7 have been found in Skåne and in other parts of Sweden, which correlates well with the observation in Paper IV that VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) strains appeared to be spreading within Sweden

Repeated sampling, i.e. longitudinal studies, produces higher and more accurate prevalence estimates than studies based on one occasion only (Synge *et al.*, 2003; Hancock *et al.*, 1997). The dairy herd study was based on only one sampling occasion and the observed prevalence of VTEC O157:H7 would probably have exceeded 8.9% if repeated sampling in the herds had been performed. Also, pooling of samples may have lowered prevalence, whereas the new enrichment protocol, introduced in 2005 with mTSB, might have increased diagnostic sensitivity and led to higher observed prevalence. However, the observed prevalence of 8.9% is within

the range of what has been reported from other studies from Europe (see Table 8, page 35).

An increase in herd size from 20 to 100 cows was identified as a risk factor in the dairy herd study and this variable was included in the final fitted multivariate model with an OR of 3.5. In a study on Canadian feedlots a significant correlation was found between prevalence rate and the number of pens occupied by cattle within the feedlots; in addition there was a correlation with cattle density in the pens (Vidovic & Korber, 2006). Other studies on dairy and beef cattle farms have not reported any correlation between herd size and VTEC O157 prevalence (Cobbaut *et al.*, 2009; Nielsen *et al.*, 2002; Wilson *et al.*, 1998; Garber *et al.*, 1995). The reason for this might be that the other studies have included few farms with herd size < 50 cows. Another explanation could be that the assumption in Paper II is correct, i.e. that the increased risk associated with number of cows is not linear and that this study was able to detect the increased risk due to the specific adjustments in the statistics with the herd size variable. Furthermore, the formula used in the statistics was probably not the optimal function for describing this relationship. Based on the knowledge we have today, probably the best formula would have been a function based on the prevalence of high-shedders in the herd, where one or more high-shedders in a herd would indicate a considerable likelihood of a circulating, and therefore persistent, VTEC O157 infection.

The observation that the risk of a farm being VTEC O157:H7 positive increased if median age was lowered from 10 months to 3 months (OR= 2.1 in multivariate model) is consistent with what other studies have reported, that prevalence is higher in younger animals (Paiba *et al.*, 2003; Nielsen *et al.*, 2002; Heuvelink *et al.*, 1998b).

Several studies have, like in Paper III (OR=1.9), reported significant results indicating that presence of pigs on dairy farms is a risk factor for a cattle herd being positive for VTEC O157:H7 (Gunn *et al.*, 2007) (OR=2.4) (Schouten *et al.*, 2004) (OR= 3.4), (Cernicchiaro *et al.*, 2009) (OR=15.5). Conversely, one study claims that the risk was reduced if pigs were present on a farm with beef suckler cows (Synge *et al.*, 2003) (OR=0.2). No explanation as to why in several studies pigs are associated with an increased risk that cattle farms would be VTEC O157:H7 positive has been presented. There are theories that some unidentified management routines in mixed herds with both cattle and pigs, can increase the risk of VTEC O157 infection in cattle. Manure management, housing practices and supposedly greater movement of animals in mixed herds than in non-

mixed herds, are all factors mentioned in these discussions (Cernicchiaro *et al.*, 2009).

4.2 Pig studies

The apparent prevalence found in Swedish slaughter pigs (Paper III) was very low (0.08%) and the two identified VTEC O157:H7-positive pigs were traced back to their farms of origin; both of them kept ruminants and pigs. The prevalence is comparable to what has been observed in Norway (Johnsen *et al.*, 2001) and Great Britain (Milnes *et al.*, 2008) (see Table 12, page 41)..

Repeated sampling on the four VTEC O157:H7 positive farms that kept both ruminants and pigs showed that the strains isolated from pigs and ruminants presented identical or very similar PFGE pulsotypes. The bacterium could be detected in pig samples on sequential sampling occasions and there was evidence that direct or indirect contact with ruminants was of importance in maintaining VTEC O157:H7 infection in pigs. On one of the farms VTEC O157:H7 prevailed, and could be detected in pig faecal samples for 11 months. It was also demonstrated that if VTEC O157:H7 positive young pigs were allocated to an environment free from ruminants, they gradually ceased shedding VTEC O157 and the bacterium could not be detected in any of the faecal samples collected 9 weeks after the pigs had been moved from the herd of origin. The conclusion from Paper III was that the prevalence in the Swedish pig population was very low and that pigs seemed unlikely to contract a VTEC O157:H7 infection unless they had direct or indirect contact with ruminants.

One explanation for the low prevalence seen in pigs could be that intimin (*eaeA*) and the TTSS which is essential for colonization in cattle do not improve colonization of VTEC O157:H7 in pigs (Jordan *et al.*, 2005). However, pigs ought to be considered as a potential reservoir for VTEC O157:H7. Experimental studies have shown that pigs do not have any innate resistance to infection; they can shed the bacterium for periods up to 4 months and can also transmit infection to other pigs in a herd (Cornick & Vukhac, 2008; Cornick & Helgerson, 2004; Booher *et al.*, 2002).

Some studies have detected higher prevalences in pigs than in cattle. In a Chilean study the prevalence of VTEC O157 in 120 slaughtered pigs was 10.8% whereas 136 sampled steers rendered a prevalence of only 2.9% (Borie *et al.*, 1997). In another study from Mexico 60 faecal samples were collected from each of four cattle farms and four pig farms; and the prevalences were 2.1% in pigs and 1.25% in cattle (Callaway *et al.*, 2004b). Moreover, in an investigation of public amenity premises in England and

Wales, a significant association was found between presence of VTEC O157 and the number of species sampled, size of enterprise, presence of young cattle and presence of adult pigs (Pritchard *et al.*, 2000), implying that the presence of adult pigs contributes to greater risk of VTEC O157:H7 infections for humans on these premises.

The results in paper III do not indicate that pigs, at least not in Sweden, are an important source of infection for humans, though the relationship between pigs and cattle is puzzling. It seems that keeping both pigs and cattle on mixed farms increases the risk of pigs shedding the bacterium to a detectable degree. On the other hand, our results (as well as results in other studies, see above), indicate that keeping pigs together with cattle on mixed farms increases the risk of cattle shedding VTEC O157:H7. More studies are needed to explain this apparently contradictory relationship.

4.3 Characterization of VTEC O157:H7 isolates

The strains collected in the different cattle prevalence studies during 1996-2002 were all isolated by random sampling and were therefore considered to reflect the VTEC O157:H7 strains prevalent in the Swedish cattle population during that period. The subtyping results from the first slaughterhouse survey (Paper I), had already presented a varied picture regarding verotoxin composition, PFGE pulsotypes and phage types in the 37 strains obtained. When comparing strains from the prevalence studies isolated during 1996-2002 (Paper IV), the conclusion was that there was a wide variation in strains present in the cattle population. Ten different phage types were identified as well as several not previously described types (RDNC types) and all these could be further divided into different PFGE pulsotypes. However, three different phage types predominated, PT4 (28%), PT8 (33%) and PT14 (18%).

The fact that a diverse picture was evident even in the first slaughterhouse survey leads to speculation as to how long VTEC O157:H7 have prevailed in the Swedish cattle population. It seems unlikely that such a diverse composition of strains could have been introduced over a short period of time. Therefore, strains of VTEC O157:H7 probably prevailed in the Swedish cattle population long before 1995-96, i.e. before the increase in human incidence was observed. This would imply that the VTEC O157:H7 strains present before 1995 were less virulent or not so effectively transmitted to humans as new strains putatively introcuded after 1995-96, though it could mean that VTEC O157:H7 infections in humans were

under-diagnosed in Sweden before 1995-96, when new diagnostic tools were introduced.

Among the 18 farms associated with human cases during 1996-2002 a specific variant of VTEC O157:H7 was predominant as it was found on 16 (89%) of the 18 farms. These strains were of phage type 4, carried two VT2 genes (vtx_2 and vtx_{2c}) and presented similar pulsotypes when typed by pulsed field gel electrophoresis (PFGE). During the same period, strains of this variant were also isolated in the slaughterhouse studies (~27% of isolated strains) and dairy herd study (21% of positive farms). Moreover, VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains (called SMI H variants by SMI) also accounted for more than two-thirds of the VTEC O157:H7 isolates from Swedish domestic cases during 2001-2007 (personal communication, Sven Löfdahl, SMI).

As it was suspected that VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains, or other strains isolated from farms linked to human cases, were more virulent, a subset of strains (n=45) was analysed by a microarray assay and PCRs targeting selected virulence genes. Generally speaking the results of that study supported our previous conclusions. The observed variability among strains isolated in prevalence studies in terms of pulsotype, phage type and VT2 type was to some extent reflected by the variable absence (or presence) of virulence genes, while strains belonging to VTEC O157 (PT4;vtx_2;vtx_{2c}) presented virtually identical virulence gene patterns with a complete set-up of LEE genes and other major virulence genes. However, no specific virulence markers distinguishing these strains from the other strains could be observed (Söderlund *et al.*, In manuscript)

In Paper V the same subset of strains (n=45) as used by Söderlund and colleagues was subjected for further subtyping, using PFGE, MLVA and two SNP typing methods. In addition the vtx_2 and vtx_{2c} genes were subtyped by partial sequencing. It was found that all VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains belonged to a specific hyper-virulent clade of strains, clade 8, that is associated with more severe illness in humans (Manning *et al.*, 2008). The utilized SNP typing method (Riordan *et al.*, 2008) proved to be a reliable and convenient tool for rapid identification of clade 8 strains and this method can be very useful in the future for rapid identification of strains belonging to the variant VTEC O157:H7 (PT4;vtx_2;vtx_{2c}).

In the partial sequencing of the vtx_2 genes it was not possible to identify any unique vtx_2variants among the VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains or other strains linked to human cases. The MLVA subtyping results correlated closely with the PFGE results regarding clustering of strains, though for a small subset of strains the two methods distinguished differently

between the strains. Moreover, MLVA was able to divide some strains with indistinguishable PFGE (*Xba*I) pulostypes into distinct MLVA variants, which also tallied closely with the epidemiological history of the strains. However, the converse was also observed, e.g. that PFGE was able to divide strains with the same MLVA variant into distinct PFGE patterns that correlated well with epidemiological history. It was also found, consistent with results obtained by Hyytiä-Trees and colleagues, that when combined *Xba*I-*Bln*I PFGE data was used, this led to better concordance between the MLVA and the PFGE results (Hyytia-Trees *et al.*, 2006). The two methods can be considered as complementary and the best information is obtained if they are run in parallel.

In the prevalence studies on slaughtered cattle, during 1996-2002, four VTEC O157:H7 strains of PT4 were isolated that carried only the vtx_2 gene (no vtx_{2c} gene). Subsequently, in 2007, a VTEC O157:H7 (PT4;vtx_2) strain was isolated from one farm associated with a human case. These strains presented PFGE pulsotypes similar to those of VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) and it was therefore suspected that they were of clade 8. When the strain associated with the human case and two isolates from the prevalence studies were SNP typed, with the method according to Riordan and colleagues, it could be confirmed that they all belonged to clade 8. These results are consistent with the decription of clade 8 strains carrying either a single vtx_2 gene, or vtx_2 and vtx_{2c} in combination (Manning *et al.*, 2008).

The fact that variant VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) belongs to the hyper-virulent clade of VTEC O157:H7 strains, is consistent with its predominance among human cases and farms linked to human cases since 1996. The two large Swedish food-borne outbreaks caused by VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains have both resulted in high frequencies of HUS, the sausage outbreak (30% HUS), (Sartz et al., 2007) and the lettuce outbreak (8% HUS) (Söderström et al., 2008), which also is typical of clade 8 strains (Kulasekara et al., 2009; Manning et al., 2008).

It has been described that clade 8 strains can produce significantly larger amounts of VT2 than lineage II strains (VTEC O157:H7 strains that are more frequently isolated from cattle than humans and thereby considered as less virulent for humans) (Zhang *et al.*, 2009). However, other strains than clade 8 also have the same, or even greater capacity, to produce VT2 (Zhang *et al.*, 2009; Manning *et al.*, 2008). Thus, the high virulence seen in clade 8 strains must also be caused by some other factor/factors not yet identified.

In a recent study a clade 8 strain isolated from a food-borne outbreak in the USA, "the spinach outbreak", was sequenced. The authors identified seven putative virulence determinants in the genome of this bacterium and suggested that an intact gene for anaerobic nitric oxide reductase, *norV*, could be correlated to the greater virulence of these strains (Zhang *et al.*, 2009).

The most frequent PFGE pattern found from human VTEC O157:H7 isolates in Sweden has been designated by SMI "SMI-H". This PFGE pattern has been prevalent in Sweden since it first was observed in 1996 in isolates from Halland. By comparing the PFGE pattern of "SMI-H" with PFGE patterns in American publications, Dr. Sven Löfdahl at SMI was able to detect a similarity in the banding pattern between American strains and "SMI-H". When a TIF file with this PFGE pattern was sent to the Center of Disease Control and Prevention (CDC) Atlanta, USA, they could confirm that the PFGE "SMI-H" was identical to the most frequently observed PFGE pattern among isolates from American patients, "EXHX01.0047". Furthermore, this PFGE banding pattern "EXHX01.0047" has previously been shown to be identical to the most common PFGE pattern observed in isolates from Argentinian patients "AREXHA26-011 (personal communication Peter Gerner-Smith, CDC, Atlanta, USA; Löfdahl, 2008). These are only preliminary data and the observations require further verification, but it is intriguing that VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains with the same pulsotypes are commonly isolated from human cases in all these three countries.

4.4 VTEC O157:H7 colonisation in cattle

Interestingly, in humans, VTs from VTEC O157:H7 elicit an enhanced immune response and thereby make the endothelial cells more vulnerable to the damaging effects of VTs (reviewed by Palermo *et al.*, 2009; and Proulx *et al.*, 2001). By contrast in cattle, it has been suggested that VTs modulate the bovine immune response in the intestines in order to improve colonization (Menge *et al.*, 2003; Hoey *et al.*, 2002; Magnuson *et al.*, 2000). In Scotland the dominant strains of VTEC O157:H7 are of phage type 21/28. These strains are responsible for 72% of human cases in Scotland and are associated with more severe disease (e.g. HUS) than other VTEC O157:H7 strains in Scotland. VTEC O157:H7 of PT21/28 are isolated from ~50% of Scottish cattle sampled (reviewed by Chase-Topping *et al.*, 2008). The odds for cattle shedding phage type 21/28 are found to be more than twice as high in high-shedding cattle as in low-shedders. This could

mean that these VTEC O157:H7 strains are more effective in colonizing the terminal rectum than other VTEC O157:H7 strains (Chase-Topping *et al.*, 2007). It can be hypothesized that VTEC O157:H7 (PT4;vtx_2;vtx_{2c}) strains likewise could be isolated more frequently from high-shedding cattle in Sweden. However, further studies are needed to investigate such a relationship.

4.5 Halland county

At the regional hospital in Halland, VTEC O157:H7 was very rarely isolated from human stool samples before 1995. From November 1994 to November 1995 a screening study for EHEC was undertaken. All stool samples from patients with bloody diarrhea under the age of 15 were screened for VTEC O157:H7 by a newly introduced culturing method. During the first 9 months of the study, all samples (approx. 10,000) proved negative for VTEC O157:H7. In autumn 1995 the first four strains of VTEC O157:H7 were isolated from children with diarrhea and in June, two additional strains were isolated. All strains were defined by DNA subtyping as belonging to variant VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) (personal communication Torvald Ripa M.D, Assistant Professor at the Department of Clinical Microbiology & Infection Control, Halmstad Hospital, Halland, Sweden). The fact that no VTEC O157:H7 strains were detected during the first 9 months of the study leads to speculation that VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) could have been introduced as a new human pathogen into Halland in 1995.

Since 1996 Halland county has had the highest domestic incidence of VTEC in all Sweden varying during 1997-2008 between 2.1 and 16.6/100,000 (mean incidence 7.0/100,000 inhabitants) (see Fig. 8, page 32). During 1996-2002, 11 (61%) of the 18 cattle farms that were linked to human VTEC O157 cases were located in Halland and all isolated strains from these farms belonged to the variant VTEC O157:H7 (PT4;vtx_2,vtx_{2c}).

In the first slaughterhouse prevalence study of 1996-1997, strains of VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) were isolated only in cattle from Halland and constituted, in this study, approx two-thirds of the VTEC O157:H7 isolates from Halland. In the subsequent prevalence studies and dairy herd study this variant has been isolated in other regions of Sweden. Moreover, this spread coincided with the observation that strains of VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) were more frequently isolated from farms linked to human cases in other parts of Sweden, than Halland. One might therefore have reason to speculate that VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) was introduced

into the cattle population of Halland around 1995 and has since spread to cattle population in other parts of Sweden.

Interestingly, a similar hot spot of high VTEC O157:H7 prevalence in cattle and high incidence in human cases is found in Grampian region, in northeast Scotland. Grampian has an infection rate of 9.2 human cases/100,000 per inhabitants and an apparent prevalence of 9.2% in cattle and 6.5% in sheep. In Grampian however the predominant strain is a VTEC O157:H7 of phage type 21/28 (Strachan *et al.*, 2005).

4.6 Future perspectives

To be able to reduce the human incidence of VTEC O157:H7 infections in Sweden, an approach that aims to reduce its prevalence in the cattle population is needed. This is important as exposure from the environment is a common infection route in sporadic human cases (Strachan *et al.*, 2006). In addition, other measures are needed, such as continuous information-campaigns describing how people can reduce the risk of infection when visiting farms, by practising hygiene routines etc. Also, efforts to ensure that the bacterium does not enter the food chain are of great importance.

However, if efficient measures should be introduce in cattle farms there are still some knowledge gaps that need to be filled, such as:

- What are the most important routes of infection/introduction of VTEC O157:H7 in Swedish cattle herds (other than introduction of VTEC O157:H7 positive animals).
- What measures would be most effective and feasible for reducing prevalence, even possibly to eradicate, the bacterium in VTEC O157:H7 positive cattle herds.
- Is it possible to reduce the prevalence, or eliminate the presence, of so-called "high-shedders" in cattle herds, as these animals account for the greater part of VTEC O157:H7 shed and may be responsible for maintaining the infection in cattle herds.
- Should measures in cattle herds be concentrated on certain VTEC variants that appear to be more virulent for humans, such as VTEC O157:H7 (PT4;vtx_2,vtx_{2c}) and if so, how should such bacteria be defined and detected.

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