Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2020:16
Faculty of Veterinary Medicine and Animal Science

Transmission and dynamics of VTEC O157:H7

A story about the complex associations between pathogen, host and environment

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2020

Acta Universitatis agriculturae Sueciae
2020:16

ISSN 1652-6880
ISBN (print version) 978-91-7760-550-8
ISBN (electronic version) 978-91-7760-551-5

Print: SLU Service/Repro, Uppsala 2020

Transmission and dynamics of VTEC O157:H7 - A story about the complex associations between pathogen, host and environment

Abstract

Verotoxin-producing *Escherichia coli* serotype O157:H7 (VTEC O157:H7), is a zoonotic pathogen often transmitted from cattle to humans. In Sweden, domestic transmission of a highly virulent subtype of VTEC O157:H7, originating in regional clusters of infected cattle farms, is increasing. To reduce the risk of transmission to humans a comprehensive picture of infection dynamics between and within farms are urgently needed.

The aim of this thesis was to provide a holistic view of drivers of transmission and susceptibility from a regional, farm and animal perspective by combining epidemiology, microbiology, bioinformatics and animal welfare.

The risk of introduction of VTEC O157:H7 on cattle farms was studied by collecting environmental samples in spring and fall from 80 farms. Information about farm characteristics, biosecurity and between farm contacts were collected by a questionnaire. On 4 farms, a more thorough environmental sampling with detailed analysis of strains was carried out during summer (between the spring and fall sampling). The results showed frequent transmission of VTEC O157:H7 between farms and that transmission occurs through human and animals contacts.

To investigate drivers of colonisation and transmission on farm level, individual samples from calves on 12 dairy farms with VTEC O157:H7 (established through environmental sampling) were collected. In addition to collecting information about pen and calf environment, a novel approach, using indicators of animal welfare and behaviour to study individual differences, was used to explore differences between colonised and non-colonised calves. The results suggest that social and active individuals are more likely to be colonised by the pathogen while animals showing signs of poor health and welfare were less likely to be colonised. Colonised animals shedding high levels of the bacteria were important for transmission but environmental exposure also increased risk of transmission within pens.

Keywords: EHEC, verotoxin, shigatoxin, calves , cattle, on-farm measures, super-shedder

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Smittspridning av VTEC O157:H7 – en skildring av komplexa samband mellan bakterie, värd och miljö

Abstract

Verotoxin-producerande *Escherichia coli* av serotypen O157:H7 (VTEC O157:H7) är en zoonos som ofta sprids från nötkreatur till människor. I Sverige ökar antalet fall av en virulent typ av VTEC O157:H7som förekommer i hög grad bland gårdar i vissa områden. Ökad förståelse för spridningen mellan och på gårdar behövs för att minska spridningen till människor.

Målet med denna avhandling var att ge en övergripande bild av hur spridningsdynamik och andra faktorer påverkar förekomst av bakterien på regional-, gård- och individnivå genom att kombinera forskningsområdena epidemiologi, mikrobiologi, molekylära metoder och djurvälfärd.

Spridning mellan gårdar studerades genom att samla in miljöprover från 80 gårdar under vår och höst. Information om besättningarna samt kontakter och andra riskfaktorer samlades in med en enkät och på 4 gårdar genomfördes ytterligare provtagningar under sommaren. Resultaten visar tät smittspridningen i området och att smittan kan spridas mellan gårdarna genom kontakter mellan både djur och människor.

För att undersöka risk-faktorer för kolonisering och spridning inom gårdar provtogs kalvar från 12 besättningar där bakterien påvisats genom miljöprovtagning vid två tillfällen. I tillägg till att samla in information om box och miljö användes indikatorer för välfärd och observation av beteenden för att undersöka individuella skillnader mellan koloniserade och icke koloniserade djur. Resultaten visar att kolonisering av VTEC O157:H7 var vanligare bland sociala och aktiva djur medan tecken på nedsatt hälsa och välfärd inte var kopplade till kolonisering. Djur som utsöndrade höga mängder bakterier (så kallade super-utsöndrare) var viktiga för smittspridningen men även exponering från miljön var en risk.

Keywords: EHEC, verotoxin, shigatoxin, kalv, nötkreatur, bekämpning, super-utsöndring

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Preface

> *"To understand the whole it is necessary to understand the parts. To understand the parts, it is necessary to understand the whole. Such is the circle of understanding"*
>
> Ken Wilbert, The Eye of Spirit

The more we learn and the more advanced our technologies become, the more we realise that we are living in an increasingly complex reality. We are moving from recognising infectious disease as simply the presence of a pathogenic organism towards a much more intricate pattern where the need and importance of considering the interactions between pathogen, host and the environment is becoming increasingly clear. A bacteria that exemplifies complexity in multiple ways is verotoxin-producing *Escherichia coli* serotype O157:H7 (VTEC O157:H7). It is able to cause severe disease in humans, but not in everyone who gets infected. It can persist and multiply in the environment, as well as establish itself in the gastrointestinal tract of ruminants and in particular cattle. As opposed to humans, cattle do not get sick, but there is unexplained variation in which cattle are colonised and which are not. This thesis is an effort to explore factors influencing VTEC O157:H7 on herd and individual level with the aim of filling knowledge gaps in some of the parts, thereby increasing the understanding of the whole.

Dedication

To Kenzo.

For enduring the most boring weeks of his life during the work of this thesis and for always showing an inspiring fighting spirit and admirable integrity.

Contents

List of publications

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

I Tamminen, L.M.*, Söderlund, R., Wilkinson, D. A., Torsein, M., Eriksson, E., Churakov, M., Dicksved, J., Keeling, L.J., Emanuelson, U. (2019). Risk factors and dynamics of verotoxigenic Escherichia coli O157:H7 on cattle farms: An observational study combining information from questionnaires, spatial data and molecular analyses. *Preventive Veterinary Medicine*, 170, pp. 104726.

II Tamminen, L.M., Dicksved. J., Eriksson, Keeling, L.J., E. Emanuelson, U. Untangling the role of environmental and host-related determinants for on-farm transmission of verotoxin-producing Escherichia coli O157:H7 *(manuscript)*

III Tamminen, L.M.*, Hranac, C.R., Dicksved. J., Eriksson, E. Emanuelson, U., Keeling, L.J. (2020). Socially engaged calves are more likely to be colonised by VTEC O157:H7 than individuals showing signs of poor welfare. *(submitted)*

IV Tamminen, L.M., Keeling, L.J., Svensson, A., Briot, L., Emanuelson, U. Considerations for using hair cortisol as an indicator of welfare in dairy calves *(manuscript)*

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* Corresponding author.

The contribution of Lena-Mari Tamminen to the papers included in this thesis was as follows:

I Involved in the designing the questionnaire. Organised and performed most analysis of data (except spatial clustering). Drafted the manuscript and finalised it together with co-authors. Corresponded with the journal.

II Involved in formulating the research idea, planning and organising the study. Contacted farmers and coordinated (and performed part of) environmental samplings of farms. Performed sampling and observation of individual animals. Performed analysis of the data, drafted the manuscript and finalised it with input from co-authors.

III Involved in formulating the research idea, planning and organising the study. Contacted farmers and coordinated (and performed part of) environmental samplings of farms. Performed sampling and observation of individual animals. Performed analysis of the data (with input from co-authors), drafted the manuscript and finalised it with input from co-authors.

IV Actively involved in formulating the research idea and development of the protocol for hair cortisol analysis. Performed sampling and observation of individual animals as well as preparation of hair samples together with master student. Performed analysis of the data, drafted the manuscript and finalised it with input from co-authors.

List of tables

List of figures

Abbreviations

CI	95 % Confidence interval
CV	Inter- and intra-assay coefficients of variation
eae	Intimin
EHEC	Enterohemoragic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Gam	Generalised additive model
Gb3	Globotriaosylceramide
Glm	Generalised linear mixed-effects model
HPA	Hypothalamic–pituitary–adrenocortical
HUS	Haemolytic uremic syndrome
IMS	Immnuomagnetic separation
LEE	Locus of enterocyte attachment
MLVA	Multi-locus variable number tandem repeat analysis
mTSB	Modified tryptic soy broth
OR	Odds Ratio
PBS	Phosphate buffered saline
PT	Phage type
RAMS	Recto anal mucosal swab
SMAC	Sorbitol MacConkey
SNP	Single-nucleotide polymorphism
SVA	Swedish National Veterinary Institute
VTEC	Verotoxin-producing *Escherichia coli* serotype
vtx	verotoxin
wgs	Whole genome sequencing
WQ	Welfare Quality

1 Introduction

1.1 A story[1]

For children the opportunity to come out to the countryside and visit a dairy farm is a great learning experience. Imagine a group of children, perhaps a pre-school class, visiting a dairy farm and the farmer proudly showing them around. The children are excited to learn about how milk and meat are produced and the highlight of the visit is meeting the animals, particularly petting the cute calves. The day is a success and after the visit the farmer is approached, in the local supermarket and other public places, by grateful parents describing the children's joy after the visit. But suddenly, just a few days later, the tune changes drastically. People suddenly avoid the farmer at the supermarket and other planned farm visits are cancelled. The farmer hears that some of the visiting children have become terribly sick, some are even hospitalised and in a critical condition. The doctors are saying that they have caught a bacteria called "EHEC" from the animals. A wave of guilt and worry washes over the farmer. Have the children really become sick because of the farm visit? Does this mean that children in the household and the staff are in danger? There is also fear of what will happen with the animals now that public health agencies want to investigate the farm. The animals are perfectly healthy and high-producing! In fact, nothing has changed on the farm and plenty of previous visiting groups have passed through without anyone getting sick before. Can it really be the farm animals? If so, how did it go wrong this time? Where did the bacteria come from? And most importantly, how do you deal with a problem when there are no symptoms?

[1] This story is purely fictional but inspired from meetings with farmers experiencing the introduction of a highly virulent verotoxin-producing *E. coli* in their area or on their farms.

1.2 The beginning of this story

Although the farmer in the previous story is fictional, the zoonotic pathogen verotoxin-producing *Escherichia coli* serotype O157:H7 (VTEC O157:H7) is often found on farms in relation to outbreaks of disease among humans, often involving children. During the last 40 years the pathogen has emerged as an important risk to public health due to the severe disease and the high risk of life-threatening complications. In Sweden, the number of cases of gastrointestinal disease in humans due to VTEC O157:H7 attributed to the cattle population remains high despite national efforts to control transmission. Despite extensive research and many scientific publications, important gaps in our understanding, and therefore our ability to implement an efficient control program, remain. Within this project a multidisciplinary approach, combining epidemiology, microbiology, ethology and bioinformatics, is used to fill some of these gaps. We also combine studies of different levels, i.e. between farms, within farm as well as between and within animal, to create a comprehensive picture of pathogen dynamics on Swedish farms. Our approach enables new perspectives, for example on the role of animal behaviour in disease transmission and exposure to the pathogen, but also supports and increases the confidence in previously suggested risk factors and theories. But let us start from the beginning.

1.3 Verotoxin-producing *E. coli* O157:H7

The nomenclature used to describe verotoxin-producing *Escherichia coli* (VTEC) and disease caused by it can easily confuse anyone. For example, the acronyms VTEC, EHEC and STEC are used frequently to describe the same pathogen and an understanding of the history, and relationship between VTECs and other *E. coli* is required to understand the differences between the acronyms. As the name suggests, VTEC of serotype O157:H7 is an *E. coli* that have acquired the ability to produce a particular toxin that is interchangeably called verotoxin or shigatoxin (more about this below). As all *E. coli,* it is a gram-negative, rod-shaped, facultatively anaerobeic bacterium belonging to the family *Enterobacteriaceae* (Gally & Stevens 2017). It is distinguished as serotype O157:H7 by diversity of the O-antigens (part of a lipopolysaccharide in the outer membrane) and the H-antigens (flagellar proteins), a method used to differentiate between different types of *E. coli* since the 1940s (Kauffmann 1947; Orskov *et al.* 1977). It is an important member of the group enterohemoragic *E. coli* (EHEC), a group of pathogenic *E. coli* able to cause bloody diarrhea and severe complications (Kaper & Nataro 1998).

1.3.1 The rise of VTEC

In 1982, two unusual outbreaks of gastrointestinal disease characterised by severe abdominal pain, bloody diarrhoea and little or no fever occurred in the states of Oregon and Michigan in the United States. At least 47 people became ill and epidemiological investigations revealed that the illness was associated with eating hamburgers at restaurants belonging to the same fast-food chain (Riley et al. 1983). The ill persons were infected with a rare type of *E. coli* of serotype O157:H7, which did not behave as previously recognised enteropathogenic *E. coli* (EPEC), and it was suggested that a yet unknown type of enterotoxin may have caused the serious illness (Riley et al. 1983).

Riley *et al.* (1983) were indeed correct about an enterotoxin causing the disease but it was not completely unknown at the time of the outbreak. In fact, two research groups, working in parallel, had already come across it. In 1977, Konowalchuk *et al.* found that a group of EPEC produced an unknown toxin with the ability to kill vero cells. Due to this ability, it was named verotoxin. Around the same time, O'Brien and LaVeck (1983) also identified a new toxin produced by an EPEC (of serotype O26). They found that this toxin was very similar to the toxin produced by the bacteria *Shigella dysenteriae* and therefore this toxin was called shiga-like toxin (O'Brien & LaVeck 1983). After the outbreak in 1982 it became clear that these toxins were the same and that the same toxin was produced by the *E. coli* causing the outbreak (Johnson *et al.* 1983; O'Brien *et al.* 1983). However, both names are still being used interchangeably in literature today and agreement on which would be most appropriate to use has caused debate (Calderwood *et al.* 1996; Karmali *et al.* 1996). In Sweden, the term verotoxin has been traditionally used within the veterinary field while shigatoxin has been the preferred term in human medicine. To keep to tradition the term verotoxin (vtx) will therefore be used in this thesis.

After the outbreaks of severe disease in Oregon and Michigan, the importance of VTEC was further acknowledged when Karmali *et al.* (1983) linked verotoxin to the severe complication haemolytic uremic syndrome (HUS), a syndrome characterised by trombocytopenia, hemolytic anemia and kidney failure. Since then the history of outbreaks, disease and public health costs has only enhanced the importance of VTEC worldwide and prompted a large research interest (Kaper & O'Brien 2014). Thus, our knowledge and understanding of the group of VTEC has increased substantially since the outbreak in 1982, but many questions remain.

1.3.2 The origin of VTEC O157 and non-VTEC O157

As O'Brien and LaVeck (1983) observed, serotype O157:H7 is just one of multiple serotypes of *E. coli* with the ability to produce verotoxin. However, when Karmali *et al.* (2003b) classified VTECs into five seropathotypes, based on incidence, involvement in outbreaks and association with severe disease serogroup O157 stood out. Due to the high incidence and common occurrence in outbreaks serotypes O157:H7 and O157:NM were the only serotypes classified as seropathotype A (the most important/severe). Phylogenetic analysis has also suggested that VTEC O157:H7 stands out from other verotoxin-producing serotypes (often referred to as non-O157) (Whittam 1998; Hazen *et al.* 2013). The serotype O157:H7 and its inferred ancestor O155:H7 is categorised as EHEC1, a group of relatively closely related strains that separated from a common ancestor as long as 4.5 million years ago (Reid *et al.* 2000). The group EHEC2 contains other serotypes able to cause disease (like O26, O103) and these are less closely related (Abu-Ali *et al.* 2009). Instead, it appears that this group has acquired their virulence factors in different ways and at different time points (Reid *et al.* 2000).

Horizontal gene transfer allows distantly related *E. coli* to exchange genes (through plasmids, phages and pathogenicity islands) between each other driving adaptation to new environmental challenges (reviewed by Lawrence 2002 and Dobrindt 2005). Genes frequently exchanged between bacteria are part of the accessory genes, while stable genes (within family, species or subtypes) make up the core genome. The genome of *E. coli* contains between 4200-5500 genes and of these ~2000 genes are core genes, i.e conserved among all strains (Rasko *et al.* 2008; Touchon *et al.* 2009; Kaas *et al.* 2012). Thus, the variable, i.e. the accessory, genome makes up more than half of the genome. Variation in this part of the genome is huge since these genes come from genepool of more than 18 000 genes (Touchon *et al.* 2009; Kaas *et al.* 2012). An example of the importance of this type of evolution is the large German outbreak where an enteroaggregative *E. coli* of serotype O104:H4 was able to cause severe disease and HUS by acquiring the ability to produce verotoxin type 2 (as reviewed by Denamur 2011).

1.3.3 Within serotype variation

The flexible and adaptive capabilities of *E. coli* also means that there can be significant variation also within serotypes. For example strains can carry genes coding for different types of verotoxins (Scheutz *et al.* 2012). Toxins are grouped into two branches; verotoxin type 1 (vtx1) and type 2 (vtx2) (Scheutz *et al.* 2012). Vtx1 is very similar to the toxin produced by *S. dysenteriae* and is

generally associated with milder disease and fewer cases of the complication HUS than vtx2, although there are exceptions (EFSA 2013). Some strains produce both vtx1 and vtx2 and there are also subcategories within vtx1 and vtx2 (also associated with differences in virulence) that appear in different combinations (Scheutz 2014).

Virulence is, however, complicated and producing virulent verotoxins is not enough if other important virulence mechanisms are lacking. For example, missing the locus of enterocyte attachment (LEE), which enables attachment to host cells, may lead to inability to attach to cells and cause disease (McDaniel et al. 1995). Hence, not all VTECs fulfil the criteria for being defined as an EHEC (able to cause enterohaemoragic disease in humans). There are also examples of strains that have caused disease without LEE (Kaper *et al.* 2004) which emphasises the importance of genome flexibility and the pathogens potential to find new ways of causing disease.

Analysis of Dutch clinical isolates of varying serotypes has shown that toxin-type does not cluster with core genome, indicating that vtx production was highly influenced by horizontal gene transfer (Ferdous *et al.* 2016). Reid *et al.* (2000) observed the same pattern for vtx while the virulence factor LEE did cluster with the core genome. Hence, there appears to be differences in the importance of horizontal gene transfer and the role of common ancestors for acquisition of different genes (Gordienko *et al.* 2013).

Variation within O157:H7

An overview of the most well recognised virulence factors and localisation in the genome of VTEC O157:H7 are presented in Figure 1. However, as in other serotypes there is variation also within serotype O157:H7. By developing a single-nucleotide polymorphism (SNP) typing system, Manning and colleagues (2008) were able to distribute 519 VTEC O157:H7 strains into nine evolutionary clades with different associations with human disease. Although many clades were associated with human outbreaks, for example clade 3 was behind the first outbreak in Michigan and Oregon, one stood out with higher rates of hospitalisation and frequency of the complication HUS; the clade 8 lineage. Analysis of isolates from human cases infected in Sweden, collected between 2008-2011, has similarly shown that a high proportion of the persons had been infected with clade 8 and that 10 out of 11 cases of HUS were caused by clade 8 (Söderlund *et al.* 2014). The association between clade 8 and severe disease has been suggested to be due to overexpression of vtx2 (Neupane *et al.* 2011). Studies suggest that acquisition and loss of virulence genes is highly dynamic within VTEC O157:H7, leading to development and regression of pathogenic strains (Kyle *et al.* 2012; Dallman *et al.* 2015; Byrne *et al.* 2018).

1.3.4 Classification, identification and characterisation of VTEC O157:H7

Much of the recently gained understanding of the importance of within and between serotype variation has been possible thanks to more advanced analytical methods. There are now multiple options for detection and characterisation depending on which depth of information is desired. Generally, methods of analysis can be divided into culture-based methods, immunological methods and molecular methods.

Detection/culture based methods

Culture of bacteria has been historically important and remains the gold standard for establishing presence of viable VTEC O157:H7 in a sample. VTEC O157:H7 will, like other *E. coli*, grow on ordinary blood agar but when the goal is to detect O157:H7, agars that use this strains unique biochemical properties (inability to ferment sorbitol or produce β-glucuronidase) are helpful (Ojeda *et al.* 1995; Kaper & Nataro 1998).

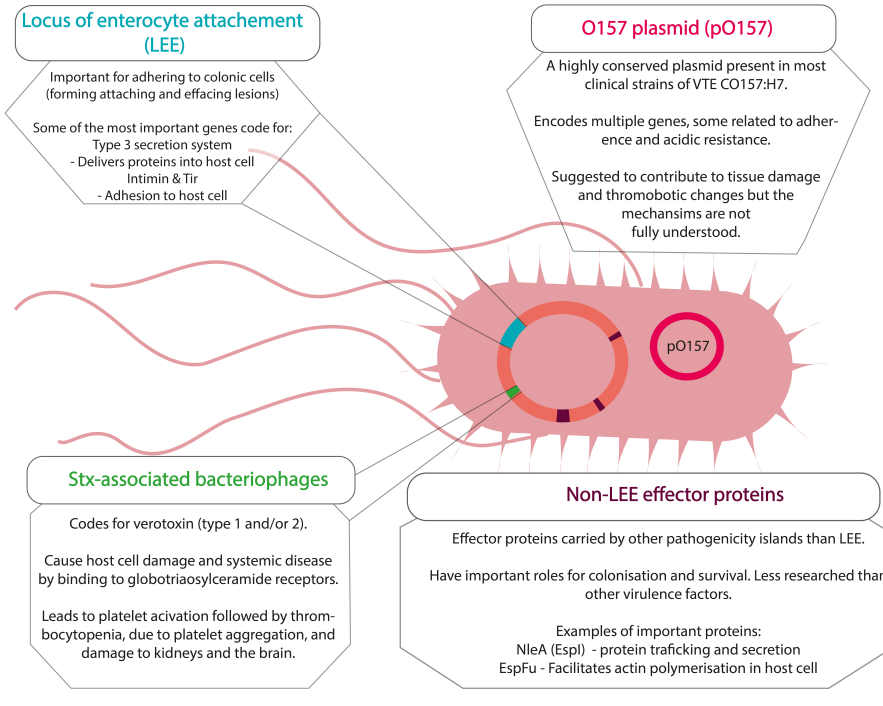

Figure 1. Important virulence factors of verotoxin-producing *Escherichia coli* O157:H7 and their localisation in the bacterial genome. Source: Croxen & Finlay 2010; Mellies & Lorenzen 2014; Gally & Stevens 2017. Illustration: Lena-Mari Tamminen.

One of the most common is Sorbitol MacConkey (SMAC) agar. This agar is selective for the family *Enterobacteriacae* and the non-sorbitol fermenting colonies of VTEC O157:H7 are distinguishable by their lack of colour compared to other sorbitol fermenting *E. coli*. However, strains of VTEC O157:H7 able to ferment sorbitol have been identified and there are VTECs of other serotypes than O157:H7 that are not able to ferment sorbitol (Gunzer *et al.* 1992; Schmidt *et al.* 1999). Adding cefixime and tellurite (CT-SMAC) inhibits growth of non-vtx producing *E. coli* and increases rate of isolation of VTEC O157:H7 from cattle, but there are also indications that it may inhibit the growth of some strains of VTEC O157 (Zadik *et al.* 1993; Karch *et al.* 2005). Similarly, novobicin can increase selection of O157:H7 (Okrend *et al.* 1990).

Another alternative is to use a chromogenic agar (like CHROMagar or rainbow agar). This agar uses the inability of *E. coli* O157 to produce β-glucuronidase. VTECs, including VTEC O157:H7, and non-vtx producing *E. coli* can be differentiated by colour (vtx-positive isolates are mauve-coloured) (Kaper & Nataro 1998; Hirvonen *et al.* 2012). However, sorbitol fermenting strains of VTEC O157:H7 may not grow on this type of agar either (Hirvonen *et al.* 2012). As there are no completely selective agars for O157:H7 it is recommended that further confirmation of suspected colonies is carried out after culture (Kaper & Nataro 1998).

Phage typing is a culture-based method that has been extensively used to subtype VTEC O157:H7. Phage type (PT) of a strain is determined by culturing the strain on an agar diffused with different lytic bacteriophages to produce a susceptibility profile (Van der Merwe *et al.* 2014). Different PTs have been associated with different virulence in VTEC O157:H7 (Lynn *et al.* 2005; Mora *et al.* 2007).

Immunological methods

There is a variety of immunological methods that can be used in different steps of analysis of VTEC O157:H7. For example, sensitivity in culture-based methods can be further increased by using immunomagnetic separation. Antibody coated paramagnetic beads are used to bind, pick up and separate O157:H7 from other bacteria in a sample before plating on for example SMAC agar (Karch *et al.* 1996). This concentrates the O157:H7 in the sample and reduces the risk of other bacteria present in the sample outcompeting them.

For confirmation of cultured colonies multiple assays and commercial kits are available, ranging from ELISAs, latex reagents and labelled antibodies, that detect surface proteins (mainly O and H antigen) but also vero-toxins (Kaper & Nataro 1998). Some of the ELISAs can be used directly on fecal samples and thereby saves time (Dylla *et al.* 1995; Park *et al.* 1996). The downside is that

there is a risk for cross-reactivity with other closely related bacteria, like *Citrobacter freundii, Escherichia hermanni* and *Salmonella Urbana* (Park *et al.* 1996). Bacterial vtx-production may on the other hand be influenced by for example culture conditions (Boone *et al.* 2016).

Molecular methods for characterisation and subtyping

Molecular methods analysing bacterial DNA are, with decreasing prices and increased availability, becoming the standard for detection and characterisation of VTEC within research, diagnostics and outbreak situations (Newell & La Ragione 2018). Already they have provided us with the increased understanding of the variation within serotypes and virulence mechanisms described above as well as possibilities for detailed investigation of outbreaks as well as bacterial phylogeny (Eppinger & Cebula 2015; Land *et al.* 2015). Although a detailed description of these methods is beyond the scope of this thesis, an overview of some of the common molecular methods and level of detail they provide is presented in Figure 2.

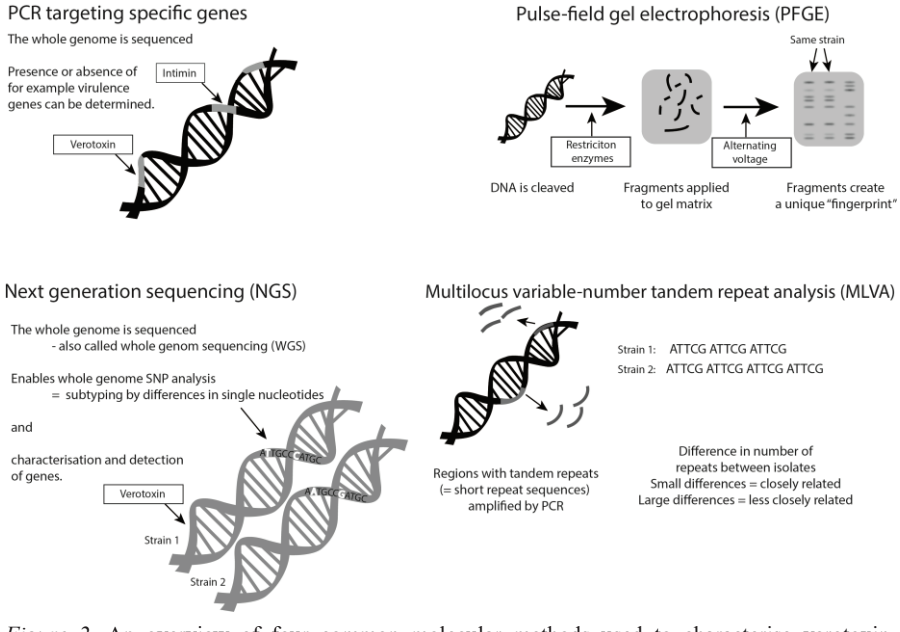

Figure 2. An overview of four common molecular methods used to characterise verotoxin-producing *Escherichia coli* O157:H7. Source: Söderlund (2015). Illustration: Lena-Mari Tamminen.

1.4 VTEC and human disease

VTEC has been estimated to cause 2 801 000 acute illnesses per year worldwide but the estimated incidence between regions varied significantly (1.4 to 152 cases per 100 000 inhabitants) (Majowicz *et al.* 2014). The subregions (as defined by the World Health Organization) with highest estimated incidence were EMR B and EMR D[2]. Countries that have reported a high incidence, i.e. cases per 100 000 inhabitants, in 2018 were for example New Zealand (18.9) (ESR 2020) and Ireland (20.0) (ECDC 2020). The Swedish incidence of VTEC during 2018 was 8.7, the third highest European notification rate reported to the European Centre for Disease Control (ECDC). This continues the increasing trend in incidence of cases that has been observed since 2006 (Folkhälsomyndigheten 2020). In addition, 40 cases of HUS (the highest number of annual cases reported so far) were reported in 2018 and half of these occurred in children less than 10 years of age (Folkhälsomyndigheten 2020). A study has estimated that the economic burden (sum of direct and indirect costs) of VTEC in Sweden during 2006 was 1.3 million euros for VTEC. Compared to the cost of *Campylobacter*, a more common cause of gastrointestinal disease estimated to cost 26.1 million euros, this is relatively small. However, although the total public health burden is larger for *Campylobacter*, the burden per case, i.e. the consequences for an individual infected as well as the cost per case, is much higher for VTEC due to the potentially severe consequences of infection (Toljander *et al.* 2012).

Although other serotypes can cause disease, VTEC O157:H7 is the serotype most commonly associated with human disease and was the most commonly reported serotype in Sweden during 2018 (EFSA 2013; Folkhälsomyndigheten 2020). In a Swedish study of VTEC in Jönköping county, O157:H7 was found to be the dominating serotype causing bloody diarrhoea (Bai *et al.* 2018) and the subtype clade 8 has been identified as the major cause of cases with the complication HUS (Söderlund *et al.* 2014). In addition to sporadic cases, often associated with farm visits or contact with cattle faeces, clade 8 has caused large national outbreaks with high proportion of infected persons developing HUS (Table 1).

[2]. EMR B: Bahrain, Cyprus, Iran (Islamic Republic of), Jordan, Kuwait, Lebanon, Libyan Arab Jamahiriya, Oman, Qatar, Saudi Arabia, Syrian Arab Republic, Tunisia, United Arab Emirates; EMRD: Afghanistan, Djibouti, Egypt, Iraq, Morocco, Pakistan, Somalia, Sudan, Yemen.

Table 1. *Overview of larger outbreaks of verotoxin-producing* Escherichia coli *in Sweden during the course of this project. Source: Folkhälsomyndigheten (2020).*

Year	Number of cases	HUS cases	Type of VTEC	Source
2018 (July-September)	116	14 (12 %)	O157:H7 Clade 8 (*stx2a, stx2c, eae*)	Unknown/Foodborne*
2016 – 2017 (September- February)	26	6 (23%)	O157:H7 Clade 8 (*stx2a, stx2c, eae*)	Meat (Cattle)
2016 (July-September)	8	3 (38%)	O157:H7 Clade 8 (*stx2a, stx2c, eae*)	Farm contact (Cattle)
2015-2016 (November –May)	70	0	O103:H2 (*stx1, eae*)	Unknown/Foodborne
2015-2016 (September-April)	57	0	O26:H11 (*stx1a, eae*)	Unkown/Foodborne

*Outbreak also included person to person spread by e.g. recreational swimming

1.4.1 Pathogenesis in human disease

Only a small number of virulent VTEC is a sufficient infectious dose for disease in humans (Griffin & Tauxe 1991; Newell & La Ragione 2018) and post-outbreak calculation has suggested that even less than 50 bacteria may be enough (Tilden *et al.* 1996). Symptoms vary between no signs of infection, abdominal pain, mild or bloody diarrhoea to the severe complication HUS, a potentially fatal syndrome including trombocytopenia, hemolytic anemia and kidney failure (Karmali *et al.* 1983; Tarr *et al.* 2005).

While anyone can be infected by VTEC O157:H7 it is generally, as in the introductory story, children and elderly that are most susceptible to the complication HUS (Gould *et al.* 2009). The serious complication is a result of the verotoxins entering the bloodstream and binding to the Gb3 receptor on the surface of host cells, for example on platelets. This leads to cell damage, secretion inflammatory chemokines and cytokines as release of thrombin which activates thrombosis (Karpman & Ståhl 2014). In the kidney this causes glomerular cell damage and in some cases the toxin can cause severe neurologic dysfunction (Karpman & Ståhl 2014). A summarized description of the pathogenesis, course of infection and treatment options is presented in Figure 3.

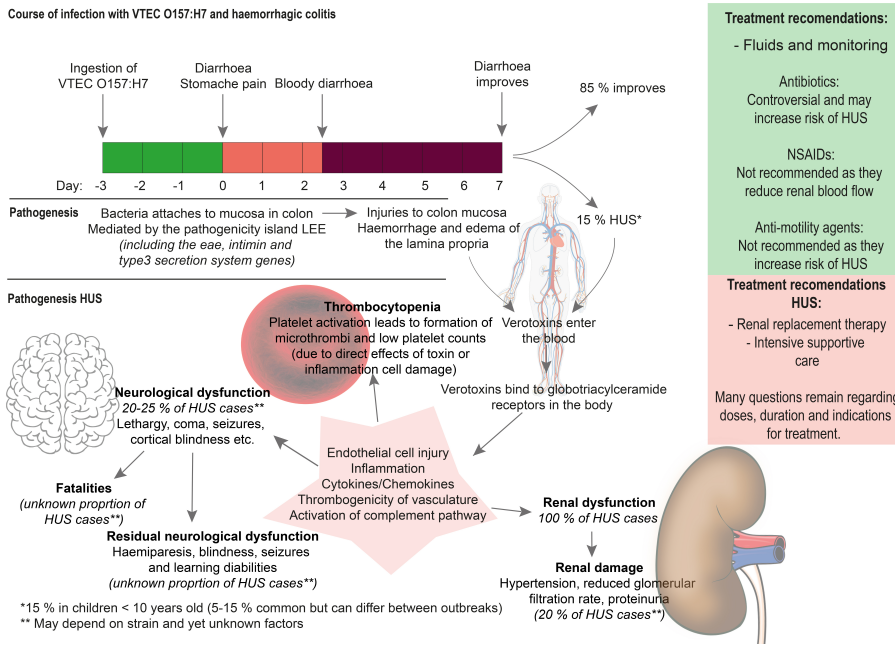

Figure 3. Infection of verotoxin-producing *Escherichia coli* O157:H7 in humans. Modified from Karpman & Ståhl (2014), Tarr (2005) and Trachtman et al. (2012). Illustration: Lena-Mari Tamminen.

1.4.2 The link between cattle and human disease

Cattle are considered the main reservoir of VTEC O157:H7 and a pattern where isolates from cattle are closely related to clinical human isolates has been observed in many studies (Zhang *et al.* 2007; Bono *et al.* 2012; Franz *et al.* 2012; Jung *et al.* 2013; Strachan *et al.* 2015; Arimizu *et al.* 2019). Phylogeographic analysis of bovine and human isolates from four continents suggests that the common ancestor of O157:H7 arose in the Netherlands around 1980 and was then spread around the world in a pattern that fits very well with trade routes of cattle (Franz *et al.* 2019). According to the analysis by Franz *et al*. (2019), VTEC O157:H7 from Europe was introduced in Sweden in 1982 and that the virulent clade 8 type was introduced from the United States around 1990. This corresponds relatively well with the first human case of VTEC O157:H7 in Sweden, which occurred in 1988. The first case was followed by a small number of sporadic cases until the first large outbreak occurred in 1995 (Ziese *et al.* 1996).

There is also a relationship between cattle density and cases of VTEC O157:H7 in humans, and a higher risk of HUS in animal contact related

outbreaks has been observed in the United States (Kistemann *et al.* 2004; Frank *et al.* 2008; Heiman *et al.* 2015). In addition to the examples presented in table 1, several large Swedish outbreaks have been associated with the Swedish cattle population. For example, the so far largest outbreak of VTEC O157:H7 (135 cases, 11 HUS) was caused by lettuce contaminated by cattle pasturing upstream of the water irrigation point and in 2002 an outbreak with a very high incidence of HUS (12/39 cases) was cause by contaminated beef sausages (Sartz *et al.* 2008; Söderström *et al.* 2008). The role of controlling VTEC O157:H7 in cattle for preventing human cases is emphasized in the national strategy for prevention of EHEC signed by the Swedish Public Health Agency, the Board of Agriculture, the National Food Agency, SVA, as well as the National Board of Health and Welfare (Socialstyrelsen, 2014).

However, not all variants of O157:H7 present in the cattle population appear to cause problems. Söderlund *et al.* (2014) observed more variation in isolates from the cattle population compared to clinical cases. Other studies have also observed lineages from cattle that do not appear to be associated with human disease (Zhang *et al.* 2007; Bono *et al.* 2012; Franz *et al.* 2012). Analysis of genomes of bovine and human isolates of O157:H7 has indicated that only 10 % of bovine isolates have zoonotic potential (Lupolova *et al.* 2016).

There are also indications that exposure to cattle may have a protective effect against disease caused by VTEC O157:H7. For example, increased immunity and less clinical disease in farm resident children compared to non-farm resident children has been observed in the United States (Belongia *et al.* 2003). A larger proportion of rural populations have been shown to have antibodies against vtx compared to urban populations but it is unknown to which extent these antibodies represent exposure to pathogenic O157:H7 (Haack *et al.* 2003; Karmali *et al.* 2003a). Haack *et al.* (2003) argued that the strain must be pathogenic to evoke an antibody response, but considering the variation of virulence within the VTEC it is possible that mild infections may induce antibody protection that reduces the risk of severe infection. Indeed less pathogenic lineages carrying vtx2c have been identified from both cattle and healthy people (Kawano *et al.* 2012).

1.4.3 Transmission of VTEC O157:H7

From being considered a food borne pathogen, first associated with undercooked meat, many pathways of transmission for VTEC O157:H7 have been recognised (Figure 4). Large outbreaks are often foodborne and associated with cattle products, like meat and unpasteurized milk, or contaminated vegetables, such as salad or spinach (Michino *et al.* 1999; Howie *et al.* 2003; Grant *et al.* 2008;

Heiman *et al.* 2015). Sporadic cases often occur through contact with shedding animals, an environment where animals have been or contact with cattle faeces in other ways (Locking *et al.* 2001; Crump *et al.* 2002). A recent meta-analysis focusing on sporadic cases identified raw/undercooked meat (population attributable fraction 19%), person to person spread (15%), contact with animals (14%) and visiting farms (12%) as the most important routes (Kintz *et al.* 2017). Although food borne outbreaks tend to be dramatic and involve many cases, the risk visiting a pasture has been estimated to be associated with a 100 times greater risk compared to eating a burger (Strachan *et al.* 2006). Spread through vegetables and green leafs have also been highlighted to be a particularly important public health risk as these products are often consumed raw which increases the number of live bacteria ingested (Griffin & Karmali 2017). Tarr *et al.* (2018) found that some lineages of VTEC were more likely to spread through raw milk while other types were more often associated with transmission through vegetables and fruits. These differences may reflect bacterial ability to survive in different types of environment and changes in the bacterial population may lead to changes in the routes of transmission.

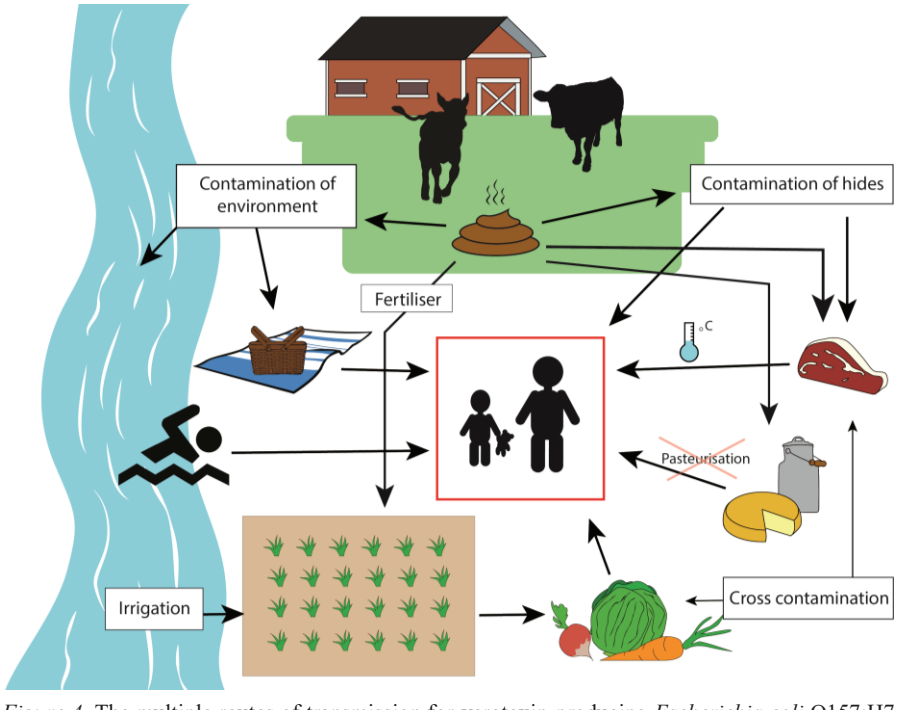

Figure 4. The multiple routes of transmission for verotoxin-producing *Escherichia coli* O157:H7 between cattle and humans include contaminated products as well as indirect transmission through the environment or direct contact with cattle. Modified from Chapman et al. (2018). Illustration: Lena-Mari Tamminen.

1.5 VTEC O157:H7 in the cattle population

As described in the pathogenesis section (p 24) vtx binding to Gb3 receptors are responsible for causing symptoms in humans (Schüller *et al.* 2007). Cattle have been suggested to have a different distribution of Gb3 receptors than humans (Pruimboom-Brees *et al.* 2000) which may be the reason why colonisation rarely causes symptoms in cattle (Kolenda *et al.* 2015). However, young calves infected with high doses of VTEC O157:H7 can develop diarrhoea and colonisation of the intestine induces a local inflammation and damage to intestinal cells (Dean-Nystrom *et al.* 1997; Nart *et al.* 2008). Thus, the bacteria should not be considered a commensal part of the microbiota of cattle.

Prevalence of VTEC O157:H7 among Swedish cattle has been investigated several times by collection of faecal samples from cattle at slaughterhouses in different regions. The first study, performed between 1996 and1997, found that 1.2 % of the slaughtered cattle were positive for the pathogen (Albihn *et al.* 2003). In samples collected between 1998 to 2000 a higher proportion, 8.3 % of the sampled cattle were positive (Eriksson *et al.* 2005). Marked regional differences were observed, especially the county of Halland stood out with a prevalence of 23.3% combined with the highest incidence of human cases. A following longitudinal study, comparing farms in four regions of Sweden between 2009 and 2013, found regional differences in prevalence of clade 8 (only found in Falköping and Halland) (Widgren *et al.* 2015). Prevalence of positive faecal samples at slaughter since 2000 has varied between 2.2-3.5%. In the 2014-2015 and the 2017-2018 sampling, strains belonging to clade 8 were only found in samples from the counties Öland and Skåne, indicating that the virulent strain had moved to a new region (Erik Eriksson, Swedish National Veterinary Institute).

1.5.1 Farm dynamics

Between farm transmission over large distances appear to be driven by trade of cattle and, just as VTEC O157:H7 spread across the world through common cattle trade routes, modelling suggests that it spread along trade routes within Sweden (Widgren *et al.* 2016; Franz *et al.* 2019). In addition, purchase/introduction of new animals has been identified as a risk factor for establishing the pathogen on farms in Sweden and other countries (Schouten *et al.* 2004; Herbert *et al.* 2014; Widgren *et al.* 2015). However, there are also signs of local transmission, as infected neighbouring farms increases the risk of a farm being positive and nearby farms often share related strains (Zhang *et al.* 2010; Herbert *et al.* 2014; Widgren *et al.* 2015).

Prevalence of VTEC O157:H7 among cattle often follows a seasonal pattern. In Sweden a peak during summer and fall (July-November) has been observed in faecal sampling at slaughterhouses (Albihn *et al.* 2003). This corresponds well to the seasonal pattern observed in Scotland and the Netherlands which are countries with comparable climate to Sweden (Schouten *et al.* 2004; Gunn *et al.* 2007; Smith *et al.* 2016; Henry *et al.* 2019). Studies performed in the United States and Australia have identified that climate related variables, such as temperature, relative maximum soil temperature, wind speed, humidity and rain also influences shedding and prevalence of VTEC O157:H7 (Williams *et al.* 2014; Benjamin *et al.* 2015; Lammers *et al.* 2015)

Although a majority of farms appear to clear infection in 4-6 months after introduction, there are many examples of farms that remain positive for VTEC O157:H7 over long periods (Hancock et al., 1997; Herbert et al., 2014; Rice et al., 1999; Widgren et al., 2015). Strains of VTEC O157:H7 that are more likely to cause human disease also appear more likely to persist on farms (Carlson *et al.* 2009; Herbert *et al.* 2014). Dairy farms have been observed to have a higher prevalence and remain positive for a longer time period compared to beef farms (Cobbaut *et al.* 2009; Widgren *et al.* 2015; Smith *et al.* 2016).

Larger herds (total number of animals) also appear to be associated with risk of infection according to several studies (Eriksson *et al.* 2005; Herbert *et al.* 2014; Benjamin *et al.* 2015). However, there are some studies that have shown the opposite or no effect (Wilson *et al.* 1993; Cobbaut *et al.* 2009; Cho *et al.* 2013). Other examples of herd level risk factors that have been suggested are wild bird density (starlings and geese), presence of pig on the farm and spreading slurry on grazing lands (Synge *et al.* 2003; Eriksson *et al.* 2005; Gunn *et al.* 2007; Cernicchiaro *et al.* 2012).

On infected farms, VTEC is often found among younger animals (Eriksson *et al.* 2005; Kuhnert *et al.* 2005). Contacts between adult animals and calves as well as other groups of animals on farm is associated with increased risk and keeping groups of animals together has been suggested to be the most cost effective on-farm measure to reduce prevalence of the pathogen (Ellis-Iversen *et al.* 2008; Cernicchiaro *et al.* 2012; Lyons *et al.* 2013). Other common on-farm measures suggested to reduce prevalence of VTEC O157:H7 on infected farms include improving hygiene, like maintaining dry bedding and reducing faecal contamination of bedding and water troughs (Lejeune & Wetzel 2007; Ellis-Iversen *et al.* 2008; Tamminen *et al.* 2018). A major cleaning of the barn has been shown to decrease vtx2 found in milk filters on Finnish dairy farms (Jaakkonen *et al.* 2019). However, it should also be noted that washing may also spread the pathogen among animals inside the barn, for example flushing

alleyways with water has been associated with an increased risk (Garber *et al.* 1999).

Other interventions to reduce presence of VTEC O157:H7 on dairy farms include changing dietary practices, adding feed additives (e.g. probiotics), phage therapy and vaccination, but so far their impact remain limited (Besser *et al.* 2014).

1.5.2 Colonisation and shedding

As in humans VTEC O157:H7 colonises the large intestine of cattle. More specifically the lymphoid dense tissue of the rectoanal-junction (Naylor *et al.* 2003). Thus, the bacteria has to survive through the passage of the gastrointestinal tract, including the rumen, as well as compete with members of the microbiota during passage and at the colonisation site (reviewed by Ducarmon *et al.* 2019). This process is highly dependent on cues from the host and other members of the microbiota as recently reviewed by (Pifer & Sperandio 2014).

Colonisation of the terminal rectum is associated with increased shedding levels (Low *et al.* 2005; Davis *et al.* 2006) and colonised animals have been suggested to be responsible for shedding a large proportion of all VTEC O157:H7 shed into the environment. Both mathematical modelling of Scottish data and a study of fecal shedding at slaughter have suggested that a small proportion of colonised animals (<10 %) are responsible for 95-99% of all VTEC O157:H7 shedd into the environment (Omisakin & MacRae 2003; Matthews *et al.* 2006b). Shedding levels higher than 10^3 and 10^4 colony forming units (cfu)/gram feces have been suggested to indicate “super-shedding” due to colonisation (reviewed by Chase-Topping *et al.* 2008). The high number of bacteria shed by super-shedders increases the risk of transmission of VTEC O157:H7 to humans by increasing hide and carcass contamination of groups of animals (Cobbold *et al.* 2007; Stephens *et al.* 2009). These high shedders have also been suggested to drive transmission to other animals in the pen which leads to new animals becoming colonised and keeping the pathogen circulating within farms (Matthews *et al.* 2006a; Cobbold *et al.* 2007; Spencer *et al.* 2015; Widgren *et al.* 2018).

In addition, colonisation has been associated with an increased duration of shedding (Rice *et al.* 2003; Cobbold *et al.* 2007; Lim *et al.* 2007). However, longitudinal studies with more frequent sampling have reported intermittent shedding, with daily variation, of colonised animals (Robinson *et al.* 2004; Lammers *et al.* 2016). A proposed reason for the intermittent shedding is that colonisation is associated with formation of biofilm, which, when it has become

large enough, is released in chunks - so called biofilm sloughing (reviewed by Munns *et al.* 2015). This would explain how cattle could shed high levels of the bacteria on one sampling whereas later the same day it is not detected at all. However, due to the short duration of super-shedding observed in some studies, the role of colonised and super-shedding individuals in pathogen transmission and persistence has been questioned (Munns *et al.* 2014; Williams *et al.* 2015).

It has been suggested that strains that are more virulent to humans are also better equipped to colonise cattle intestines and are shed at higher levels (Chase-Topping *et al.* 2007; Carlson *et al.* 2009).

1.5.3 Individual heterogeneity and similarity

Environmental exposure of VTEC O157:H7 influences the risk of colonisation and shedding in cattle and studies have reported a synchronised increase of shedding on group level, so called super-shedding events (Williams *et al.* 2014; Lammers *et al.* 2015). However, there is considerable differences in shedding levels and duration of shedding during these reported events and other studies show similar heterogeneity (Robinson *et al.* 2004; Jonsson *et al.* 2009; Sheng *et al.* 2016).

The heterogeneity between individuals exposed to the same environment and same strain of VTEC O157:H7 indicates that there are host related differences influencing colonisation (as reviewed by Munns *et al.* 2015). To some extent, variation may be related to amount of bacteria the animal is exposed to as a higher dose is associated with increased risk of colonisation (Sheng *et al.* 2016). However, even a low dose can lead to high shedding levels in some individuals (Besser *et al.* 2001). It also appear that the number of shedding periods vary. In a longitudinal study of beef cattle followed for a year from 4-6 months of age, 82 % of the animals shed only once while other animals shed longer or multiple periods (Rhades *et al.* 2019).

The colonisation process of VTEC O157:H7 is complex and dependent on signals from the host and other member of the host microbiome (Pifer & Sperandio 2014; Baümler & Sperandio 2016). Shedding and colonisation of VTEC O157:H7 has been associated with lower diversity of gut microbiota and differences in host gene expression in the terminal rectum which may explain some individual variation observed (Xu *et al.* 2014; Mir *et al.* 2016; Wang *et al.* 2017). However, considering the close interactions between microbiota, VTEC O157:H7 and enterocytes (including the immunomodulatory effects of the pathogen), it cannot be excluded that these differences are in fact a result of colonisation. Bacteriophages, i.e. viruses that infect bacteria, may also influence

shedding dynamics. Hallewell *et al.* (2014) sampled 6 super-shedders and 5 low-shedders daily during 5 weeks and found that low-shedder had higher prevalence of phages and more T4-like phages, which had strong lytic abilities against O157:H7.

Modelling suggest that both between animal variability (a small proportion of more susceptible animals) or within animal variability (all animals have potential to shed but variability arises from transmission dynamics) can generate similar patterns as observed in observational studies (Chen *et al.* 2013). If the latter is true, all animals may have potential to become colonised or super-shedders at some time or in some settings and instead of looking for the subpopulation of colonised individuals focus should be on when animals become colonised. It has also been proposed that stress hormones, like noradrenalin, may have direct effects on the pathogen, promoting colonization and shedding (as reviewed by Freestone *et al.* 2008). Increased risk of shedding has also been observed following weaning, long haul transportation and feed deprivation (Cray & Casey 1998; Rugbjerg *et al.* 2003; Bach *et al.* 2016). There are also unexplored potential explanations for heterogeneity observed between animals. Modelling has linked heterogeneity in social contacts between calves to transmission dynamics (Turner *et al.* 2008). In addition, personality and animal wellbeing has impacts on animal behaviour, and thereby how it handles changes and stress induced by for example transportation, weaning or other factors (Wiepkema *et al.* 1987; Sapolsky 1994; Lecorps *et al.* 2018; Neave *et al.* 2018).

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2 Aims and objectives

The transmission and dynamics of VTEC O157:H7 in cattle are complex and involve risk factors associated with host, pathogen and environment. The aim of this thesis is to increase the understanding of this complexity and the role of these factors in pathogen persistence, transmission and host susceptibility. The overall goal is to synthesise the complexity and identify target areas for preventive on farm measures to reduce prevalence of VTEC O157:H7.

The specific objectives were:

- To identify factors related to transmission of VTEC O157:H7 between farms
- To describe prevalence and dynamics of VTEC O157:H7 in dairy herds with known presence of the pathogen
- To identify drivers of colonization of individual animals
- Explore the hypothesis that chronic stress increases susceptibility to colonisation

3 Overview and comments on materials and methods

This section will be used to provide an overview, as well as comments and reflections on the methods used across all studies with references to the papers where applicable. Detailed descriptions of materials and methods can be found in the respective papers.

3.1 Study design

The focus of paper I was farm persistence and between farm transmission and here 80 farms on the island of Öland, a region where VTEC O157:H7 had been recently detected at the start of this project, were selected by convenience and sampled twice. Information about herd characteristics and between sampling activities were collected by postal questionnaires around the time of the second sampling. For paper II-IV individual sampling of calves from 12 dairy farms where presence VTEC O157:H7 had been established by environmental samples was performed. In paper II, transmission dynamics between two sampling occasions were evaluated in relation to pen-level risk factors, age and sex of animals. Paper III focused on the first individual sampling and animal-based indicators and behaviour to identify differences between calves colonised by VTEC O157:H7 and those not. Non-colonised individuals housed with colonised individuals were compared to control for environmental factors and explore individual differences. In paper IV results of hair cortisol samples collected from the animals were used to explore the associations between animal based indicators and stress. Before the first individual sampling, a thorough environmental sampling to identify groups of animals shedding VTEC O157:H7 was performed. The different parts of the study with references to the respective papers are outlined in Figure 5.

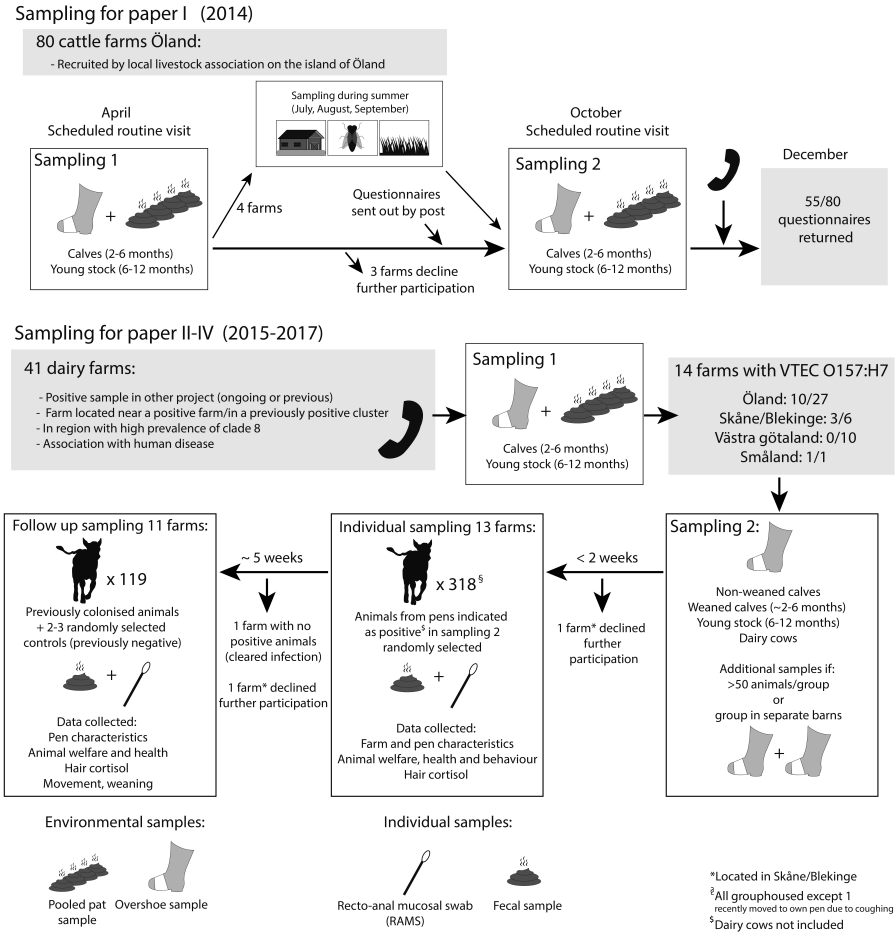

Figure 5. Overview of sampling performed within the project.

3.2 Detection and enrolment of participating farms

Identification of farms with VTEC O157:H7 is difficult, as animals do not show any symptoms of carrying the pathogen nor is there any routine sampling of the bacteria on farms, except in association with epidemiological investigations of human cases suspected to be connected to specific farms. We were also interested in focusing on strains of O157:H7 able to cause disease in humans (like clade 8) and not the less virulent strains that appear in the cattle population. As a previous study has shown that farms often clear strains of O157:H7 over time (although some farms remain positive over longer periods) (Widgren *et al.* 2015), detecting and enrolling farms with pathogenic strains was a significant challenge.

Thanks to the national slaughter prevalence study performed by the Swedish Veterinary Institute (SVA) around the start of the project we knew that clade 8 had recently been detected on the island of Öland. There was also some additional information available about clade 8 prevalence on Öland as the company Farm and Animal Health (Sv. Gård och Djurhälsan) had performed environmental sampling of 80 farms in the spring of 2014 for initiating a study on vaccine efficiency. However, soon after initiation, the vaccine trial was cancelled due to high mortality in the first vaccinated calves. At this point we were invited to collaborate and together with the Farm and Animal Health the study on transmission dynamics presented in paper I was developed.

As described in Paper I, the 80 farms in the study were recruited by the local livestock association (VÄXA). The staff visited farms across the island and combined sampling in the project with routine visits. They continued recruiting until 80 farms were reached and these farms were sampled in spring and fall 2014. The help from the local staff in encouraging farmer participation was invaluable as there was some hesitance to participate due to the risk of being associated with having VTEC O157:H7.

The close collaboration with Farm and Animal Health was also crucial for recruiting farms to the studies in paper II-IV. During the time of the project Farm and Animal Health conducted sampling of farms in the regions Öland, Skåne and Blekinge as well as of farms associated with human cases in other regions. Through these samplings, farms where the pathogen had already been identified could be enrolled in the project. To increase the number of farms in the project we also contacted farmers on Öland in areas where Farm and Animal Health was not monitoring the infection status as well as an area in Falköping where several farms positive for clade 8 had been previously identified (Widgren *et al.* 2015). Farms were continuously recruited and sampling carried out between fall 2015 and spring 2017. Farmers were first contacted by phone (after permission to share contact details had been procured by Farm and Animal Health or VÄXA) and informed about the project. If environmental sampling had not already been performed in other projects this was scheduled. To save time the initial environmental sampling was in many cases performed by staff from VÄXA according to standard protocol for identifying farms connected with human outbreaks provided by SVA.

3.3 Sampling of VTEC O157:H7

3.3.1 Sampling to identify positive farms

Environmental sampling in paper I, and the initial environmental sampling of farms in paper II-IV to establish presence of VTEC O157:H7, consisted of pooled pat samples and overshoe samples from young stock (6-12 months) and calves (<6 months). Sampling was done as previously described and validated by Widgren *et al.* (2013). In short, overshoe samples were collected by walking around the pen area with a gauze soaked with phosphate buffered saline (PBS) fitted over plastic overshoes (Figure 6). While walking around the pen a pooled pat sample was collected by sampling from 15-20 fresh faecal pats around the pen. Samples were sent to SVA by postal service.

Figure 6. Overshoe samples used in the environmental sampling.

On farms positive for VTEC O157:H7 that agreed to take part in the individual study (paper II-IV) a more thorough sampling was performed in the pens of all groups of animals to identify in which buildings/pens the pathogen was present. At minimum, separate overshoe samples from the pens of non-weaned calves, weaned calves, young stock and adult animals were taken. If any of these groups consisted of more than 50 animals housed in different pens or groups of animals

housed in separate buildings additional samples were taken. Overshoe samples were sent to SVA by postal service or kept on ice and transported by car.

3.3.2 Individual sampling

After results from the second environmental sampling were analysed, the farm was visited for sampling of individual animals. Up to 30 animals were sampled on this visit and pens from which positive environmental samples had been acquired were targeted. A sample size calculation based on results from pilot sampling on a positive farm, which indicated that the prevalence of colonised animals was 15 %, specified that minimum 21 animals should be sampled (assuming test sensitivity 0.9) to identify at least one colonized animal and 20 animals was defined as a minimum number of animals sampled in a group. However, on some farms the number of animals in pens indicated as positive by environmental sampling was smaller than 20. In these cases all animals were sampled. Sampling in larger groups was systematically randomised. On arrival the calf standing nearest to the observer was selected and then every second or third calf (depending on total number of animals in pen) was selected for sampling.

The aim was to sample animals from all pens that had been positive in the environmental sampling. However, there were practical constraints that prevented sampling from being carried out as planned on some farms. Generally animals were restrained either individually (using feeders and other structures in the pen) or in groups. If available on the farm temporary fencing panels were brought into the pens. However, on some farms the pens had no practical or safe way of restricting animal movement for sampling. This problem mostly occurred for older and larger animals housed in large groups in pens without possibility to reduce the accessible pen area. In one case sampling of younger animals could not be performed as animals managed to jump out of the pen. When it was not possible to approach and restrain animals without risk of injury to the animals and samplers, sampling in the pen was aborted/not performed.

First a fecal sample was collected from the rectum of the calf and placed in a plastic jar. Following this, a foam coated cotton swab was used to swab the recto-anal junction – approximately 2-5 cm from the rectum. The area was swabbed for 1 minute before the recto anal mucosal swab (RAMS) was put into a Falcon tube with 2.5 ml of sterile phosphate buffer. Samples were then stored in a cooler and either sent by postal service or transported in the cooler to the SVA for analysis the following day. Sampling was started in the younger groups of animals and plastic gloves were changed between each calf.

Follow up sampling

Animals that were positive for VTEC O157:H7 in the first individual sampling were resampled again after about 5 weeks. On this occasion, 2-3 controls (previously negative individuals) in the same age as the positive animals and housed with a colonised animal were also sampled in the same way as described for the first sampling.

3.3.3 Motivation for targeted sampling

The motivation for using a targeted sampling regime, both to identify positive farms as well as identify positive groups of animals was dual. The obvious reason was to improve cost efficiency and increase the probability of identifying positive farms as well as colonised and shedding animals. In addition, the idea was to enable comparison between farms and animals that were at risk of being infected (and avoid introducing noise by including farms and individuals that had not exposed to the pathogen). For Paper I this was achieved by limiting the study population to farms on Öland where the pathogen was present and transmission ongoing. Similarly, to avoid inclusion of unexposed individuals the study population in the second part of paper II and paper III was narrowed down to only include animals that were housed together with colonised animals.

3.4 Microbial analysis of VTEC O157:H7

3.4.1 Detection in environmental samples

Environmental samples (pair of overshoe samples or pooled faecal samples) were pre-enriched in modified tryptic soy broth (mTSB) supplemented with novbiocin (20 mg/ml) for 18-24 hours in 41.5 °C ± 0.5 °C before immunomagnetic separation (IMS) (Dynabeads anti-E. coli O157; Dynal/Thermo Fisher) was performed. Paramagnetic beads were then plated on CT-SMAC agar (0.05 mg/l cefixime and 2.5 mg/l of potassium tellurite) and incubated in 37 °C for 18-24 hours. Analysis started within 2 days of sampling. Due to logistic reasons, there were farms where the collection of the first environmental sampling and the more thorough sampling was performed at the same time. In these cases the additional samples were kept in 2°C while the first samples were analysed and only analysed if the first samples were positive for VTEC O157:H7.

3.4.2 Detection in individual samples

The RAMS and fecal samples collected in the study were handled differently. RAMS were vortexed for 1 minute and 20 ml of mTSB supplemented with 20 mg/l novobiocin was added to 2 ml of the sample. After pre-enrichment (for 18-24 hours in 41.5°C ± 0.5C°) IMS using paramagnetic beads (Dynabeads anti-E. coli O157; Thermo Fisher) was performed and the beads were plated on CT-SMAC agar (0.05 mg/l cefixime and 2.5 mg/l potassium tellurite) and incubated for 18-24 hours in 37°C. Confirmation on two isolates per farm was performed using latex agglutination and PCR as described above.

Fecal samples on the other hand were stored in 2°C during analysis of the RAMS (approximately 2-3 days). Samples from RAMS positive calves were analysed by direct plating and to enable quantification of shedding levels a tenfold dilution of 10 grams of feces was made and plated on CT-SMAC agar. Plates were then incubated for 18-24 hours in 37°C.

The combination of pre-enrichment and IMS for the RAMS was used to achieve a high sensitivity and reduce the risk of false negative calves (Rice *et al.* 2003; Davis *et al.* 2006). As RAMS positivity has been shown to be correlated with positive fecal samples (Rice *et al.* 2003; Greenquist *et al.* 2005; Davis *et al.* 2006) and a pilot sampling on two farms, where only RAMS positive animals were found to be shedding, it was decided to only analyse the fecal samples from RAMS positive calves. Another motivation for this decision was that we were interested in the shedding of colonised animals and not VTEC O157:H7 passing through the gastrointestinal tract without colonisation. As the interest was the actual shedding levels of the individuals, enrichment was avoided although this results in lower sensitivity (Davis *et al.* 2006).

3.4.3 Characterisation and subtyping

All samples from which non-sorbitol fermenting *E. coli* was cultured were confirmed by agglutination of 5 suspected colonies using a latex kit (DR 622; Oxoid). In paper I, positive colonies were also tested biochemically using the API 20 E system (bioMérieux) and analysed with PCR to identify the presence of genes coding for verotoxin 1 and 2 (*vtx1* and *vtx2*) and intimin (*eaeA*) (Gannon *et al.* 1997; Paton & Paton 1998). In paper II and III, two positive isolates (as determined by agglutination) from each sampling occasion were analysed with PCR to detect presence of genes coding for *O157*, *vtx1, vtx2* and *eaeA* (Nielsen & Andersen 2003; Perelle *et al.* 2004).

In paper I, additional characterisation was also performed. Belonging to clade 8 was determined by real-time PCR and multi-locus variable number tandem repeat analysis typing (MLVA) was performed as previously described by

Söderlund *et al.* (2014). Whole genome sequencing (wgs) was performed on 30 isolates of clade 8 recovered from 4 farms during 2014. DNA was extracted with DNeasy Blood & Tissue kit (Qiagen), sequencing libraries were prepared using the Nextera XT kit and the sequencing were done on the Illumina MiSeq system with 2 x 250 bp paired-end reads. Detailed description of analysis and processing of raw reads is found in paper I.

3.5 Risk factors

3.5.1 General overview

Risk factors analysed in this thesis are related to between farm, within farm as well as between and within animal dynamics. As a first step (Paper I) we explored local transmission of VTEC O157:H7 on the island of Öland. Although studies have shown that local transmission occurs (Herbert *et al.* 2014; Widgren *et al.* 2015), how it occurs remains unexplored. Öland is one of Sweden's most cattle dense regions and, considering the association between high number of cases and high cattle density (Innocent *et al.* 2005), understanding of dynamics within such an area is of public health importance.

Many studies have investigated risk factors on farm and pen level and while similar results are common for some factors there are also conflicting results as well as risk factors that are unconfirmed by additional studies. Farm, pen and management related factors are also complexly interrelated and, when all are not accounted for, correlations and confounding may affect estimates of the included variables. In paper II we included pen-level variables suggested to be important for prevalence and transmission in previous studies and analysed their impact on colonisation together to account for confounding and possible correlation. We also follow up on the role of suggested risk factors for new infections after five weeks (in pens where colonised individuals were identified) to validate the repeatability of the results.

The last part of the project (including Paper III and IV) focused on animal level determinants to address the individual heterogeneity observed in colonisation of VTEC O157:H7. As in paper II, a targeted sampling design and selection of cases and controls from the same pens was used to enable comparison of individual differences. By combining observations of animal-based welfare measures and behaviour, we explored possible drivers of transmission and host factors related to increased susceptibility or resistance (Paper III) and provided new perspectives on animal-level risk factors associated with colonisation. In addition, hair cortisol analysis was used to explore the often

proposed, but poorly established, association between stress and colonisation. Although hair cortisol has been suggested to be a promising objective way to measure stress we evaluate methodological aspects and cortisol levels in relation to welfare parameters (Paper IV) and discuss our results in relation to these findings to clarify the difficulties of making inference about animal stress.

3.5.2 Farm characteristics and management

In Paper I, coordinates representing the farm building were retrieved from national registry for productions sites (Swedish Board of Agriculture through the national database Geodata). Information about risk factors was collected through questionnaires sent to farmers by postal service around the time of the second sampling. The questionnaire was developed together with representatives from Farm and Animal Health. The first edition was reviewed by a veterinarian specialized in cattle medicine and herd health and some questions were rephrased before it was sent out. It contained mostly closed questions, with space for free comments, about farm characteristics (number of animals, type of production etc.), contacts with other farms (on pasture, co-use of agricultural machines) as well as visits to and on other farms. Documents for retrieving a small economical compensation for participating in the project was also sent together with the questionnaire with the hope that farmers would complete both and return them together. If no reply had come about a month after the documents had been sent, farmers were contacted by phone to remind them of the questionnaire and possibility to get financial compensation.

During this phone call, farmers were also asked if they needed some clarifications about the questionnaire. It appeared that farmers had not had problems answering questions but they found the questions about which other farms they or their animals were in contact with time consuming to fill in. This was due to many farms having a large number of contacts but also that they had to actively ask their neighbours about their farm identification number (Farm ID) or write their names. The issue of this also became clear when looking at the returned questionnaires. On these particular questions there were multiple occasions where one farmer had filled in contact with another farm while this farm had not mentioned the other farm. There was also farmers that had written down the names of the farmer his animals had contact with instead of farm ID. In most cases we were able to tie the names to farm ID but there were occasions when this could not be done. Thus, these questions could only be used for descriptive purposes.

On the farms included in paper II-IV a structured interview based on a questionnaire was performed to provide background information about the

farms. This included questions about number of animals on the farm, management, feeding and cleaning routines different groups of animals and farmer perception of health and welfare.

3.5.3 Pen characteristics

On the 12 farms positive for VTEC O157:H7 (included in paper II-IV), characteristics of pens indicated to be positive in the environmental sampling were collected on the day of the individual sampling. The size of the pen was measured with a laser telemeter and the number of animals in the pen counted. The number of drinkers per pen and the cleanliness of them was assessed based criteria from the Welfare Quality protocol (1 = drinker and water clean to 3 = water and drinker dirty) (Welfare Quality® 2009). The bedding material (fecal contamination and wetness) was visually assessed and type of pen was also noted. On the first visit wetness of bedding and fecal contamination were scored together in a single measure (clean: limited faeces visible, dry bedding; some dirt: faecal contamination of bedding material clearly visible and/or bedding wet in part of the pen; very dirty: faecal contamination visible and/or bedding wet in the whole pen). During the sampling of the first visits, it was noted that although bedding sometimes appeared clean and dry from a distance it could be very wet under the surface. Thus, for the follow up sampling, this measure was expanded and cleanliness and wetness assessed separately.

Initially animals per square meter was used as a measure for stocking density. However, during analysis of the data it was suspected that number of animals did not describe the stocking density very well. As animal size increases with age number of animals per square meter does not mean the same for young animals as for older animals. Instead, a stocking density measure that reflected change of weight as animals get older was calculated. First, an average number of kilograms within the pen was created by multiplying average age (in days) of calves within the pen with the average daily weight gain (estimated to 0.81 kg) and the total number of animals in the pen. This number was then divided with the area of the pen (m^2). Although this measure was not an exact measure of the kilograms within pen it represents at least a more meaningful estimation of stocking density than the first measure.

3.5.4 Individual assessment

The heterogeneity in colonisation indicates that there are host differences, either intrinsic or extrinsic that influence the susceptibility or exposure. Within the field of animal welfare, it has been well documented that individuals differ in

their behaviour as well as coping, and by using validated animal-based measures developed to assess welfare these individual differences can be studied (Broom 1986; Wiepkema *et al.* 1987; Duncan 2005; Lecorps *et al.* 2018). As welfare and stress are closely linked (although poor welfare does not necessarily mean high stress or vice versa)(Veissier & Boissy 2007), considering the association between welfare and colonisation may also add some clarity to the connection between colonisation and stress.

The protocol used for individual observations used in paper III and IV was developed based on existing protocols for assessing health and welfare in dairy cows and calves. The basis was protocols and background material developed within the Welfare Quality (WQ) project (Welfare Quality® 2009). However, due to the wide scope of the existing WQ protocol it was trimmed and simplified for easier and faster scoring. Part of this development was inspired by observing a certified assessor perform a herd health and welfare assessment within VÄXAs welfare scheme "Ask the cow" (Louise Winblad von Walter, VÄXA, personal communication). The VÄXA protocol contains similar measures but is designed to be performed faster than the WQ assessment. In addition, previous studies on dairy calf behaviour and welfare were used to select and define behaviours as well as some measures. Descriptions of the different measures and their origins are presented in Paper III, supplementary material S1). All observations were carried out by the author with support from students and on some occasions staff from VÄXA.

Performing the welfare and behaviour assessment

Individual assessment in the form of undisturbed behavioural assessment was started as soon as the animals to be sampled in the positive pens had been identified and their ID-number noted. Before the visit farmers had been asked what time they would start activity in the barn where the animals to be sampled were kept and the start of the visits were planned to coincide with this time so animals would be active (Bokkers & Koene 2001). The aim was to start observations when the farmer started work in the barn (normally feeding the calves) which was communicated to the farmer during the phone call. However, on some occasions the farmer had started feeding the calves earlier with the intention of being helpful and not being in the way of the observations. Hence, some farms were observed post-feeding. Still, low activity was rarely a problem in the groups that were fed during or before the visit. Instead low levels of activity were seen more often in groups that had continuous access to feed throughout the night and did not appear as excited about new food being provided.

Undisturbed behavioural observations were carried out for a total of 20 minutes per pen divided into 5 minute intervals. During this time the observers were careful about standing still, not making noise and standing preferably at least 1 m from the pen fence. Movement between pens was done slowly and as quietly as possible. The observed animals appeared to take notice of the persons watching them in the beginning of the first observation period but generally lost interest when nothing interesting happened. The difficult situations arose when it was not possible to stand at least 1 m away from the pen or the observer had to move closer to them to move between pens as this peaked their interest. For pens where it was not possible to see all calves all the time, the pen was observed from another location for another 20 minutes.

It is obvious that observing a pen for 20 minutes does not represent all behaviours calves perform during a day. It is also uncertain how repeatable this behaviour would be if the observations wold have been performed several days in a row. However, due to the time consuming sampling this was the maximum time available. (Visits still lasted between 5 a.m to 8 p.m on occassions). The time for observation in the WQ is 10 minutes for a segment of 25 animals but this time was increased to enable observation of more behaviours. Nevertheless, the observations should be considered as a snapshot taken on one day during an active period. The limitation in observed time should mainly be associated with a risk of type II errors (failure to observe a difference when it is there) as a lower frequency of observed behaviours decreases the power of the study. Also, if calves have very different behavioural patterns, for example individual differences in when during the day grooming behaviour is performed, this would similarly decrease the ability to differentiate between grooming and not grooming calves and make an association more difficult to identify.

3.5.5 Analysis of hair cortisol

Using cortisol to measure the activity of the hypothalamic–pituitary–adrenocortical (HPA) axis in itself is nothing new. Concentration of the hormone has been analysed in blood, saliva, faeces and urine for many years in studies of welfare and acute as well as chronic tress (as reviewed by Mormède *et al.* 2007). Analysing hair cortisol content offers a non-invasive possibility to measure retrospective levels of circulating cortisol, as cortisol is incorporated in the hair while it grows, which is not influenced by daily fluctuations and stress around sampling (Lee *et al.* 2015; Burnard *et al.* 2017). When the project began hair had been used to analyse cortisol from cattle but in studies with a smaller number of animals (González-de-la-Vara *et al.* 2011; Moya *et al.* 2013; Burnett *et al.* 2014).

The concentration of cortisol was determined using an ELISA kit designed for salivary cortisol (Salimetrics Europe Ltd, Art 1-3002) according to the manufacturer's instructions (validated by Moya *et al.* 2013). Final cortisol content in hair was calculated using the formula suggested by Meyer *et al.* (2014) and inter- and intra-assay coefficients of variation (CV) calculated according to manufacturer instructions (Salimetrics 2018).

A detailed description of the hair cortisol extraction is presented in Paper IV and the steps visualised in Figure 7. To enable extraction of a larger number of samples the protocols suggested in previous studies on cattle required some changes. To be able to pulverise the large number of samples in a standardised way, freezing of hair followed by bead beating with three chrome steel (3.2 mm in diameter, BioSpec Products, Cat. No. 11079132c) in the tubes was done. The freezing step was added to achieve a more homogenous pulverisation thanks to input from researchers working with dog hair at Linköping University (Roth *et al.* 2016).

During the processing of samples, it became clear that the washing procedure did not remove all dirt and hair with severe faecal contamination also appeared to have changed structure. As the washing procedure is known to impact cortisol extraction (Davenport *et al.* 2006) and it was suspected that the changes in structure might also have an impact it was decided to score the samples of remaining dirt to enable control for this in the analysis. As dirt on dark hair

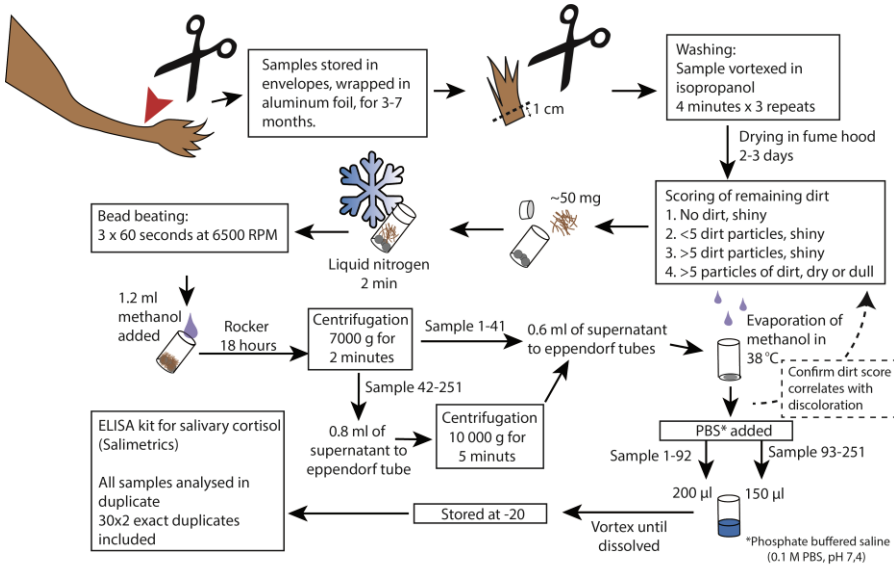

Figure 7. Description of the preparation and extraction of hair cortisol.

would potentially be more difficult to spot the dirt score was correlated with discoloration of the final solution of extracted cortisol. The impact of dirt is discussed in detail in paper IV.

After drying of the first batch (samples 1-41) it was also clear that one centrifugation did not remove all the hair particles and some were transferred with the supernatant to the final sample. To ensure this did not happen an additional centrifugation was added. The extra centrifugation did not impact cortisol content in the final sample (Paper IV). In the initial protocol, 200 µl of PBS was used to dissolve the extracted cortisol. However, the dried cortisol was hard to dissolve with this amount of PBS. When the volume was decreased slightly (to 150 µl) for a test batch the process was easier. As CV values of this batch were smaller the protocol was changed to 150 µl PBS.

3.6 Statistical analysis

Data was entered in Excel and all statistical analysis performed in the statistical software R (R Core Team 2018). Several different approaches were used and details of each analysis, as well as R-packages used, are outlined in the papers I-IV.

To summarise, Fisher's exact test, Wilcoxon rank test or Chi-square tests were used in the univariable analyses in paper I, II and IV. Paper I and II included generalised linear mixed-effects models with a logit link (glm), to assess risk factors for presence of VTEC O157:H7 on farms and risk factors for colonisation. Paper I also included analyses of spatial clustering (using Cuzick-Edwards' kNN (k nearest neighbours) and Ripley's K function tests). In paper II, the glm was complemented with generalised additive models (gam) to investigate non-linear associations. In paper III cluster analysis, elastic net regression and principal component regression were combined to study individual risk factors. These methods were used to enable analysis without reducing the data. This was done to provide a holistic perspective where associations between the variables could be used to enable interpretation of possible underlying meanings. The different methods also have different strengths as they are not dependent on the same assumptions. In paper IV, gam and elastic net regression were used to analyse how hair cortisol concentrations were influenced by methodological changes, age and welfare indicators.

There were a small proportion of missing values in the welfare observations (for more details see paper III and IV). In most cases observations of one or two variables of an individual were missing. As the number of animals in the study was limited, and one missing value would mean that the individual could not be included in the elastic net regression, imputation of the missing values was

performed using non-parametric random forest imputation as described in Paper III and IV.

Some additional analyses (not presented in the papers) are included in this thesis. Causal pathways to visualise assumptions of causality in paper II were created using DAGitty v3.0 (www.dagitty.net). Stratified analysis of risk factors from paper II was performed using generalised mixed models with a logit link in the package lme4 (Bates *et al.* 2015). Analysis of the association between hair cortisol and colonisation was performed using generalised additive models in the package mgcv (Wood 2004, 2011). Figures were created using the packages ggplot2 and ggeffects (Wickham 2016; Lüdecke 2018).

4 Results and discussion

4.1 Between farm transmission on Öland

The results from paper I indicate that local transmission on the island of Öland was common and strains were frequently exchanged between farms. One of the risk-factors for introduction of the infection was purchase of animals, which is a recognised risk factor in previous studies and the suggested underlying driver for transmission over large distances (Widgren *et al.* 2015, 2016; Franz *et al.* 2019). Trade of animals is also a well-known risk for introduction of other infectious diseases on dairy farms and avoiding purchase of animals was the measure most commonly mentioned when the farmers participating in Paper II-IV were asked how they protect animals on their farm from infectious disease (Table 2).

Still, the results from paper I indicate that avoiding purchase of animals will not be enough if the farm is located in an area with high cattle density where VTEC O157:H7 circulates. The analysis of risk factors as well as more detailed analysis of isolates using wgs, point to human activities (visitors travelling between farms) being responsible for introducing the pathogen on farms. A previous study of small scale dairy farms in Mexico have correspondingly observed a genetic pattern that matched shared forage storage and milking staff (Rosales-Castillo *et al.* 2011).

Still, humans may not literally have to carry the pathogen between barns. As sharing of agricultural machines was a risk factor for being positive in the fall sampling, moving vehicles between farms may be enough. For example flies, known to be able to spread VTEC O157:H7 (Ahmad *et al.* 2007) and found to carry VTEC O157:H7 in paper I, may pick up the pathogen from faecal contamination on vehicles or travel in vehicles between farms.

Table 2. *Characteristics of the farms included in the individual sampling (paper II-IV). Farm size includes the total number of cattle on the farm. Sampled animals is the number of animals sampled for verotoxin-producing* Escherichia coli *O157:H7 and the proportion colonised as determined by recto anal mucosal swabs. Information about calf mangement of calves, cleaning routines and biosecurity measures were collected in a structured interview. Last 13 rows reflect farmers spontaneous answers to the questions "How is introduction of infectious agents prevented" and "How is transmission of infectious agents between animals prevented?". (Yes) = mentioned but applied with exceptions.*

Farm	F1	F2	F3	F4	F5	F6	F7 visit 1	F7 visit 2	F8	F9 visit 1	F9 visit 2	F10	F11	F12
Farm size	330	375	650	300	422	380	360		350	700		135	130	250
Sampled animals	24	26	20	20	28	26	17	20	25	29	20	25	20	18
Proportion colonised	25%	8%	5%	10%	11%	39%	29%	20%	12%	17%	15%	12%	30%	17%
Calves in single pens (weeks)	8-12	1	~2	0	8	~2	2-3 days		2-4	6		~2	2-3	4
Weaning age (weeks)	8-12	14	8	8-10	8	10-12	12		8	8		8-10	12-16	12
Yearly cleaning	Yes	Yes	Yes	No	Every second year	Yes	Yes		Yes	"Most years"		Yes	Yes	Some buildings
Regular use of disinfectant	No	Slaked lime	Slaked lime	Slaked lime	Slaked lime	No	Slaked lime		Yes + Slaked lime	Yes + Slaked lime		No	No	Yes
How is introduction of infection to the herd prevented:														
Avoid purchase of animals			Yes	Yes	Yes	(Yes)	(Yes)			Yes		Yes		(Yes)
Protective clothes for visitors	(Yes)	Yes	Yes		Yes	Yes	Yes		Yes	(Yes)				Yes

Farm	F1	F2	F3	F4	F5	F6	F7 visit 1	F7 visit 2	F8	F9 visit 1	F9 visit 2	F10	F11	F12
No contact with other farms on pasture	Yes													
How is transmission between groups of animals prevented:														
Rinse boots between groups	Yes	Yes	Yes	(Yes)	Yes	Yes	(Yes)		Yes	(Yes)				Yes
Desifection at entrance									Yes					
Clean feed through		Yes				Yes							Yes	
Control rats and wildlife													Yes	
Desinfect between groups of animals					Yes									
Dont move sick animals					Yes									
Wash hands after handling sick animals	Yes													
Avoid contact between young and old animals	Yes													

Animal contacts (nose-nose contact on pasture) were also an important risk factor. However, it should be kept in mind that farms on Öland differ from farms in other regions of Sweden. On Öland, pastures are often adjacent to pastures of other farms and animals are often transported from the main farm to different pastures during summer. There are often only simple fences (traditional stone walls) around pastures separating the animals (Figure 8). This means that there is an unusually high rate of contacts between farms and the extent of animal to animal contacts between farms located on different parts of the island becomes very clear in the questionnaire study (See Paper I, Figure 4). The high contact rate likely explains how the strain of clade 8 spread so efficiently across the island and why the proportion of farms with clade 8 is high compared to other national studies including other regions (Eriksson *et al.* 2005; Söderlund *et al.* 2014; Widgren *et al.* 2015).

Another interesting observation from paper I was animals picking up strains of clade 8 previously found on neighbouring farms on pasture and introducing it on farms. It is possible that strains from neighbouring farms were acquired through animal contacts on pasture but another explanation could be contact with a common environmental reservoir like flies, birds and wild game as has been discussed previously. Differentiating whether environmental presence of the same strains found in cattle is a reservoir of infection or a spill-over from circulation among cattle is beyond this study to ascertain. However, considering that farm and cattle density appear to be of importance for transmission the results of this thesis supports the latter.

Figure 8. Pasture on Öland with the traditional stone walls separating pastures of animals from different farms in the background. (Photo: Lena-Mari Tamminen)

4.1.1 Is it important to differentiate between introduction and persistence?

In paper I it was found that contact with a known positive farm (positive for VTEC O157:H7 in the spring sampling) was a risk factor for persistence (positive on both sampling occasions) as well as new infection (a previously negative farm becoming positive in the fall sampling). It was also the risk factor most strongly associated with being positive in the fall sampling regardless of previous status in the spring sampling (OR 6.8, CI 1.6-32.3). This suggests that some of the farms that were positive in both the spring and the fall sampling were infected with a new strain during summer and hence were not really farms where infection persisted. The results from the whole genome sequencing of strains from four of the farms also exemplifies that different strains were circulating among the farms between the spring and fall sampling (Paper I, Figure 5). However, being positive in both the spring and fall sampling was also associated with farm size (large farms more likely to be positive) and combining milk and meat production. These risk factors are not clearly related to introduction of new strains. In addition, the farms positive in both samplings had significantly fewer neighbours within 5 km compared to the farms that cleared infection. There are several examples of farms that have remained positive over time despite a turnover of animals (Lahti et al., 2003; Tamminen et al., 2018) as well as farms where circulation of the same strain over time has been confirmed using PFGE (Joris *et al.* 2013; Herbert *et al.* 2014).

If the underlying pattern behind observed persistence is in fact frequent introductions to a farm, on-farm measures applied to prevent persistence will be an unnecessary cost to the farmer as the farm would likely clear the infection if new introductions stopped. However, on a farm where an environmental reservoir or circulation of infection within groups of animals is occurring, external biosecurity measures will not reduce the prevalence. Not separating these two scenarios also risks introducing noise to studies of transmission and prevalence on farms, just as we are likely observing in paper I.

4.2 Within farm prevalence and transmission

The separate environmental samples collected from young calves, weaned calves, young stock and dairy cows in this study showed a large variation between farms. On the 12 farms where thorough environmental sampling was performed, only 1 had positive samples from all groups of animals. On all farms, the pathogen was found among calves between 2-6 months of age. On 6 farms, groups including animals up to 12 months were also positive in the environmental sampling. However, these groups were often difficult to

distinguish as animals were housed in overlapping age constellations. Dairy cows and non-weaned calves were positive only on part of the farms (n = 2 and 5 respectively). Low prevalence among dairy cows and non-weaned calves is consistent with previous studies (Mechie *et al.* 1997; Rugbjerg *et al.* 2003; Gunn *et al.* 2007; Cho *et al.* 2009). In addition to the variation between groups observed in the environmental sampling, the individual sampling showed variation in colonised animals between pens. Colonised and shedding animals were generally not found in all pens on a farm and there were several examples of farms where differences in prevalence were observed within the same age groups if animals were housed in separate buildings. Thus, it appears possible to keep transmission from occurring between groups of animals despite most of the farms not having strategies for preventing disease spread between groups (Table 2). Avoiding transmission between groups of animals on farms by keeping groups together has previously been suggested as a cost effective measure to reduce prevalence of VTEC O157:H7 on farms (Ellis-Iversen et al. 2008; Lyons et al. 2013). However, there are indications that other vectors may participate in spreading the bacteria between groups on a farm. For example, a cat on the farm, identified as a potential risk factor for being positive for VTEC O157:H7 in paper I (although only significant with 90 % confidence), may both bring the pathogen to the farm and circulate it within the farm as cats tend to move freely among groups of animals. As mentioned above, flies and birds may also play a possible role in transmission on farms. But, since VTEC O157:H7 was generally associated with a subset of animals on the farm this does not appear to be a huge problem.

4.2.1 Management and susceptibility – closely connected potential drivers of transmission

Interdependencies between predictors

The risk factors included in paper II were selected based on associations with colonisation and shedding in previous literature. Analysis of risk factors on pen level from the first individual sampling showed an interaction between stocking density and age, suggesting that with low stocking density the risk of colonisation increased with age while in high stocking density the risk of colonisation decreased with increasing age (Paper II, Figure 1). The effect was most notable as a large difference in risk of colonisation in young animals housed in high density compared to low density. However, in the follow up sampling (5 weeks after the initial sampling) this association was no longer observed. The average age of animals in the second sampling was higher (130

days compared to 122) but most importantly there was a smaller proportion young animals (below 100 days) compared to the first sampling (Figure 9 A). Thus, the second sampling may not have had the power to detect an increased risk at young age. Stocking density, on the other hand, was observed to be an important risk factor for colonisation, both in the first and the second sampling but the effect was estimated to be slightly larger in the first sampling compared to the second (OR 1.99 and 1.31 respectively). A potential reason for this difference can be found when considering a causal network of the variables included in the two models (Figure 10).

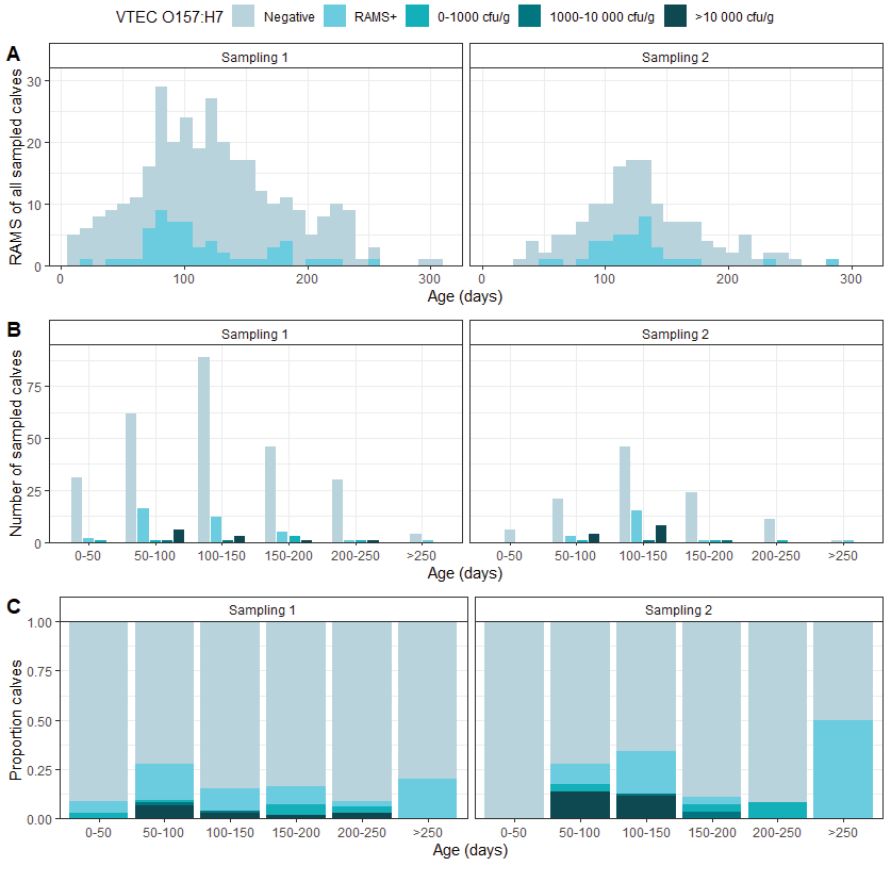

Figure 9. Distribution of age of sampled calves and results of individual sampling for verotoxin-producing *Escherichia coli* O157:H7. A: Number of sampled calves and results from recto anal mucosal swab (colonised/non-colonised). B: Number of calves and results of individual sampling including faecal shedding levels. C: Proportion colonised and shedding within age groups.

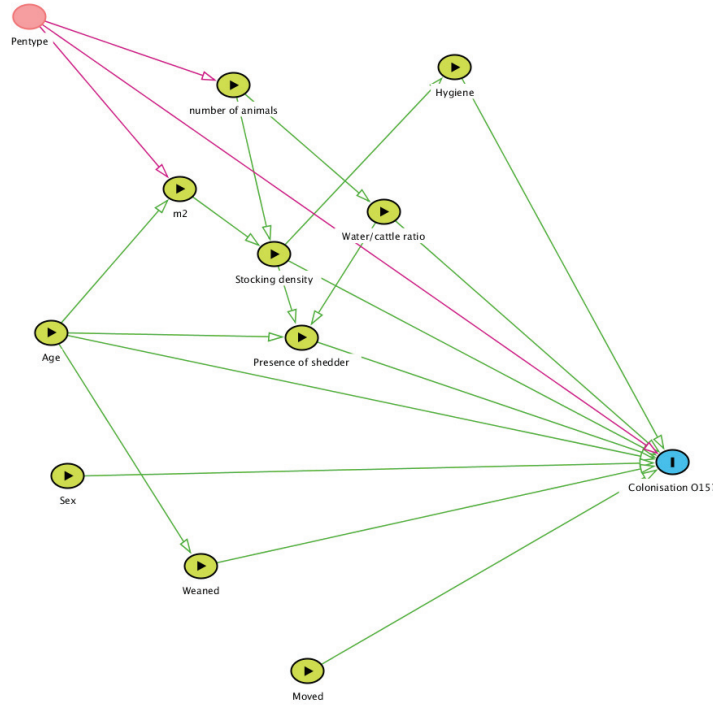

Figure 10. Causal diagram describing causal assumptions of risk factors for colonisation of verotoxin-producing *Escherichia coli* O157:H7 analysed in paper II. *(m2=Pen size)*

As seen by the arrows in the figure, associations between the risk factors; age, stocking density, pen hygiene as well as water to cattle ratio and the presence of super-shedders were assumed. (Based on the assumption that these variables influence the risk of individual colonisation, they likely influence the risk of colonisation of peers and through this effect the presence of super-shedders). If part or all of the effects of these variables on colonisation is mediated through increased presence of super-shedders their effect will be reduced or blocked when the presence of super-shedder is included in the model (Dohoo *et al.* 2014). Thus, the estimate of stocking density in the second model in paper II likely is a better estimation of the direct effect on the risk of colonisation of an individual (as the effect of stocking density on other calves in the pen was accounted for).

Considering this there may be an alternative explanation for why the interaction between age and stocking density does not influence colonisation when presence of super-shedders are accounted for. It is possible that the effect of the interaction is related to the presence of shedders, i.e. that the presence of

shedders is more common in high stocking density and young animals. The presence of shedders was closely connected to the outcome (colonisation) in the first individual sampling, since analysis of shedding was only performed on samples from colonised individuals. Thereby it was not independent from the response variable and not included in the risk factors for colonisation analysed in the first sampling. In Figure 11, the association between colonisation, shedding levels, age and stocking density from the first and second sampling are visualized. In sampling 1 there does appear to be a group of animals around 100 days shedding higher levels although there were also older animals shedding. In sampling 2 high shedding also occurred around 100 days and it is noteworthy that the highest shedding animals are housed in higher stocking density. The shedding pattern of calves in relation to their age is presented in Figure 9 (B & C). Out of the RAMS positive calves a low frequency of animals were shedding (19 individuals in the first sampling and 17 individuals in the second). It appear as if the proportion of RAMS positive calves shedding high levels of bacteria was higher in the age group 50-100 days in sampling 1 and 50-150 days in sampling 2. Thus, there are signs of an association between young age and increased shedding but this should be interpreted with caution due to the small number of shedding individuals.

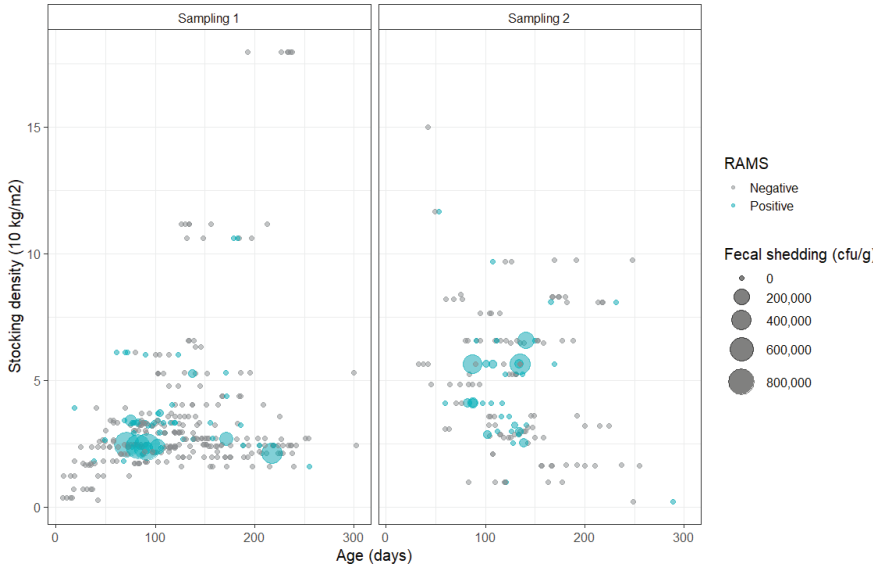

Figure 11. The association between age (x-axis) and stocking density (y-axis) and colonisation/shedding of verotoxin-producing *Escherichia coli* O157:H7 of the sampled dairy calves. Coloured dots indicate colonised calf (as detected by recto anal mucosal swabs) and size of dots indicates shedding level.

Age and weaning may influence exposure in different ways

Age was found to have a small negative coefficient in the elastic net regression (Paper III) which supports a decreased risk of colonisation with increasing age. This is in agreement with many studies where prevalence of VTEC O157:H7 has been observed to decrease with age (Kuhnert *et al.* 2005; Gunn *et al.* 2007; Mir *et al.* 2015). However, as mentioned above, non-weaned calves have been associated with a lower prevalence (Garber *et al.* 1995; Hancock *et al.* 1997; Rugbjerg *et al.* 2003). This is consistent with the result that VTEC O157:H7 was found in the environment of non-weaned calves on only 5 of the sampled farms. However, analysis of individual risk factors for colonisation in the first sampling (paper III) and transmission among positive groups of animals (Paper II, part 2) did not indicate a protective effect of drinking milk or an effect of weaning. Most of the farms included in this study kept calves in single crates for around 2 weeks before group housing (Table 2) which is common practice in Sweden (although exceptions occur). Common practice in many other countries, like the United States, is to keep calves in single crates until weaning around 2 months of age. As grouping of calves before weaning has been identified as a risk factor regardless of weaning age (Garber *et al.* 1995), the practice of early group-housing may explain the discrepancy between our study and previous studies. This indicates that other factors, sometimes associated with weaning, such as changes in the management of young animals and being introduced to other animals, influence the risk of shedding and colonisation. This is consistent with studies in the UK where the effect of age and weaning was not significant after accounting for management related factors (Synge *et al.* 2003; Smith *et al.* 2016) and that VTEC O157:H7 has been identified in high prevalence in very young, group housed calves on New Zealand (Browne *et al.* 2018).

The first months of the life of a dairy calf is a dynamic period with changes in feeding, housing and social contacts. Social grooming behaviour, one of the important risk factors on individual level (Paper III), increases rapidly during first week calves are housed together (Abdelfattah *et al.* 2018; Horvath & Miller-Cushon 2018) and it is important for maintaining social contacts (Færevik *et al.* 2007). Mixing of calves has been observed to lead to a marked increase in behaviours directed towards other calves but the effect disappeared with increased number of regroupings (Veissier *et al.* 2001). In another study, regrouping has been shown to be associated with increased grooming mainly of familiar calves (Horvath & Miller-Cushon 2018). Thus, a reason for the decreasing proportion of colonised animals observed with increasing age, in this and other studies, may be related to reduced transmission as groups and social contacts stabilise with age.

4.2.2 Importance of super-shedding for transmission

The second part of paper II emphasises the importance of super-shedders as the risk of colonisation in the follow up sampling was much larger in animals housed with a super-shedder compared to being housed with a non-shedding colonised animal (OR 9.8, CI 3.9-50.6). This finding supports the importance of super-shedders on transmission of VTEC O157:H7 proposed in previous studies (reviewed by Chase-Topping *et al.* 2008). The selection of animals in the follow up sampling included only animals housed with a colonised individual in the first sampling, which means that all animals should have had the opportunity to be exposed to some level of the pathogen. Despite that it has been shown that low doses are enough to induce colonisation and shedding (Besser *et al.* 2001) the presence of a super-shedder played an important role in the dynamics. However, we cannot, based on these results, identify whether the presence of a shedder was related to individual characteristics of the particular individual or group level characteristics. The presence of a super-shedder may indicate a pen where some necessary, unmeasured, requirements for transmission are fulfilled, like contacts within pen (as suggested by Turner *et al.* 2008) or environmental contamination (Gautam *et al.* 2015). Considering that the individual risk factors for colonisation in these pens were related to social interactions as well oral exposure, through for example grooming, supports a combination of both explanations (Paper III).

It is well known that high shedding can lead to large and unpredictable fluctuations in environmental prevalence of the pathogen and that other animals become infected with strains that are being shed in high levels by pen mates (Chase-Topping *et al.* 2007; Cobbold *et al.* 2007; Stephens *et al.* 2009; Henry *et al.* 2019). However, recent longitudinal studies questioned the role of super-shedding individuals as periods of high shedding were rare and occurred during short periods (Munns *et al.* 2014; Lammers *et al.* 2015). Instead of individual super-shedders they suggest that synchronised shedding of many animals is driving transmission. In this study, the proportion of colonised animals was relatively stable, few animals per farm were found to be colonised and even fewer super-shedding (1-2 individuals). Although we cannot know the dynamics between the two sampling occasions, it is clear that the presence of a super-shedder has an important role in the dissemination of the pathogen.

4.2.3 Transmission dynamics in poor and good hygiene conditions

In our study, pen hygiene was not a significant risk factor for presence of colonised individuals in the first individual sampling and, while moisture was indicated to be important in the follow-up sampling, faecal contamination was negatively associated with colonisation (Paper II). In addition, analysis of risk factors on individual level (Paper III) did not suggest associations between poor hygiene and colonisation. Instead, calves with poor cleanliness scores (below hocks and body) were less likely to be colonised by VTEC O157:H7. This contradicts recent suggestions that contamination of the environment and ingestion of faecal material is the major driver for transmission (Gautam *et al.* 2015; Spencer *et al.* 2015). Variables most strongly associated with colonisation were self-licking and licking other calves which may be related to direct transmission between animals as VTEC O157:H7 is commonly found on the hides of animals housed with a super-shedder (Arthur *et al.* 2009; Stephens *et al.* 2009).

Still, the most important route of transmission may depend on context. This was illustrated when the animals in the first individual sampling in paper II were stratified by pen hygiene. The calves housed in clean pens differed slightly from calves in pens with poor hygiene as animals were housed in lower stocking density (average 24 kg/m^2 compared to 30 kg/m^2) and were slightly younger. Reanalysis of risk factors for the separate groups (using univariable analysis and multivariable analysis as performed in Paper II) revealed interesting differences (Table 3). For example, the interaction between age and stocking density was only significant for calves housed in clean pens and not for calves in pens with poor hygiene. As explained in paper II, the interaction means that in clean pens young animals housed in high stocking densities were more likely to be colonised by the pathogen but that the risk decreased with increasing age. The stratified analysis suggests that age and stocking density did not influence individual susceptibility to colonisation in a dirty pen. This could be interpreted as direct transmission between individuals being less important in pens with high environmental exposure. Thus, in a dirty environment calves are probably infected through environmental exposure and direct contacts while in a clean pen the only way to become colonised is through contact with another individual. The reduced risk with increasing age in the clean pens support the suggestion by Gautam *et al*. (2015) that direct transmission is less effective than from the environment.

Table 3. *Risk factors for colonisation by verotoxin-producing* Escherichia coli *O157:H7 in dairy calves in the first sampling stratified by pen hygiene.*

Colonisation of VTEC O157:H7* in:	Clean pens: (Score 1)		Uni†- variable analysis		Multi¶- variable analysis	Dirty pens: (Score 2-3)		Uni†- variable analysis		Multi¶- variable analysis
	RAMS-	RAMS+	*p*	β	*p*	RAMS-	RAMS+	*p*†	β	*p*
Number of calves	112	18				149	38			
Shedders >10^3 cfu/g		3					1			
Shedders >10^4 cfu/g		4					9			
Sex = M (%)	40 (35, 7)	2 (11,1)	0,06	-1,42	0,10	50 (33,6)	7 (18,4)	0,08	-0,70	0,18
Age (months) (median [IQR])	3,82 [1,80, 6,11]	2,93 [2,35, 6,08]	0,97	1,15	0,02	4,10 [3,23, 5,07]	3,47 [2,81, 4,07]	0,01	-0,02	0,95
Stocking density (10 kg/m2) (median [IQR])	2,43 [2,09, 2,92]	2,43 [2,36, 3,73]	0,15	1,52	0,01	2,92 [2,33, 4,04]	3,34 [2,92, 3,41]	0,04	0,50	0,21
Animals in pen (median [IQR])	11,00 [6,00, 18,25]	7,00 [4,00, 16,50]	0,26	0,00	0,97	9,00 [7,00, 13,00]	8,00 [7,00, 18,50]	0,66	-0,03	0,64
Pentype (%)			0,56					0,06		
Straw	64 (57,1)	13 (72,2)				54 (36,2)	22 (57,9)			
Deepstraw	35 (31,2)	4 (22,2)		-1,55	0,22	79 (53,0)	14 (36,8)		0,13	0,85
Loose housing	13 (11,6)	1 (5,6)		-0,94	0,74	0	0			
Slatted floor	0	0				16 (10,7)	2 (5,3)		-1,10	0,58
Water/cattle ratio (median [IQR])	0,09 [0,06, 0,17]	0,17 [0,06, 0,25]	0,45	0,11	0,98	0,11 [0,10, 0,14]	0,14 [0,11, 0,20]	0,00	10,24	0,07
Stocking density:Age (months)				-0,42	0,01				-0,06	0,29

*As determined by detection of bacteria in recto-anal mucosal swabs (RAMS); †Fisher exact test or Wilcoxon rank sum test; ¶Generalised linear mixed model with a logit link.

In the analysis of risk factors for colonisation in the follow up sampling (Paper II part 2), being housed in a pen with faecal contamination of bedding reduced risk while a wet bedding was associated with increased risk. It is known that moisture and faecal contamination influences persistence and growth of VTEC O157:H7 in the environment (as discussed in paper II). But poor hygiene will not only influence VTEC O157:H7 – it will also have an impact on the health, well-being and behaviour of animals in the pen, for example cleaning frequency of pens has been associated with calf diarrhoea (Klein-Jöbstl *et al.* 2014). Sick individuals will modify their behaviour to overcome disease (Hart 1988) which may impact exposure to agents like VTEC O157:H7. Behavioural changes in calves include reduced self-grooming, feeding and social interactions and have been even observed in early and mild stages of respiratory disease (Borderas *et al.* 2008; Cramer *et al.* 2016; Hixson *et al.* 2018). Sick calves are also less likely to approach novel objects or a stationary human (Cramer & Stanton 2015), possibly indicating a less exploratory behaviour. This fits very well with the finding that individual risk factors not associated with colonisation in paper III were associated with diarrhoea, coughing and nasal discharge (signs of respiratory disease) as well as other indicators of poor welfare.

4.3 Stress, colonisation and susceptibility

The hypothesis that stress is related to colonisation of VTEC O157:H7 has been suggested in several studies and reviews (Cray & Casey 1998; Chase-Topping *et al.* 2007; Rostagno 2009; Munns *et al.* 2015). However, few studies have actually explored the association deeper than connecting changes in management associated with stress (such as dietary or heat stress, weaning, movement and transport) and suggesting that increased stress makes calves more susceptible to colonisation and shedding (Cray & Casey 1998; Chase-Topping *et al.* 2007; Bach *et al.* 2016; Stenkamp-Strahm *et al.* 2018). But, as described for weaning above, it is not necessarily the increased susceptibility of the host, but the increased exposure to the pathogen due to changes in environmental exposure, behaviour or social contacts, which is the actual risk.

In this study, three approaches to investigate stress were used. Firstly, the associations between colonisation and indicators of poor welfare were explored. Although stress and welfare are not the same, they are closely linked (Veissier & Boissy 2007), and animals experiencing poor welfare should be more likely to also experience stress. As mentioned above, colonisation was associated with social and active calves that were grooming themselves and others, while animals that were showing signs of poor health and welfare were less likely to be colonised (Paper III).

Secondly, comparing hair cortisol concentrations of the colonised and non-colonised groups (Figure 12) using a generalised additive model (including the variables amount of buffer, faecal contamination of hair as well as age identified as important influencers in paper IV) showed no significant association. The model indicated an interaction between age and hair cortisol, suggesting that high hair cortisol and increasing age was connected with colonisation, but this was not statistically significant ($p = 0.12$). This could indicate an association between colonisation and hair cortisol in older animals but there were too few old animals in the study to explore this. Considering that previous studies on cows have showed associations between increased hair cortisol and clinical disease (Comin *et al.* 2013; Burnett *et al.* 2015) and increased risk of shedding of VTEC O157:H7 in downer cows (Byrne *et al.* 2003), supports the possibility that hair cortisol may be associated with colonisation in older animals.

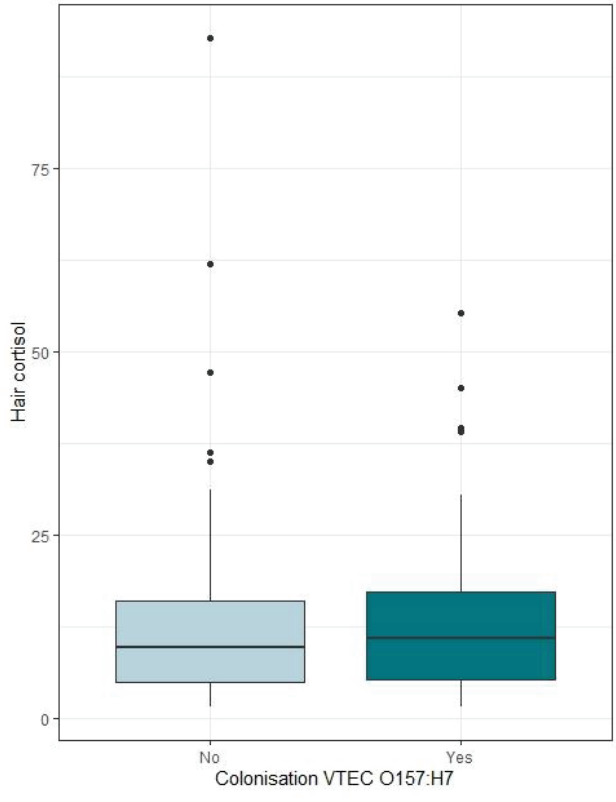

Figure 12. Hair cortisol concentration (pg/µl) of calves colonised by verotoxin-producing *Escherichia coli* O157:H7 (as determined by recto-anal mucosal swabs) and non-colonised (negative) calves.

Thirdly, individual reactivity and fearfulness, indicators of coping styles that are linked to vulnerability to stress (Koolhaas *et al.* 1999), were assessed in paper III. A previous study looking into temperament and shedding of VTEC O157:H7 in calves compared excitable, intermediate and calm calves (classified by a temperament index), found that calm animals were more likely to shed VTEC O157:H7 than other calves (Schuehle Pfeiffer *et al.* 2009). In our study, a similar association between reduced risk of colonisation and high reactivity was observed (Paper III), further supporting that personality and/or coping style influences colonisation.

Schuehle Pfeiffer *et al.* (2009) also analysed serum cortisol levels but could not find an association between shedding and non-shedding animals as there was no difference in cortisol levels between the intermediate and calm groups of animals. However, the excitable group had higher serum cortisol levels. This is inconsistent with the results of the hair cortisol analysis and welfare measures in this study (paper IV) where high reactivity was associated with low hair cortisol. This may be a result of the different methods used for cortisol analysis. Serum cortisol represents immediate changes while hair cortisol represents average hair cortisol levels during hair growth (Lee *et al.* 2015). There are also other important regulators of coping, like serotonin, that are unaccounted for in both studies which may explain the discrepancies observed (Koolhaas *et al.* 2007). In addition, as discussed in paper IV, the cortisol levels of a calf in a poor non-stimulating environment may be difficult to differentiate from an individual in a good environment exposed to acceptable, stimulating challenges (Korte *et al.* 2007).

Combining the results of the three approaches there is no clear indication that stress is related to increased risk of colonisation. This is consistent studies focusing on increased susceptibility colonisation due to heat and handling stress, where no effect of either were observed (Brown-Brandl *et al.* 2009; Sheng *et al.* 2016). Instead results indicate that animals showing signs of coping well are more likely to be colonised while animals showing signs of poor welfare and disease were less likely to be colonised. However, there are signs of interesting differences in personality and behaviour between colonised and non-colonised calves and we propose that these are related to different exposure to the pathogen. An additional important aspect of the identified risk factors is that the risk factors associated with colonisation, i.e. being a socially engaged and active calf, may be associated with a more efficient dissemination of an infectious agent. A super-spreader is a description of an individual who has more opportunities to infect others, through for example through a high number of contacts (Chase-Topping *et al.* 2008). Although super-shedding and super-spreading by definition are independent traits (the first referring to interactions

between host and pathogen while the latter refers to interactions between hosts), risk factors identified for colonisation in this study suggest that colonisation, and thereby super-shedding, and super-spreading of VTEC O157:H7 are associated.

4.4 Validity, bias and methodological considerations

4.4.1 Study population and external validity

Identification and selection of farms

Almost from the beginning of the project a major concern and limitation has been obvious – identification of farms positive for VTEC O157:H7 to enrol in the study. As animals do not show any symptoms and Sweden does not have an active national surveillance, apart from a slaughter prevalence study including a small proportion of slaughtered animals every second year, finding farms infected with VTEC O157:H7 was difficult. Previous studies had shown a regionally high prevalence in Falköping (Widgren *et al.* 2015) and the slaughter prevalence study performed by SVA indicated that the pathogen was present on the island of Öland and the counties Skåne and Blekinge (Erik Eriksson, SVA, personal communication) which is why these areas were targeted for environmental sampling. Environmental samplings of farms around Falköping (guided by the results from Widgren *et al.* 2015) were all negative, which supports the national slaughter prevalence study suggesting that clade 8 in this areas has decreased.

Thanks to the collaboration with Farm and Animal Health, a number of farms where VTEC O157:H7 were found in a parallel research project as well in association with human disease farms were enrolled. Most of these farms were located on Öland but one was located in Småland county and would not have been identified in any other way. The samples collected from Skåne and Blekinge were also guided by results from Farm and Animal Health and performed in an area where several farms had been positive just weeks before our sampling. However, most farms had cleared the pathogen. This region also included the only farm that cleared the infection during the time between environmental sampling and individual sampling. This may indicate that there were differences between Öland and Skåne/Blekinge, either in the circulating strains ability to persist on farms or in transmission between farms.

Regarding the effect of potential differences between circulating strains there is much to learn about survival and persistence of different types of VTEC O157:H7. Considering the flexible genome of VTEC O157:H7, strain

differences are expected and can be present even in closely related strains. However, we still lack the knowledge to fully understand the impact of these differences. It may be a strength to study farms in an area where one dominating strain is present (like on Öland) as this decreases variation and noise introduced by strain differences. In addition, all strains in our study belonged to a virulent subtype and understanding how these subtypes behave is a priority compared to other, less virulent, subtypes.

While the majority of farms were located on the island of Öland, and thus somewhat similar, the farms included a variety of farm sizes, housing systems and management and should therefore include a representative selection of Swedish dairy herds. However, the unique regional characteristics, like high rate of contacts between farms and high cattle density, raises the question of how relevant the risk factors identified in this study are for other regions. We argue that although all routes of transmission that were identified in paper I may not apply to all regions, it remains relevant to identify them. The unique characteristics and frequent contacts between farms on Öland may have given us the opportunity to discover routes that would have been difficult to identify in other regions with fewer contacts.

Our study design was cross-sectional and thus we do not know how long the pathogen had been circulating on each farm. As discussed in Paper II animals infected with VTEC O157:H7 develop an immune response, although there appear to be strains that can overcome these responses partly or completely (Hoffman *et al.* 2006; Corbishley *et al.* 2014). Depending on how long the pathogen had been circulating on each study farm, the dynamics may be different. For example, sampling a farm just as infection hade been introduced in a naïve population there could be more colonised animals and increased levels of shedding leading to different risk factors than entering a farm with a long-lasting presence. Thus, there is a risk that the study design biased the results.

Selection of animals for individual sampling

Farms in papers II-IV represent a selection of farms where VTEC O157:H7 had been identified and environmental sampling was used further target groups of positive animals on the farm. The targeted sampling approach was used to avoid sampling and analysing animals not exposed to the pathogen and increasing the number of colonised animals included in the study. While previous large scale studies have struggled with identifying enough calves, e.g. only 34 calves out of 1324 calves were positive in a recent large study from the United States (Stenkamp-Strahm et al. 2018), we identified at least one colonised individual on all sampled farms except one.

In addition to using environmental sampling to narrow the individual sampling to a population exposed to the bacteria, calves for follow-up sampling in paper II and analysis in paper III were selected based on a case-control approach where only individuals housed with a colonised individual were included in the analysis. This enabled us to control for environmental exposure and decreased the risk of introducing noise by including animals that had not been exposed to the pathogen. However, there is also a risk that this approach may have led to over-matching and that some determinants associated with negative pens were not discovered. For example, the environmental sampling directed the individual sampling to groups of young animals and only sampling among these animals may be a reason why age was not a strong determinant in our study as in other studies sampling a wider variety of age groups on farms or following animals over time (Nielsen *et al.* 2002; Mir *et al.* 2015). As the aim was to understand transmission and risk for colonisation in the presence of VTEC O157:H7, this risk was accepted.

There were also some practical constraints during sampling and there were cases when pens with older calves and young stock indicated as positive by environmental sampling could not be sampled. These cases were commonly a large pen where animals had space to run that lacked of restraining facilities. To avoid injury of both humans and animals sampling was not performed in these situations. However, this may have skewed sampling so older animals sampled in our study represent animals kept in smaller pens than young stock generally are. Still, this should mainly be a potential bias in the first part of paper II where it may explain why the effect of high stocking density was only observed in young animals.

4.4.2 Choice of methods

There is a large variation in methods that have been used to study colonisation and super-shedding. Some studies uses faecal counts, others RAMS (with or without enumeration), and enrichment, culture and confirmation protocols vary. The most sensitive method for detecting colonisation has been suggested to be RAMS (Cobbold et al. 2007), which were used to analyse all individual samples in this study. Spencer *et al.* (2015) estimated a median sensitivity of 0.78 (95% CI 0.73–0.82) for the RAMS and 0.46 (95% CI 0.42–0.51) for the faecal test. Spencer et al. (2015) did not use immunomagnetic separation during analysis of the RAMS, as in this study, and we therefore expect an even higher sensitivity. For the analysis in paper II and III we also chose to focus on colonised individuals and not shedding levels as studies have shown that shedding patterns can be intermittent and RAMS positive animals may have shed high levels just

before the sampling. However, there is a risk that a RAMS may have been contaminated by VTEC O157:H7 that is just passing through the gastrointestinal system without colonisation. This would lead to wrongly classifying a non-colonised individual as colonised and reduce the specificity of the test.

Enumeration of faecal shedding was only done for RAMS positive calves as RAMS are considered to indicate colonisation while low fecal shedding has been observed to occur also in non-colonised animals (Davis *et al.* 2006; Cobbold *et al.* 2007). In addition, in a pilot study, including 40 animals from two farms where all faecal samples were enumerated, VTEC O157:H7 was only found in faecal samples from animals with positive RAMS. Faecal samples were stored in 2°C during analysis of the RAMS. As it was a concern that this might lead to reduced levels of VTEC O157:H7 in the samples, we performed a pilot study where levels of VTEC O157:H7 where analysed before and after storage in the fridge. There were no indications of reductions in levels of VTEC O157:H7, instead, a small increase was noticed in some samples. This may have been due to VTEC O157:H7 increasing in numbers but more likely due to an uneven distribution of VTEC O157:H7 in the sample or a reduction of other bacteria that may have competed with VTEC O157:H7 when plated on the CT-SMAC agar.

Super-shedding has been proposed to be shedding more than 10^4 cfu/g faeces (Chase-Topping *et al.* 2008) but a recent study has proposed that 10^3 may be sufficient to influence transmission (Spencer *et al.* 2015). In paper II we chose the latter definition but also explored the effect of animals shedding more than 10^4 cfu/g faeces. Using 10^3 better explained the risk of new infections, compared to using the higher number 10^4 (AUC 81% compared to 77%), which supports the suggestion by Spencer *et al.* (2015). However, as only 3 animals shed between 10^3 and 10^4 more studies confirming this is needed.

4.4.3 Assessment of risk factors/determinants

There are also some methodological aspects regarding the determinants included that warrant attention. Collection of information in paper I was done by a postal questionnaire and there is a risk that not all farmers perceived the questions in the same way. In addition, the formulation of some questions was not optimal. For example, in the question about farm contacts farmers were asked to write down contact farms. When going through the contact patterns it became clear that there were several cases where one farmer had indicated contact with another farm which had not stated that farm as a contact. These discrepancies indicate that farmers varied in how thoroughly they filled in the answers to these questions. It might have been useful to ask farmers to estimate the number of contact farms first, before asking for detailed contacts.

The animal-based assessments in paper III and IV were all performed by one person and consequently inter-observer reliability is not an issue in this study. However, although assessments were practiced before the study began, there is a risk that intra-observer reliability changed over the two years the study was performed. Still, considering that colonised and non-colonised animals were compared within pens (and assessed on the same day) the differences observed within groups should be relevant and not due to a possible drift in the assessment.

The behavioural observations were performed for 20 minutes during an active time of the day (when activity in the barn started in the morning). This means that the behaviours observed reflect a snapshot in time and are not representative of all behaviours performed during one day. However, this snapshot represents individual differences within the pen. Longer observation time had likely increased the frequency of observed behaviour and potentially added associations that we did not have the power to detect.

Finally, one of the aims was to explore the hypothesis that chronic stress increases the risk of colonisation. Due to the difficulties of objectively measuring stress, we combined three different approaches including welfare assessment, fearfulness and reactivity as well as hair cortisol. However, neither of these are perfect measures of chronic stress and only provide indications about stress experienced by the calves. With increased understanding of the HPA-axis and coping in relation to calves personalities, it may become possible to better interpret the meaning of these findings.

4.4.4 Statistical methods

A variety of different models and analyses have been used within this project and especially linear and logistic regression. One important assumption for these types of models are independence of the predictors (Dohoo *et al.* 2014). In all papers there were dependencies between observations and these were generally handled by including random effects to control for clustering. In paper I, this was done by including farm in the multivariable analysis, where two sampling occasions per farm were included in the model, and pen was included as a random effect to control for clustering in samplings on individual level. However, random effects was not possible to include in the elastic net regression (paper III and IV) and in these models pen (paper III) and herd (paper IV) were included as a fixed effects. There are some recognised drawbacks with controlling for clustering using fixed effects – first of all it is not possible to include other pen/herd level predictors, which limited the analysis to within-pen/farm level variables. In addition, inference made from such a model is specific to the actual pens and not the general population and having pen as a

fixed effect increases the number of predictors. The elastic net regression technique can handle the large number of predictors and as a clear aim of paper III and IV was to explore individual differences within pen/farm accounting for this variation was considered appropriate. However, this means that interpretation of estimates is slightly different in this model compared to the others. It should also be remembered that the estimates in an elastic net regression are biased (shrunk by a penalty-term to control variance inflation) and not directly translatable to OR (James *et al.* 2013).

The assumption of linearity is another important consideration in regression analysis and in paper II and IV the usefulness of gams to identify non-linear associations is clear. Especially the interaction observed in paper II was interesting and provided important insights and this model might have been useful when exploring risk factors using multivariable analysis in paper I as well.

Different approaches were used for model building in the different papers. For the first part of paper I, the glm was reduced using stepwise backwards selection using Akaike Information Criterion (AIC) while in paper II model building was guided by a causal diagram. The model in the second part of paper II was reduced using likelihood ratio test. In both studies all removed variables were reintroduced one by one to control for confounding by studying the change of estimates. Although stepwise selection using AIC is often used to reduce models (Dohoo *et al.* 2014), and convenient due to the relatively large number of variables in paper I, the structured approach in paper II is preferred as more care can be taken to intervening, mediating and confounding variables.

In paper III, we wanted to study the combined effects of the highly interdependent variables. Adding the principal component regression was an alternative that was not constrained by correlation between variables or the assumption of normal distribution in the creation of the components and made it possible to explore how variables may have had context dependent meaning. It was also interesting to combine principle component regression, driven by variance of the observations, with a clustering method, which identifies homogenous subgroups among observations, to visualise the associations from different perspectives. There are of course alternatives that could have been used. For example network analysis and exploratory factor analysis would have been interesting options (James *et al.* 2013). In paper IV the main question was related to the association between hair cortisol to identify indicators of welfare and interdependencies of variables were not explored further. However, adding analyses, like principle component regression and cluster analysis, could potentially provide deeper understanding of hair cortisol concentration and, for example, personalities of calves.

5 Conclusions, reflections and future perspectives

5.1 The end of this story

In the work of this thesis, new insights into the complex associations involved in transmission and persistence of the pathogen between and within farms have been gained and possible target areas for on farm measures to reduce prevalence have been identified. The main conclusions are:

- Frequent transmission of virulent strains between nearby farms occur in cattle dense areas with frequent contacts. Many neighboring farms increase the risk of infection on a farm and the routes of transmission are related to both human and animal contacts between farms.
- On infected farms, VTEC O157:H7 is most often found among calves and young stock and only occasionally among dairy cows. Transmission dynamics within farms is influenced by direct contacts between animals, presence of super-shedding animals as well as pen hygiene. The most influential driver is context dependent, which means that different farms may require different measures.
- Drivers of colonisation of individual animals include social and active behaviour, related to increased exposure of the bacteria, while indicators of poor health and welfare decrease the risk. This indicates that the variation in colonisation observed within groups is related to different levels of exposure.
- Although individual differences and personality appears to influence risk, there are no signs of an association between chronic stress and increased susceptibility to colonisation of VTEC O157:H7.

5.2 What can we tell the farmer in the beginning of our story?

Although VTEC O157:H7 is a public health issue and not a pathogen of cattle, farmers are the key when it comes to reducing farm prevalence and reducing transmission from animals to humans. Farmers are the ones best placed for preventing sporadic human cases caused by direct contacts with animals by informing visitors, especially children, to wash their hands after contact with the animals. Thus, it is important that farmers are aware of this pathogen and that it can suddenly appear on the farms without animals showing any symptoms of infection. Perhaps it can also be comforting to know that the farm is probably not the only farm in the area that is affected, and that on most farms the pathogen will disappear almost as suddenly as it came. In addition, there are concrete measures that can help the farm clear the infection faster, like making sure that bedding is dry and trying to reduce stocking density in animal groups.

Due to the many potential routes of transmission, it may not be possible to remove the risk of introduction of VTEC O157:H7 on farms. However, implementation of biosecurity measures reducing human and animal contacts between other farms will decrease the risk. Such measures could include providing protective clothing for visitors, especially the ones travelling between farms, and avoiding pastures where animals can have contact with animals from other farms. If the latter is not possible, separating animals returning from pasture, giving them time to clear the pathogen before being reintroduced among animals on the farm, may be an alternative. Testing would be required to be certain that a group has cleared the pathogen (as the pathogen does not lead to symptoms of disease), but it is questionable if this additional cost to the farmer can be motivated.

Knowing that active and social animals may be the ones most likely to be colonised emphasises that the farmer has not overlooked signs of poor health in the animals spreading the bacteria. This also means that cleaning pens between groups of animals is important, even when groups of animals do not show signs of disease. Still, cleaning does not prevent the already colonised animals from shedding and hygiene efforts need to be complemented with measures that prevent transmission between groups of animals, like the well-known action of avoiding mixing of groups and preventing the pathogen from moving between pens by dirty boots, flies and other potential vectors.

Although VTEC O157:H7 is not really a problem for the animals, these measures will have the added benefit of reducing transmission of other infections and improve general health of animals. Thus, the added costs of handling this zoonotic pathogen should be associated with other benefits than reducing the risk of transmission to humans.

5.3 The never ending story

The increasing trend of human cases of VTEC O157:H7 and the common association with cattle, highlights the importance of reducing the prevalence of VTEC O157:H7 on cattle farms. During the work of this thesis we have provided new insights but the results also lead to new questions and hypothesises that should be explored in future studies.

For handling of infected farms these include:

- On-farm persistence is linked with both a contaminated environment and presence of shedding animals. Thus, in addition to measures targeting environmental presence of the pathogen in pens, measures that reduce the shedding of individuals are needed. There is ongoing research of vaccines, phage-therapy and dietary supplements but maybe it is also time to consider how management and housing can influence oral exposure to the pathogen. For example, keeping cow and calf together, providing hay, providing milk through a teat, using a more gradual weaning process and to provide access to pasture have been observed to reduce non-nutritional oral activities of animals, and such management actions may thereby reduce exposure to VTEC O157:H7.

- Despite many suggestions of drivers of transmission and possible on-farm measures, few intervention studies where the effect of measures are studied have been performed. To enable estimations of effectiveness, costs and benefits such studies are needed.

On animal level many questions about susceptibility and resistance to colonisation, which could help development of on-farm measures remain to be explored.
For example:

- It is clear that super-shedding matters and that there are individual drivers influencing colonisation – but to which extent does individual characteristics (in behaviour, immunity and/or susceptibility) influence the risk of colonisation compared to increased environmental exposure?

- Are shedding levels really higher in younger animals or are they simply higher in naïve animals encountering VTEC O157:H7 for the first time? To which extent can previous exposure to less virulent strains circulating in the cattle population influence colonisation of VTEC O157:H7?

- Are there different drivers of colonisation in young and old animals? If young animals are more susceptible to infection, but increased colonisation resistance develops over time, it is important to identify the source of this resistance. Is it related to changes in behaviour, microbiome, stress or immunity? Can we enhance this resistance in some way?

In paper I the added benefit of whole genome sequencing for understanding transmission is clear. Thanks to decreasing costs and increased availability of molecular methods it will be possible to look into:

- If on-farm transmission occurs and is driven by colonised animals or an environmental/other on-farm reservoir?

- The differences between on-farm persistence and reoccurrence of the bacteria. To which extent does on-farm persistence actually represent new introductions?

- What intrinsic factors are important for VTEC O157:H7 and enhances strains capability to persist in environment and to colonise cattle and humans?

- Is VTEC O157:H7 dependent or inhibited by other bacteria and are there other, less virulent, bacteria that can reduce VTEC O157:H7?

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Popular science summary

Escherichia coli is a species of bacteria with considerable variation. We are surrounded by harmless types that are normal parts of the environment and our microbiomes. However, some types are able to cause disease. Verotoxin-producing *E. coli* is a group of *E. coli* associated with serious disease in humans. In particular, the ones belonging to serotype O157:H7 are often the cause of severe disease and outbreaks. The symptoms range from mild to severe bloody diarrhoea. In some cases, often when children and elderly are infected, the verotoxin produced by the bacteria enters the body. The toxin can cause damage to kidneys, blood cells and the brain, which can lead to lasting injuries and even death.

Sweden has experienced an increasing trend of disease caused by this pathogen and in many cases the source of transmission is cattle. Thus, by reducing the presence of the bacteria among cattle, transmission to humans could be prevented. However, as the animals do not show any signs of carrying the bacteria it is difficult to know when and how to react. Although the bacteria does not affect cattle, it is not a normal part of their microbiota, which means that they can clear the infection under the right conditions.

In this thesis the transmission and dynamics of the bacteria among cattle are explored from several perspectives; between farms, on farms as well as on individual level.

By comparing farms in an area where the pathogen was circulating, we have investigated how farms where the bacteria is present differ from farms that remain free. Large farms with many close neighbouring farms were more likely to be infected, especially if they had contact with other farms where the bacteria was present. There were signs of frequent transmission of the bacteria between farms and it could take many routes. For example it appeared to hitch a ride with visitors travelling between farms or with animals that picked up the bacteria on pasture. By analysing bacterial DNA we could trace the relationships between bacteria from four of the farms and saw examples of how animals on one farm

picked up the bacteria of the neighbouring farm on pasture and brought it home to the farm.

On farms where the bacteria was present it was mostly found among the younger animals – perhaps because they were more susceptible as they had not encountered the bacteria before or because of a less developed, and therefore less resistant, gastrointestinal microbiota. However, it could also be that young animals are exposed to more bacteria from the environment. Animals can be exposed both through the environment and by contact with individuals carrying the bacteria and shedding it in their faeces. After studying transmission between animals between two sampling occasions, we propose that efforts reducing the bacteria's ability to survive in the environment, for example providing a dry bedding, are important to reduce transmission. However, reducing the environmental exposure is not enough because of a small number of animals, called super-shedders, that carry the bacteria and shed high levels – more than 10 000 bacteria per gram faeces. Thus, combining environmental efforts with keeping groups of animals together to prevent naïve individuals from being exposed to shedding animals is also required.

Super-shedding occurs following a temporary colonisation and growth in the intestine. To understand how animals that become colonised by the bacteria differ from the ones that do not, we used a novel approach to study individual differences of calves – comparing behaviour and indicators of welfare. We could see that active and social animals, compared to animals showing signs of poor welfare and health, were more likely to carry the bacteria. This indicates that the reason for colonisation may simply be an increased exposure to the bacteria by interactions with other calves, such as licking and buffing.

Previous studies have suggested that stress increases the susceptibility and risk of animals to become colonised. To study this we measured cortisol, a hormone that increases with stress, in hair samples from the calves. When the hair grows, cortisol circulating in the blood is stored in the hair and by analysing the level we can get an average of the cortisol level during hair growth. When we compared hair cortisol levels of calves colonised with the bacteria to other calves there was no difference. Although the lack of association may be due to cortisol being a poor indicator of stress (for example activity, like play, increases the levels while disease can lead to lower levels) the combined picture of calves that are social, active and not more nervous or fearful indicates that stress is not an important driver at young age.

With the results of this thesis, more targeted on-farm measures to reduce the presence of the bacteria on farms can be developed.

Populärvetenskaplig sammanfattning

Escherichia coli är en bakterieart som uppvisar stor variation. Många av dess medlemmar utgör en harmlös och normal del av vår miljö och vår tarmflora. Vissa typer av *E. coli* kan dock ställa till problem och orsaka sjukdom på olika sätt. Just en sådan typ av *E. coli* är verotoxin-producerande *E. coli* av serotyp O157:H7 (VTEC O157:H7). VTEC O157:H7 är en så kallad zoonos; en bakterie som sprids från djur, i det här fallet friska idisslare och framför allt nötkreatur, till människor. Djuren blir inte sjuka av att bära på den men vi människor kan drabbas av allvarlig magsjuka i form av blodig diarré. I vissa fall, särskilt när barn och äldre infekteras, tas verotoxinet - det gift som bakterien producerar - upp i kroppen där det kan orsaka allvarlig sjukdom genom att angripa njurar, blodcirkulationen och hjärnan. I värsta fall kan den orsaka men för livet och även leda till dödsfall.

I Sverige har vi de senaste åren haft ett ökande antal fall orsakade av denna bakterie och smittkällan är ofta nötkreatur. Genom att minska förekomsten bland djuren skulle smittspridning till människor kunna förhindras, men eftersom djuren inte visar några symptom, är det inte så lätt att veta när och hur man ska agera. Men trots att kor och kalvar inte påverkas synligt är den inte en del av djurens normala tarmflora, vilket betyder att de kan bli fria från den med rätt förutsättningar.

I den här avhandlingen undersöktes spridning och förekomst av bakterien bland nötkreatur från flera olika perspektiv; inom ett område, inom en gård och mellan djur på gården.

Genom att jämföra gårdar inom ett område där bakterien cirkulerade, har vi studerat vilka gårdar som riskerar att få in bakterien och hur den sprids. Större gårdar med många nära grannar visade ökad risk att smittas, särskilt om man hade kontakt med en annan smittad gård. Det verkar även som att bakterien kan spridas på många olika sätt mellan gårdarna. Den kan ta landsvägen och färdas med exempelvis besökare eller lifta med djur som varit på bete. Genom analys

av bakteriernas DNA kunde vi släktspåra bakterier på fyra gårdar och såg bland annat att djur plockar upp granngårdens bakterie på bete, kanske genom kontakter med djur eller via miljön, och tog med sig bakterien hem till gården.

Bland djuren på en infekterad gård fanns bakterien oftast bland de yngre djuren – kanske för att dessa är mer mottagliga då de inte stött på bakterien tidigare? Det kan även handla om att yngre djurs tarmflora inte lika utvecklad och därmed inte lika motståndskraftig. En annan möjlig orsak är att yngre djur får i sig större mängder bakterier. Djur kan exponeras för bakterien både via miljön och genom kontakt med andra individer som utsöndrar den i sin avföring. Efter att ha jämfört smittspridning mellan kalvar med 5 veckors mellanrum föreslår vi att det är viktigt att försvåra bakteriens överlevnad till exempel genom att se till att djuren har torrt strö. Åtgärder i miljön räcker dock inte eftersom enstaka djur som bär smittan kan utsöndra mycket stora mängder bakterier – mer än 10 000 bakterier per gram träck. Det är därför viktigt att kombinera miljöåtgärder med att undvika kontakter mellan djurgrupper för att förhindra att de utsöndrande djuren sprider smittan till nya djur.

Högutsöndring uppstår när bakterien tillfälligt etablerar sig och växer till i tarmen. Detta sker bara hos en liten andel av djuren på en gård. För att förstå hur djur som bär på smittan skiljer sig från andra använde vi oss av ett nytt angreppssätt – att jämföra beteende och välfärdsindikatorer. Vi såg att det var aktiva och sociala djur, till skillnad från sjuka och nedsatta djur, som oftare bar på bakterien. Detta tyder på att dessa djur helt enkel träffar på smittan i högre grad när de socialiserar och interagerar med andra kalvar, t.ex. genom att slicka och buffa på varandra.

Tidigare studier har även föreslagit att stress ökar risken att kalvar ska bli bärare och utsöndra bakterien. För att studera detta närmare mätte vi kortisol, ett hormon som ökar vid stress, i kalvarnas päls. När pälsen växer lagras kortisol som cirkulerar i blodet och man får ett medelvärde av kalvens kortisolnivåer under pälsens tillväxt. När vi jämförde kalvar som bar på bakterien med de som inte bar på bakterien såg vi ingen skillnad i kortisolnivåer. Det kan till viss del bero på att kortisolnivåer kan påverkas av många faktorer. Till exempel ökar kortisol även vid aktivitet, såsom lek, och kan sjunka eller stiga om djuren är sjuka. Eftersom djuren som bar på smittan inte var mindre välmående, snarare tvärt om, och inte visade tecken på att vara nervösa, rädda eller oroligare än andra kalvar, tror vi inte att stress är viktigt i ung ålder för om kalvar koloniseras eller ej.

Med den ökade kunskap vi fått genom avhandlingsarbetet kan vi utforma åtgärder som kan vara till nytta för lantbrukare som drabbas av bakterien, både för att minska smittan bland sina djur och förhindra att den sprids till besökare eller via andra vägar.

Acknowledgements

The work of this thesis was carried out at the Department of Clinical Sciences at the Swedish University of Agricultural Sciences. Funding was generously provided by the Swedish research council Formas (grant 221-2014-1553). In addition, sampling to part of the thesis was supported by funding from the Swedish Board of Agriculture.
A study stay at Massey University was supported by SLU's grant for internationalisation and presentations at international conferences were supported by travel grants from the Royal Swedish Academy of Agriculture and Forestry (KSLA).

There are so many people that have made this thesis possible. Most importantly, there would not have been any results if it had not been for the amazing **farmers** participating in the studies. I am so grateful to you for participation, taking the time, arranging restraining for the animals so the sampling could happen and patiently answering questions.

I have also been lucky to have five great, supporting supervisors to guide me through this work. Although it has sometimes been challenging to get everyone in the same room, your contributions and expertise in different fields have made this project truly multidisciplinary.

First and foremost: Thank you **Ulf Emanuelson** for being an extraordinary main supervisor and an inspiring person. You have supported me from the day I walked into your office with a vague idea of a project and a wish to do a PhD and I am so grateful for everything that has happened since. Every time I've gotten lost (and managed to over-think something) your guidance and enthusiasm has gotten me back on track and made the way ahead much clearer. I know I am not always (generally not) great at keeping things simple but I

promise to try. Still, after everything you've taught me about the world of epidemiology I'm not worried about facing a complex reality. :)

Linda Keeling, thank you for all the guidance in the world of animal welfare, coming out with me to Lövsta to practice animal-based assessment and helping me to put meaning and words to my results and thoughts. I am also grateful for all the time you have taken to provide feed-back on writing and invitations to connect with other welfare people, both at SLU as well as on the dance floor in Norway.

Johan Dicksved, somehow you always seem to know when I am in need of support or good advice, about the project in particular or science and academia in general. Thank you for your patience with me in the lab, the inspiring discussions as well as work on our side projects. I am so excited to work with you to finish the microbiota study.

Erik Eriksson – You have been my lifeline with your knowledge about all things of VTEC and constantly keeping an eye out for new farms to recruit. Even though you have been busy, you have always gotten back with an answer, arranged sampling material when a farm suddenly emerged and made sure everything has worked out with the analysis process. I am so happy that you decided to join the supervisor team, you have been invaluable.

Stefan Alenius – Thank you for bringing me on to your VTEC project and always being encouraging and supportive.

Although not an official supervisor, it feels like I have had an unofficial one looking out for me. Thank you **Robert Söderlund** for sharing your expertise in bioinformatics and for your friendship. I have enjoyed our inspiring discussions and beers in small towns around Sweden. I look forward to continuing working together and maybe realising some of those ideas we have discussed!

This work would not have been possible without the help of Gård och Djurhälsan and VÄXA. In particular I want to thank **Elöd Szanto** for all the help with recruiting farms and for teaching me so much about VTEC O157:H7 on farms. Another key player was **Isabell Karlsson,** thank you for your help in arranging sampling and recruiting farmers. Your enthusiasm and positivity is contagious. In addition, I want to thank **Andrea Holmström** and **Anita Jonasson** for giving me the opportunity to do hands-on work with on-farm measures on infected farms.

I also want to thank **Griselda, Stefan** and everyone else at "**EHEC-labb**" at for excellent assistance and analysis of my samples.

I have also gotten plenty of help within the different parts of the project by my co-authors.
Reed, thank you for help with coding, statistics as well as making sense of sometimes confusing results. Also for the warm welcome when I came to Massey and for bringing me along to Castle point, all the hikes and other events. I am so grateful for the time in NZ and our friendship.
Thank you **David** for getting me started on bioinformatics, constructive feedback on my writing and for explaining principal component regression in a way that finally made sense. Also thanks to **Mikhail** for help with spatial analyses, **Anna** at KV-lab for development of the hair sample analysis and **Maria** for development of questionnaires.

Of course, work gets easier when you have a group with great colleagues that support and inspire you.
Thank you **Karin A** for being such a good friend, our dog-walks and coffee-runs are the highlight of the day. Thank you for all the support and cheers during the past weeks.
Karin S, although I know the single office has probably been good for my productivity I miss my roommate and our chats about every day life. Thank you for your friendship and support through this time.
Dorota, I am so glad that we took that statistics course together and got to know each other. I could not have found a better companion to tackle the mysteries of R with.
Karolina, for sharing the burdens and joys of statistics, DAGs and being a PhD-student as well as the ups and downs in life. Kenzo wanted me to add that he is extra grateful for your small animal skills.
Nils, thank you for always being ready to share solid practical solutions to all problems that may appear, either at work or life in general, and making sure that we make the most of the time at conferences.
Isa, for always thinking about others and friendly advice. I miss the days when we had the phytotherapy project together.
Haseeb, Gabriella, Ann: Thank you for enthusiastic support during these last weeks. I look forward to getting started on our next project together!
Marjolein, you will always be part of the Epi-group to me. You are a really inspiring person and a wonderful friend.

Of course, this also includes all present and former colleagues from “IME”.
In particular, I want to thank **Lina** and **Hanna** for the support and good days at the ruminant clinic. I have learned heaps from both of you and so happy to have you as friends.
I also want to thank **Camilla** for excellent supervision during the work of my master-thesis, which became the start of this PhD.
I would also like to mention **Agneta, Jonas, Cecilia, Catarina, J-F** and **Sara.** Thank you for all the fikas, support and interesting discussions!

In addition to a great research group, no PhD is completed without the support and inspiration of fellow PhD-students.
Katrina – I am not sure how I would have made it through these last weeks without you. Thank you for the support, food and for widening my tunnel vision when needed. After that first Gotland summer with laughter as well as blood (yup - a lot of it), sweat and tears, I knew you were a keeper and I am so glad that we have ended up in the same place again.
Ida - Thank you for all the fika and for always being there with good advice about how to tackle all things in life. Be it writing, renovations, Kenzos health or other things I always feel so much better after talking to you.
Gunilla – I miss to accidentally walk in on you changing in the office and our dog walks. But I am so happy that you won’t be too far away.
Dinah and **Niclas** for the friendship, quizzes and chats about present and future life, as well as reminiscing about past days in veterinary practice.
Lisa, for all the excellent work within Husdjurssektionen as well as the fikas and chats about life.
Sara L, for your inspiring enthusiasm and contagious energy.
Emma, for sharing your experiences these past weeks and making me feel like I can make it.
Arata, Sabrina and Jun - thank you for the Zuu-sessions, chats and parties. I look forward to seeing you at the next ISVEE.

Also former and present PhD colleagues at SLU: **Malin Å, Josef, Torun, Daniel, Theo, Elisabeth, Ninni, Denise, Malin Ö, Elin, Celina** and **Emeli.** Thank you for making every day at work enjoyable!

I also want to thank **Ylva** for making the PhD-education run smoothly and **Anette, Annika, Zelal, Elinora** and **Sussie** for help with all other administrative questions.
Sanna, for your energy, all the work within SVF and giving me the opportunity to smell some horses.

Stefan W for help with questions about spatial analysis, good times, interesting discussions about VTEC and good times at conferences.
Tomas Jinnerot for help with prepping sequences and support at the bioinformatics course.
Guo for help with analysis of hair cortisol and PCR.
The students who made the samplings possible: Linnea, Ellen, Claire-Lise, Laurie, Daniel and Ulf.

Även ett stort tack till alla vänner som finns där när det gäller. Jag så glad för alla delade stunder, alla resor, middagar, spelkvällar, quizz-kvällar och andra festligheter.

Men ett extra stort tack måste gå till Eva – för ingen kan få en på bättre humör än du. Tack för att du alltid finns där, alla fantastiska kvällar, tröst i svåra stunder och härligt häng i vardagen.

Även ett stort tack till min kära familj för stöd och pepp, nu och alltid. Vad det än handlar om, finns ni alltid där, engagerade och fulla av goda råd. Det är oklart när lillasyster blev så klok dock... Extra tack till mamma och pappa för alla middagar på väg till eller från provtagning på Öland, såklart även för medföljande studenter, medarbetare och annat löst folk. Ni är bäst.

Slutligen, Kristoffer och Kenzo. Tack för att jag får dela mitt liv med er. Vi gör inte som alla andra men vad gör det när man är världens bästa team.

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2020:16

The aim of this thesis was to provide a holistic view of drivers of transmission and susceptibility of the zoonotic pathogen VTEC O157:H7 from a regional, farm and animal perspective. The results show frequent transmission between farms in a cattle dense area and that transmission occurs through human and animals contacts. A small proportion of colonised "super-shedders" were important for transmission and colonisation was associated with being a social and active calf.

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Online publication of thesis summary: http://pub.epsilon.slu.se/

ISSN 1652-6880
ISBN (print version) 978-91-7760-550-8
ISBN (electronic version) 978-91-7760-551-5