

Helsinki, Finland

and

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Division of Microbiology and Biotechnology  
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Helsinki, Finland

# **Foodborne human isolates of *Salmonella* and Shiga toxin -producing *Escherichia coli* of domestic origin in Finland**

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ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in Auditorium 1041, Biocenter 2, Viikinkaari 5, on 9.10.2015, at 12 noon.

Helsinki 2015

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This thesis is published in YEB series, 21/2015  
Cover Photo: Taru Lienemann  
Layout by Tinde Päivärinta, PSWFolders Oy

ISBN 978-951-51-1480-8 (printed)  
ISBN 978-951-51-1480-5 (PDF)  
ISSN 2342-5423 (printed)  
ISSN 2342-5431 (PDF)

Hansaprint, Vantaa 2015

*To my family*

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# ABSTRACT

*Salmonella* is one of the most commonly reported foodborne pathogens and enterohaemorrhagic *Escherichia coli* (EHEC) is one of the most dangerous. They both spread by zoonotic and person-to-person transmission routes. Most *Salmonella* infections are characterized by mild-to-moderate self-limited diarrhea but also serious disease resulting in death has been reported. Bloody diarrhea is a common symptom of human EHEC infection and the infection may lead to severe post-infection disease such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and even death.

The purpose of this thesis was to investigate the diversity of *Salmonella* and EHEC strains isolated from domestically acquired infections using several pheno- and genotyping methods for surveillance and outbreak investigation purposes as well as evaluate certain epidemiological typing methods in Finland. All *Salmonella* and EHEC isolates of domestic origin during 2007-2014 in Finland were studied. Serotyping, phage typing, antimicrobial susceptibility testing, phenotype microarray, pulsed-field gel electrophoresis (PFGE) and multilocus variable-number tandem repeat analysis (MLVA) were applied as epidemiological typing tools for *Salmonella* isolates. EHEC isolates were analyzed using serotyping, phage typing, antimicrobial susceptibility testing, virulence gene detection (*stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae*, *hlyA* and *saa*) and PFGE.

During the study period, the number of domestically acquired *Salmonella* infections has decreased about one fifth compared to the previous decade whereas the number of domestic EHEC infections have increased about one third. The incidence of *Salmonella* infections was highest in 2012 (7.5/10<sup>5</sup> population) and lowest in 2014 (5.4/10<sup>5</sup> population). The incidence of EHEC infections was highest in 2013 (0.33/10<sup>5</sup> population) and lowest in 2008 (0.07/10<sup>5</sup> population). 15% of all *Salmonella* strains and 70% of all EHEC strains were considered domestically acquired. A total of 131 different *Salmonella* serovars were detected. The most common serovars were Typhimurium (32%), Enteritidis (15%) and group B (6%). Among Typhimurium strains, phage types DT1 (37%), RDNC (18%) and DT104 (9%) were the most common ones. The most frequently detected phage types among the domestically acquired *S. Enteritidis* infections were PT8 (17%), PT1B (14%) and PT4 (13%). The majority of domestic Typhimurium and Enteritidis (60%) strains were susceptible to tested antimicrobials. During the study period, a total of 188 infections caused by EHEC were detected. Most of them were caused by serotype O157:H7 (60%). The majority of O157 strains (63%) were unable to ferment sorbitol. PT8 was the most common phage type among the sorbitol-negative and PT88 among sorbitol-positive O157 strains. Among non-O157, 22 distinct O:H serotypes were detected. The most common ones were O26:H11, O103:H2 and O145:H-. The majority of domestic EHEC strains (81%) were susceptible to all tested antimicrobials: 96% of O157:H7/H- and 60% of non-O157 strains. All O157 strains carried *stx*<sub>2</sub> (40% in combination with *stx*<sub>1</sub>), *eae* and *hlyA* genes. In contrast, 55% of non-O157 strains had *stx*<sub>1</sub> gene and 76% carried *eae* and *hlyA* genes.

The MLVA method was found to be a powerful epidemiological tool for *S. Typhimurium* with discriminatory power similar to PFGE. In addition, MLVA was faster, cheaper and the results

were easier to compare between laboratories. The domestic *S. Typhimurium* strains were divided into 170 distinct MLVA types (diversity index 0.891). In MLVA, the three most common profiles (3-16-NA-NA-0311, 3-15-NA-NA-0311 and 3-17-NA-NA-0311) counted for 47% of the strains showing that the lack of locus STTR6 and locus STTR10p was characteristic for domestic *S. Typhimurium*. However, XbaI-PFGE remains a useful genotyping method for investigations of other *Salmonella* serovars and EHEC strains. The interpretation of XbaI-PFGE profiles can be challenging as demonstrated by a Finnish nationwide outbreak caused by *S. Newport* and *S. Reading* -contaminated iceberg lettuce. The *S. Reading* strains had four different XbaI-PFGE profiles. Based on epidemiological information, all these different variants of the outbreak causing strains were considered as outbreak-related.

The sources of the most EHEC outbreaks remained undetermined. In one out four EHEC O157 outbreaks, unpasteurized milk was found as the source of the infections. Although 40% of the domestic EHEC strains were non-O157, only strains of serogroup O157 caused outbreaks in Finland. However, non-O157 strains caused several family clusters and were linked with HUS. In 2009, a sorbitol-fermenting EHEC O78:H:*stx*<sub>1c</sub>:*hlyA* was detected in blood and fecal samples of a neonate. This EHEC serotype had not been seen in Finland prior to this family-related outbreak and bacteremia caused by EHEC is exceptionally rare.

Taken together, *Salmonella* and EHEC infections are a major public health concern. This thesis provides new information about the characteristics of *Salmonella* and EHEC strains isolated from domestically acquired infections in Finland and evidence that effective surveillance is needed for early detection and prevention of the spread of *Salmonella* and EHEC infections. In particular, typing methods used should be internationally harmonized and the results made comparable.

# TIIVISTELMÄ

*Salmonella* on yksi yleisimmistä elintarvikevälikteisistä bakteeripatogeeneista, enterohemorraginen *Escherichia coli* (EHEC) yksi vaarallisimmista. Molemmat bakteerit ovat eläimen ja ihmisen välityksellä leviäviä ja tarttuvat myös henkilöstä toiseen. Useimmat salmonellojen aiheuttamat infektiot ovat lieviä, itsestään parantuvia suolistoinfektioita, mutta myös vakavia, kuolemaan johtavia tartuntoja todetaan. Veriripuli on yleinen oire erityisesti pikkulasten EHEC-infektiossa. Infektio saattaa johtaa myös vakaviin jälkitauteihin kuten hemolyyttisüreemiseen oireyhtymään (HUS), tromboottiseen trombosytopeeniseen purppuraan (TTP) ja jopa kuolemaan.

Tässä työssä tutkittiin salmonella- ja EHEC-kantojen ominaisuuksia useilla ilmiäsuun (fenotyyppi) ja perimään (genotyyppi) perustuvilla menetelmillä. Lisäksi selvitettiin tiettyjen genotyypitysmenetelmien soveltuvuutta epidemiologiseen seurantaan ja tartuntalähteiden jäljittämiseen. Erityisesti oltiin kiinnostuneita niistä salmonella- ja EHEC-kannoista, joiden tartunta oli saatu Suomessa. Tutkimus käsitti kaikki kotimaiset vuosina 2007-2014 eristetyt salmonella- ja EHEC-kannat. Salmonella-kantojen epidemiologiseen tyyppittämiseen käytettiin neljää fenotyypitysmenetelmää, serotyypitystä, faagityypitystä, mikrobilääkeherkkyysmäärittystä ja mikroarray testiä sekä kahta genotyypitysmenetelmää, pulssikenttägeelelektroforeesia (PFGE) ja multilocus variable-number tandem repeat analysis (MLVA) testiä. EHEC-kantojen tyyppitykseen käytettiin O:H-serotyypitystä, faagityypitystä, mikrobilääkeherkkyysmäärittystä, virulenssigeenien määrittystä (*stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae*, *hlyA* and *saa*) ja PFGE -menetelmää.

Tutkimusaikavälillä kotimaassa saatujen salmonellainfektioiden määrä laski noin viidenneksen verrattuna edelliseen vuosikymmeneen kun taas EHEC-tartuntojen lukumäärä nousi noin kolmanneksen. Kotimaisten salmonellatartuntojen ilmaantuvuus oli korkeimmillaan vuonna 2012 (7.5/10<sup>5</sup> asukasta) ja alimmillaan vuonna 2014 (5.4/10<sup>5</sup> asukasta). Kotimaisten EHEC-infektioiden ilmaantuvuus oli korkein vuonna 2013 (0.33/10<sup>5</sup> asukasta) ja matalin vuonna 2008 (0.07/10<sup>5</sup> asukasta). Kaikista salmonellatartunnoista 15 % ja EHEC-tartunnoista 70 % oli kotimaista alkuperää. Kotimaisia salmonellatartuntoja aiheutti yhteensä 131 eri serotyyppiä. Niistä yleisimmät olivat Typhimurium (32 %), Enteritidis (15 %) ja ryhmä B (6 %). Kotimaisten *S. Typhimurium* -kantojen yleisimmät faagityypit olivat FT1 (37 %), NST (18 %) ja FT104 (9 %). Kotimaista alkuperää olevien *S. Enteritidis* -kantojen yleisimmät faagityypit olivat FT8 (17 %), FT1B (14 %) ja FT4 (13 %). Suurin osa kotimaisista Typhimurium- (60 %) ja Enteritidis-kannoista (60 %) olivat herkkiä testatuille mikrobilääkkeille. Tutkimusaikavälillä todettiin 188 EHEC-tartuntaa, joista valtaosan aiheutti serotyyppi O157:H7 (60 %). Suurin osa EHEC O157-kannoista (63 %) oli sorbitoli-negatiivisia. Faagityyppi FT8 oli yleisin sorbitoli-negatiivisten ja faagityyppi FT88 sorbitoli-positiivisten O157 kantojen keskuudessa. Non-O157 kannat tyyppittyivät yhteensä 22 eri O:H serotyyppiin. Yleisimmät non-O157 serotyypit olivat O26:H11, O103:H2 ja O145:H-. Suurin osa kotimaista alkuperää olevista EHEC-kannoista (81 %) oli herkkiä testatuille mikrobilääkkeille: 96 % kaikista O157:H7 ja 60 % non-O157 kannoista. Kaikki O157 -kannat kantoivat geenejä *stx*<sub>2</sub> (40 %:lla kannoista oli myös *stx*<sub>1</sub> geeni), *eae* ja *hlyA* kun taas valtaosa non-O157 kannoista kantoi geeniä *stx*<sub>1</sub> (55 %) ja 76 %:lla kannoista oli geenit *eae* ja *hlyA*. MLVA -menetelmä osoittautui toimivaksi epidemiologiseksi tyyppitysmenetelmäksi, ja sen erottelukyky oli yhtä hyvä kuin aiemmin käytetyn PFGE -menetelmän. Lisäksi MLVA oli



nopeampi ja halvempi kuin PGFE menetelmä ja tulokset olivat helpommin kansainvälisesti vertailtavissa. Kotimaista alkuperää olevat *S. Typhimurium* -kannat jakaantuivat 170 eri MLVA-tyyppiin (diversiteetti-indeksi 0,891). Noin puolet (47 %) kannoista kuului kolmeen yleisimpään MLVA -tyyppiin (3-16-NA-NA-0311, 3-15-NA-NA-0311 ja 3-17-NA-NA-0311). Lokuksien STTR6 ja STTR10p puuttuminen oli tyypillistä kotoperäisille *S. Typhimurium* -kannoille. XbaI-PFGE oli kuitenkin hyödyllinen menetelmä muiden salmonellan serotyyppeiden ja EHEC-kantojen genotyyppittämiseen. XbaI-PFGE tulosten tulkitseminen saattaa olla haastavaa, minkä myös maanlaajuinen *S. Newport/S. Reading* -epidemia vuonna 2009 havainnollisti. Kyseisessä epidemiassa *S. Reading* kannat jakaantuivat neljään eri PFGE -tyyppiin, joiden kuitenkin katsottiin epidemiologiseen tietoon perustuen liittyvän samaan epidemiaan.

Useimpien EHEC-epidemioiden lähteet jäivät tuntemattomiksi. Tutkimusaikavälillä havaittiin neljä EHEC -bakteerin aiheuttamaa epidemiaa ja useita perheensisäisiä rypäitä, joista yhdessä lähteeksi todettiin pastöroimaton maito. Vaikka 40 % kaikista kotoperäisistä EHEC -infektioista oli non-O157 serotyyppeiden aiheuttamia, vain serotyyppi O157 aiheutti epidemioita Suomessa. Non-O157 kannat aiheuttivat kuitenkin perheensisäisiä tartuntoja ja yhdistettiin vakaviin jälkitauteihin (HUS). Vuonna 2009 sorbitoli-positiivinen EHEC O78:H:*stx*<sub>1c</sub>:*hlyA* eristettiin vastasyntyneen verestä ja ulosteesta. Kyseistä serotyyppiä ei ollut havaittu Suomessa aikaisemmin ja EHEC -bakteerin aiheuttamat verenmyrkytykset ovat kansainvälisestikin erittäin harvinaisia. *Salmonella* ja EHEC aiheuttavat merkittäviä kansanterveysongelmia. Tässä työssä saatiin merkittävää uutta tietoa Suomessa todettujen kotimaista alkuperää olevien salmonella- ja EHEC-infektioiden aiheuttajakakteereiden ominaisuuksista. Tehokas salmonella- ja EHEC-seuranta auttaa havaitsemaan alkavat epidemiat ja estämään niiden leviämisen. Erityisesti uusia tutkimusmenetelmiä kehitettäessä tulisi huomioida, että tyyppitysmenetelmä on kansainvälisesti harmonisoitu ja tulokset vertailukelpoisia.

# ACKNOWLEDGEMENTS

This work was carried out at the Bacterial Infections Unit, National Institute for Health and Welfare (THL), Helsinki, Finland. I wish to thank the current and former Director Generals of the THL Professor Juhani Eskola and Professor Pekka Puska, and the Heads of Department Professor Mika Salminen, Professor Petri Ruutu and Professor Pentti Huovinen, and the Heads of Unit Adjunct Professor Jari Jalava, Dr. Saara Salmenlinna and Research Professor Anja Siitonen for the opportunity to carry out my research at THL and for providing excellent working facilities for this study. I also thank Professor Per Saris at the Department of Food and Environmental Sciences, University of Helsinki for his positive and flexible attitude towards my studies.

I would like to express my deepest gratitude to my supervisor Research Professor Anja Siitonen, for her continuing support and guidance throughout the project. I really appreciate our discussions together, and her trust in my abilities. She is incredibly inspiring to work with, encouraging, hard-working and I am amazed by her knowledge in the field of *Salmonella* and EHEC research. Adjunct Professor Kaisa Haukka, my other supervisor, played an invaluable role in the design and realization of the “Food-Bug”-project. She is “a true scientist” with an innovative mind and she was of great personal support throughout my thesis work.

I sincerely thank the official reviewers of my thesis Adjunct Professor Merja Rautio, HUSLAB, and Adjunct Professor Antti Hakanen, University of Turku, for taking time to comment on my thesis and for their expertise. I also thank all my co-authors: Manal Abu Oun, Muna Anjum, Sandra Guedes, Jani Halkilahti, Jari Hirvonen, Markku Kuusi, Aino Kyyhkynen, Taina Niskanen, Ruska Rimhanen-Finne, Kai Rönholm, Eeva Salo, Mari Taimisto, Eveliina Tarkka and Martin Woodward, for their collaboration and their input to the publications. I want to give special thanks to Susanna Lukinmaa-Åberg and Ruska Rimhanen-Finne, the members of my official follow-up and support group, for the advice and help they gave me, both in work and in private life. I also wish to thank all the colleagues who participated to “Food-Bug”-project founded by Academy of Finland. I acknowledge all my current and former colleagues at the Bacterial Infections Unit, all of you have contributed to this thesis in one way or another. It has been a pleasure and privilege to work with you. I warmly thank all current and former fellow Ph.D. students and researchers Ulla-Maija Nakari, Leila Sihvonen, Anni Vainio, Lotta Siira, Outi Nyholm, Silja Mentula, Salla Kiiskinen, Tuula Siljander, Salha Ibrahim, and the researchers in the neighbouring laboratories as well, for their friendship, the great discussions and black humour in the cafeteria. Matjut Eklund and Tarja Heiskanen who taught me all about the typing methods for EHEC and Aino Kyyhkynen, Anna Wiklund, Nina Aho, Ritva Taipalinen and Marja Veckström who showed me how to handle *Salmonella* are acknowledge for their skilful laboratory work. I thank Jani Halkilahti for his help with all my IT problems and Kirsi Mäkisalo and Sari Enckell for taking care of the paper work.

Finally, I would like to thank my parents, Raila and Asko, and all my friends for their support and for always being proud of me no matter what happens, and the two men in my life; Michael and Eerik, without whose love and understanding I never would have made it through.

Helsinki, 2015  
*Taru Lienemann*

# LIST OF ORIGINAL PUBLICATIONS

The original publications are hereafter referred to as studies and by their roman numerals (I, II, III and IV). In addition, some unpublished data are presented in this thesis.

1. Kauko, T., Haukka, K., Abu Oun, M., Anjum, M., Woodward, M., Siitonen, A. Phenotype MicroArray™ in Metabolic Characterization of *Salmonella* Serotypes Agona, Enteritidis, Give, Hvittingfoss, Infantis, Newport and Typhimurium. Eur J Clin Microbiol Infect Dis. 2010; 29(3):311-317.
2. Lienemann, T., Niskanen, T., Guedes, S., Siitonen, A., Kuusi, M., Rimhanen-Finne, R. Iceberg lettuce as suggested source of a nationwide outbreak caused by two *Salmonella* serotypes, Newport and Reading, in Finland in 2008. J Food Prot. 2011;74(6):1035-1040.
3. Lienemann, T., Halkilahti, J., Kyyhkynen, A., Haukka, K., Siitonen, A. Characterization of *Salmonella* Typhimurium isolates from domestically acquired infections in Finland by phage typing, antimicrobial susceptibility testing, PFGE and MLVA. BMC Microbiol. 2015; 2;15:131.
4. Lienemann, T., Salo, E., Rimhanen-Finne, R., Rönholm, K., Taimisto, M., Hirvonen, J., Tarkka, E., Kuusi, M., Siitonen A. 2012. Shiga toxin- producing *Esherichia coli* serotype O78:H(-) in family, Finland, 2009. Emerg Infect Dis. 2012;18(4):577-581.

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# ABBREVIATIONS

A	Ampicillin
A/E	Attaching and effacing lesions
C	Chloramphenicol
CDC	Centers for Disease Control and Prevention, USA
Cp	Ciprofloxacin
Ct	Cefotaxime
DAEC	Diffusely adherent <i>Escherichia coli</i>
DNA	Deoxyribonucleic acid
DEC	Diarrheal <i>Escherichia coli</i>
DI	Discrimination index
DT	Definite phage type
EAEC	Enteraggregative <i>Escherichia coli</i>
ECDC	European Centre for Disease Control
EHEC	Enterohamorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
ETEC	Enterotoxigenic <i>Escherichia coli</i>
Evira	Finnish Food Safety Authority (Elintarviketurvallisuusvirasto in Finnish)
ExPEC	Extra-intestinal pathogenic <i>Escherichia coli</i>
FSCP	Finnish salmonella control program
G	Gentamicin
H antigen	Flagellar antigen
HUS	Hemolytic uremic syndrome
I	Imipenem
kb	Kilo-base
M	Mecillinam
MAEC	Meningitis-associated <i>Escherichia coli</i>
MDR	Multi-drug resistance
MLST	Multilocus sequence typing
MLVA	Multilocus variable-number tandem-repeat analysis
MST	Minimum spanning tree
NIDR	National Infectious Diseases Register
NT	Not typeable
Nx	Nalidixic acid
LEE	Locus of enterocyte effacement
Lpf	long polar fimbriae
LPS	Lipopolysaccharide
O antigen	Somatic surface antigen
R	O Rough
PCR	Polymerase chain reaction
Pef	Plasmid-encoded fimbriae
PFGE	Pulsed-field gel electrophoresis
PM	Phenotype Microarray
pSLT	Salmonella virulence plasmid
PT	Phage type

RDNC	Reacts but does not confirm
S	Streptomycin
sor+	Sorbitol fermenting
sor-	Sorbitol non-fermenting
SMAC	Sorbitol MacConkey agar
SNP	Single nucleotide polymorphism
SPI	Salmonella pathogenicity island
SSI	Staten Serum Institute
STEC	Shiga toxin -producing <i>Escherichia coli</i>
Stx	Shiga toxin
Su	Sulphonamide
T	Tetracycline
THL	National Institute for Health and Welfare (Terveyden ja hyvinvoinninlaitos in Finnish)
Tm	Trimethoprim
TTP	Thrombotic thrombocytopenic purpura
UPGMA	Unweighted pair group method with arithmetic mean
UPEC	Uropathogenic <i>Escherichia coli</i>
VNTR	Variable number of tandem repeats
VTEC	Verocytogenic <i>Escherichia coli</i>
WHO	The World Health Organization
WGS	Whole genome sequencing



# 1 INTRODUCTION

*Salmonella enterica* and enterohemorrhagic *Escherichia coli* (EHEC) are important foodborne bacteria and infections spread via contaminated food and water that make millions of people ill worldwide annually [1, 2]. These bacteria are spread by zoonotic transmission route or through direct person-to-person contact causing gastrointestinal infections in humans [3, 4]. The consequence of these infections is a serious impact on the economy in terms of lost productivity and medical expenses. After *Campylobacter*, *Salmonella* infections are the most common cause of diarrheal diseases in the industrialized countries while EHEC infections are rare but often linked with severe disease and sequelae [5, 6]. Various serovars of *S. enterica* cause about 2,000 infections in Finland yearly; of them, 15-20% are considered of domestic origin. Serovars Typhimurium and Enteritidis are the most common [7]. Symptoms of salmonellosis range from a self-limiting mild diarrhea to severe bacteremia and infections might be fatal especially to infants, the elderly and immunocompromised individuals [8]. In addition, even asymptomatic infections may result in post-infectious complications such as reactive arthritis. In contrast to *Salmonella* infections, only about 10-100 EHEC infections are detected annually in Finland. Of them, the majority (ca. 80%) are considered domestically acquired [7]. The main symptoms of EHEC infections are watery and bloody diarrhea but the disease may progress to severe post-infectious complications such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and even death [4].

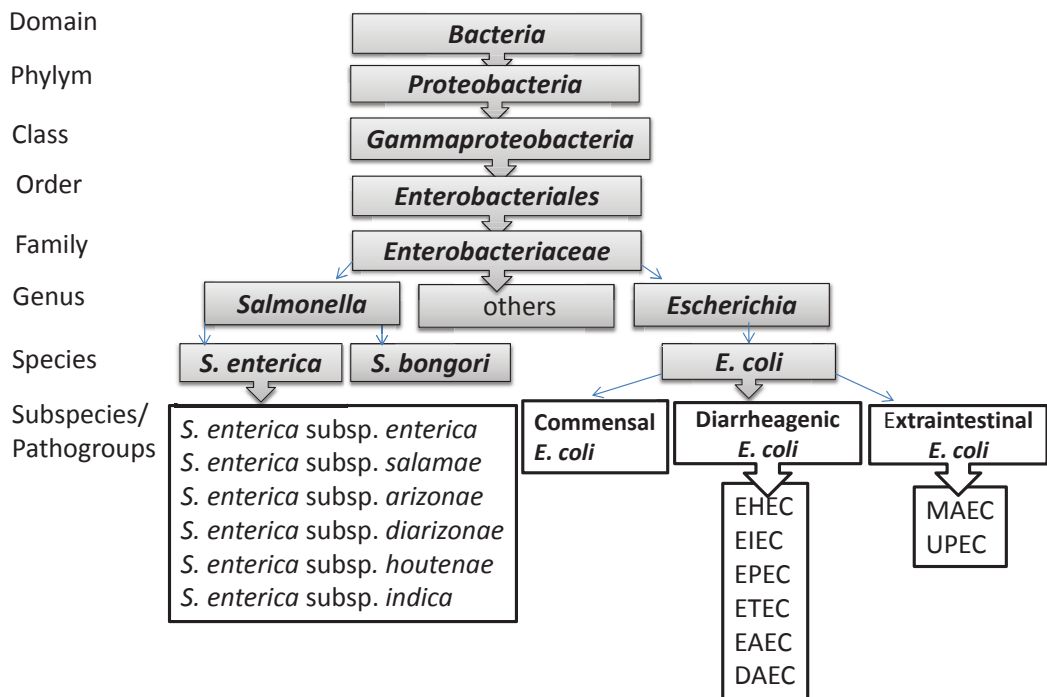
The overall annual number of *Salmonella* infections has decreased. However, incidence has increased for some domestic serovars and decreased for others. Presumably at least some of the domestic cases have been associated with contaminated imported foodstuffs, changes in human lifestyle and industry. In contrast to the declining trend of *Salmonella* infections, the numbers of EHEC infections have risen. Although *Salmonella* and EHEC can cause foodborne outbreaks, the majority of the reported cases are sporadic [9]. The sources and transmission routes of previous domestic cases of gastrointestinal disease have remained largely unknown. Therefore, in order to monitor changes among domestic foodborne bacteria, to detect family-related clusters and large outbreaks, and to compare bacterial genotypes internationally, accurate and internationally harmonized typing methods are required.

The purpose of this study was to characterize domestic *Salmonella* and EHEC strains in detail using several pheno- and genotypic methods and trace back the sources of human infections. Several typing methods were designed and set up. The study aimed also to evaluate typing methods for routine use in reference laboratories and for epidemiological outbreak investigations.

## 2 REVIEW OF THE LITERATURE

### 2.1 Nomenclature, classification and general characteristics of *Salmonella*

The history of the *Salmonella* species dates back to 1885, when the organism today known as *Salmonella* was first isolated from pigs by Dr. D.E. Salmon and Dr. T. Smith [10]. Later in 1896, Dr. F.M. Widal found that the serum of a typhoid patient agglutinated the typhoid bacillus which was the base for the serological diagnosis of *Salmonella* Typhi infection in humans [11]. Initially, the bacterium was named in honor of Dr. Salmon by Lignières in 1900. The nomenclature for the genus *Salmonella* is complex because of the ever-changing nomenclature system, newly detected species and different systems used to refer to this genus [12]. The *Salmonella* nomenclature has evolved over time and at the early stages each *Salmonella* serovar was considered a separate species [13]. However, this one serovar – one species concept was found to be misleading since most serovars cannot be distinguished by biochemical tests. Various other taxonomy proposals have been made based on the clinical role of the different strains, their biochemical characteristics and their genomic relatedness [14-17] leading to the current nomenclature system with only two species including more than 2,600 serovars [18] which are distinguished by antibody interactions with the somatic O and flagellar H and in some cases capsular polysaccharides (Vi antigen) surface antigens. The exact reasons for this high level of surface-antigen diversity are still unknown. In order to avoid confusion between *Salmonella* serovars and species, the name of the serovar starts with a capital letter and is not italicized. The former *S. typhimurium* is now written in the form *S. enterica* supsp. *enterica* serovar Typhimurium or briefly *S. Typhimurium*.



**Figure 1:** Taxonomy of *Salmonella* and *Escherichia coli* species.



Table 1: Comparison of characteristics of *Salmonella* species and some *E. coli* types (Modified from Pui et al., 2011; [19]).

Species	<i>Salmonella enterica</i> subsp.						<i>Salmonella bongori</i>	<i>E. coli</i> O157	<i>E. coli</i> non-O157
	<i>enterica</i> I	<i>salamae</i> II	<i>arizonae</i> IIIa	<i>diarizonae</i> IIIb	<i>houtenae</i> IV	<i>indica</i> VI			
<b>Subspecies Classification</b>	<i>enterica</i> I	<i>salamae</i> II	<i>arizonae</i> IIIa	<i>diarizonae</i> IIIb	<i>houtenae</i> IV	<i>indica</i> VI			
<b>Habitat</b>	Warm-blooded animals	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment	Cold-blooded animals & environment	Warm-blooded animals	Warm-blooded animals	
<b>Infective dose</b>	high (usually 10 <sup>5</sup> -10 <sup>7</sup> cells)	high	high	high	high	high	low (usually 1-100 cells)	low	
<b>Morphological characteristics</b>									
Gram staining	-	-	-	-	-	-	-	-	
Motility	+ (except <i>S. Gallinarum</i> and <i>S. Pollurum</i> )	+	+	+	+	+	+/-	+	
Shape	rod	rod	rod	rod	rod	rod	rod	rod	
Size (width x length, µm)	0.7-1.5 x 2-5	0.7-1.5 x 2-5	0.7-1.5 x 2-5	0.7-1.5 x 2-5	0.7-1.5 x 2-5	0.7-1.5 x 2-5	0.25-1 x 2	0.25-1 x 2	
<b>Growth characteristics</b>									
Temperature optimum (°C)	35-37	35-37	35-37	35-37	35-37	35-37	35-37	35-37	
pH optimum	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	6.5-7.5	
<b>Selected biochemical characteristics *</b>									
β-Glucuronidase	d	d	-	+	-	d	-	+	
Galacturonate	-	+	-	+	+	+	+	-	
Gelatinase	-	+	+	+	+	+	-	-	
Hydrogen sulfide	+	+	+	+	+	+	-	-	
Indole test	-	-	-	-	-	-	+	+	
Lactose fermentation	-	-	-	+	-	d	+	+	
Malonate fermentation	-	+	+	+	-	-	-	-	
Sorbitol fermentation	+	+	+	+	+	-	d	+	
<b>Serotypes</b>	>1500	502	95	333	72	13	22	>450	
<b>Some selected serotypes</b>	Typhimurium, Enteritidis, Paratyphi, Typhi, Choleraesuis	9,46:z:z39	43:x29:-	6,7:l,v:1,5,7	21:m,t-	59:z36:-	13,22:z39:-	O157:H7	O26:H11/H-, O103:H2/H-, O145:H28/H-

\* + =more than 90% positive reactions; - = less than 10% positive reactions; d= different reactions given by different serovars or strains

*Salmonella* species belong to the same proteobacterial family as e.g. *Escherichia coli*, *Shigella*, *Yersinia* and others in the family of *Enterobacteriaceae* (Fig. 1). *Salmonella* diverged from *E. coli* approximately 100-160 million years ago and acquired the ability to invade host cells [20, 21]. According to current taxonomy, the genus of *Salmonella* is divided into two species, *S. enterica* and *S. bongori* (Table 1). The species *S. enterica* is further subdivided into six subspecies named (or numbered) as follows: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) [22]. Of these six subspecies, subspecies I is most often associated with salmonellosis in warm-blooded animals [23]. The other subspecies usually originate from cold-blooded animals and the environment [24]. Each of the subspecies contains multiple serovars which are listed in the White-Kauffmann-Le Minor scheme [18]. The World Health Organization (WHO) Collaborating Center for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France is responsible for updating the list when new serovars are recognized [18, 25, 26]. According to the current nomenclature, serovar specific names are only given to the serovars of subspecies I. The unnamed species of other subspecies are designated based on their antigenic formulas determined according to the White-Kauffmann-Le Minor scheme. In addition, the *Salmonella* strains can be divided into typhoid *Salmonella* and non-typhoid *Salmonella*, based on clinical symptoms. The former strains are the causative agents of enteric fever and they include serovars *S. Typhi* and *S. Paratyphi* A, B, C. The latter group includes the remaining enteric *Salmonella* strains.

*Salmonella* are generally considered as facultative anaerobe, Gram-negative, motile (chicken-adapted serovars Gallinarum and Pullorum are an exception), non-lactose fermenting, oxidase negative, urease negative, citrate positive and potassium cyanide negative, rod-shaped bacteria which are about 2-5 x 0.7-1.5 µm in size [27]. *Salmonella* species can adapt to extreme environmental conditions. For example, *Salmonella* can grow at temperature between 5-47°C with an optimum temperature of 35-37°C [28]. They are sensitive to heat and killed at temperature of 70°C or higher. *Salmonella* grow in a pH range of 4.5 to 9.5 with an optimum between pH 6.5 and 7.5. Acid-adapted *Salmonella* species have raised a concern regarding the safety of fermented foods such as cured sausages and fermented raw milk products. They prefer high water activity (*aw*) between 0.99 and 0.94 but can also survive at *aw* <0.2 as found in dried foods [29].

## 2.2 Salmonellosis in humans

*Salmonella* infections in humans are in general zoonotic, but some serovars such as *S. Typhi* and *S. Paratyphi* A and B colonize only humans [30]. Salmonellosis may cause significant social and economic costs due to lost productivity and through their impact on industry and agriculture. Most *Salmonella* infections are sporadic, and only about 5-20% of them are associated with outbreaks [31]. There is a clear seasonal trend among *Salmonella* infections, peaking after warm summer months [32-34].

### 2.2.1 Risk factors and sources for *Salmonella* infections

About 95% of human salmonellosis results from the ingestion of contaminated foods [35], particularly foods of animal origin such as poultry, eggs, pork, and dairy products [36-39]. The consumption of raw eggs has been especially identified as the primary risk factor for human *S. Enteritidis* infection [37, 40]. In contrast to *S. Enteritidis*, transmission routes for *S. Typhimurium* are more diverse and less well-known. Several risk factors for *S. Typhimurium* have been identified, including consumption of beef [41] pork [42], dairy products made with unpasteurized milk [43], exposure to animals [44] and playing in sandboxes [40]. In addition, imported food items have been identified as the most important source for sporadic domestic cases, responsible for 12% of the cases in Denmark [45] and 6.4% of the cases in Sweden [46]. Other vehicles for salmonellosis include fresh fruits and vegetables [47-49], spices and herbs [50], and water [51, 52]. Also, reptiles [24], direct person-to-person transmission [53, 54] and direct animal contact [55] have been implicated. Also, travel has been recognized as relevant for the burden of human salmonellosis in countries with low levels of *Salmonella* in domestic animals including cattle, swine and poultry [9]. Unusual vehicles for human salmonellosis include smoked salmon [56] and certain foods with low water activity such as peanut butter [57]. The knowledge of the sources or vehicles for *Salmonella* infections are mostly based on case-control studies and outbreak investigations. These results are of interest because they highlight the multiplicity of food items and *Salmonella* serovars that have been associated with human disease.

### 2.2.2 Occurrence and epidemiology of *Salmonella* infections

*Salmonella* infections are a significant public health concern around the world [1, 58] and the incidences vary between 15-54 per 100,000 inhabitants in developed countries (Table 2). Only a small proportion of cases are detected and actually reported. According to one study, in industrialized countries as few as 1% of clinical cases are reported [59]. The annual global burden of non-typhoid *Salmonella*-mediated gastroenteritis has been estimated as high as 93.8 million cases, with 155,000 deaths [1]. Although more than 2,600 potentially infectious *Salmonella* serovars have been reported, most human infections are caused by limited number of serovars [60]. In most developed countries, the serovar *Enteritidis* and *Typhimurium* are the most commonly reported causatives of human salmonellosis while other serovars are more prevalent in specific regions e.g. serovars *Stanley* and *Weltevreden* in Southeast Asia [61-63]. In the United States (USA) alone the estimated annual incidence of *Salmonella* infections is approximately 1.4 million human infections with at least 22% of cases requiring medical treatment and leading to 600 deaths [35]. In the USA, annual incidences of 15 illnesses per 100,000 inhabitants were reported in 2013 [64]. The three most common serovars were *Enteritidis* (19%), *Typhimurium* (14%) and *Newport* (10%). In the EU, a total of 82,684 confirmed human salmonellosis were reported in 2013 and an annual incidence of 20 illnesses per 100,000 inhabitants was detected [58]. This represents a decrease of salmonellosis by 8% compared to 2012. The incidence of *Salmonella* was lower in the whole EU level than in Finland and Sweden which might be due to different reporting systems [7]. Particularly, a decrease in the number of *S. Enteritidis* has been reported in the EU. Of all the serovars, *S. Enteritidis* (40%) and *S. Typhimurium* (20%) were the most frequently reported. As a result of the harmonized reporting system and also due to several large outbreaks, monophasic *S. Typhimurium* which is an emerging variant of biphasic *S. Typhimurium* lacking one flagellar phase was the third most commonly reported serovar in the EU [58].

**Table 2:** Incidence of *Salmonella* infections in certain industrialized countries.

Geographic area/country	Incidence of <i>Salmonella</i> /100,000 inhabitants	Major serotypes associated with human disease	Reference
Australia	54	Typhimurium, Enteritidis	[65]
EU*	24	Enteritidis, Typhimurium	[58]
Finland separately	40	Enteritidis, Typhimurium	[7]
Canada	18	Enteritidis, Typhimurium	[66]
USA	15	Enteritidis, Typhimurium	[67]

\* Data based on 25 reporting countries from the EU including Finland

### 2.2.2.1 Epidemiology of *S. Enteritidis*

Two major changes have occurred in the epidemiology of non-typhoidal salmonellosis during the 1980s and 2000s: the emergence of foodborne human infections caused by *S. Enteritidis* and by multiresistant strains of *S. Typhimurium*. *S. Enteritidis* was responsible for a worldwide pandemic during the 1980s and 1990s. The infections were associated with the consumption of raw or undercooked eggs and have caused large outbreaks worldwide [68, 69]. In Europe, about 70% of the outbreaks caused by *S. Enteritidis* during the 90s were related to eggs and egg products [70]. The source of the pandemic is understood to have been the rapid contamination of a few companies' flocks and the ability of the bacterium to colonize the reproductive tract of the birds and infect eggs [3]. This theory is supported by the fact that Finland and Sweden which have the most extensive salmonella control programs and Australia which has strict rules on the import of animal products, have remained largely free from colonization of domestic poultry [71, 72]. The spatial and temporal distribution of different *S. Enteritidis* phage types varies between the continents and multiple clones of *S. Enteritidis* emerged simultaneously in geographically separate countries during this pandemic. Different phage types (PT) dominate in different continents. For example, PT8, PT13a and PT13 are most common in North America [73, 74], while PT4 has been the dominant phage type in the Western Europe and Japan [69, 75-77] and PT1 in Baltic countries, Poland and Russia [78, 79]. Furthermore, PT14b represents a recently emerging phage type in Southern European countries [80]. The strains of *S. Enteritidis* have remained more susceptible to antimicrobials than some other *Salmonella* serovars e.g. Typhimurium [81].

### 2.2.2.2 Epidemiology of certain multiresistant *S. Typhimurium* strains

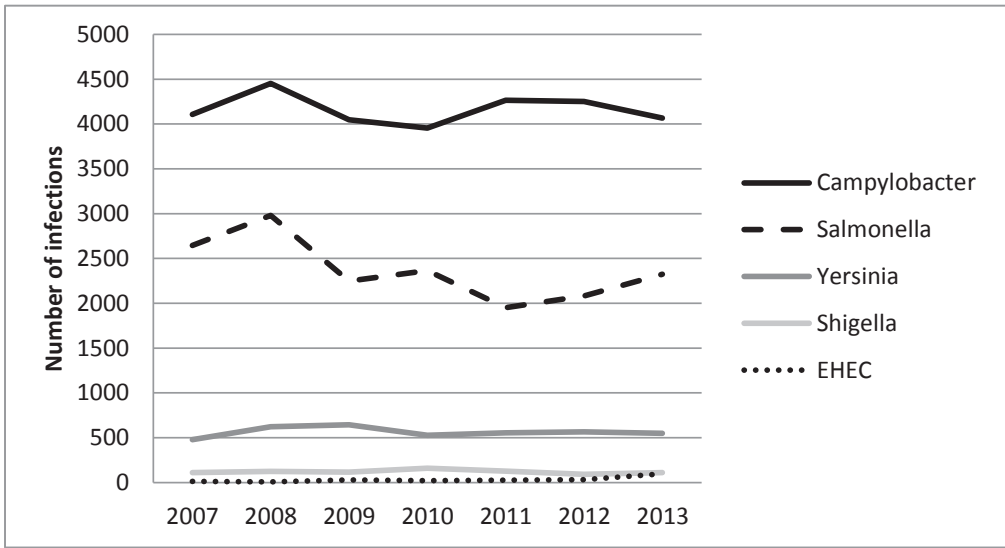
A much higher rate of resistance has been reported among the strains of *S. Typhimurium* than among those of *S. Enteritidis*. For example, a multiresistant *S. Typhimurium* definitive phage types (DTs) DT193 in the 70s [82] and DT104 in the late 80s which originated from cattle in the United Kingdom emerged as a global health problem and have since then become common in other animal species such as poultry, pigs and sheep [83]. The multiresistant DT104 strains are generally resistant to five different drugs: ampicillin (A), chloramphenicol (C), streptomycin (S),

sulfonamide (Su) and tetracycline (T), referred as resistance type ACSSuT [84]. Genes associated with ACSSuT resistance are located in a chromosomal *Salmonella* genomic island 1 (SGI-1) [84, 85]. In addition to DT104, a multiresistant monophasic *S. Typhimurium* (with antigenic structure 4,[5],12:i:-) was rarely identified before the mid-1990s but has now been recognized as an emerging pathogen in the EU. Two major clonal lines of monophasic *S. Typhimurium* have emerged in the EU: so-called European and Spanish clones. The European clone, which has emerged since 2000, harbors a chromosomal region responsible for resistance against at least four antimicrobials referred as R-type ASSuT [39, 86, 87]. The Spanish clone, first reported in Spain in the late 1990s, harbors plasmid-mediated resistance against up to seven antimicrobials: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamide (Su), tetracycline (T), trimethoprim (Tp) and gentamicin (G) [88]. Both of these clones are believed to have evolved from a traditional biphasic *S. Typhimurium* and lack the second-phase flagellin-encoding gene or the ability to express it resulting in a monophasic variant [89]. The majority of the monophasic *S. Typhimurium* strains in the European clone belong to the phage types DT193 or DT120 [86, 90, 91] while the Spanish clone consists of strains belonging mostly to the phage type U302 [92]. In Finland, a significant increase of cefotaxime nonsusceptibility has been registered among the strains of monophasic *S. Typhimurium* isolated from patients with travel history to Asia as well [93]. The exact reasons for successful colonization of these multidrug resistant strains are unknown. However, several factors such as improved mechanism to survive in the host or acquisition of bacteriophages encoding antimicrobial resistance to additional drugs and virulence factors needed for the fitness might have had an impact [94].

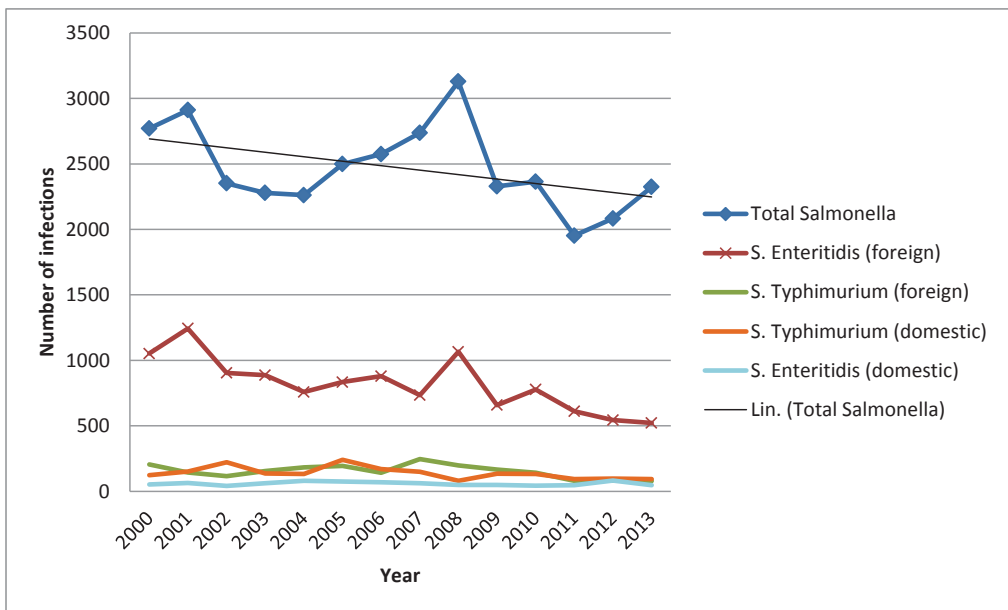
### **2.2.3 Human *Salmonella* infections in Finland**

In Finland, the average annual incidence for all reported salmonellosis cases was 44-59 cases per 100,000 inhabitants during 2000-2013, including 6-7.5 cases per 100,000 inhabitants of domestically acquired infections (statistics of THL). The overall occurrence was highest among 20-29 year-olds and lowest among over 75 year-olds individuals. With a total of 1,952-3,129 reported cases in 2007-2013, human salmonellosis was the second most common bacterial human intestinal disease (after *Campylobacter*) reported in Finland (Fig. 2). Since 2011, the overall trend of *Salmonella* infections has been decreasing in Finland (Fig. 3). This decline can be most clearly observed among the *S. Enteritidis* infections that have been acquired abroad.

During 2000-2013, more than 200 different serovars caused salmonellosis in Finland (statistics of THL). Of them, 136 were linked to domestically acquired infections. Of all strains, the most common serovars were Enteritidis and Typhimurium. In 2014, the most common serovars associated with domestically acquired infections were Typhimurium, Enteritidis, monophasic *S. Typhimurium*, Infantis and Newport. Among *S. Typhimurium*, phage type DT1 has been the most common DT since the 1960s. In addition to human infections, DT1 has been detected among domestic production animals (cattle, pigs and turkeys) and it has come to be considered endemic to Finland [95]. However, in the past years the incidence of monophasic *S. Typhimurium* (antigenic structure 4,[5],12:i:-), has increased. In 2013, the number of infections caused by domestic monophasic *S. Typhimurium* was as high as domestic DT1. In general, the majority of domestic monophasic *S. Typhimurium* are of resistance type ASSuT while domestic DT1 strains are fully susceptible to tested antimicrobials.



**Figure 2:** Trends of common enteric bacteria in Finland (2007-2013), data from the National Infectious Disease Register (NIDR), THL.



**Figure 3:** The trend of salmonellosis in Finland (statistics of THL, 2000-2013).

### **2.2.4 Infectious dose, disease and treatment of *Salmonella***

How large a dose of *Salmonella* is necessary for infection depends on host resistance, food composition [96] and the virulence and physiological state of the ingested bacterium. Therefore, the infectious dose for salmonellosis is variable. It is thought to be typically between  $10^6$ - $10^8$  colony forming units for humans. However, infective doses as low as 10 cells have been reported [97, 98]. Children, elderly or immune compromised people and pregnant women are more susceptible to developing salmonellosis than healthy adults [97, 98].

Human *Salmonella* infections can lead to four clinical conditions: (1) enteric (typhoid) fever, (2) acute gastroenteritis, (3) invasive infections and (4) asymptomatic fecal excretion [99]. Enteric fever is a serious disease which occurs only in humans and is due to *S. Typhi* or *S. Paratyphi*. Commercial vaccines against *S. Typhi* are currently available [100]. Acute gastroenteritis is the most common manifestation of non-typhoid *Salmonella* infection. Nearly all *S. enterica* subsp. *enterica* serovars can cause human gastroenteritis but the most of the human infections are limited to only a few serovars. The incubation time may vary from 4 to 72 hours, with an average of 12-36 hours, after the ingestion of contaminated food or water. Symptoms are acute onset of fever, chills, nausea, occasionally vomiting, abdominal cramping and diarrhea [8]. The fever usually ends within 72 hours and the diarrhea is usually self-limiting, lasting 3-7 days, although some patients have symptoms for as long as 14 days [101]. Therapy should be directed at preventing dehydration. However, the usage of antimicrobial therapy is justified in patients with bacteremia, infants aged <3 months and in immunocompromised patients [8]. The mortality of salmonellosis is low, and less than 1% of reported salmonellosis cases have been fatal [60, 102]. In Finland, reactive arthritis occurs in 4.4%-12% of patients after *Salmonella* infection [103-105]. This sequela most commonly affects young adults, and more frequently the white population, possibly due to the higher frequency of the HLA-B27 tissue allele in this ethnic group [106]. There are probably as many asymptomatic infections as symptomatic, but the real number of these remains unknown. People continue to excrete *Salmonella* bacteria for 3-4 weeks after either symptomatic or asymptomatic infection [107]. The carrier state can be prolonged for up to one year. Children may excrete *Salmonella* for even longer than adults [108]. Furthermore, vaccines against two out of 2,600 *Salmonella* serovars (Enteritidis and Typhimurium) do exist which are effective in poultry but not in humans or other animal reservoirs such as cattle or pigs [109].

### **2.2.5 Pathogenesis of *Salmonella* and the main virulence factors**

In order to cause disease, ingested *Salmonella* cells first need to overcome several non-specific barriers such as bactericidal action of lactoperoxidases, the pH of the stomach and intestinal mucoid secretion and peristalsis. Secondly, they need to overcome host-specific defense mechanisms which include antibacterial actions of phagocytic cells coupled with the immune response. To cause a disease, *Salmonella* possess numerous virulence factors. The majority of the genes encoding these factors are located in five highly conserved salmonella pathogenicity islands (SPI-1-5) in the chromosome, while others are found in a virulence plasmid (pSLT).

In general, it is a characteristic of non-typhoid *Salmonella* that they are able to colonize the gut. The attachment of the bacterium to the host's epithelial receptors is mediated by lipopolysaccharide (LPS), flagellin and fimbriae and by other large adhesins or autotransporter proteins encoded within SPI-3 and SPI-4 [110-112]. *Salmonellae* can enter host cells by invasion

or phagocytosis [113]. Invasion proteins and the invasion locus (*inv*) in SPI-1 play an important role in the invasion [114, 115]. *Salmonella* invade and survive in macrophages leading to inflammation of the intestines and to gastroenteritis. The survival of the *Salmonella* inside the macrophage is supported by factors encoded within, SPI3, SPI-2 and pSLT plasmid [113]. The virulence of *Salmonella* is dependent on many virulence determinants. It has been estimated that about 4% of the *Salmonella* genome (which encode over 200 virulence genes) is required for fatal infection in mice [116]. The need for so many virulence factors could reflect the complexity of *Salmonella* pathogenesis.

#### *Lipopolysaccharide and capsular polysaccharide*

The serovar-specific lipopolysaccharide chains (LPS) play an important role in inhibiting the potentially lytic attack of the host complement system as they hinder the insertion of certain complement factors into the inner cytoplasmic membrane which would otherwise initiate bacteriolysis [117]. Thus, the short LPS on rough variants are considered less virulent [117]. The capsular polysaccharide Vi (virulence) antigen have been detected in most strains of *S. Typhi*, in some strains of *S. Paratyphi C* and seldom in *S. Dublin* [118-120]. The Vi locus is encoded in the SPI-7 that is not present in non-typhoid *Salmonella* serovars [119].

#### *Flagella and fimbriae*

Flagella contribute to virulence through the mobility of the organism within its environment, allowing it to move towards attractants and away from repellents (chemotaxis), and by aiding in adhesion to and invasion of host surfaces [121]. The flagellar filaments are surface appendages of *Salmonella* and are composed of approximately 20,000 subunits of a unique protein, known as flagellin [122]. To date, 13 different fimbrial loci have been identified among *Salmonellae* [123], many of which are required for biofilm formation (e.g. curli fimbriae and plasmid-encoded fimbriae Pef) [124], attachment to host cells (e.g. type I fimbriae, curli fimbriae, Pef, long polar fimbriae Lpf and Std) [125-127], intestinal fluid accumulation (e.g. curli fimbriae and Pef) [125] and intestinal persistence in mice [128].

#### *Salmonella pathogenicity islands (SPI-1 to 5)*

SPI-1 and SPI-2 are the best-characterized of the five SPIs. SPI-1 is about 40 kb in size and contains at least 29 genes which encode several components of type III secretion system (T3SS) or its regulators and its secreted effectors enabling *Salmonella* to efficiently penetrate the intestinal epithelium [129]. In addition, several chaperons which protect SPI-1 proteins from degradation are encoded in SPI-1. SPI-2 contains more than 40 genes including a two-component regulon (*ssrAB*) and a T3SS system which is structurally and functionally distinct from that of SPI-1 [130, 131]. It is divided in two segments: the smaller part which is involved in tetrathionate reduction and the larger part which enable *Salmonella* to survive and replicate within epithelial cells and macrophages [130, 132]. SPI-3 is a 17-kb mosaic structure at the *selC* tRNA locus which encodes proteins with no known functional relationship to each other. The genes encoded in *mgtCB* operon, allow *Salmonella* to transport magnesium at low  $Mg^{2+}$  conditions which is required for intramacrophage survival during systemic dissemination [133]. MisL is involved in both attachment and long-term persistence [134]. SPI-4 is a 25-kb mosaic structure and contains six ORFs, arranged in a single operon *siiABCDEF* and plays a role during the interactions with intestinal epithelium and long-term persistence [111, 135]. These genes may encode a type I



secretion system [135]. In addition, it has been speculated that SPI-4 is involved in the secretion of a cytotoxin when *Salmonella* induces apoptosis of infected macrophages [136], however, the main function of SPI-4 remains to be determined. SPI-5 is involved in accomplishing several pathogenic processes during infection. The SigDE operon encodes SigD (SopB), an effector involved in fluid secretion in intestinal mucosa and SigE (PipC), its presumed chaperone [137, 138]. The genes *pipB* and *pipA* are presumed to contribute to systemic infection in mice [139].

#### *pSLT* plasmid

Some *Salmonella* serovars of clinical importance harbor a serovar-specific virulence plasmid which varies in size: 95 kb for serovar Typhimurium, 60 kb for Enteritidis, 80 kb for Dublin [140]. They all contain a highly conserved 8-kb region of five genes, the *spvRABCD* locus. The SpvR is a transcriptional activator, and induces *spvABCD* expression in the stationary phase in response to nutrient limitation. Two genes, *spvB* and *spvC*, encode factors for plasmid-mediated virulence of serovar Typhimurium [141, 142]. Furthermore, the *pSLT* plasmid also contains a fimbrial operon (*pef*) that encodes an adhesion involved in colonization of the small intestine [143]. It is noteworthy that highly infectious *S. Typhi* lacks this virulence plasmid [144].

### 2.3 Nomenclature, classification and general characteristics of *E. coli*

The history of the *Escherichia coli* species dates back to 1885, when it was first isolated from the feces of a healthy infant [145]. In 1919, the commensal *E. coli*, was named in honor of its discoverer a German paediatrician Dr. T. Escherich. *E. coli* species belong to the same proteobacterial family as *Salmonella*, *Shigella*, *Yersinia* and others, the family of *Enterobacteriaceae* (Fig. 1). *E. coli* are facultative anaerobic, Gram-negative, rendered motile by peritrichous flagella, or non-motile, lactose fermenting rod-shaped bacteria which are about 2-6 x 1-1,5 µm in size [146].

Based on the clinical pathogenesis, *E. coli* is classified into 3 major groups: (1) commensal *E. coli*, (2) diarrheagenic *E. coli* (DEC) and (3) extra-intestinal pathogenic *E. coli* (ExPEC). Commensal *E. coli* is considered beneficial for maintaining a healthy intestinal ecosystem. They usually colonize the gastrointestinal tract in a few hours after birth, becoming a part of the normal flora and co-existing in symbiosis with the host [4]. However, some *E. coli* strains have acquired specific virulence factors, often encoded by mobile genetic elements, which allow them to adapt into new niches and cause a wide spectrum of diseases. DEC isolates are categorized into six specific pathogroups based on virulence properties, mechanisms of pathogenicity and clinical syndromes (Table 3). The major pathogroups within DEC are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) [147, 148]. *Shigella* species should be classified within the species EIEC [149]. However, due to the clinical significance of *Shigella* the traditional nomenclature is still maintained [4]. ExPEC strains cause urinary tract infections (by uropathogenic *E. coli*, UPEC), neonatal bacterial meningitis (by meningitis-associated *E. coli*, MAEC) and sepsis. The pathogroup specific *E. coli* nomenclature and classification is summarized in Table 3. In addition, the unique mosaic O104:H4 strain that caused a large fenugreek-related outbreak in Germany 2011 might represent a new pathogroup designated as enteroaggregative-heamorrhagic *E. coli* (EAHEC) [150].

The enterohaemorrhagic *E. coli* (EHEC) was first described in Canada in the late 1970s [151]. During an extensive outbreak associated with a consumption of poorly cooked minced meat in the USA in 1982, it was first demonstrated that Shiga toxins produced by EHEC are linked to bloody diarrhea [152, 153]. In the same year, it was discovered that a post-diarrheal disease, hemolytic-uremic syndrome (HUS), could be caused by EHEC O157:H7 [154]. Most strains of EHEC possess some biochemical and physiological characteristics which are uncommon to other *E. coli* including the production of Shiga toxins, inability to ferment sorbitol within 24 hours, inability to produce  $\beta$ -glucuronidase, carrying of an attaching and effacing (*eae*) gene and the inability to grow well at temperature  $>45^{\circ}\text{C}$  [4]. The EHEC strains can be further characterized by serotyping which is based on differences in antigenic structure on the bacterial surface: O-antigen, H-antigen, and sometimes also K-antigen (Kapsel) and F-antigen (Fimbriae). Serologically, EHEC bacteria can be divided into two main groups: strains of EHEC O157 and non-O157. Of these, most widespread are EHEC O157 strains. This might be due to their higher virulence. However, over 400 non-O157 serotypes have been associated with human disease [155]. Although EHEC is one of the best-characterized bacterium in clinical microbiology laboratories, researchers often refer to these pathogens interchangeably as enterohemorrhagic *E. coli* (EHEC), shiga toxin-producing *E. coli* (STEC), or verotoxin-producing *E. coli* (VTEC). For consistency, in this study all STEC strains are called EHEC and the Stx nomenclature will be used.

## **2.4 EHEC infections in human**

The EHEC O157 and non-O157 are zoonotic bacteria. Human infection may be acquired through the consumption of contaminated food or water, by direct transmission from person-to-person or from colonized animals or fecally-contaminated environments to humans [164]. In contrast to infections caused by ETEC and EPEC, EHEC infections are mainly found in developed countries. The infections are most common among children less than 5 years. Also, severe complications such as HUS are more commonly reported in children and the elderly whereas asymptomatic carriage is more common in the age groups between. Major EHEC outbreaks have resulted in greater public awareness, but sporadic infections cause the largest disease burden as most EHEC infections are sporadic. In a recent study, it was shown that about 20% of the infections were secondary infections [165].

### **2.4.1 Sources and risk factors of EHEC infections**

Ruminants, particularly healthy cattle, are a major reservoir for human infections caused by sorbitol-negative EHEC O157:H7. Similar to those, sorbitol-positive EHEC O157:H7 and non-O157 are also often associated with cattle and ruminants [166, 167]. In addition to cattle, EHEC are detected in a wide spectrum of animals e.g. sheep, goats, deer, moose, swine, horses, dogs, cats, pigeons, chickens, turkeys and flies [168]. In contrast to humans, most EHEC infections of animals are clinically asymptomatic [169]. Identified risk factors for EHEC infection include living in or visiting a place with farm animals [170, 171], consumption of undercooked beef and consumption of cold sliced meat [172]. In addition, unpasteurized milk, yogurt and cheese made of unpasteurized milk are commonly reported as sources of EHEC O157:H7 infections [173-178]. Furthermore, numerous plant products have been reported as vehicles to human disease, including apple cider and vegetables such as lettuce, radishes, alfalfa sprouts and spinach [179-

Table 3: Nomenclature, characteristics of diarrhoeagenic *E. coli* and enterohaemorrhagic *E. coli* toxin subtypes.

Pathogroup	Meaning	Description	Characteristics	Main virulence factors*	Reference
EPEC	Enteropathogenic <i>Escherichia coli</i>	A group of <i>E. coli</i> that cause diarrhoea, characterized by the ability to form Intimin-mediated attaching and effacing (A/E) lesions, presence of genes for bundle-forming pili and lack of <i>stx</i> genes.	Discovered in 1945, EPEC is common among the children in developing countries but also linked to infant diarrhea in industrialized countries. Watery to bloody diarrhea.	Pathogenicity island LEE including T3SS system, Intimin, Tir, EspABDE; EPEC adherence factor (EAF) plasmid including bundle-forming pilus (Bpf), plasmid-encoded regulator (Per)	[156]
(aEPEC)	atypical Enteropathogenic <i>Escherichia coli</i>	A subgroup of EPEC which lack genes for bundle-forming pilus.	More common in industrialized countries than EPEC	Lacks EAF plasmid	[4]
ETEC	Enterotoxigenic <i>Escherichia coli</i>	A group of <i>E. coli</i> that cause diarrhoea by producing of enterotoxins (LT and ST).	Discovered in 1961. ETEC is considered the most common agent for tourist diarrhea of adults in industrialized countries and the leading cause of infants diarrhoea in developed countries. Watery diarrhoea.	Colonization factor antigens (CFA), Heat-labile toxin (LT), Heat-stable toxin (STa, STb)	[147, 157]
EIEC	Enteroinvasive <i>Escherichia coli</i>	A group of <i>E. coli</i> that cause diarrhoea, characterized by the presence of invasion genes also found in <i>Shigella</i> .	Discovered in 1971, occurrence high in poor countries. Watery to bloody diarrhea.	Vir/lcs cluster; Invasion plasmid (pINV) including IpaABCD	[158, 159]
STEC	Verocytotoxic <i>Escherichia coli</i> / Shiga Toxin-producing <i>Escherichia coli</i>	VTEC and STEC are synonyms of a group of <i>E. coli</i> that are associated with ability to cause diarrhoea, characterized by the Verocytotoxin (Vtx)/ Shiga toxin (Stx) production.	Discovered in 1977	Pathogenicity island LEE; T3SS system, Intimin, Tir, EspABDE; Shiga Toxins: Stx1, Stx2, pO157 plasmid; Enterohaemolysin (EHEC-Hly), Serine protease, ToxB	[151]
(EHEC)	Enterohaemorrhagic <i>Escherichia coli</i>	VTEC/STEC patients that have the symptoms of bloody diarrhoea/haemorrhagic colitis. The infections can lead to severe sequelae such as hemolytic uremic syndrome (HUS) characterised by acute renal failure, hemolytic anaemia, thrombocytopenia (TTP) or neurological disorder.	Discovered in 1982, increasing incidence worldwide, common agent for bloody diarrhea and severe sequelae.	<i>stx1</i> , <i>stx2</i> , <i>eae</i> , <i>EHEC-hlyA</i> and <i>saa</i>	[160, 153]
EAEC	Enter aggregative <i>Escherichia coli</i>	A group of <i>E. coli</i> that cause diarrhoea, characterized by the ability to adhere the epithel cells in a stacked-brick pattern (aggregative adherence).	Discovered in 1987, common agent of acute mucoid and prolonged diarrhea in children and adults both in developing and industrialized countries, increasingly an agent of outbreaks worldwide.	Aggregative adherence fimbriae (AAFs), EAEC flagellin, Toxins: Pic, ShET1, EAST, Pet	[161]
DAEC	Diffusely adherent <i>Escherichia coli</i>	A group of <i>E. coli</i> that are characterized by their diffuse adherence on the epithel cells. Heterogeneous with respect to plasmid content.	Discovered in 1993, cause diarrhea particularly in < 1-year old children and in the developed countries.	Fimbrial adhesin F1845 and adhesin AIDA-1	[4]

\* The chromosomal virulence factors are presented underlined and those located in plasmids are not underlined.

182]. Recently, novel vehicles such as cookie dough have been reported [183]. Lake water and water from private wells have been associated with human infection as well [184-186].

#### 2.4.2 Occurrence and epidemiology of EHEC infections

Since its discovery in 1982, EHEC has been recognized as an emerging pathogen in industrialized countries causing mostly sporadic infections or small family-related clusters, although large foodborne outbreaks have also been reported. The incidences of EHEC infections vary among geographic areas including some high EHEC O157:H7 incidence regions such as some regions of Argentina [187, 188], Canada [189], Ireland [190] and Scotland [191] (Table 4). Strains of EHEC serotype O157:H7 are the primary cause of bloody diarrhea and HUS in many countries. There are currently no exact data available on the frequency of EHEC infection in Argentina, but the incidence of EHEC O157-mediated HUS is among the highest in the world and HUS is considered endemic in Argentina [187]. The dominance of EHEC O157:H7 as a cause of illness might be misleading because of a lack of detection methods and inconsistent testing frequencies for other non-O157 serotypes. Despite these limitations, the incidence of non-O157 as disease agent in human EHEC infections has steadily increased worldwide. For example in some EU countries, EHEC non-O157 are more prevalent over EHEC O157:H7 [192].

**Table 4:** Incidence of EHEC infections in certain regions and the most common EHEC non-O157 serogroups in the region.

Geographic area/ country	Incidence of EHEC (O157 and non-O157)	Major non-O157 serogroups associated with human disease	Reference
Australia	0.4	O26, O111	[193]
EU*	1.1-1.9	O26, O91, O103, O104, O111, O113, O128, O145, O146	[58, 194]
Finland	0.2-1.8	O26, O103, O145	[195-197]
Sweden	4.9	O26, O103, O121	[58]
Canada	3.0-6.0	O26, O91, O103, O111, O121	[189, 198]
Japan	2.0-3.0	O26, O111, O121, O103, O145	[199, 200]
Argentina	10.4-12.2	O8, O26, O113, O145, O174	[188, 201]
USA	0.9-1.2	O26, O45, O103, O111, O121, O145	[202, 203]

\*) Data based on 25 reporting countries from the EU including Finland and Sweden.

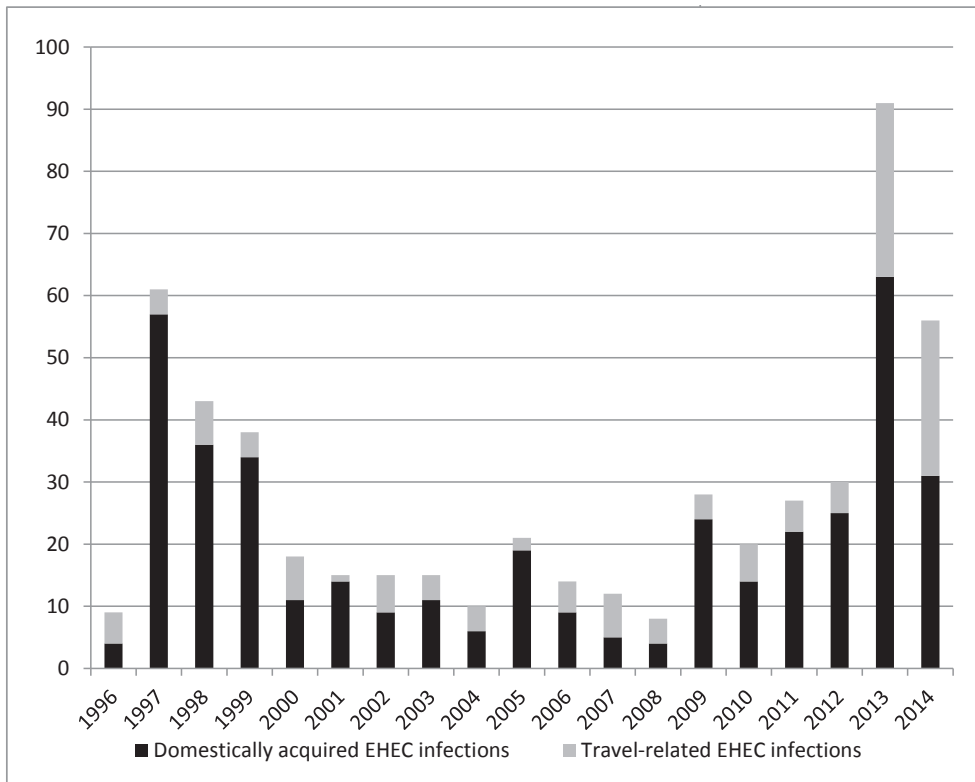
EHEC has been estimated to cause more than 265,000 illnesses each year in the USA, and more than 3,600 hospitalizations and 30 deaths [203]. In 2010, the annual incidence rate of reported EHEC infection was 0.9 cases per 100,000 in the USA [204]. Strains of O157:H7 are still the most frequently isolated EHEC strains from patients in the USA, although, the number of infections caused by non-O157 has been estimated to vary from 30% to 50% [202, 205]. Among non-O157 infections, the most common O-groups in the USA were O26 (37%), O103 (24%), and O111 (17%) and about 20% of EHEC O157 infections were associated with recognized outbreaks [204]. Similar incidence values as in the USA have also been reported in the EU, although regional

variations within the continents do occur. Nevertheless, the EHEC incidence remains in the USA and in the EU over 2-fold higher than the annual incidence in, for example, Australia (0.4 cases per 100,000 population) [193]. In the EU, an increasing trend of confirmed EHEC cases in 2008-2011 has been reported. During that time an annual incidence rate in the EU has varied between 0.96-1.15 per 100,000 inhabitants, except in 2011 when 2.5 cases per 100,000 inhabitants were reported. The increase in 2011 was due to a large outbreak caused by an enteroaggregative Shiga toxin-producing *E. coli* O104:H4 in Germany that resulted in 3,842 infected people [206]. When excluding Germany, the highest EHEC notification rates in 2011 among European countries were observed in Ireland, the Netherlands and Sweden. The prevalence of non-O157 serotypes has been higher than those of O157 in some European countries. For example, 82% in Germany [192, 207], 80% in the Netherlands [208] and 74% in Denmark [209] of the infections have been caused by non-O157 serotypes. Furthermore, EHEC non-O157 has been associated with 10-30% of reported HUS cases in Germany, Italy and UK [210]. EHEC of serotype O26:H11/H<sup>-</sup> has emerged as the most common non-O157 EHEC strain in human diseases in the EU [207, 211-214] and the United States [202, 215]. It has also been increasingly isolated from patients in South America [187], Asia [216], and Australia [217]. Another feature among the European EHEC strains is the ability of some *EHEC* O157 strains to ferment sorbitol. These sorbitol-positive EHEC O157 strains often express a non-motile phenotype (H<sup>-</sup>), although H7 gene is amplified in the PCR analysis. These strains have caused several outbreaks in the EU but they have seldom been reported from other continents [166, 218]. Thus, rare serotypes with new virulence properties, as experienced with the German outbreak of EAHEC O104:H4, is worthy of note.

As with *Salmonella*, a seasonal variation has been noted throughout the world with a tendency of EHEC infections to occur in the late summer during the warm months [219]. The reasons for this phenomenon are not known, however it has been speculated that it may be related to infection trends in animal hosts or to storing food at inappropriate temperatures.

### **2.4.3 EHEC infections in Finland**

Most of the EHEC infections in Finland are sporadic or family-related secondary infections [197]. Of all infections, about 70-80% are considered domestically acquired (Fig. 4) but the number of infections that were acquired abroad has increased during the previous two years. During 1998-2008 the annual incidence of EHEC infections in Finland had been low ranging from 0.2 to 0.6 per 100,000 populations [195, 197]. Since 2009 an emerging trend of EHEC infections has been observed, and in 2013 a new record was established as almost 100 EHEC infections were reported. The annual incidence rate rose into 1.8 per 100,000 populations in 2013 [7]. During 2009-2014, about 70% were domestically acquired infections. Since the beginning of the surveillance in 1996, EHEC serotype O157:H7 and its variant O157:H<sup>-</sup> have every year caused the majority of the domestically acquired EHEC infections in Finland.



**Figure 4:** Number of domestic and travel-related EHEC infections 1996-2014 (statistics of THL).

#### 2.4.4 Infectious dose, disease and treatment

In contrast to the relatively high infectious dose for *Salmonella*, the infectious dose of EHEC is low: less than 10 cells have been reported to cause a disease [220]. More than 400 EHEC serotypes have been associated with human disease, causing illnesses that range from symptom-free carriage to bloody diarrhea, HUS and death. EHEC strains are usually non-invasive and HUS is rarely complicated by bacteraemia, but some serotypes invade into the bloodstream [221]. After a mean incubation period of 3 days (can vary from 1 to 8 days) infected individuals develop watery diarrhea, vomiting (30-60% of cases) and abdominal pain with cramps. About 30% of patients have mild fever, usually observed in the early stages of the disease [191]. About 1-3 days after onset, over 70% of the patients develop bloody diarrhea though lower frequencies have also been reported. The bloody diarrhea is more common in infections caused by EHEC O157:H7 [191]. Most patients (95%) recover spontaneously within a week of onset, whereas some of the patients progress to HUS or other complications. The proportion of patients who progress to severe bloody diarrhea and/or HUS varies for different strains of EHEC, as well as by the age and immunological status of the infected patient [222]. In sporadic cases, 3-7% of cases progress into HUS [219] while in specific outbreaks a HUS incidence of up to 20% has been reported [223]. HUS occurs 5-13 days after the onset of diarrhea and is characterized by hemolytic anemia with fragmented erythrocytes, thrombocytopenia and acute renal failure [224]. HUS mortality has been reported to be 3-5% [224]. Although HUS caused by serotype O157:H7 has been associated with the highest mortality, serotypes O26:H11 and O145:H28 can lead to fatal HUS as well [207].

To support this, recent studies have indicated that there is no statistically significant differences in the Stx proteins encoded by EHEC O157 and non-O157 strains or in their level of toxicity [225] and there appears to be no significant difference in the long-term outcome of HUS caused by non-O157 EHEC and EHEC O157 [226]. Thus, it is possible that factors other than Stx may contribute to chances of developing HUS [227].

The current treatment for EHEC infection is supportive and the infected patients should be monitored for dehydration, anuria and other symptoms that might suggest HUS [228]. Drugs commonly used in the hospital to treat bacterial enteric infections such as antibiotics, anti-motility agents, anti-inflammatory medicines should be avoided for treatment of EHEC-infected patients [222, 229].

#### **2.4.5 Pathogenesis of EHEC**

After ingestion, EHEC adheres to and primarily colonizes the large intestine and forms attaching and effacing (A/E) lesions on the mucosal epithelium [230]. A/E lesions are characterized by loss of the epithelial micro-villi, intimate attachment of the bacteria to the cell, and accumulation of polymerized actin filaments in high concentrations and a formation of a pedestal-like structure in the epithelium [147]. All the proteins needed for the formation of A/E lesions are encoded by a chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE) [231]. In contrast to some *Salmonella* serovars, *Shigella* species and EPEC, EHEC bacteria are generally unable to invade the HEP-2 cell lines and thus, are considered usually non-invasive [232]. The released Stx toxins bind to their target receptor, a glycolipid receptor globotriaosylceramide (Gb3), and leading to an increase in proinflammatory cytokines from host cells and to irreversible inhibition of protein synthesis [233]. The intestinal vascular injury leads to necrosis and intestinal perforation that, together with the inflammatory response, cause the bloody diarrhea [222]. Stx toxins can also be absorbed across the gut epithelium into the bloodstream by a process of receptor-mediated endocytosis where they bind to polymorphonuclear leukocytes [234]. At target organs such as kidney and brain, Stx toxins bind to Gb3 receptors. For example, renal glomerular endothelium expresses high levels of Gb3 in humans, and Shiga toxin production results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, all typical characteristic of HUS [154, 235]. In the brain the Stx toxins can cause endothelial damage and thrombotic disorders leading to neurological symptoms [236].

#### **2.4.6 Virulence factors of EHEC**

The primary virulence determinants of EHEC strains are chromosomally encoded. These include the Shiga toxin (Stx) and its variants as well as the locus of enterocyte effacement (LEE) encoding A/E lesion [237]. Also, plasmids such as pO157 and pO113 may play an important role in the pathogenesis of EHEC [238]. In addition, new putative virulence factors could be detected via next-generation sequencing methods in the future.

##### **2.4.6.1 Main virulence factors in chromosome**

###### *Shiga toxins (Stx) and stx variants*

The defining characteristic of EHEC is the production of Shiga toxin (Stx) [4]. The name Shiga toxin originates from the DNA sequence homology with the Stx of *Shigella dysenteriae* [239].

Shiga toxins belong to a family of AB<sub>5</sub> toxins, characterized by an enzymatically active A subunit of about 32 kDa non-covalently linked to a pentamer of five identical receptor-binding B subunits each of about 7.7 kDa [147]. Both toxins are encoded by the *stx* genes located in a temperate bacteriophage that is inserted as a prophage at specific insertion sites into the EHEC chromosome [240]. For example, the strains of O157:H7 can carry several distinct phages and at least five different insertion sites, of which *yehV* and *wrbA* are most commonly occupied [241]. The pentameric B unit of most Stx variants binds specifically to the glycolipid receptor globotriaosylceramide (Gb<sub>3</sub>), except that the variants Stx<sub>2e</sub> and Stx<sub>2f</sub> prefer to bind to the longer globotetraosylceramide (Gb<sub>4</sub>) [239]. Two major groups, Stx<sub>1</sub> and Stx<sub>2</sub>, showing about 60% nucleotide sequence identity with each other, have been detected [242]. Moreover, these two toxin groups can be further divided into several variants. The nomenclature is not conclusive and new variants of Stx are described. The Stx<sub>1</sub> has three variants Stx<sub>1a</sub>, Stx<sub>1c</sub>, and Stx<sub>1d</sub>. The Stx<sub>2</sub> group is more heterogeneous and can be divided into seven variants: Stx<sub>2a</sub>, Stx<sub>2b</sub>, Stx<sub>2c</sub>, Stx<sub>2d</sub>, Stx<sub>2e</sub>, Stx<sub>2f</sub>, Stx<sub>2g</sub> [162, 243]. In addition, a strain can produce either Stx<sub>1</sub> or Stx<sub>2</sub> or both toxins [242]. Generally, Stx<sub>2</sub> has been more frequently associated with severe disease and HUS than Stx<sub>1</sub> [243, 244]. Several studies have shown that *stx* gene variants *stx*<sub>2</sub> and *stx*<sub>2c</sub> are more often associated with HUS [163, 245, 246], however, strains carrying the gene *stx*<sub>2d</sub> [247, 248] and *stx*<sub>2e</sub> [243] have also been isolated from humans with HUS.

#### *LEE associated virulence factors*

The factors responsible for the histological events associated with A/E lesions of EHEC are encoded in the LEE pathogenicity island in the chromosome [4]. The LEE consists of approximately 41 genes, divided into five major operons (LEE1-5), that encode for a type 3 secretion system (T3SS), regulators, chaperones, and effector proteins [231]. The first gene encoded in LEE1, regulator (Ler), acts as the major transcription factor of the pathogenicity island, regulating expression of the genes in the entire LEE area [249]. In addition, the up-regulation of the genes on LEE occurs through quorum sensing (QS) mechanisms which allow even a small number of bacteria to form A/E lesions and attach [250]. The major feature of T3SS encoded by genes in LEE is the translocation of a variety of virulence factors from the bacterium into the host cell via a filamentous needle complex formed by EspABD (“EPEC-secreted proteins”). The EHEC translocates its own receptor Tir (transmembrane intimin receptor) which is inserted into the host cell’s plasma membrane where it acts as an adhesion receptor for adhesion protein called intimin. Intimin is an important bacterial outer membrane protein encoded by the gene *eae* and its variants. The Intimin–Tir interaction mediates the attachment [251]. Currently 17 intimin subtypes have been described and named after the Greek alphabet [252]. Subtypes *eae*-α, *eae*-β and *eae*-γ have been commonly isolated from the strains which have caused HUS [252]. A subset of Type III effector proteins e.g. Map, EspF, EspG, EspH, EspB and SepZ translocate into the host cell where they cause a variety of reactions resulting in diarrhea [251].

#### *2.4.6.2 Main virulence factors in plasmids*

##### *pO157*

A large virulence plasmid pO157 is found in almost all clinical O157:H7 strains isolated from humans. The production of the O157:H7 enterohemolysin is associated with this plasmid [253]. The pO157 plasmid is about 94 kb in size and contains an EHEC *hly* operon which consists of *hlyC*, *hlyA*, *hlyB*, and *hlyD* genes and is about 60% homologous with the



alpha-hemolysin gene of *E. coli* [4]. Like alpha-hemolysin, enterohemolysin is able to form pores in membranes of erythrocytes and lyse certain eukaryotic cells [253]. In the case of enterohemolysin, hemolysis is observed on blood agar after overnight incubation, in contrast to the rapid 4h hemolysis associated with alpha-hemolysin [254]. The exact role of enterohemolysin in EHEC pathogenesis is not fully clear. In addition to enterohemolysin, the sequence analysis of pO157 has revealed some putative virulence genes [255]. These include a catalase-peroxidase (*katP*) [256], a type II secretion system apparatus (*etp*) [257], a serine protease (*espP*) [258], a putative adhesin (*toxB*) [259], a zinc metalloprotease (*stcE*) [260], and an *eae* conserved fragment (*ecf*) [261]. Although sorbitol-positive and sorbitol-negative EHEC O157/H7/H strains share several virulence factors, their plasmids differ from each other. The plasmid of sorbitol-fermenting EHEC O157 is about 30 kb larger (ca. 30%) and the major differences are the absence of *katP*, *espP*, and *toxB* genes [262].

### *pO113*

STEC auto-agglutinating adhesion (Saa) and STEC autotransporter contributing to biofilm formation (Sab) are both plasmid-encoded adhesins that have been identified primarily in the EHEC LEE-negative O113:H21 strains and might contribute to pathogenesis [263, 264]. Several variants of the *saa* gene have been identified and it has been hypothesized that variations at the 3' end influence the adhesive affinity, and that shorter variants are less adhesive than longer variants [264].

## **2.5 Foodborne disease outbreaks caused by *Salmonella* and EHEC**

### **2.5.1 Certain large outbreaks caused by *Salmonella***

Over the past five years, the number of outbreaks caused by *Salmonella* has been decreasing in concordance with a steady decrease in reported human salmonellosis cases in the EU and in the USA. This is largely due to declining egg-related outbreaks [194]. Major outbreaks of foodborne salmonellosis in the last decades are of interest because they highlight the diverse range of food items and diversity of *Salmonella* serovars that cause human disease.

In 2004-2005, a large outbreak caused by *S. Bovismorbificans* associated with consumption of raw minced pork was reported in Germany. About 400 persons were infected and one died during the outbreak [38]. In 2005, *S. Hadar* contaminated vacuum-packaged chicken infected more than 2,000 in Spain [265]. In 2008, a large multi-state outbreak of *S. Saintpaul* infections associated with contaminated jalapeno peppers occurred in the USA. More than 1,442 people were infected and 2 died [266]. One of the largest food recalls in the USA history occurred in 2009 when *S. Typhimurium* contaminated peanut butter caused an outbreak where more than 700 persons fell ill and 9 died [57].

### **2.5.2 Certain outbreaks caused by *Salmonella* in Finland**

During 2007-2014, a few *Salmonella* outbreaks have been reported annually in Finland. Most of them were small or medium outbreaks and sources are mostly unknown. The majority of *Salmonella* outbreaks which have led to outbreak investigations are foodborne. The outbreak investigations have identified some food vehicles such as iceberg lettuce in 2008, sprouts in 2007

and 2009 and frozen pre-cooked chicken cubes in 2012 (Table 5). Among others, the serovars Agona, Infantis, Isangi, Mikawasima, Poona, Typhimurium DT1, DT104, DT120, monophasic S. Typhimurium U311 and Urbana have been frequently associated with small outbreaks or clusters of unknown source in Finland.

**Table 5:** Selected *Salmonella* outbreaks with identified vehicle in Finland 2007-2013.

Year	Serotype	Xbal-PFGE/ MLVA type	No. of cases	Other countries involved	Vehicles	Reference
2007	S. Agona	SAGO39	12		Sandwich cake	Statistics of THL
2007	S. Weltevreden	SWEL4	7	Norway and Denmark	Alfalfa sprouts	[267]
2007	<i>Salmonella</i> spp.		14		Contaminated drinking water	[267]
2008	S. Newport/ S. Reading	SNWP59 and SREA4-7	107		Iceberg salad	[49]
2009	S. Bovismorbificans	SBVS4	42		Alfalfa sprouts	[269]
2012	S. Enteritidis PT1B	SENT117	53	Estonia	Frozen chicken cubes	Statistics of THL
2013	S. Typhimurium DT135	STYM243/ MLVA 2-14-11-11-312	8		Cattle farm contact	Statistics of THL

### 2.5.3 Certain large outbreaks caused by EHEC

EHEC O157:H7 and non-O157 strains have caused hundreds of outbreaks worldwide and the largest have been reported in Canada, Japan, Germany and the United Kingdom. In 1993, a large outbreak caused by EHEC O157:H7 contaminated ground beef served in a fast-food restaurant occurred in the USA. More than 700 individuals were infected, 55 had HUS and four children died [270]. In 1995, a large Australian community outbreak caused by EHEC O111:H7 and associated with the consumption of fermented sausages [271] resulted in more than 200 people being infected and 22 children had HUS. In 1996, the largest outbreak known occurred in Japan, in Sakai City, where about 8,000 persons, mostly school children, were infected by EHEC O157:H7 [272]. A total of 106 children had HUS and three of them died. White radish sprouts served as a part of a school lunch were identified as the vehicle of the EHEC infections. In 1996, Europe experienced a large O157:H7 outbreak in Central Scotland where about 500 persons had gastroenteritis after consumption of meat from a local butcher's shop and 20 died [273]. In 2005, meat products from a local butcher's shop in Wales caused the second largest O157:H7 outbreak in the UK where 157 persons were infected and one died [274]. The largest waterborne outbreak occurred in 2000 in Canada where a drinking water supply became contaminated with EHEC O157:H7. As a result, 2,300 persons became ill, 27 patients developed HUS and six died [275]. The largest non-O157 EHEC outbreak ever occurred in summer 2011 in Germany when with a multiresistant EHEC O104:H4 contaminated fenugreek sprouts infected 3,842 persons. A total of 2,987 cases of laboratory-confirmed gastroenteritis with 18 deaths and 855 cases of

HUS with 35 deaths were reported [276]. Interestingly, the genome of epidemic O104:H4 strains harbors a unique combination of genes present in enteroaggregative *E. coli* (EAEC) and in enterohemorrhagic *E. coli* (EHEC) pathotypes: two mobile elements, a phage carrying the *stx* genes and a plasmid carrying an aggregative adherence fimbria (AAF) operon and *aggR* gene, contribute to the main virulence of this hybrid strain [276, 277]. However, in contrast to regular EAEC and EHEC, the epidemic strains lack the EAEC-specific plasmid carrying AAF/III and *astA* encoding for the EAEC heat-stable enterotoxin (EAST1) and the EHEC-specific plasmid carrying the gene for enterohemolysin (*hlyA*) and LEE (locus for enterocyte effacement) pathogenity island [150].

#### 2.5.4 Certain outbreaks caused by EHEC in Finland

In 1996, an enhanced microbiological surveillance of EHEC infections was initiated in Finland. During the surveillance between 1996-2014, three outbreaks caused by EHEC with known sources or vehicles were detected (Table 6). The first domestic outbreak caused by sorbitol-negative EHEC O157:H7:PT2:*stx*<sub>2</sub>:*eae:hlyA* in 1997 was associated with swimming in a lake [185]. During this outbreak, 18 individuals were infected and two children died. Since then, two foodborne outbreaks in 2001 and in 2012 have been reported in Finland. In 2001, sorbitol-negative EHEC O157:H7:PT14:*stx*<sub>1</sub>:*stx*<sub>2</sub>:*eae:hlyA* -contaminated imported minced meat used in kebab was verified as source of a small outbreak (statistics of THL). The 2012 outbreak was caused by sorbitol-positive EHEC O157:H:PT88:*stx*<sub>2</sub>:*eae:hlyA* which was transmitted through unpasteurized milk and animal contact [278]. Several potential clusters or outbreaks have been identified but sources have remained unknown or microbiologically unverified.

**Table 6:** EHEC outbreaks with identified vehicle in Finland 1997-2014.

Year	Serotype	Phage type	Vehicle	Reference
1997	O157:H7	2	Swimming water	[185]
2001	O157:H7	14	Kebab meat	Statistics of THL
2012	O157:H	88	Unpasteurised milk	[278]

## 2.6 Epidemiological typing of *Salmonella* and EHEC

Multiple bacterial typing methods are used to differentiate bacterial strains of the same species. Phenotyping methods are those that detect characteristics expressed by the microorganism and genotyping methods are those that involve direct DNA-based analysis of chromosomal or extra-chromosomal genetic elements. Both are essential epidemiological tools but the recent development of molecular methods has especially provided new tools for surveillance, outbreak detection and infection prevention and control. The choice of a molecular method or combination of methods depends on bacterial species, reason for typing as well as time and the resources of the laboratory.

## 2.6.1 Phenotyping methods

### *Serotyping*

Serotyping is probably the most important phenotyping method in the world and it has been standardized to a level which allows the results to be compared internationally. Serovars of *Salmonella* are defined based on the antigenic structure of O (somatic) antigens of the cell surface and H1 and H2 (flagellar) antigens of the phase 1 or phase 2 and sometimes the Vi (capsular) antigens which, however, are present in very few serovars, for example in *S. Typhi*. The antigens are detected using glass slide agglutination with usually commercially produced antisera. The *Salmonella* serovar result is interpreted from the specific pattern of agglutination reactions using the White-Kauffmann-Le Minor classification scheme [13, 18]. In 2015, more than 2,600 serovars of *Salmonella* have been identified. The serotyping of EHEC strains is also based on differences in antigenic structure on the bacterial surface which include O-antigen, H-antigen, and sometimes also capsular K-antigen and F-antigen (fimbriae). In 2015, over 400 O:H-types of EHEC have been reported [155]. A minority of *Salmonella* or EHEC strains remain O-nontypeable and are referred to as “O rough”. Some strain cannot be H-typed, because they are non-motile. However, most of *Salmonella* and EHEC strains with non-motile phenotype can be H-typed by PCR or sequencing [279].

Traditional serotyping (under standardized conditions) has proven to be a robust and reproducible method. The advantages of serotyping include good discriminatory power and epidemiological concordance. However, slide agglutination is time-consuming and takes at least 3 days for *Salmonella* and up to one week for EHEC O:H-typing. Furthermore, expensive antisera of good quality are required and some strains are not typeable. The major disadvantage of serotyping is that although it provides a good discriminatory power, the typing does not provide any information on the relatedness between the different serotypes. In general, the serotype is, however, often the first sign of an ongoing outbreak and is mostly used in conjunction with other subtyping method or methods.

In addition to traditional serotyping, the serotyping of the most common *Salmonella* serovars and EHEC O-groups may be performed using conventional or real-time PCR techniques [280, 281]. Other molecular subtyping approaches available in the reference laboratories, such as pulsed-field gel electrophoresis PFGE [282] or multilocus sequence typing MLST [283] have also been suggested for *Salmonella* typing at the serovar level. Furthermore, some commercial kits based on DNA microarray technology (Check&Trace *Salmonella*, Check-Points PV) or based on microsphere suspension array technology (Luminex xMAP®, *Salmonella* serotyping assay, Luminex Corporation) are available for determination of common *Salmonella* serovars [284].

### *Phage typing*

In addition to serotyping, certain bacterial strains can be phenotypically further subtyped by phage typing. Phage types are determined by resistance or sensitivity of the strains to a standardized set of typing phages and the lytic pattern obtained allows assignment to a specific phage type. The strains exhibiting a lytic pattern that does not correspond to a known phage type are designated as “reacts but does not confirm” (RDNC) and strains that did not react with any of the typing phages are “not typeable” (NT). Phage typing is performed commonly for *Salmonella* serovars Typhimurium using 36 different phages [285] and Enteritidis using 17 different phages

[286]. So far, more than 300 definitive phage types (DTs) for *S. Typhimurium* and more than 60 for *S. Enteritidis* have been recognized [287]. In 1987, a standardized phage typing schema with 16 phages for EHEC O157 was developed and so far, more than 80 phage types have been recognized [288].

Phage typing has proven to be an important tool for strain characterization. It is cheap and does not require specific equipment and the results obtained have been applied since the mid-60s in surveillance, source attribution and outbreak investigations [45, 289]. The limitations of phage typing include occurrence of non-typeable strains and inadequate discriminatory power. Further drawbacks are that it is performed only in some of the National reference laboratories (since only these institutions have access to the defined sets of typing phages) and the interpretation of the lysis results requires considerable experience [290]. For example, some international outbreaks have been confused due to different phage type interpretation between countries [289]. Also, a phage-conversion event due to, for example, expression of temperate phages or loss or gain of a plasmid might lead to different interpretation of a certain phage type [291]. In order to guarantee the comparability among the laboratories and maintain the expertise, standardization and annual external quality assurance are important for the Reference laboratories. Despite the limitations, phage typing still is a valuable tool for the first evaluation of potential outbreak, especially for less common phage types, or identifying strains related to certain countries or sources [292, 293].

#### *Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing is an important and useful test performed by the clinical microbiology laboratory enabling an optimal antimicrobial treatment of the patient if needed. The method is also commonly used for surveillance of resistance among bacteria and for strain typing of *Salmonella* and EHEC. Resistance profiles towards a specific set of antimicrobials can be determined by the disk diffusion method into three categories: susceptible (S), intermediate (I) and resistant (R). In order to harmonize the performance and determination of breakpoints for each drug, standardized protocols have been developed by the Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The method is cheap, simple and does not require specific equipment. The drawbacks include poor discriminatory power and instability of certain resistance factors under selective pressure or storage that are carried in mobile elements such as plasmids and integrons [294]. Furthermore, there is a lack of harmonization in terms of drugs tested and criteria used for interpretation, particularly between human and veterinary laboratories [295].

#### *Other phenotyping methods*

Phenotype MicroArray™ (PM) technology (by Biolog, CA, USA) is based on simultaneous testing of large amounts of bacterial growth phenotypes [296, 297]. Preconfigured 96-well plates can be used for testing the ability to utilize carbon, nitrogen, phosphorous and sulfur substrates, the cellular response to different pH conditions, osmolytes and chemicals such as antibiotics. PM assay measures cellular respiration colorimetrically via reduction of a tetrazolium dye. Metabolized substrates generate a purple color whose intensity is monitored and recorded automatically with a camera on the Omnilog instrument [297]. The PM technology can be applied for an overview of metabolic capability or it can be tailored to address specific research needs, also related to outbreaks. For example, PM analysis of the EHEC O157:H7 strain related

to a spinach outbreak in the USA showed a rare N-acetyl-D-galactosamine-negative phenotype, which had only been found once before [298]. However, for routine surveillance of foodborne pathogens, it is too expensive and it would be challenging to cluster metabolic differences in the outbreak situation.

## 2.6.2 Genotyping methods

Phenotypic typing methods, which require time, skilled personnel and expensive sera, have led to the development of typing methods based on genotypic information. Currently used molecular typing methods include techniques such as nucleic acid amplification (polymerase chain reaction, PCR and multilocus variable-number tandem repeat analysis, MLVA), restriction endonuclease digestion (pulsed-field gel electrophoresis, PFGE) and nucleotide sequencing techniques (single gene sequencing, multilocus sequence typing, MLST and whole genome sequencing, WGS).

### *Polymerase chain reaction (PCR)*

PCR was developed by Saiki *et al.*, in 1985 and Mullis and Faloona in 1987 [299, 300]. In PCR, a known DNA sequence is amplified from the genome using specific primer pairs which hybridize to the complementary target sequence and thermostable DNA polymerase which catalyzes the assembling of nucleotides. All newly synthesized PCR-product are used as templates in the following cycle. One PCR cycle consists of three thermal steps: denaturing of the double strand DNA, annealing of the primers to each single strand and elongation of the fragment. In theory, PCR can multiply the amount of DNA molecules exponentially up to a billion-fold. The advantages of PCR include sensitivity and portability. However, the sensitivity is also the disadvantage of PCR since contaminating DNA is also amplified.

PCR methods (conventional, real-time and multiplex) have been widely used for both pathogen identification from different sample types and for detection of virulence genes for typing purpose. PCR has been applied for detection of *stx* genes which are characteristic of both EHEC O157 and non-O157 and for *invA* gene which is characteristic of *Salmonella* [301]. In addition, further PCR-based characterization of *stx* genes into different variants has improved the epidemiological investigations while certain variants have been associated with more severe disease [302]. PCR-based methods have also been used for the determination of the most common EHEC [281] and some *Salmonella* serovars [280, 303, 304]. Moreover, PCR methods for virulence gene profiling of EHEC by 5-plex PCR [279, 305] or rapid detection of different pathogroups of *E. coli* by 16-plex PCR [306] have been successfully developed. PCR assays specific for EAHEC O104:H4, which was associated with the large outbreak in Germany, have recently been developed [307].

### *Pulsed- field gel electrophoresis (PFGE)*

PFGE was initially been developed in 1984 [308]. In the last decade, it has become the primary typing tool used in identification of bacterial relatedness and in outbreak investigations [309]. There is a long tradition aiming at international harmonization of PFGE protocols for different foodborne pathogens by PulseNet [310]. Therefore, PFGE is considered as “the gold standard” among bacterial molecular typing [311, 312]. PFGE is a molecular typing method in which the whole genome of the bacteria is fragmented using rare cutting restriction enzymes (endonuclease). These enzymes recognize certain restriction sites in the genome usually yielding less than 30 fragments. For *Salmonella* and EHEC, the restriction enzyme XbaI has been the most

used. The resulting fragments, which are mostly large, are then run in an agarose gel within an electric field in which the angle is changed periodically, in order to allow the large fragments to move through the gel. Even fragments of up to 10 Mb can be separated by PFGE. The fragments move through the gel according to size and create a “fingerprint” based on the position of the bands that defines the type. Commercially available software programs such as Bionumerics (AppliedMaths, Kortrijk, Belgium) are available for accurate analysis and PFGE patterns can be shared for global comparison [313].

The advantages of PFGE include high discriminatory power and epidemiological concordance. Numerous studies have demonstrated its value in outbreak investigations of *Salmonella* and EHEC [314-316]. Moreover, it has high typeability since almost all strains can be typed. The most widely used standardized PFGE protocol is the PulseNet protocol developed for long-term surveillance and outbreak investigation of *Salmonella* and EHEC infections [310]. In addition, international PFGE databases such as PulseNet have been established which allow the rapid detection of the spread of new subtypes [315, 317, 318]. Although widely used, PFGE has several limitations. For example, the method is labor-intensive taking at least 2-3 days (in addition to sample preparation), cannot be automated, is expensive and requires skilled personal to analyze the gels [319]. Another drawback is the lack of clear rules about how to interpret PFGE data in outbreak situations or how to cluster outbreak-related strains. For example, two outbreak-related strains may show minor differences in their PFGE pattern [316] whilst identical PFGE profiles may be generated to outbreak-unrelated strains of common clones such as *S. Enteritidis* PT4 [320] or *S. Typhimurium* DT104 [321]. The differences in the PFGE profile of outbreak-related strains are thought to be due to point mutations, inserts and deletions in the genome or loss or acquisition of a plasmid. According to the generally accepted “Tenover criteria” a single point mutation in a restriction site may result in a difference of up to three bands and therefore profiles differing from each other by one to three band positions should be considered closely related [312]. More recent guidelines for foodborne outbreaks suggest that only isolates with indistinguishable profiles should be included into infections cluster, and profiles with 2-3 differences in the band position could be included if the outbreak persists for long time or if the infections are recognized as secondary infections due to person-to-person spread [309]. Therefore, the similarity of PFGE profiles should not be considered alone as a measure for genetic distance and the PFGE results should always be evaluated together with the epidemiological data.

#### *Multilocus variable-number tandemrepeat analysis (MLVA)*

MLVA is based on size polymorphisms in a certain variable number of tandem repeat (VNTR) areas within the bacterial genome [322]. These VNTR units mutate rapidly which enables the study of genetic relatedness between isolates [323]. They can locate in coding and non-coding regions in the genome. The number of repeats can be determined by PCR amplification and following fragment analysis by capillary electrophoresis. The use of fluorescently labeled primers in the capillary electrophoresis allows all amplicons to be analyzed in one run. In comparison to conventional agarose gel electrophoresis, the amplicon size can be much more accurately determined by DNA sequencer. Using computer software (freely available e.g PeakScanner), all loci can be distinctly recognized according to the dye colors, and based on the size of the PCR-product, the exact number of repeats for each MLVA loci can be calculated. Afterwards, an allele

number corresponding to the number of repeats of specific MLVA locus can be given (e.g 3-16-NA-NA-0311) and the MLVA profile can be easily internationally compared [324].

MLVA is one of the most common genotyping method used for public health surveillance and epidemiological investigations of *Salmonella* (serovars Typhimurium and Enteritidis) and EHEC O157 [321, 325-328]. Advantages of MLVA include speed, high discriminatory power and reduced handling times of pathogenic organisms. In comparison to PFGE, it is cheaper, can be automated and the generated data are easier to interpret [313]. The drawbacks of MLVA include a species-specific protocol meaning that the same MLVA scheme is not equally discriminative between species or between different *Salmonella* serovars. Currently in the EU, well-standardized and general accepted MLVA protocols exist for only *S. Typhimurium* [324] and *S. Enteritidis* [325]. Furthermore, for these two serovars a set of reference strains with verified fragment sizes is available allowing for normalization and direct comparison between laboratories. However, differences in the choice of loci and the nomenclature may confuse the inter-laboratory comparison (Table 7). Several MLVA typing scheme have been developed for the typing of EHEC O157 [329-331] and for some non-O157 serotypes [332, 333]. Also, some studies on generic MLVA for all *E. coli* isolates have been proposed [334]. Similar to PFGE, there is a need for clear guidelines on how to interpret MLVA results in outbreak situations. An acceptable difference in the number of repeats in one or more loci should be defined. The size difference in a VNTR locus does not always reflect the real number of repeats because insertion and deletions due to occasional DNA polymerase mistakes may occur during the outbreak [322, 335]. A recent study on *S. Enteritidis* suggested that PFGE together with MLVA would provide the most efficient subtyping [336].

In contrast to PCR-based MLVA typing, an alternative strategy for epidemiological typing by MLVA is the DNA sequencing of the produced amplicons. Sequencing-based methods for VNTR determination have been developed for example for *Vibrio cholera* [337]. However, sequencing is not widely used since specific equipment is required and it would be more expensive than capillary electrophoresis.

**Table 7:** Nomenclature of MLVA loci for *Salmonella Enteritidis* and *Typhimurium* in Europe and the USA.

<b>S. Enteritidis VNTR loci</b>	<b>S. Typhimurium VNTR loci</b>	<b>Europe</b>	<b>PulseNet USA</b>
SET533		SENTR7	SE9
SET2073		SE3	SE3
SET2504		SENTR4	SE1
SET3073		SENTR5	SE5
SET3511		NA	SE6
SET4617		SENTR6	SE2
	STM2730	STTR6	ST5
	STM3184	STTR5	ST6
	STM3246	STTR9	ST7
	STM3629	STTR3	ST8
	pSLT53	STTR10	STTR10



### *Multilocus sequence typing (MLST)*

MLST is based on the principles of phenotypic multilocus enzyme electrophoresis (MLEE) which first revealed the clonal nature of some bacteria [338]. In MLST, multiple (usually five to seven) highly conserved housekeeping genes are amplified by PCR and sequenced [339]. For each gene, a specific sequence type is assigned based on the alleles. Moreover, isolates can be clustered into clonal complexes based on their sequence types. Since housekeeping genes are robust and under limited selective pressure, MLST is a valuable tool in evolutionary studies and population genetic analyses [313]. Other advantages of MLST include that it has high typeability, it is reproducible, and internet-based MLST databases (e.g [www.mlst.net](http://www.mlst.net)) allow standardized nomenclature and inter-laboratory comparison. Several studies on *Salmonella* [340-342] and *E. coli* [329, 343] have revealed that the discriminatory power (especially the ability to discriminate between isolates of the same serovar) of MLST is low. Also, the relative high cost of separate gene sequencing hinders the use of MLST typing in the routine surveillance of foodborne pathogens. In order to improve the discriminatory power of traditional MLST, protocols which additionally included more rapidly changing genes such as temperate phages [290] or virulence genes [340] have been developed. In general, the discriminatory power was not higher than by traditional MLST [340, 344]. In the near future MLST might be replaced with WGS typing.

### *Future molecular typing methods*

Novel molecular subtyping methods based on single-nucleotide polymorphism (SNP) using Sanger sequencing and whole genome sequencing (WGS) have been applied for characterization of foodborne pathogens [345]. SNP method is based on random single-nucleotide mutations that happen independently over time throughout the bacterial genome. The differences in the number of SNPs between two organisms define the genomic distance between the isolates and this relationship has been used in outbreak investigations of *Salmonella* and EHEC [346, 347]. In comparison to Sanger sequencing, WGS allows the discovery of SNPs across the bacterial genome [348]. WGS can be applied for species identification [349], genotyping [350], determination core genome or virulence genes and for outbreak investigations [351-353]. Next generation sequencing approaches have made WGS widely available, and due to new and easy to handle bench-top sequencers, even small microbiological laboratories are able to do sequencing by themselves. In the near future WGS will be affordable, fast and simple to use and will be an increasingly attractive method for strain characterization and typing. The potential of WGS was demonstrated in 2011 during the German outbreak caused by multi-drug resistant EAHEC O104:H4 -contaminated sprouts. The sequencing of samples from an early stage of the outbreak suggested that the outbreak had a clonal origin [277]. Further analysis indicated that the outbreak strain had originated from an EAEC O104:H4 backbone, and by gain and loss of chromosomal and plasmid-encoded virulence factors there emerged a highly pathogenic hybrid of EAEC and EHEC [277]. In addition, the isolates from this large German outbreak were compared to the isolates from a smaller French outbreak, suggesting that there were no direct transmission between these populations, but instead both outbreaks emerged by different transmission routes from the same fenugreek sprout seed batch [354]. The major challenges of WGS will be the harmonization of the WGS pipelines and protocols (comparable and high quality data) and the extraction of needed information from the large amount of detailed information available. Moreover, once sequenced, the data can be easily reanalyzed and tailored based on interest and study design or if new software programs are available in the future. In addition, to compare

results obtained with WGS and results obtained by traditional methods, it is important to be able to evaluate the concordance of the methods [348]. As with any genotyping method used for outbreak investigations, WGS data should be linked with epidemiological data.

### **3 AIMS OF THE STUDY**

The aims of this study were:

1. to apply genotyping for outbreak investigation and trace back the sources of human *Salmonella* and EHEC infections.
2. to investigate the diversity of *Salmonella* and EHEC isolates from domestically acquired infections in Finland.
3. to evaluate MLVA method for epidemiological typing purposes of *Salmonella* Typhimurium.

## 4 MATERIAL AND METHODS

The material and methods used in this study are summarized in Table 8 and 9. Materials and methods are described in detail in the original articles (I, II, III and IV).

### 4.1 *Salmonella* and EHEC strains (I-IV, unpublished data)

**Table 8:** Bacterial strains used in this thesis (from the strain collection of THL, Finland).

Organism (serovar/serotype)	Selection criteria for isolates	Isolation year	No. of isolates	Study
<i>Salmonella</i> (Agona Enteritidis, Give, Hvittingfoss, Infantis, Newport and Typhimurium)	Strains from seven most common salmonella serovars in Finland were chosen for phenotype microarray.	1995	29	I
<i>Salmonella</i> (Newport and Reading)	All strains related to nationwide <i>S. Newport/S. Reading</i> outbreak in Finland were studied	2008	77	II
<i>Salmonella</i> (Typhimurium)	All domestic <i>S. Typhimurium</i> strains (one strain/patient, clusters or outbreaks excluded) were studied	Nov 2007- Dec 2012	375	III
EHEC (O78:H)	Family cluster: EHEC strains isolated from stool and blood of the five family members	2009	6	IV
<i>Salmonella</i>	Domestically acquired infections	2007-2014	2337	Unpublished
EHEC	Domestically acquired infections	2007-2014	175	Unpublished

#### 4.1.1 Strains isolated from domestically acquired *Salmonella* and EHEC infections

All *Salmonella* and EHEC strains isolated in Finland during 2007-2014 from patients that had not been abroad recently (within one week prior onset of symptoms) were studied. Clinical laboratories across Finland are obligated to report all *Salmonella* and EHEC findings to the NIDR at the THL and send all isolates to the Bacterial Infections Unit for verification and further typing. In Finland, all patients with EHEC infection are interviewed about their travel history, diet, symptoms and connection to farms, and the contact persons and farms are sampled.

## 4.2 Methods

**Table 9:** Methods used in this thesis. Roman numerals refer to the publications in which the methods are used and described in detail.

Methods	Organism	Described in publication
<b>Phenotyping methods</b>		
Antimicrobial susceptibility testing with disc diffusion method	<i>Salmonella</i> /EHEC	I, II, III, IV
API 20E test/sorbitol fermenting	EHEC	IV
Minimum inhibitory concentration test	<i>Salmonella</i>	III
Phage typing	<i>Salmonella</i>	I, III
Phenotype microarray	<i>Salmonella</i>	I
O:H serotyping	<i>Salmonella</i> /EHEC	I, II, III, IV
Toxin production by VTEC-RPLA	EHEC	IV
<b>Genotyping methods</b>		
16-plex PCR	EHEC	IV
5-plex PCR ( <i>stx1</i> , <i>stx2</i> , <i>eae</i> , <i>hlyA</i> and <i>saa</i> )	EHEC	IV
Multilocus variable number tandem repeat analysis (MLVA) with VNTR loci STTR3, STTR5, STTR6, STTR9 and STTR10p	<i>Salmonella</i>	III
Pulsed-field gel electrophoresis (PFGE) with XbaI	<i>Salmonella</i> and EHEC	I,II, III, IV
<i>stx1</i> gene detection by sequencing	EHEC	IV
<b>Other methods</b>		
Statistical methods	<i>Salmonella</i>	I, III
Case-control-study	<i>Salmonella</i>	II

### 4.2.1 Changes in antimicrobial susceptibility testing of *Salmonella* and EHEC during this study (I, II, III, IV and unpublished data)

During this study, the antimicrobial susceptibility to 12 antimicrobials was determined by the agar diffusion method on Müller-Hinton II agar: ampicillin (A) (10 µg), chloramphenicol (C) (30 µg), streptomycin (S) (10 µg), sulphonamide (Su) (300 µg), tetracycline (T) (30 µg), ciprofloxacin (Cp) (5 µg), trimethoprim (Tm) (5 µg), gentamicin (G) (10 µg), nalidixic acid (Nx) (30 µg), cefotaxime (Ct) (5 µg), mecillinam (M) (10 µg) and imipenem (I) (10 µg). However, for 2007-2010, the protocols and breakpoints of the Clinical and Laboratory Standards Institute (CLSI) and for 2011-2014 those of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org/>) were applied. Minimum inhibitory concentration (MIC) for ciprofloxacin (from 0.002 to 32 mg/L) was detected by E-Test (bioMérieux, Marcy l'Etoile, France) for the strains that were resistant (R) or intermediate resistant (I) to nalidixic acid. Ciprofloxacin MIC breakpoint ≤0,06 mg/L was interpreted as susceptible (<http://www.eucast.org/>). Multi-drug resistance (MDR) was defined as resistance to four or more antimicrobial groups.

#### 4.2.2 **Changes in *Salmonella Typhimurium* MLVA during this study (III and unpublished data)**

The MLVA method was set up in THL in November 2007. For that, the calibration of MLVA was performed using 33 *S. Typhimurium* strains (the MLVA calibration set was obtained from SSI (Statens Serum Institute, Denmark). Between November 2007 and December 2012 MLVA was performed as described in study III to all *S. Typhimurium* strains isolated from domestically acquired infections. Since January 2013, the MLVA method was adjusted according to the protocol of European Centre for Disease Prevention and Control (ECDC) and some changes in primer labeling was performed (Table 10). Both, the fluorescent-labeled forward primers and unlabeled reverse primers, were obtained from Applied Biosystems (CA, USA). The calibration of MLVA was repeated when primers were changed. The new fluorescent labels showed no effect on fragment size in capillary electrophoresis using the 33 reference strains. In paragraph 5.1.2 the MLVA results obtained with both sets of primer labels are presented. For the MLVA (Nov 2007-Dec 2012 described in study III and Jan 2013-Dec 2014 described in this study 5.1.2) the strain selection criteria was following: one strain per patient was chosen and known epidemics and family clusters were excluded.

**Table 10:** Fluorescence labels used in MLVA analysis at THL.

MLVA primers	Fluorescent labels during 1 <sup>st</sup> of November 2007 to 31 <sup>st</sup> of December 2012	Fluorescence labels since 1 <sup>st</sup> of January 2013 (ECDC protocol)
STTR-3 forward	NED	PET
STTR-5 forward	NED	VIC
STTR-6 forward	FAM	FAM
STTR-9 forward	FAM	FAM
STTR-10 forward	PET	NED

#### 4.2.3 **Phage typing of *EHEC O157* (done for unpublished strains)**

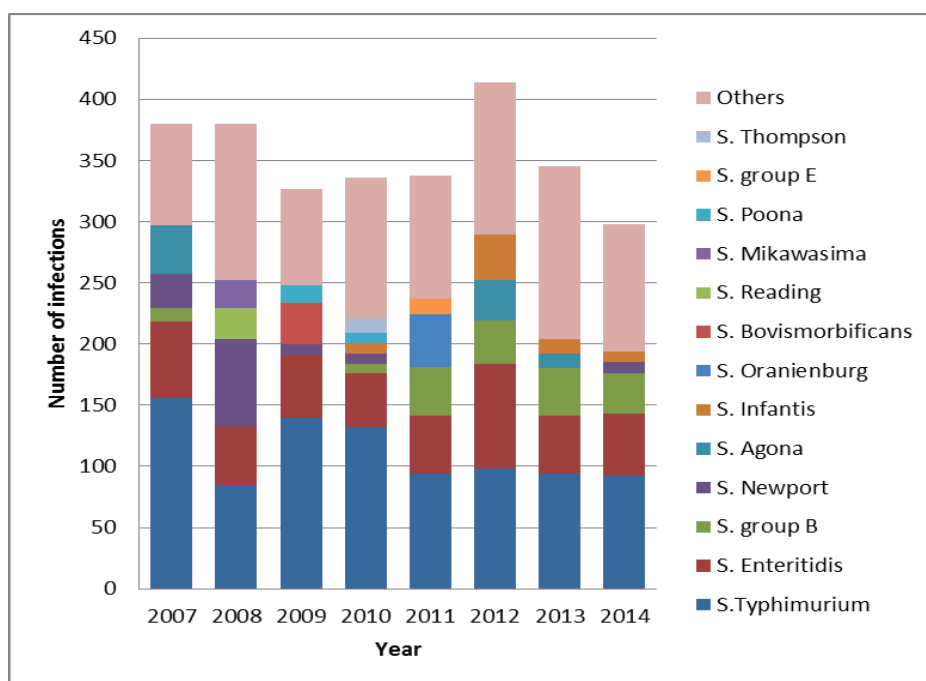
For phage typing, one bacterial colony of *EHEC O157* were grown in double-strengt nutrient broth (Difco laboratories, Dietroit, USA) for 1.5 hours at 37°C and 80 rpm, and flooded onto double-strengt nutrient agar plates (Difco). The 16 *EHEC O157* specific bacteriophages used in this study were obtained from Public Health England, formel HPA. Phage-typing was carried out as described by Ahmed et al. [288] and extended by Khakhria et al. [355]. The results were analysed using a reference table in which 66 confirmed and 14 provisional phage types and lytic reaction are listed.

## 5 RESULTS

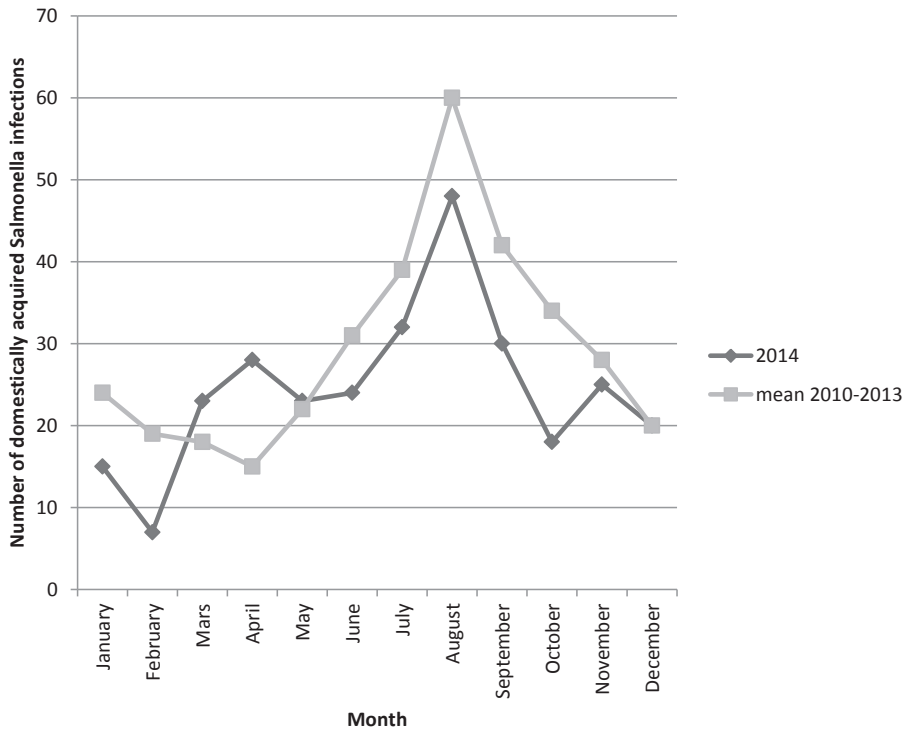
### 5.1 Pheno- and genotypic characteristics of certain *Salmonella* serovars (I-III and unpublished data)

#### 5.1.1 *The most common Salmonella serovars and phage types among domestically acquired infections 2007-2014*

During 2007-2014, there were a total of 2,818 (15%) reported domestic *Salmonella* infections among all 18,303 reported *Salmonella* infections in Finland. The incidence of domestic *Salmonella* infections varied between 5.4-7.5/100,000 inhabitants being highest in 2012 and lowest in 2014. The three most common serovars were Typhimurium (891 strains, 32%), Enteritidis (436 strains, 15%) and group B (155 strains, 6%) (Fig. 5). The majority of *S.* group B are monophasic Typhimurium. During 2007-2014, a total of 131 different *Salmonella* serovars were isolated from domestically acquired human infections but only six serovars (Typhimurium, Enteritidis, group B, Agona and Infantis) comprised 65% of the 2,818 serotyped domestic *Salmonella* strains. A clear seasonal trend in confirmed salmonellosis cases was observed, with most cases reported during the late summer months (Fig. 6).



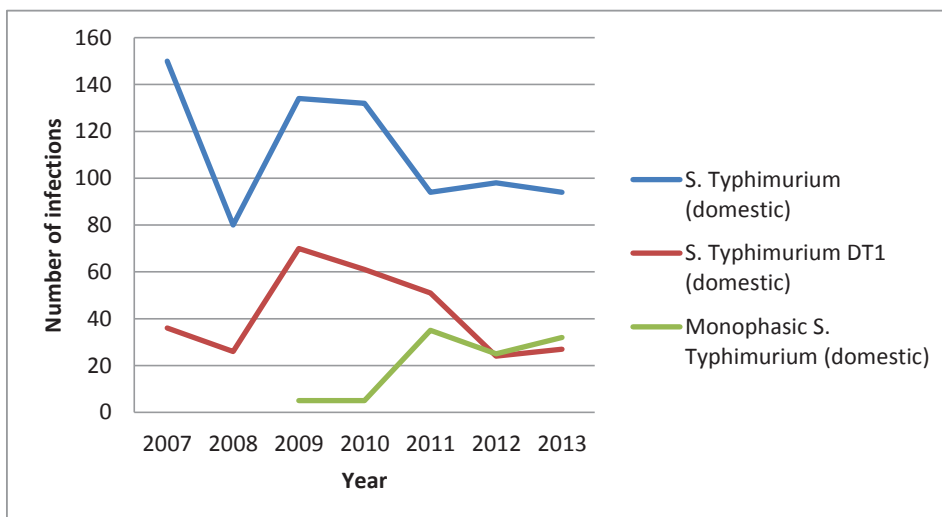
**Figure 5:** *The most common serotypes among domestically acquired Salmonella infections in Finland 2007-2014 (one strain/patient).*



**Figure 6:** *The seasonal trend of domestically acquired Salmonella infections in Finland.*

All the domestic *S. Typhimurium* and *S. Enteritidis* and strains were phage typed. Among *S. Typhimurium* strains (including clusters and outbreaks), DT1, RDNC and DT104 were the most common phage types. The most frequently detected phage types among the domestically acquired *S. Enteritidis* infections were PT8, PT1B and PT4. Since the 60's endemic phage type DT1 has caused infections and small epidemics in Finland. During 2007-2014, 37% of all domestic *S. Typhimurium* infections were caused by DT1. The number of domestically acquired *S. Typhimurium* infections has decreased during the study period. Meanwhile, the number of infections caused by domestic monophasic *S. Typhimurium* has increased since 2011 (Fig. 7). During 2012-14, a total of 84 PCR confirmed monophasic *S. Typhimurium* strains were detected among domestically acquired infections, and the most common phage types were DT193 in 2012, DT195 in 2013 and DT120 in 2014. The majority of *S. Typhimurium* and *S. Enteritidis* strains (both approximately 60%) were susceptible against all tested antimicrobials whereas only 10% of *S. group B* were fully susceptible.





**Figure 7:** Trends of domestic *S. Typhimurium* (2007-2013), endemic *S. Typhimurium* DT1 (2007-2013) and monophasic *S. Typhimurium* (2009-2013).

### 5.1.2 Distribution of susceptible and multi-resistant strains among phage types in domestic *Salmonella Typhimurium* infections between Nov 2007-Dec 2014 (III, unpublished data)

During Nov 2007-Dec 2014, a total of 546 sporadic *S. Typhimurium* strains isolated from Finns, who had not traveled abroad recently, were typed by phage typing and antimicrobial susceptibility testing. One strain per patient was included and epidemics and family clusters were excluded from the study. Among the strains, 31 distinct phage types were detected (Table 11). Of all strains, 78% (426 strains) were assigned to the five most common DTs: DT1 (43% of the strains), RDNC (16% of the strains), U277 (8% of the strains), DT104 (7% of the strains) and DT120 (4% of the strains). The majority of the strains (62%) were susceptible to all tested antimicrobials. However, 12% of the *S. Typhimurium* strains were MDR (resistant to at least four antimicrobials when a set of 12 antimicrobials were tested by the disk diffusion test). Among 18 phage types (DT2, DT7, DT7A, DT8, DT9, DT10, DT15A, DT40, DT40, DT41, DT69, DT82, DT99, DT132, DT136, U277, U287, U310), none of the strains were MDR. In contrast, all strains of phage types DT104B and U311 were MDR. Of all non-susceptible strains for nalidixic acid, 25 strains had a decreased susceptibility to ciprofloxacin (MIC values were ranging from 0.125 to 4 mg/L). Decreased susceptibility to ciprofloxacin was observed among 15 DT104 (0.125-0.25 mg/L), four U311 (0.125-0.5 mg/L), two DT120 (0.25-0.5 mg/L), two NT (0.25-0.5 mg/L), one DT104B (4 mg/L) and one U310 (0.5 mg/L) strains. In comparison, only one domestic monophasic *S. Typhimurium* strain was fully susceptible (DT193 in 2013) and 96% of all strains were MDR. Resistance to ampicillin, streptomycin, sulfonamide and tetracycline (R-type ASSuT) was the most frequent (77%). In addition, the increase of resistance to nalidixic acid was evident in 2014 (9 strains) compared to 2012-2013 (1 strain each year).

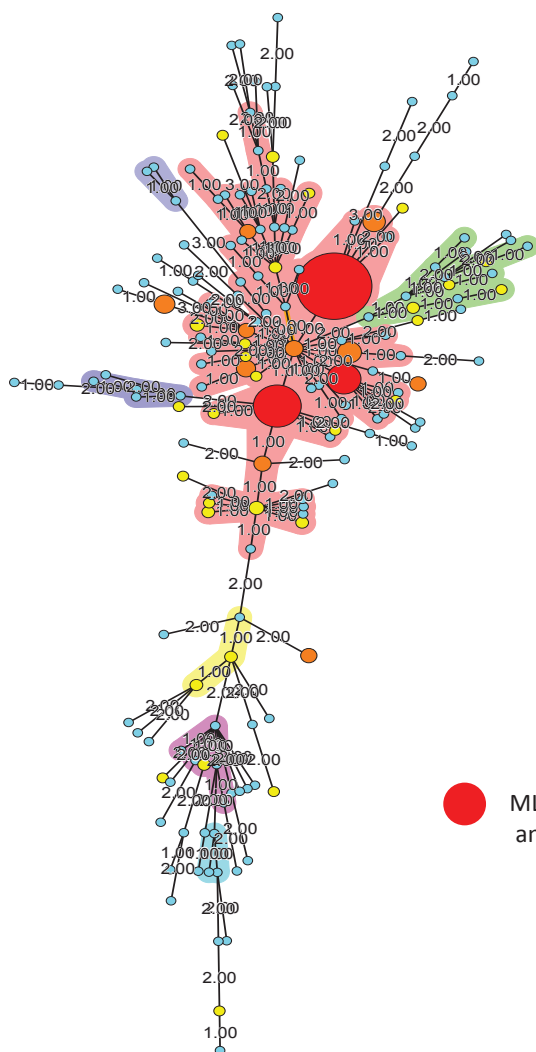
**Table 11:** Distribution of phage types and antimicrobial resistance among 546 *S. Typhimurium* strains isolated from sporadic infections in Finland, Nov 2007- Dec 2014.

Phage types (No. of strains)	No. of susceptible strains (% of strains within each phage type)	Multidrug resistance (MDR) profiles (no. of strains)*
DT1 (233)	172 (74%)	ASSuTm (1)
U277 (44)	38 (86%)	-
DT104 (40)	3 (8%)	ACSSuTNx (14), ACSSuT (6)
DT120 (23)	6 (26%)	ACSSuT (8), ASSuT (4)
DT41 (15)	12 (80%)	-
DT116 (14)	9 (64%)	ASSuTTm (2), ASSuT (1)
U302 (11)	5 (45%)	ACSSuTGM (2), ACSSuTG (1), ACSSuT (1)
DT195 (11)	2 (18%)	ASSuT (2), ACSSuTct (1)
DT104B (10)	0	ASSuT (4), ACSSuT (4), ACSSuTTm (1), CSSuTGNx (1)
DT193 (8)	0	ASSuT (1), ACSSuT (1), ASSuTTm (1)
DT2 (6)	4	-
DT12 (6)	2	ACSSuT (2)
DT10 (5)	4	-
DT135 (5)	3	ASSuTTm (2)
U282 (4)	4	-
U311 (4)	0	ACSSuT (1), ASuTNx (1), ASSuTNx (1), ACSSuTTmNx (1)
DT15A (3)	1	-
NT (3)	1	ASSuTTmNx (1)
DT8 (2)	1	-
DT9 (2)	0	-
U312 (2)	1	-
DT7, DT7A, DT40, DT69, DT99, DT132, DT136, U287, U310 (1 strain of each type)	7	-
RDNC (86)	66 (77%)	SSuT (1), ACSuTTm (1), ASSuT (1)
<b>A total of 31 DTs were identified</b>	<b>Of all strains, 341 (62%) were susceptible to all 12 antimicrobials</b>	<b>Of all strains, 68 (12%) were MDR</b>

\*ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamide (Su), tetracycline (T), trimethoprim (Tm), gentamicin (G), nalidixic acid (Nx), cefotaxime (Ct), mecillinam (M).

### 5.1.3 MLVA subtypes among domestic *Salmonella* Typhimurium strains (III, unpublished data)

During November 2007-December 2014, 546 sporadic *Salmonella* Typhimurium strains isolated from Finns who had not been abroad recently were subtyped by 5-loci MLVA. One strain per patient was included and known epidemics and family clusters were excluded from MLVA analysis. The studied strains belonged to 31 different phage types and were divided into 170 distinct MLVA types. All strains were typeable with 5-loci MLVA and the Simpson's diversity index (DI) for MLVA was 0.891. The minimum spanning tree (MST) of the MLVA results showed that the Finnish *S. Typhimurium* strains clustered together (Fig. 8). The three most common profiles (3-16-NA-NA-0311, 3-15-NA-NA-0311 and 3-17-NA-NA-0311, red circles in the Fig. 8) counted for 47 % of the strains indicating that domestic strains were homogenous. XbaI-PFGE profile STYM1 was the most common among the domestic DT1 strains and STYM8 among the U277 strains. STYM1 profile (146 strains) was divided into 12 MLVA types and STYM8 into 9 MLVA profiles (Table 12). Among STYM1 and STYM8, the MLVA profile 3-16-NA-NA-0311 was the most common.



**Figure 8:** MST of MLVA types (546 sporadic *S. Typhimurium* isolates from domestically acquired infections). Categorical coefficient was used to construct the MST. Each circle in the tree represents a different MLVA type. Heavy, short lines connecting two MLVA types denote types differing by a single MLVA locus. The halos extending over the various types denote the grouping by Bionumerics analysis. The color and size of a circle indicate the numbers of the strains with a particular MLVA type: light blue: 1 strain; yellow: 2-5 strains; orange 6-30 strains and red: >30 strains. The most common MLVA types are marked with red and orange dots.

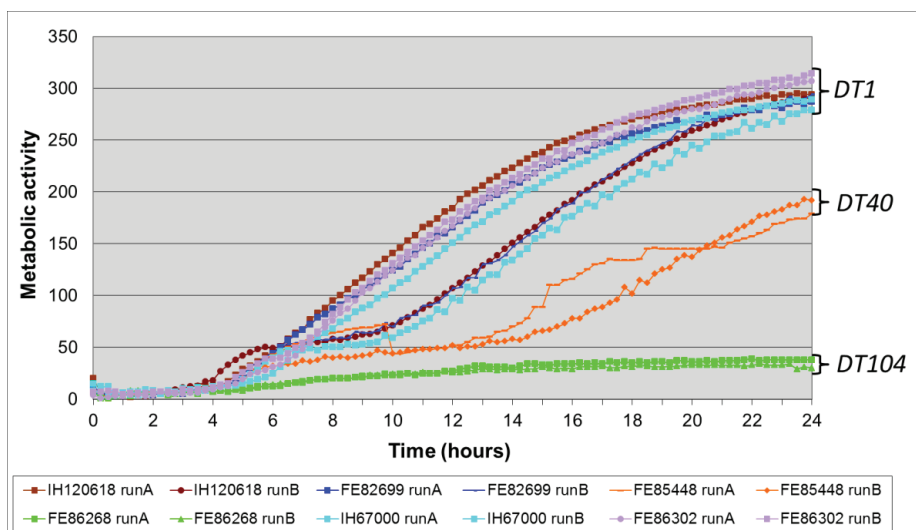
**Table 12:** MLVA types among the two most common XbaI-PFGE profiles among sporadic domestically acquired *Salmonella* infections, Nov 2007- Dec 20014.

XbaI-PFGE profile	No. of strains	MLVA type	No. of strains
STYM1	146	3-16-NA-NA-0311	85
		3-15-NA-NA-0311	20
		3-17-NA-NA-0311	9
		3-16-NA-NA-0211	7
		3-15-NA-NA-0211	7
		3-18-NA-NA-0311	6
		3-13-NA-NA-0311	4
		3-6-NA-NA-0311	3
		3-14-NA-NA-0311	2
		3 MLVA types (1 strain each)	3
	<b>total</b>	<b>12 MLVA types</b>	<b>146</b>
STYM8	34	3-16-NA-NA-0311	12
		3-18-NA-NA-0311	6
		3-16-NA-NA-0211	5
		3-15-NA-NA-0311	5
		3-14-NA-NA-0311	2
		4 MLVA types (1 strain each)	4
	<b>total</b>	<b>9 MLVA types</b>	<b>34</b>

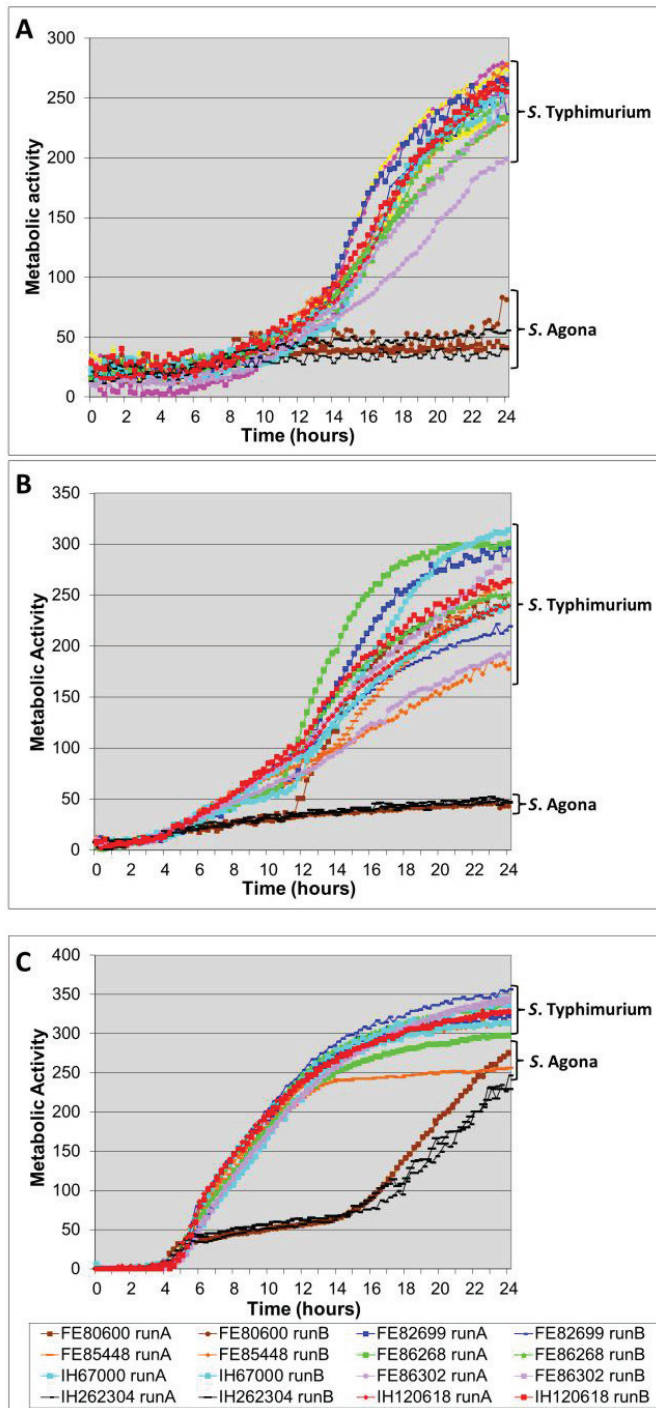
NA=no amplification

#### 5.1.4 Metabolic characteristics of certain *Salmonella enterica* (I)

Phenotype microarray (PM) plates 1-10 containing 949 substrates were tested for six *S. Typhimurium* and two *S. Agona* strains and all tests were performed in duplicate. Only a few substances led to differences in the phenotype. Among *S. Typhimurium*, 15 substrates (m-tartaric acid, m-inositol, glyoxylic acid, D-galactonic acid  $\gamma$ -lactone, L-alanine, L-rhamnose, itaconic, L-proline and the dipeptides Leu-Ala, Ala-Ala, Ala-Val Tyr-Ala, Trp-Glu, Asp-Trp Met-Phe) showed differences in the metabolic activity (In study I). Interestingly, *S. Typhimurium* phage types DT1, DT40 and DT104 differed in their carbon utilization characteristics when tested with m-tartaric acid (Fig. 9). DT104 strain could not assimilate glyoxylic acid and m-inositol as carbon sources. The strains of XbaI-PFGE profile STYM1 differed on itaconic acid, L-proline, Trp-Glu, Asp-Trp, and Met-Phe. Furthermore, *S. Agona* differed from *S. Typhimurium* on D-tagatose (Fig. 10). The studied *S. Typhimurium* strains were typeable by PM assay and reproducible respiratory activity was measured in two independent experimental run. Other studied *Salmonella* serovars showed even less metabolic diversity in PM analysis.



**Figure 9:** The ability of six different *S. Typhimurium* strains to utilize *m*-tartaric acid as a carbon source was measured in duplicate sets. The analyzed strains belong to the phage types DT1 (IH67000, FE86302, FE82699 and IH120618), DT40 (FE85448) and DT104 (FE86268). This graphic presentation uses the area under the curve (AUC) values obtained when the color change was measured in every 15 min using OmniLog software. *S. Typhimurium* DT104 was unable to utilize *m*-tartaric acid as a substrate whereas *S. Typhimurium* DT40 and DT1 were able to metabolize the substrate at intermediate and high rates, respectively.



**Figure 10:** The respiratory profiles of *S. Typhimurium* strains IH67000, FE86302, FE82699 and IH120618 (phage type DT1), FE85448 (phage type DT40) and FE86268 (phage type DT104) and *S. Agona* isolates FE80600 and IH262304 based on AUC values in duplicate measurements. *S. Agona* stains showed no respiratory activity on D-tagatose (A), D-galactonic acid  $\gamma$ -lactone (B) and delayed activity on L-proline (C).

## 5.2 XbaI-PFGE profiles in the outbreak investigation of *Salmonella* Newport and *Salmonella* Reading infections (II)

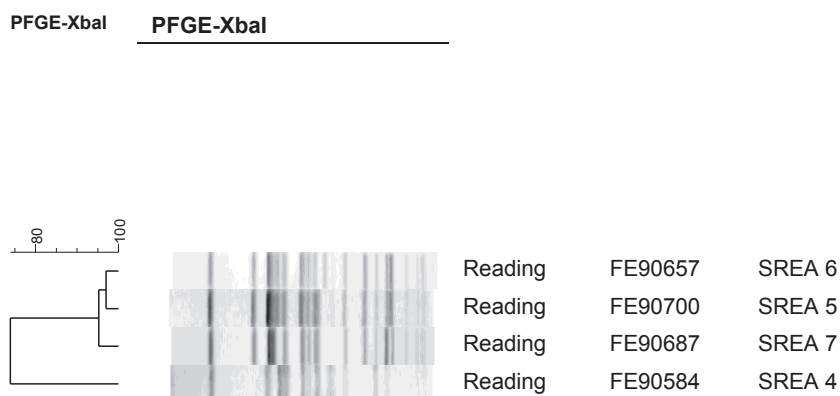
In 2008, a nationwide outbreak caused by *S. Newport* and *S. Reading* affected 107 persons in Finland. Three temporal and geographical clusters with an epidemiological link were identified, and in stool sample of one patient from Lapland cluster both serotypes were isolated (Study II, Fig. 2). A total of 77 culture-confirmed *S. Newport* cases and 30 *S. Reading* ones were identified. All 77 outbreak-related *S. Newport* strains belonged to the same XbaI-PFGE profile SNWP59 (SNWPXB.0068) (Table 13).

**Table 13:** *S. Newport* and *S. Reading* genotypes associated to the outbreak. The analyzed sample types were feces (F), urine (U) and blood (B).

Serotype	XbaI-PFGE profile	No. of strains	Sample type
<i>S. Newport</i> (6,8:e,h:1,2)	SNWP59	77	F (67), U (6), B (4)
<i>S. Reading</i> (4,12:e,h:1,5)	SREA4	3	F (2), B (1)
	SREA5	23	F (20), U (2), B (1)
	SREA6	3	F (2), B (1)
	SREA7	1	F (1)*

\* Double infection

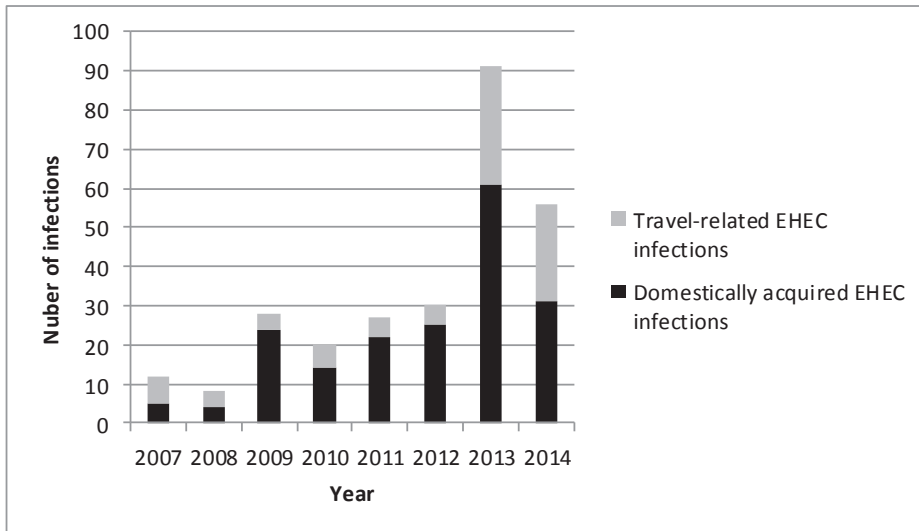
Four distinct XbaI-PFGE profiles were detected among the *S. Reading* SREA4-7, no international names available (Fig. 11). Of them, the most common XbaI-PFGE profile was SREA 5 (23 strains). The SREA5, 6 and 7 profiles differed from each other in one to two band positions in the PFGE analysis. SREA4 differed by more than 10 banding positions from SREA5, 6 and 7. The SREA5, 6 and 7 profiles showed a genetic similarity of 95%. Taking the strains with SREA4 into account, a genetic similarity of about 75% was shown in the UPGMA dendrogram (Fig. 11). One patient had a double-infection with both *Salmonella* serovars, the strains had XbaI-PFGE profiles SNWP59 and SREA7.



**Figure 11:** UPGMA dendrogram of four different *S. Reading* XbaI-PFGE profiles related to studied outbreak (SREA4-7).

### 5.3 Occurrence and characteristics of clinical isolates of EHEC in Finland (IV, unpublished data)

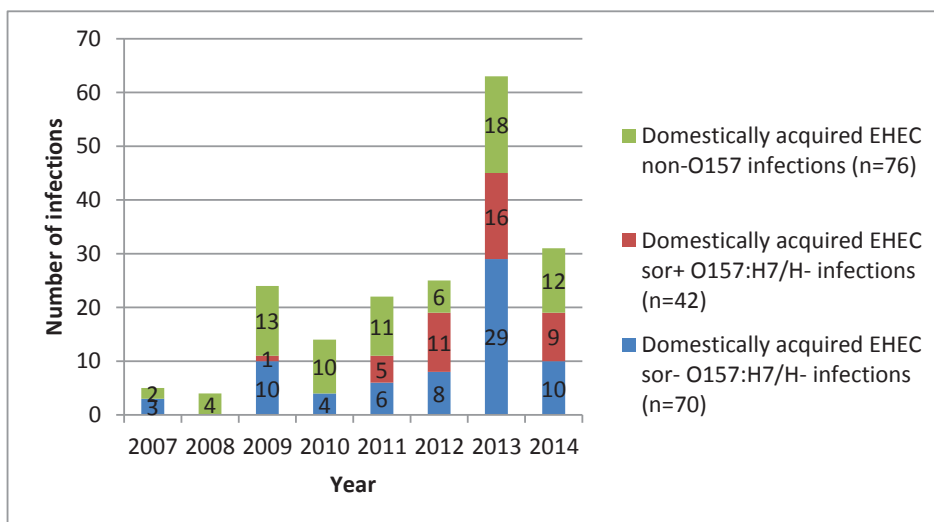
In 2007-2014, microbiologically confirmed EHEC infections were detected among 271 patients. The majority of them (188 strains, 69%) were isolated from domestically acquired infections (Fig. 12).



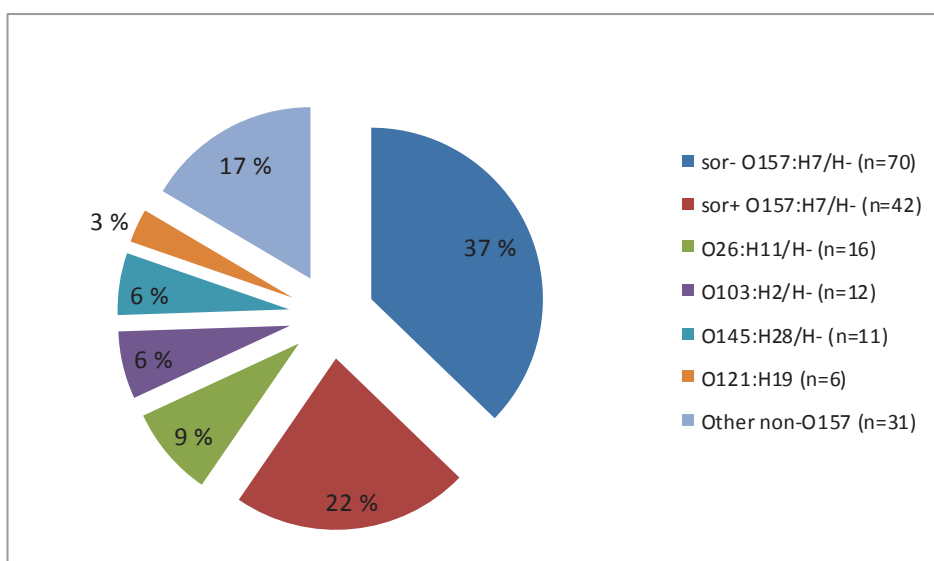
**Figure 12:** The proportions of domestically acquired and travel-related EHEC infections in Finland, 2007-2014.

Of the 188 domestically acquired infections, 112 strains (60%) were of serotype O157:H7/H<sup>-</sup> and the remaining 76 strains belonged to non-O157 serotypes (Fig. 13). The majority of the O157 strains were sorbitol-negative (70 strains) and the most common non-O157 serotypes were O26:H11/H<sup>-</sup>, O103:H2/H<sup>-</sup> and O145:H28/H<sup>-</sup> (Fig. 14).





**Figure 13:** Trends of microbiologically confirmed EHEC infections in Finland 2007-2014.

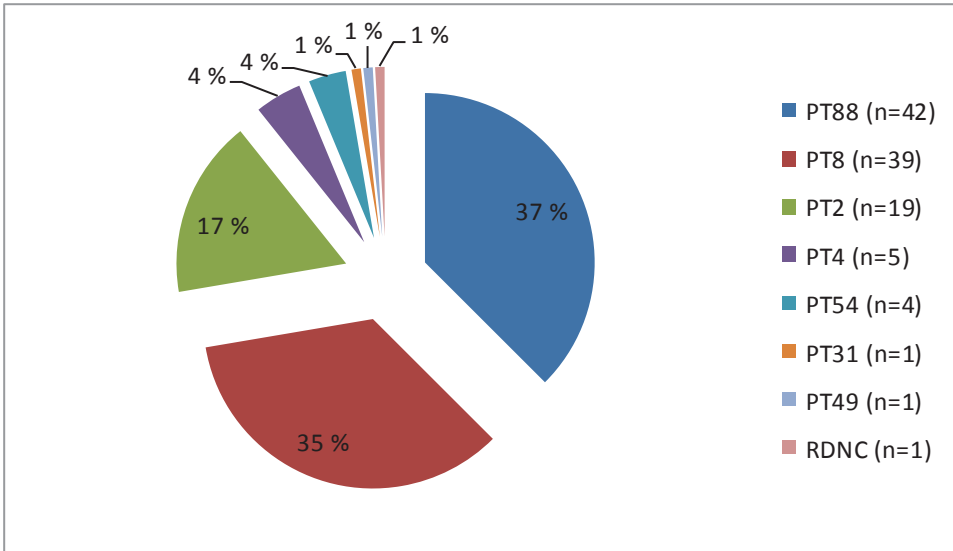


**Figure 14:** Most common EHEC serotypes isolated from domestically acquired infections.

### 5.3.1 Domestic EHEC O157 strains

Among all EHEC serogroup O157 strains, eight phage types (PTs) were identified (Fig. 15). The majority of O157 strains (63%) were unable to ferment sorbitol. The three most common phage types were PT88 (42/112, 37%) and PT8 (39/112, 35%) and PT2 (19/111, 17%). PT88 was the most common phage type among the sorbitol-positive (sor+) and PT8 among sorbitol-negative (sor-) O157 strains. The majority of the O157:H7/H- were susceptible to all tested antimicrobials (96%) and only four sorbitol negative O157 strains were MDR (R-type SSuTTmNx) when a set

of 12 antimicrobials were tested in an disk diffusion test. The strains were divided into 37 distinct XbaI-PFGE profiles. The most common XbaI-PFGE profile (1.203, 21 strains) has been detected among several small national outbreaks (Table 17 and Fig. 16)



**Figure 15:** Distribution of EHEC O157 phage types among domestically acquired infections during 2007-2014.

Only two different virulence gene combinations were detected. Of all domestic O157 strains, 67 strains (60%) carried a virulence gene combination *stx*<sub>2</sub>, *eae* and *hlyA* and 45 strains (40%) possessed the gene combination *stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae* and *hlyA* (Table 14). The strains of sor+ O157:H<sup>-</sup> showed only one virulence gene combination (*stx*<sub>2</sub>, *eae*, *hlyA*). None of the EHEC O157 strains had *stx*<sub>1</sub> gene only or *saa* gene.

**Table 14:** Virulence gene profiles of EHEC O157 strains isolated from domestically acquired infection during 2007-2014.

Domestic EHEC O157:H7/H <sup>-</sup>	No. of strains
<b>Sor+ O157:H<sup>-</sup></b>	
<i>stx</i> <sub>2</sub> , <i>eae</i> , <i>hlyA</i>	42
<b>Sor- O157:H7/H<sup>-</sup></b>	
<i>stx</i> <sub>2</sub> , <i>eae</i> , <i>hlyA</i>	25
<i>stx</i> <sub>1</sub> , <i>stx</i> <sub>2</sub> , <i>eae</i> , <i>hlyA</i>	45
<b>Total</b>	<b>112</b>

### 5.3.2 Domestic EHEC non-O157 strains

During 2007-2014, 76 human EHEC non-O157 infections of domestic origin were detected. These strains belonged to 22 distinct O:H serotypes (Table 15). The three most common non-O157 serotypes were O26:H11/H<sup>-</sup> (16 strains), O103:H2 (12 strains) and O145:H28/H<sup>-</sup> (11 strains). Two strains remained non-typeable (ONT). The majority of the non-O157 strains were susceptible to all tested antimicrobials (60%) and 11% were MDR when a set of 12 antimicrobials tested by the disk diffusion test. All domestic EHEC non-O157 strains were sor+, except four strains of serotypes O121:H19 (n=1), O117:H7 (n=2) and ONT:H49 (n=1) were sor-. Genotyping divided the EHEC non-O157 strains into 54 different XbaI-PFGE profiles. The most common XbaI-PFGE profiles were associated to known small family clusters.

**Table 15:** EHEC non-O157 serotypes of domestic origin during 2007-2014.

Non-O157 serotype	Year								Total
	2007	2008	2009	2010	2011	2012	2013	2014	
O26:H11	1			2		1	9	2	15
O103:H2		1	4			1	2	4	12
O145:H <sup>-</sup>				3	4	2		1	10
O121:H19				1	3			2	6
O78:H <sup>-</sup>			6						6
O5:H <sup>-</sup>		3							3
O146:H28							3		3
O55:H7			1				2		3
O117:H7						2			2
O182:H25				2					2
O146:H21				1				1	2
ONT								2	2
10 serotypes (1 each)	1		2	1	4		2		10
<b>Total</b>	<b>2</b>	<b>4</b>	<b>13</b>	<b>10</b>	<b>11</b>	<b>6</b>	<b>18</b>	<b>12</b>	<b>76</b>

Among the non-O157 strains, eight different virulence gene combinations were detected (Table 16). The most of the strains carried common *stx*<sub>1</sub> only (42 strains, 55%) followed by *stx*<sub>2</sub> only (32 strains, 42%). Only two strains carried both *stx*<sub>1</sub> and *stx*<sub>2</sub> genes. The most common virulence gene combinations were *stx*<sub>1</sub>, *eae* and *hlyA* (43% of the strains) and *stx*<sub>2</sub>, *eae* and *hlyA* (30% of the strains). Only one EHEC strain (ONT:H<sup>-</sup>) carried the gene *saa*. Among 15 strains of the most common non-O157 serotype O26:H11, two virulence gene profiles were detected, of which the combination *stx*<sub>1</sub>, *eae*, *hlyA* was the most common (10 strains) and remaining five strains carried the genes *stx*<sub>2</sub>, *eae*, *hlyA*. In contrast to the strains of O26:H11, one strain of serotype O26:H<sup>-</sup> carried both *stx*<sub>1</sub> and *stx*<sub>2</sub> genes. All 12 strains of serotype O103:H2 and 10 strains of O145:H<sup>-</sup> showed a single virulence gene combination *stx*<sub>1</sub>, *eae* and *hlyA* and *stx*<sub>2</sub>, *eae*, *hlyA*. In comparison to O145:H<sup>-</sup> which possessed the genes *stx*<sub>2</sub>, *eae* and *hlyA*, one O145:H28 strain had the genes *stx*<sub>1</sub>, *eae* and *hlyA*.

**Table 16:** Virulence gene profiles of EHEC non-O157 strains isolated from domestically acquired infection during 2007-2014.

Domestic EHEC non-O157	No. of strains
<i>stx</i> <sub>1</sub>	3
<i>stx</i> <sub>p</sub> , <i>hlyA</i>	6
<i>stx</i> <sub>1</sub> , <i>eae</i> , <i>hlyA</i>	33
<i>stx</i> <sub>2</sub>	6
<i>stx</i> <sub>2</sub> , <i>eae</i>	2
<i>stx</i> <sub>2</sub> , <i>eae</i> , <i>hlyA</i>	23
<i>stx</i> <sub>2</sub> , <i>hlyA</i> , <i>saa</i>	1
<i>stx</i> <sub>1</sub> , <i>stx</i> <sub>2</sub> , <i>eae</i> , <i>hlyA</i>	2
<b>Total</b>	<b>76</b>

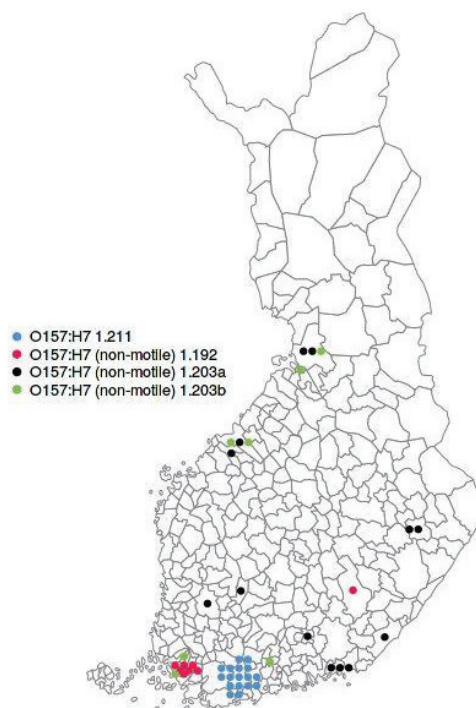
### 5.3.3 Detected outbreak and family clusters caused by EHEC in Finland, 2007-2014

Most of the EHEC infections in Finland are sporadic or family-related secondary infections. During 2007-2014, 15 family-related small clusters and four outbreaks were detected (Table 17). On October 2009, a family-related cluster (5 patients) caused by a rare EHEC O78:H<sup>-</sup> strain was observed. The strain harbored *stx*<sub>1c</sub> and *hlyA* genes and was detected in blood and stool samples of a 2-week-old neonate who had bacteremia and HUS and in the stool samples of his asymptomatic family members. All strains were susceptible to tested antimicrobials and showed indistinguishable XbaI-PFGE profiles (Fig. 1 in study IV). The source of the infection was not known. In June 2012, eight persons (six children and two adults) contracted an EHEC infection (sorbitol-positive, O157:H<sup>-</sup>, phage type PT88, XbaI-PFGE profile 1.192) in the Turku region (Fig. 16). Five out of six children were hospitalized because of HUS. The patients had visited a local farm and/or consumed unpasteurized milk from that farm. An identical EHEC strain was found in the cattle feces on the farm and in environmental samples from the farm. In April-May 2013, 13 persons were infected with an EHEC (sorbitol-positive, O157:H<sup>-</sup>, PT88, XbaI-PFGE profile 1.203a) nationwide in Finland (Fig. 16). The majority of the patients (11/13) were children and seven of them developed HUS. Interviews conducted did not reveal any particular food common for the cases. According to the expert network of the ECDC, no similar bacterial strain had been found in other EU countries or in the USA. The second outbreak in 2013 occurred in Lohja, where an outbreak caused by sorbitol-negative EHEC O157:H7, PT2, XbaI-PFGE profile 1.211 strain was isolated from feces of eight day care children and from nine of their family members (Fig. 16). None of them developed HUS. Day care food was identified as connecting factor between the patients, but the source of the infections remained unidentified. On Dec 2013, sorbitol-negative EHEC O157:H7:PT8:*stx*<sub>p</sub>,*stx*<sub>2</sub>,*eae*,*hlyA* strain was isolated from feces of 5 family members with farm contact and from a asymptomatic person who had worked in that farm in Loviisa region. The source remained unknown. Between Dec 2013 and Jan 2014, a sorbitol-positive EHEC O157:H<sup>-</sup>, PT88, strain was isolated from feces of 12 patients nationwide. Eight of them had an identical XbaI-PFGE profile 1.203b (Fig. 16). The source of infections remained unidentified.

**Table 17:** Family-related clusters and outbreaks detected in Finland during the period of 2007 to 2014.

Serotype	Phage type	Virulence genes	XbaI-PF-GE	No. of patients	Clusters	Suspected source
O157:H	PT8	<i>stx<sub>1</sub>, stx<sub>2</sub>, eae, hlyA</i>	1.29	3	Family cluster	Unknown
O5:H		<i>stx<sub>1</sub>, eae, hlyA</i>	O5a	3	Family cluster	Unknown
O103:H2		<i>stx<sub>1</sub>, eae, hlyA</i>	O103p	3	Family cluster	Unknown
O78:H		<i>stx<sub>1</sub>, hlyA</i>	O78a	5	Family cluster	Unknown
O26:H11		<i>stx<sub>2</sub>, eae, hlyA</i>	O26j	2	Family cluster	Unknown
O157:H	PT88	<i>stx<sub>2</sub>, eae, hlyA</i>	1.151	2	Family cluster	Unknown
O121:H19		<i>stx<sub>2</sub>, eae, hlyA</i>	O121c	2	Family cluster	Unknown
O157:H7	PT8	<i>stx<sub>1</sub>, stx<sub>2</sub>, eae, hlyA</i>	1.122	2	Family cluster	Unknown
O157:H	PT88	<i>stx<sub>2</sub>, eae, hlyA</i>	1.192	8	Local epidemic	Unpasteurized milk / animal contact
O157:H	PT88	<i>stx<sub>2</sub>, eae, hlyA</i>	1.203	13	Nationwide epidemic	Widely sold food
O157:H7	PT2	<i>stx<sub>2</sub>, eae, hlyA</i>	1.211	17	Local epidemic	Day care food
O157:H7	PT8	<i>stx<sub>1</sub>, stx<sub>2</sub>, eae, hlyA</i>	1.164	5+1	Family cluster & farm worker	Farm contact
O157:H7	PT8	<i>stx<sub>1</sub>, stx<sub>2</sub>, eae, hlyA</i>	1.29	3	Family cluster	Unknown
O26:H11		<i>stx<sub>1</sub>, eae, hlyA</i>	O26r	3	Family cluster	Unknown
O26:H11		<i>stx<sub>2</sub>, eae, hlyA</i>	O26s	2	Family cluster	Unknown
O103:H2		<i>stx<sub>1</sub>, eae, hlyA</i>	O103s	3	Family cluster	Unknown
O157:H	PT88	<i>stx<sub>2</sub>, eae, hlyA</i>	1.203	8*	Nationwide epidemic	Unknown
O157:H7	PT8	<i>stx<sub>1</sub>, stx<sub>2</sub>, eae, hlyA</i>	1.29	2	Family cluster	Unknown
O121:H19		<i>stx<sub>2</sub>, eae, hlyA</i>	O121e	2	Family cluster	Unknown

\* Isolation date 13.12.2013-10.02.14

**Figure 16:** Geographical locations of detected outbreaks caused by EHEC in Finland (2007-2014).

## 6 DISCUSSION

Foodborne diseases cause a wide spectrum of illnesses and are a public health problem worldwide, also in industrialized countries. *Salmonella* is the second most common agent of foodborne disease and EHEC is rare but is associated to severe disease. Furthermore, these bacteria are able to gain additional virulence factors by horizontal gene transfer which might lead even more virulent subtypes and severe disease. It has been estimated that EHEC causes 2.8 million illnesses, leads to 3,890 cases of HUS and 230 deaths annually worldwide [2]. The global burden of *Salmonella* is higher than that of EHEC, with an estimated 93.8 million infections and 155,000 deaths each year [1]. In order to efficiently detect and prevent human *Salmonella* and EHEC infections, the development of rapid and sensitive subtyping methods is important. In the outbreak situations subtyping results of human and non-human strains should be comparable using standardized protocols.

### 6.1 Occurrence of domestically acquired *Salmonella* and EHEC infections

In Finland, as in many other countries, *Salmonella* is, after *Campylobacter*, the second most common cause of foodborne infections. During 2007-2014, about 85% of all notified salmonellosis cases in Finland were travel-related and the annual incidence of domestically acquired *Salmonella* infections was low varying between 5.4 and 7.5/100,000 population, depending on how many domestic outbreaks were annually reported. The low salmonella incidence among the Finns reflects the low incidence in domestic production animals and food [356]. Similar low incidence rates for domestically acquired *Salmonella* infections have been reported in Sweden [357]. Finland and Sweden had the lowest sero-incidences based on serological measurements of *Salmonella* LPS antibody in human, including asymptomatic individuals, compared to eight other countries included into study of Falkenhorst et al. [358]. In contrast, both Finland and Sweden have reported higher incidences of all culture-confirmed (including travel-related) *Salmonella* than the EU average [58]. Low incidence among domestic and high among travel-related infections could indicate that Finland and Sweden have the most accurate surveillance systems and active salmonella control programs. The aim of the Finnish salmonella control program (FSCP) is to keep the annual prevalence of *Salmonella* below 1% in the specimens collected from the animals included in the control program. The FSCP covers egg and meat (beef, pork and poultry) production and concerns all *Salmonella* serovars. Despite the accurate surveillance in Finland, about 200-300 domestically acquired human *Salmonella* infections are reported each year demonstrating that the sources of domestic *Salmonella* infection might be other than those covered by the FSCP.

*S. Enteritidis* is the most common *Salmonella* serovar followed by *S. Typhimurium* worldwide [58, 62, 194]. In Finland, serovar *Typhimurium* was the most common among domestically acquired infections, causing 32% of human salmonellosis. However, when also taking strains isolated from foreign infections into account, *S. Enteritidis* was the most common serovar in Finland as well. In 2014, the number of domestic *Typhimurium* infections decreased 2% when compared to the mean value from 2011-2013. Despite a decreasing trend, about 100 domestic *Typhimurium* infections and a few outbreaks are still reported annually in Finland. Exact reasons

for the drop in the number of infections caused by domestic Typhimurium are unknown. *S. Enteritidis* caused 15% of human *Salmonella* infections in Finland and the number of reported *S. Enteritidis* has dropped 20% when compared to the mean value from 2010-2012. Many countries in Western Europe have witnessed a substantial decrease in *Salmonella* findings, particularly of *S. Enteritidis*, compared to the late 1990s [359, 360]. The decrease is most likely a sum of several control methods and due to increased use of vaccines in the poultry and egg production in the EU [359]. However, some studies have suggested that the reduction of human *S. Enteritidis* and *S. Typhimurium* experienced in the EU and North America might have occurred because of the number of animal infections by these serovars has fallen and the proportion of infections caused by antigenically distinct serovars such as Dublin in cattle and *S. diarizonae* found in sheep, has risen [361]. As these serovars are either host-adapted or seldom causes of disease in humans, the drop of human infections generally could be explained. Also, several codes of practice for hygiene have been introduced to public which might have led to a better awareness of *Salmonella*. On the other hand, an increase of other serovars e.g. MDR monophasic *S. Typhimurium* has been reported in Europe, North and South America, and Asia [362-365]. However, there are no recognized reservoirs of *S. Enteritidis* or monophasic *S. Typhimurium* among domestic production animals in Finland.

As in many other countries [2, 58], the annual incidence of EHEC in Finland has increased being lowest in 2008 (0.2/100,000 population) and highest in 2013 (1.8/100,000 population). In general, the large outbreak caused by EHEC O104:H4 -contaminated sprouts that occurred in Germany 2011 enhanced awareness of EHEC. As a consequence, many countries have improved their EHEC diagnostics towards more sensitive PCR-based methods which target *stx* genes rather than serogroup [366]. An increased proportion of detected non-O157 serotypes in some countries might be due to the use of novel, more sensitive methods [366, 367]. The exact reasons for the generally increasing trend of domestically acquired EHEC infections in Finland remain unknown but four domestic EHEC O157 outbreaks contributed into the increase in 2012-2013. In addition, there have been some changes in Finnish EHEC diagnostics. For example, currently the majority of EHEC screening is performed by a single clinical microbiology laboratory by a PCR-based method and EHEC is detected together with other common enteropathogenic bacteria without a clinical suspicion or additional request from a physician [368]. The PCR is a sensitive method which could explain at least some of the increase. Due to the sensitivity, occasionally the results cannot be culture confirmed.

### **6.1.1 Occurrence of *S. Typhimurium*, *S. Enteritidis* and EHEC phage types**

Historically, epidemiological subtyping of *S. Typhimurium* and *S. Enteritidis* has relied on phage typing. However, this method has only limited discriminatory power and it is performed only by a few reference laboratories. Furthermore, in 2008 an outbreak caused by *S. Typhimurium* highlighted problems in phage typing as different naming of the lysis pattern of outbreak-related strains hindered the outbreak investigations (U288 in Denmark, RDNC in Norway and U302 in Sweden) [369]. This international outbreak was eventually recognized by MLVA typing. In this study, the three most common *S. Typhimurium* phage types among domestically acquired infections were DT1, U277 and DT104, accounting together 65% of the strains. This finding correlated with previous studies conducted in Finland in which DT1 was found to be the most common in Finland [370]. DT1 is endemic in Finland and it has been the most common phage

type since the 60's (statistics of THL). In Sweden and Norway, hedgehogs have been associated with infections in children under 5 years old caused by DT1 [371, 372]. However, no association with the age of the patients was found in this study. Other phage types dominate in other countries. For example, phage types DT135 and DT9 are common in Australia [323], whereas DT193, DT195 and DT120 are common in Belgium [373] and DT104 in United Kingdom (UK) and Canada [374]. Approximately, 10% of the domestic strains were non-typable by traditional phage typing. In Finland, during 2007-2014, the most common *S. Enteritidis* phage types were PT8, PT1B and PT4, accounting together for 44% of the domestically acquired *S. Enteritidis* infections. In contrast to *S. Typhimurium* phage type DT1 which has been the most common phage type since the 60s [375], the most common PTs among *S. Enteritidis* have varied depending on how many domestic outbreaks have occurred annually. In the 1990s, the most commonly reported phage types in Finland were PT1 and PT4 [370]. The increase of PT1B was associated with an outbreak caused by precooked frozen chicken in 2012. As among *S. Typhimurium*, the occurrence of certain *S. Enteritidis* phage types varies between the continents. For example, PT1 is common in the Baltic countries, Russia and Korea [79, 376] whereas PT4 is the most common in the Western European countries [377, 378] and PT8 in the North America [73, 74].

The strains of EHEC O157 (60%) dominated over non-O157 serotypes in Finland and in other countries [379]. In this study, the phage types PT8, PT88 and PT2 were the most common ones. According to previous studies, PT8 and PT2 are commonly found in Europe and they frequently cause outbreaks [380-383]. During the 90s PT2 was the most common in Finland [384]. The emerge of PT88 which is most commonly associated with sor+ EHEC O157:H- strains has been noticed previously in Finland and in some other European countries [218, 385-387].

### **6.1.2 Antimicrobial susceptibility of *Salmonella* and EHEC**

Susceptibility testing against certain antimicrobials is commonly performed in clinical microbiology laboratories. The test provides important information for a clinician to choose optimal treatment for the patient, if needed. It is also used for surveillance purposes as a standard typing method. In this study, the majority of domestic *S. Typhimurium* and *S. Enteritidis* (both 60%) strains were susceptible to all tested antimicrobials. Also, the studied EHEC strains (81%) were mainly susceptible to all tested antimicrobials: 96% of O157:H7/H- and 60% of non-O157 strains. Similar results have been obtained in Spain in which 41% of non-O157 strains were resistant to more than one of the tested 26 antimicrobials [388]. The finding is consistent also with the results obtained in British Columbia where higher levels of resistance were observed among non-O157 than among O157 strains, although they tested 19 antimicrobials [389] [389]. The most domestic *Salmonella* and EHEC strains were fully susceptible to the tested antimicrobials. For example, 74% of DT1 strains which considered endemic phage type in Finland were fully susceptible to the tested antimicrobials. However, 12% of all *S. Typhimurium* strains and 7% of the EHEC strains were MDR in this study. Decreased sensitivity to ciprofloxacin was rare (4.5%) among the domestic *S. Typhimurium* strains. In comparison to human isolates, less than 1% of the tested samples from domestic production animal (cattle, swine, poultry and eggs) were *Salmonella* positive and MDR was rare [95]. Interestingly, the XbaI-PFGE profile STYM7 which was the most common profile among isolates from domestic infections caused by MDR *S. Typhimurium* DT104 in this study is also the most common XbaI-PFGE profile among MDR DT104 infections of foreign origin [390]. Since MDR strains were detected among Finns



with no travel history one might speculate that at least some of the infections could be due to imported foods sold in supermarkets and restaurants. In Sweden, imported foods have been estimated to cause 6.5% of domestic *Salmonella* infections [46]. In addition to imported foods, some of the infections could be secondary infections from the patients who have traveled abroad. Furthermore, an association between decreased sensitivity to ciprofloxacin and foreign travel has been previously suggested in Finland [391] and in Denmark [392]. Even when the antimicrobial susceptibility testing is not used for patient treatment purpose, it can be used for bacterial typing and for estimating the origin of the infections.

## 6.2 Molecular epidemiology of domestic *S. Typhimurium*

XbaI-PFGE showed a genetic similarity of more than 70% of the studied *S. Typhimurium* strains. During the study period, STYM1 (also known STYMXB.0098), was the most common XbaI-PFGE profile (27%). This profile was found among 6 phage types, DT1 being the most common. Furthermore, STYM1 divided into 12 MLVA types. 15% of the *Typhimurium* strains had same type using four different subtyping methods. They were susceptible to the tested antimicrobials, had phage type DT1, XbaI-PFGE profile STYM1 and MLVA type 3-16-NA-NA-0311. This lack of diversity may suggest that these strains originated from the same reservoir or source. In comparison, DT1 with XbaI-PFGE profile STYM1 caused 66% of human infections in 2000-2003 in Finland [370]. This particular XbaI-PFGE and MLVA type has been associated with cattle, wild birds and hedgehogs in Finland [375, 393, 394]. In agreement with these results, the same DT1 with MLVA type 3-16-NA-NA-0311 has been isolated from hedgehogs in Sweden and Norway where it has been linked to infections among children [371, 372]. In this study, no association with age and DT1 infections was detected.

One third of the domestic *S. Typhimurium* strains were of MLVA type 3-16-NA-NA-0311. This particular MLVA type was divided into seven phage types and 19 XbaI-PFGE profiles. Moreover, MLVA types with a single locus difference to type 3-16-NA-NA-0311 occurred making cluster definition challenging. This was demonstrated in the minimum spanning tree of MLVA types as domestic *S. Typhimurium* strains showed no particular clustering but instead they formed a “stardust” shaped picture. This might indicate that there a common reservoirs in Finland. In comparison to the situation in Finland, more diversity among *S. Typhimurium* has been observed by MLVA elsewhere [323, 395, 396].

The most common phage types in Finland are rather homogenous and thus difficult to subtype using PFGE or MLVA. Molecular subtyping by XbaI-PFGE or MLVA alone or both together were not discriminatory enough for the further characterization of the endemic infections caused by DT1. Thus, the sources of DT1 infections remained unidentified. In particular, the lack of MLVA loci STTR6 and STTR10p among domestic *S. Typhimurium* strains diminished the discriminatory power of MLVA. Similar to our finding, the absence of loci STTR6 and STTR10p has been previously reported by other researchers [395, 397]. Despite lacking information for two MLVA loci, a diversity index of 0.891 (CI 95% 0.845-0.925) was calculated for MLVA. The source of sporadic human infections remained unknown.

### 6.3 Epidemiology of domestically acquired EHEC

In Finland, the epidemiological typing of EHEC relies on a combination of pheno- and genotyping methods including O:H-serotyping, phage typing of O157:H7 strains, XbaI-PFGE and virulence gene profiling. These methods are internationally standardized and nationally and internationally comparable [288, 310, 311].

Of all O157 strains studied, more than 30% were sor+ and non-motile, as in Germany [398]. In this study, all sor+ O157:H<sup>-</sup> strains carried the virulence gene combination *stx*<sub>2</sub>, *eae* and *hlyA*. Sor+ O157:H<sup>-</sup> strains were suggested to have caused three outbreaks in Finland of which one outbreak was associated with a visit to cattle farm and/or consumption of unpasteurized milk [195]. Previous studies have implicated that cattle or cattle products occasionally harbor sor+ O157:H<sup>-</sup>, although the primary reservoir for this serotype is unknown [399].

In this 7-year study (2007-2014), about 40% of the strains belonged to non-O157 serotypes. In comparison, the proportion of non-O157 serotypes was 47% in 1998-2002 [197]. This difference might be due to O157-related outbreaks that occurred in Finland during the study period. The most common non-O157 serotypes in Finland were O26:H11/H<sup>-</sup>, O103:H2 and O145:H28/H<sup>-</sup>. These non-O157 serotypes are common in other countries as well [366, 381]. Furthermore, the strains of the serogroups O26, O103 and O145 have been associated with severe disease and HUS in Finland and elsewhere [202, 207, 367], particularly the strains of the serotype O26:H11/H<sup>-</sup> have been linked with HUS [400]. Despite the fact that 40% of the domestic EHEC infections were caused by non-O157 serotypes and that some non-O157 serotypes have been detected in feces of domestic cattle [401], the non-O157 strains were not associated with outbreaks in Finland. Non-O157 strains have been a growing concern as in several countries: they have been identified as a significant cause of disease, including HUS, and it has been suggested that their incidence may exceed that of EHEC O157 [6, 155, 212, 367].

EHEC O157 does not generally ferment sorbitol. Thus, this biochemical feature is used for the detection of these bacteria in many laboratories in culturing on sorbitol-MacConkey agar (SMAC). Thus the detection of sor+ EHEC strains remains complex and the strains of sor+ O157:H<sup>-</sup> and the majority of non-O157 are still estimated to be under-diagnosed [402, 403]. In addition, sor- non-O157 strains were identified in this study. Four sor- strains belonging to serotypes O121:H19, O117:H7 and ONT:H49 were found. Such strains are rare but they have been identified, for example, in Japan [404]. In 2009, EHEC O78:H<sup>-</sup>:*stx*<sub>1c</sub>:*hlyA* was detected in blood and stool samples of a neonate in Finland. This serotype had not been detected prior to this family outbreak and bacteraemia caused by EHEC is extremely rare. A recent study reported bacteraemia caused by EHEC O128ab:H2 as well [221]. In comparison to our finding, EHEC O128ab:H2 was isolated from a 27-year old male and it carried both *stx*<sub>1</sub> and *stx*<sub>2</sub> as well as *hlyA* genes. This finding emphasizes the importance of early detection of EHEC infections caused by non-O157 strains. Furthermore, these findings highlight the diversity of EHEC strains and the need for diagnostic methods that detect both sor+ and sor- EHEC strains.

#### 6.3.1 Virulence properties of EHEC

Virulence gene profiling (*stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae*, *hlyA* and *saa*) was used for the characterization of the EHEC strains. Since Shiga toxin 2 has been associated with more severe outcomes of disease

and HUS early characterization of a virulence profile can provide valuable information about potential disease severity [243, 405]. Furthermore, the gene profiles can be used as a subtyping method to cluster strains [406]. Of the sor+ O157:H<sup>-</sup> strains, all were found to carry only *stx*<sub>2</sub>, similar to previous studies in Finland [218] and elsewhere [166, 385, 407]. In contrast to sor+ O157:H<sup>-</sup>, 65% of the sor- O157:H7 strains carried the *stx*<sub>2</sub> gene in combination with *stx*<sub>1</sub> in this study. This finding is in agreement with results from other studies [246, 408]. On the other hand, some studies have reported that only 30-40% of the studied sor- O157:H7 strains contained both *stx* genes [409, 410]. None of the domestic sor+ or sor- O157 strains carried the *stx*<sub>1</sub> gene only or the *saa* gene. Virulence profiles in non-O157 strains displayed more variability than O157 strains. In this study, the non-O157 strains were divided into eight different virulence gene profiles, most commonly in the following combinations *stx*<sub>1</sub>, *eae*, *hlyA* (43%) or *stx*<sub>2</sub>, *eae*, *hlyA* (30%), similar to previous studies [366, 411]. However, dominance of *stx*<sub>2</sub> among non-O157 strains has also been detected as about half of the studied strains carried only *stx*<sub>2</sub> [381]. According to some studies, the carriage of the *stx*<sub>1</sub> gene only was the most common among O26:H11 and O103:H2 [409, 412]. This is in line with the results of this present study in which 69% of the O26:H11 and 100% of the O103:H2 strains carried only the *stx*<sub>1</sub> gene. However, a recent report from UK has shown that the majority of the O26 strains carry both the *stx*<sub>1</sub> and *stx*<sub>2</sub> genes [366] or *stx*<sub>2</sub> only in Poland [413]. In Finland, such a shift in the virulence gene profile of the O26 strains was not detected. Although the carriage of only *stx*<sub>1</sub> was common among non-O157 in this study, all strains of O145:H28/H<sup>-</sup> carried only *stx*<sub>2</sub> gene. Similar result for O145 has been reported [381] but in another study *stx*<sub>1</sub> has been also detected [409]. About 15% of the strains of non-O157 serotypes were *eae* and *hlyA* negative, in contrast to the O157:H7 strains which all carried the genes *eae* and *hlyA*. Although intimin has been recognized as an important adhesion protein among EHEC, strains lacking *eae* might be even more virulent and possess another mechanism for colonization as was evident in the large German outbreak caused by *eae*-negative EHEC O104:H4 [277]. Previously, the *saa* gene, responsible for producing autoagglutinating adhesin, has been associated in development of HUS [264]. In this study, however, only one EHEC strain (ONT:H<sup>-</sup>) carried *saa* gene. It is important to note that the 5-plex PCR [281] used in this study does not detect all *stx*, *eae* and *hlyA* variants found in EHEC.

## 6.4 Usefulness of traditional and newer methods in surveillance (I, II, III and IV)

The goal of any typing method is to characterize bacterial strains and group the strains together in a way that is epidemiologically useful. Rapid and effective typing is needed for laboratory-based surveillance and detection of beginning outbreaks. Strains with identical subtypes are more likely to be associated with a common source than those with different typing profiles. The most important criteria for a subtyping method are typeability, reproducibility, discriminatory power and epidemiological concordance. Additional criteria include speed, low costs and ease of interpretation. Since none of the subtyping methods will fulfill all the criteria, compromises must be made and a combination of methods should be selected. New typing methods such WGS has been used surveillance and outbreak investigation [414]. Although WGS provides the ultimate typing resolution and will replace some traditional methods in the near future, it is still important to maintain the know-how of traditional methods and comparability between the methods.

### 6.4.1 Evaluation of phenotyping methods

Serotyping, phage typing and antimicrobial susceptibility testing are important traditional subtyping methods for *Salmonella* and EHEC, and are all internationally standardized methods. Serotyping has proven to be a robust and reproducible method for both bacteria [415]. For over 70 years serotyping has been an important method in public health monitoring. The advantages of serotyping include ease of performance and good epidemiological concordance. Some *Salmonella* serovars are host-specific or occur in certain geographical regions or continents [416] whereas serotype results of EHEC can give important information about the source of infection or severity of the infection [307]. However, slide agglutination is time-consuming and expensive antisera of good quality are required. Additional subtyping is required for the most common *Salmonella* serovars and EHEC serotypes. Phage typing, for example, is a traditional subtyping method for *Salmonella* serovars Typhimurium [285], Enteritidis [286] and for EHEC O157 [288]. It is cheap but non-typeable strains do occur. In this study, non-typeable strains were detected among the strains of *S.*Typhimurium (16%). Furthermore, phage typing is not always reproducible between laboratories. For example, an international outbreak has been hindered due to different phage type interpretation between countries [289]. In addition, phage typing has limited discriminatory power and it requires specific phage collection [417]. Still, phage typing is a valuable tool for the first quick evaluation of potential outbreaks, especially for less common phage types, and for identifying strains related to certain countries or sources [292, 293]. In particular, when phage typing results are combined with results of other typing method such as PFGE or MLVA a good epidemiological concordance can be achieved. Antimicrobial susceptibility testing is also important traditional typing method which has been applied for routine typing and to understand source and transmission dynamics of resistant strains [418]. The method is cheap, simple and does not require specific equipment. The drawbacks include poor discriminatory power and instability of certain resistance factors under selective pressure or during storage [294]. Furthermore, there is a lack of harmonization in terms of antimicrobials tested and criteria used for interpretation, in particularly between human and veterinary laboratories [295]. Nevertheless, epidemiological antimicrobial susceptibility testing is widely used. Since the majority of domestic *Salmonella* and EHEC in Finland are susceptible to antimicrobials this method gives information about the potential foreign origin of the studied strain if it contains resistance markers.

Novel phenotyping methods that are not internationally harmonized can be applied for the characterization of foodborne *Salmonella* and EHEC strains or research [297]. Phenotyping microarray (PM) plates with various substances can be used to study the ability of bacteria to metabolize certain compounds [419]. Differences in the metabolic profile might be characteristic of new clones or hybrids or genetic features. For example, PM analysis of the EHEC O157:H7 strain related to a spinach outbreak in the USA showed a rare *N*-acetyl-D-galactosamine-negative phenotype, which had only been found once before [298]. Variation in motility and biofilm formation of *S.* Typhi has been studied using the PM method [420]. However, in this study PM technology was applied only for certain *Salmonella* strains. The PM results were reproducible but only a few metabolic differences were found between different *Salmonella* serovars. Therefore, PM is not useful method for grouping *Salmonella* strains in epidemiologically meaningful way. For routine surveillance of foodborne pathogens the PM technology is too expensive.

#### 6.4.2 Comparison of PFGE and MLVA methods

PFGE has been shown to be a highly discriminatory genotyping method for subtyping of *Salmonella* and EHEC isolates and it has been considered as “gold standard” [309, 311]. The advantages of PFGE include that it is robust and internationally standardized. Furthermore, the naming of the PFGE profiles is harmonized and the most common profiles or profiles associated with outbreaks have international names [390]. However, identification of infection clusters and source tracking by PFGE are complicated by common and widely distributed PFGE profiles [421, 422]. Furthermore, PFGE is a labor-intensive and time-consuming technique and results might be challenging to interpret [309, 312]. The XbaI restriction enzyme is typically used as a primary enzyme for determining genetic relatedness of *Salmonella* and EHEC strains and BlnI can be used to either confirm XbaI results or to provide additional resolution [423]. For example, XbaI-PFGE has showed only limited discrimination for *S. Enteritidis* but the use of multiple restriction enzymes can increase the discriminatory power of PFGE [424]. However, in agreement with previous studies [310, 311, 316], only XbaI was used in this study for PFGE analysis for both *Salmonella* and EHEC. In this study, relatively high discriminatory ability of XbaI was observed for *S. Typhimurium* (DI>0.8). Furthermore, the XbaI-PFGE analysis was used for investigation of outbreak caused by *S. Newport*/*S. Reading* in Finland. All studied *S. Newport* strains had indistinguishable XbaI-PFGE profiles whereas four distinct XbaI-PFGE profiles for *S. Reading* (SREA4-7) were detected. The differences in the PFGE profiles of the outbreak-related strains are thought to be due to point mutations, inserts and deletions in the genome or loss or acquisition of a plasmid [309]. Since the profiles SREA4 and SREA7 differed by more than 10 banding positions it is likely that the vehicle (iceberg salad) had been contaminated with *S. Reading* with diverse genotypes. One might also expect that if five or more colonies per patient had been investigated even more genotypic variation might have been observed. Only four outbreaks caused by EHEC were detected but in two of them an identical XbaI-PFGE profile 1.203 occurred. Still, no for all cases common source or transmission route was identified. The most domestic EHEC infections were sporadic or related to family clusters, as in other studies [197, 209]. In recent outbreak investigations and research studies, PFGE results have been complemented with MLVA [320, 425]

Molecular techniques like MLVA are generally considered to improve surveillance and detection of outbreaks and their sources [373, 406]. In this study, the discriminatory power of MLVA was equal to XbaI-PFGE among the strains of *S. Typhimurium*. However, compared to PFGE, MLVA is faster, cheaper and the interpretation of the results is unambiguous [322] but the time required and cost per strain depends on the fragment analysis platform and equipment used. The possibility of sharing MLVA results across country borders as a string of five to seven numbers is one of the strengths of MLVA. Although an increasing number of studies indicate that MLVA can provide improved discrimination, only two MLVA protocols, namely, for *S. Typhimurium* [322] and *S. Enteritidis* [292, 325], have been harmonized in the EU. Therefore, PFGE still remains the best choice for the remaining *Salmonella* serovars and EHEC. Although not used in this study, MLVA for *S. Enteritidis* and EHEC O157 have been widely used [377, 406]. In the near future WGS will most probably replace PFGE and MLVA. Although, MLVA is as sensitive as WGS, WGS provides an ultimate resolution and more information at one run [426]. Even the serotyping of *Salmonella* which was earlier thought to be a limiting factor in utilization of WGS has been solved as a free software which recognizes more than 2,300 serovars was launched at the beginning of 2015 [427].

## 7 CONCLUSIONS AND FUTURE CONSIDERATIONS

In this thesis, knowledge of microbiological pheno- and genotypic characteristics of human *Salmonella* and EHEC strains isolated from domestically acquired infections in Finland was obtained. During 2007-2014, 2,818 microbiologically confirmed *Salmonella* infections and 188 EHEC infections were characterized. Only 15% of all microbiologically confirmed *Salmonella* but 70% of all EHEC infections were considered domestically acquired. The number of domestically acquired *Salmonella* infections decreased about one fifth compared to the previous decade whereas the number of domestic EHEC infections increased about one third. The incidence of domestic *Salmonella* infections was highest in 2012 ( $7.5/10^5$  population) and lowest in 2014 ( $5.4/10^5$  population). The incidence of domestic EHEC infections was highest in 2013 ( $0.33/10^5$  population) and lowest in 2008 ( $0.07/10^5$  population).

1. The most common *Salmonella* serovars were Typhimurium (32%), Enteritidis (15%) and Group B (6%). EHEC serotype O157:H7/H<sup>-</sup> dominated in Finland (60%). The most common non-EHEC serotypes were O26:H11/H<sup>-</sup>, O103:H2/H<sup>-</sup> and O145:H28/H<sup>-</sup>.
2. More than 60% of all domestic *S. Typhimurium* and strains were fully susceptible against 12 tested antimicrobials. The majority of domestic EHEC strains (81%) were susceptible to all tested antimicrobials, more specific, 96% of the O157:H7/H<sup>-</sup> and 60% of the non-O157 strains.
3. MLVA provided an inexpensive, high-throughput, discriminatory and accurate typing method for the surveillance of *S. Typhimurium*. The results were easy to compare between laboratories.
4. The discriminatory power of MLVA was reduced because 70% of the domestic *S. Typhimurium* strains lacked MLVA loci STTR6 and STTR10p. Still, the calculated discriminatory power was equally high ( $DI \geq 0.8$ ) for PFGE and MLVA methods. Since January 2013, MLVA has been the primary genotyping method for *S. Typhimurium* at THL. In outbreak situations or if needed for clarification, MLVA results can be complemented with PFGE (or in the future with WGS) at THL.
5. About 15% of the sporadic *S. Typhimurium* strains were of identical type with four different typing methods. They were susceptible to 12 antimicrobials, of phage type DT1, XbaI-PFGE profile STYM1 (STYMXB.0098) and MLVA type 3-16-NA-NA-0311.
6. Domestic EHEC non-O157 strains showed more variability in the virulence gene profiles than EHEC O157 strains. All O157 strains carried  $stx_2$  (40% in combination with  $stx_1$ ), *eae* and *hlyA* genes. In contrast, 55% of the non-O157 strains had  $stx_1$  gene and 76% carried *eae* and *hlyA* genes and only one strain had *saa* gene.
7. In many cases the sources and transmission routes of the domestic infections caused by *Salmonella* and EHEC remained undetermined. However, some of the domestic *Salmonella* infections were associated with contaminated imported foodstuffs such as pre chopped

lettuce (*S. Newport/S. Reading*, 2008), imported sprout seeds (*S. Bovismorbificans*, 2009) and precooked chicken cubes (*S. Enteritidis*, PT1B, 2012). Only EHEC serotype O157:H7/H<sup>-</sup> was associated with outbreaks in Finland. In one of the outbreaks, consumption of unpasteurized milk or animal contact was the source of the infections.

8. In 2009, a rare EHEC O78:H<sup>-</sup>:*stx*<sub>1c</sub>: *hly*A was detected in blood and stool samples of a 2-week-old neonate who had bacteremia and HUS. The strain was also found in the stool samples of his asymptomatic family members. This finding emphasizes the importance of early detection of EHEC infections caused by non-O157 strains especially in young children.

*Salmonella* and EHEC infections pose a major public health concern and they cause substantial costs to society. Although the overall number of *Salmonella* infections has decreased in Finland, the number of certain *Salmonella* serovars (e.g. monophasic *S. Typhimurium*) and EHEC has increased. In order to detect and prevent outbreaks and limit the spread of infections, accurate and cost-effective typing methods are of importance. New typing methods are being developed for *Salmonella* and EHEC and in the near future they probably will replace many traditionally used methods. For example, WGS is getting an increasingly attractive method for routine surveillance of both the pathogens studied in this research because costs for sequencing have dropped, bench-top sequencing machines enable fast typing and user friendly software and web-based tools do exist. However, although typing by WGS provides a high resolution, as with any typing method it is important to remember that additional epidemiological information is still necessary for correct interpretation of the results.

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