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CATTLE AND REINDEER AS POSSIBLE SOURCES OF \textit{ESCHERICHIA COLI} O157 INFECTION IN HUMANS

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ACADEMIC DISSERTATION
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ABBREVIATIONS

A/E, attaching and effacing lesion
AFLP, amplified-fragment length polymorphism
aslA, gene encoding enteroaggregative heat-stable enterotoxin
aw, water activity
BCIG, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
BFP, bundle forming pilus
BHI, brain heart infusion
BPW, buffered peptone water
BPW-VCC, buffered peptone water supplemented with vancomycin, cefsulodin, cefixime
CDT-E. coli, cytotoxial distancing toxin producing E. coli
CFA, colonisation factor antigen
cfu, colony forming unit
cGMP, cyclic guanulate monophoshate
CI, confidence interval
CNF 1, cytotoxic necrotising factor 1
CR-SMAC, sorbitol Mac Conkey agar supplemented with cefixime and rhamnose
CT-SMAC, sorbitol Mac Conkey agar supplemented with cefixime and tellurite
DAEC, diffusely adherent E. coli
D values, decimal reduction time
eaE, E. coli attaching and effacing gene
EAEC, enteraggregative E. coli
EAF, EPEC adherence factor
EAST1, enteroaggregative heat-stable enterotoxin
EEB, EHEC enrichment broth
EHEC, enterohaemorrhagic E. coli
ethA, enterohaemolysin gene
EIEC, enteroinvasive E. coli
EPEC, enteropathogenic E. coli
Esp, E. coli secreted protein
ETEC, enterotoxigenic E. coli
fliC, gene encoding H7 flagella
Gb3, globotriaocylceramide
G-C, guanine-cytosine
GNn, gram negative broth with novobiocin
HACCP, hazard analysis of critical control points
HC, haemorrhagic colitis
HC agar, haemorrhagic colitis agar
HUS, haemolytic uraemic syndrome
IMS, immunomagnetic separation
IL, interleukin
ISO, International Organization for Standardisation
katP, gene encoding catalase peroxidase
kb, kilo base (pair)
kDa, kiloDalton
LEE, locus for enterocyte effacement
LPS, lipopolysaccharide
LT, heat labile enterotoxin
Mb, mega base (pair)
MDa, mega Dalton
mEcN, modified E. coli broth with novobiocin
MEMB, modified eosin methylene blue agar
MPN, most probable number method
MRS, Man-Rogosa-Sharp agar medium
MTSBca, modified tryptone soya broth with casa-amino acids
MTSBn, modified tryptone soya broth with novobiocin
MUG, 4-methylumbelliferyl-β-D-glucuronide
NM, nonmotile
NMKL, Nordisk Metodikkommitté for livsmedel
OR, odds ratio
PATS, polymorphic amplified typing sequences
PBS, phosphate buffered saline
PFGE, pulsed field gel electrophoresis
PMN, polymorphonuclear leucocyte
ppm, parts per million
PT, phage type
RAPD, random amplification of polymorphic DNA
rdnc, phage type reacts but does not conform
rb, gene encoding region for O-antigen
RFLP, restriction fragment length polymorphism
rh, relative humidity
SD, standard deviation
SF O157:H-, sorbitol fermenting nonmotile E. coli O157:H-
SMAC, sorbitol MacConkey agar medium
ST, heat stable enterotoxin
Stx, shiga toxin
stx, shiga toxin gene
Tir, translocated intimin receptor
TNF, tumour necrosis factor
TSA, tryptone soya agar
TSB, tryptone soya broth
TTP, thrombotic thrombocytopenic purpurae
uidA, gene encoding β-glucuronidase
UPB, universal pre-enrichment broth
VCC, vancomycin, cefsulodin, cefixime
VRB, violet-red-bile agar medium
VT, vero cytotoxin
z value, degrees required for the thermal destruction curve to traverse one log cycle
ABSTRACT

The survival of *E. coli* O157:H7 during the manufacture of dry sausage was studied using two different commercial starter cultures. *E. coli* O157:H7 was not eliminated during the 49-day long manufacturing process, including fermentation, drying and storage. The pathogen numbers declined more using a starter culture containing *Staphylococcus carnosus* Mill and *Lactobacillus curvatus* Lb3 than with a starter containing *Staphylococcus xylosus* DD-34, *Pediococcus acidilactici* PA-2 and *Lactobacillus bavaricus* MI-401 (p<0.0015 and p<0.0002). The reasons for this disparity could not be elucidated.

The prevalence of *E. coli* O157 in Finnish slaughter cattle was studied by collecting 1448 cattle faecal samples during June-December 1997 at 14 major cattle abattoirs. *E. coli* O157 was isolated from 19 samples (1.31%; 95% CI 0.73-1.90%). The prevalence of *E. coli* O157 was highest in July (8/204; 3.92%) and September (6/244; 2.46%). No *E. coli* O157 was detected in November and December, nor from faecal samples from the northernmost region where cattle density is low. The 19 isolates belonged to ten different pulsed-field gel electrophoresis (PFGE) genotypes. The most common PFGE genotype (1.42) was detected in eight isolates. Four PFGE genotypes (1.1; 1.6; 1.12; 1.14), 32% of the isolates, were indistinguishable from those isolated from humans in Finland, suggesting that at least some human infections are of bovine origin.

The prevalence of *E. coli* O157 in reindeer intended for meat production was examined. Faecal samples from 1387 animals and 421 meat samples were taken at ten reindeer slaughterhouses and at two slaughter ing sites between October 1998 and March 1999. *E. coli* O157 was not isolated from either the faecal or meat samples. The prevalence of *E. coli* O157 in reindeer faeces was less than 0.22% (CI 95%) suggesting that reindeer are not a reservoir of *E. coli* O157 in Finland.

Four sporadic cases of *E. coli* O157:H7 infection and one family outbreak could be traced to five different Finnish dairy farms. These five case patients (age range 2-17 years, median age 3 years) were hospitalised with bloody diarrhoea and two of them developed haemolytic uremic syndrome (HUS). The phage and pulsed-field gel electrophoresis types of the human and bovine isolates from the corresponding farms were indistinguishable. The proportion of cattle excreting the type that caused the human infection varied from 3.2-68.7% when sampled soon after the human cases, and from zero to 5.3% about a year or so later. On most of the farms the animals excreted the pathogen intermittently. Although the infection could be traced back to the farms, it could not be established whether the infection route was unpasteurised milk or direct or indirect contact with cattle.

A longitudinal study in one Finnish cattle finishing unit was performed to investigate excretion and sources of *E. coli* O157 in bulls from post-weaning until slaughter. Three groups of 31 to 42 calves were sampled in a lorry before entering the farm, and then 4-7 times at approximately monthly intervals while on the farm. All calves sampled in the livestock transporter were negative for *E. coli* O157 upon arrival, whereas positive animals were detected a day later. During the fattening period the infection rate of *E. coli* O157 varied between 0 and 38.5%. Seasonal variation in shedding was detected in bulls at slaughter, but no absence in shedding at the farm was observed during the cold months. *E. coli* O157 was detected from 9.7-38.9% of the faecal samples taken at slaughter, while only two rumen and one carcass surface samples were found positive. Faecal samples taken at the abattoir had lower counts (≤0.4/g) than those at the farm (p<0.05). Most farm isolates belonged to one PFGE genotype (79.6%) and the rest to closely related PFGE genotypes, which suggests a common source of infection. *E. coli* O157 seemed to persist well on barn surfaces. This study indicated that the finishing unit rather than the introduction of new cattle seemed to be the
source of *E. coli* O157 at the farm, although the possibility of transporter surfaces being the infection site could not be ruled out.
LIST OF ORIGINAL PUBLICATIONS

I Lahti E., Johansson T., Honkanen-Buzalski T., Hill P. and Nurmi E. Survival and detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the manufacture of dry sausage using two different starter cultures. Food Microbiology 2001; 18:75-85


III Lahti E., Hirvelä-Koski V. and Honkanen-Buzalski T. Occurrence of *Escherichia coli* O157 in reindeer (*Rangifer tarandus*). Veterinary Record 2001; 148:633-634


1 INTRODUCTION

During recent decades shiga toxin producing (STEC) or vero-cytotoxin producing (VTEC) _Escherichia coli_ O157:H7 has emerged as a serious human pathogen causing diarrhoea, bloody diarrhoea (haemorrhagic colitis, HC) and haemolytic uremic syndrome (HUS). The infection is especially severe in children under five years and elderly people (Ostroff et al. 1989a; Griffin and Tauxe 1991; Griffin and Tauxe 1991). The first reported outbreak occurred in 1982 in the USA, when HC and HUS were associated with eating undercooked hamburgers at restaurants of the same chain in the states of Michigan and Oregon (Riley et al. 1983). In 1986 children visiting or living on a dairy farm suffered diarrhoea and some developed HUS; _E. coli_ O157:H7 was isolated from both the cattle of the farm and the children (Martin et al. 1986; Borczyk et al. 1987). Several outbreaks have occurred since then, the biggest by far, in Japan in 1996, involving thousands of people (Michino et al. 1999). Most infections are thought to be sporadic, however. Outbreaks and sporadic cases have been associated with food items, such as various meat and dairy products, green produce like sprouts and salads, acid products like dry sausage, apple juice and mayonnaise, drinking water, with direct and indirect contact with cattle, swimming, or person-to-person transmission (Griffin and Tauxe 1991; Besser et al. 1993; Armstrong et al. 1996; Tilden et al. 1996). The infectious dose is very low, with possibly just a few cells enabling secondary transmission (Tilden et al. 1996; Tuttle et al. 1999). The pathogen also survives in low pH environments, which together with the low infective dose demands a good level of hygiene in food production (Glass et al. 1992).

The virulence of _E. coli_ O157 is not fully understood, but shiga toxins (Sx) or vero cytotoxins (VT) are considered important in the pathogenesis along with the property of causing attaching and effacing lesions (A/E) (Nataro and Kaper 1998; Paton and Paton 1998). STEC O157 belongs to a group of pathogenic _E. coli_ called enterohaemorrhagic _E. coli_ (EHEC). More than 100 different STEC serotypes have been associated with human illness (Johnson et al. 1996a). _E. coli_ O157 is considered the most important; it has caused most EHEC outbreaks and is associated with the most severe illnesses. However, it is important to note that the other STEC serotypes are under diagnosed because the analysis of typical _E. coli_ O157 is easier than that of non-O157 STEC due to the former’s property of sorbitol negativity and β-glucuronidase negativity (Paton and Paton 1998).

Cattle are considered the main reservoir of STEC O157 (Armstrong et al. 1996). Cross-sectional studies performed at slaughterhouses have found prevalences of faecal shedding of _E. coli_ O157 ranging from less than one to 26% (Chapman et al. 1989; Chapman et al. 1993; Chapman et al. 1997; Rice et al. 1997; Conedera et al. 1997; Heuvelink et al. 1998a; Bonardi et al. 1999; van Donkersgoed et al. 1999; Elder et al. 2000; Bonardi et al. 2001; Chapman et al. 2001a; Tutenel et al. 2002; Paiba et al. 2002). Studies performed on farms in countries such as Canada, Denmark, Norway and the USA have found animal prevalences of _E. coli_ O157 shedding ranging from less than one to 11 percent (Wells et al. 1991; Hancock et al. 1994; Garber et al. 1995; Zhao et al. 1995; Faith et al. 1996; Rice et al. 1997; Hancock et al. 1997b; Richards et al. 1998; Vold et al. 1998; Heuvelink et al. 1998b; Syng and Paiba 2000; Syng et al. 2001; Gannon et al. 2002; Sanchez et al. 2002; Nielsen et al. 2002). Results of different studies are difficult to compare, however, because the earliest studies in particular used less sensitive detection methods, often direct culture without enrichment, and varying sampling strategies, sample sizes and numbers of samples taken. In farm studies often only a portion of animals may have been sampled, and at feedlots pat samples have been used which give lower prevalences of shedding. However, it has been estimated that _E. coli_ O157 is prevalent on most US farms (Hancock et al. 1997b; Hancock et al. 1997c; Laegreid et al. 1999). Regional variations might exist because of differences in the production systems, farm
management practices and feeding regimes (Hancock et al. 2001). Non-O157 STEC are commonly excreted by cattle: 18-42% excrete them at slaughter (Borie et al. 1997; Miyao et al. 1998; Pradel et al. 2000; Leung et al. 2001; Khan et al. 2002; Samadpour et al. 2002b). Other ruminants, like sheep, can harbour *E. coli* O157 too, but the prevalence is lower than in cattle (Heuvelink et al. 1998a; Chapman et al. 2001a; Paiba et al. 2002). *E. coli* O157 is not species-specific, having been isolated from a wide range of animals including swine, dogs, horses, rats, birds, raccoons, opossums, rabbits and flies (Trevena et al. 1996; Wallace et al. 1997; Chalmers et al. 1997; Shere et al. 1998; Heuvelink et al. 1999; Iwasa et al. 1999; Cizek et al. 1999; Pritchard et al. 2001; Renter et al. 2003).

*E. coli* O157 is not considered a primary cattle pathogen although it has been sporadically isolated from calf diarrhoea (Daniel et al. 1998). Non-O157 STEC serotypes of O5:nonmotile (NM), O26:H11, O26:NM, O103:H2 and O111:NM are causative agents of calf diarrhoea (Gyles 1998; Stevens et al. 2003). In an experimental study *E. coli* O157:H7 caused mild diarrhoea to neonatal or fasted calves (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1999a). *E. coli* O157 is thus shed by healthy cattle, and the shedding is intermittent. Young animals, especially weaned calves, excrete *E. coli* O157 more often and at higher numbers than adult cattle (Wells et al. 1991; Hancock et al. 1994; Garber et al. 1995; Zhao et al. 1995; Hancock et al. 1997a; Wilson et al. 1998; Nielsen et al. 2002). The bacterium is excreted only in faeces but it has been isolated from mouth and tonsils and throughout the gastrointestinal tract (Cray and Moon 1995; Buchko et al. 2000) although the predilection site seems to be the hindgut (Cray and Moon 1995; Grauke et al. 2002; Naylor et al. 2003). Conflicting results have been obtained regarding the influence of diet on shedding (Kudva et al. 1995; Diez-Gonzalez et al. 1998; Buchko et al. 2000; Tkalcic et al. 2000). A single genotype or several genotypes have been detected on each farm (Lee et al. 1996; Rice et al. 1999; Nielsen and Scheutz 2002), suggesting complex ecology of the organism (Faith et al. 1996; Keene et al. 1997a).

*E. coli* O157 persists in the environment, in water and on surfaces (Wang and Doyle 1998; Rahn et al. 1999; Randall et al. 1999). It is sensitive to heating and most disinfectants but seems to be difficult to remove altogether from the local environment (Oie et al. 1999; Stringer et al. 2000; Small et al. 2002). *E. coli* O157 is able to grow outside the gastro-intestinal tract if conditions are favourable. Low infective dose, and the ability to persist in the environment and cause serious disease in humans pose great challenges to farmers, slaughterhouses, dairy plants and food production facilities.
2 REVIEW OF THE LITERATURE

2.1 General properties of Escherichia coli

Escherichia coli, a gram-negative non-spore-forming rod, is a commensal of warm-blooded animals and humans, and the most common facultative anaerobic bacterium in the gut (Donnenberg and Whittam 2001). The species E. coli was first described by Escherich in 1885 as Bacillus coli, a commensal intestinal inhabitant (Pupo et al. 1997). Later the genus was renamed Escherichia, the bacterium E. coli and the family Enterobacteriaceae. The genus Escherichia contains several species, although their true relatedness has been questioned (Pupo et al. 1997). The cause of dysentery was first named as Bacillus dysenteriae, because at that time B. coli was known as a non-pathogenic organism (Pupo et al. 2000). Shigella and E. coli are very closely related and could be considered as the same species (Pupo et al. 2000; Wang et al. 2001).

The evolution of E. coli has been dynamic; 18% of the genome has been obtained from other bacteria by horizontal transmission (Lawrence and Ochman 1998). The horizontal transfer sequences have a different guanine-cytosine (G-C) content from the ancestral genome, and they often contain virulence genes and are inserted into the genome at specific sites as pathogenicity islands (Donnenberg and Whittam 2001). The G-C content of the entire E. coli genome varies from 50 to 51% (McDaniel et al. 1995).

A typical two-layer cell wall of a gram-negative bacterium surrounds the cell. The outer membrane contains two types of lipids, lipopolysaccharides (LPS) and phospholipids, as well as a set of proteins. LPS comprises three regions: lipid A, an oligosaccharide core, and a polysaccharide O antigen. The O antigen is very variable and is used for serotyping. The cytoplasmic membrane borders the cytoplasm. Periplasm fills the space between the outer and cytoplasmic membrane, and contains a layer of murein or peptidoglycan. E. coli often have fimbria on the surface of the cell that the bacterium uses for adhering to intestinal epithelial cells.

Pathogenic variants of E. coli cause diarrhoeal diseases, diseases in the urinary tract, or other extraintestinal infections like meningitis and septicaemia (Nataro and Kaper 1998). Diarrhoeagenic E. coli can be divided into five pathotypes on the basis of their pathogenicity mechanisms: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohaemorrhagic (EHEC), enteroinvasive (EIEC), enterocaggregative (EAEC) (Czeczulin et al. 1999). The sixth pathotype includes diffusely adherent E. coli (DAEC) (Nataro and Kaper 1998; Donnenberg and Whittam 2001) and the seventh pathotype includes cytotoxlated distending toxin (CDT) - producing E. coli (Nataro and Kaper 1998; Clarke 2001).

2.1.1 Enteropathogenic E. coli (EPEC)

EPEC are a major cause of human infantile diarrhoea, especially in the developing world (Nataro and Kaper 1998), but they also cause disease in a wide range of mammalian species, including rabbits, pigs and dogs (Kenny 2002). Healthy adults can also become infected if the inoculum is high (Nataro and Kaper 1998). The reservoirs of EPEC for humans are thought to be symptomatic or asymptomatic children, or asymptomatic adults. A number of serotypes have been associated with EPEC. Typical human EPEC serotypes, like O55:H6/H-, O86:H34, O111:H2/H-, and O114:H2, have not been isolated from animals, whereas atypical EPEC serotypes have been isolated and animals might be the reservoir (Trabulsi et al. 2002). Characteristic for EPEC is the production of attaching and effacing lesions (A/E) where the bacteria adhere intimately to the enterocyte surface resulting in the loss (effacement) of intestinal absorptive microvilli (Donnenberg et al. 1993). The initial attachment of EPEC to intestinal epithelial cells is mediated by bundle forming pilus (BFP), a type IV fimbria that is
encoded on a large EPEC adherence factor (EAF) plasmid (Nataro and Kaper 1998; Donnenberg and Whittam 2001). EPEC also produces a large toxin that inhibits lymphocyte activation (Donnenberg and Whittam 2001). EPEC strains form densely packed three-dimensional clusters on the surface of the eukaryotic cell, a characteristic pattern called localised adherence. After adherence to epithelial cells, genes located in a 35 kb chromosomal pathogenicity island called locus for enterocyte effacement (LEE) are activated. LEE carries 41 genes, which encode a type III secretion system, proteins secreted via the system and several other proteins of unknown function (Donnenberg and Whittam 2001). A LEE-encoded protein translocated intimin receptor (Tir) is translocated via the type III secretion system into the plasma membrane of the host cell. An outer membrane protein called intimin (94-97 kDa) encoded by a gene eae (E. coli attaching and effacing) located in LEE inserts into Tir and binds the bacterium more closely to the epithelial membrane. E. coli secreted proteins (EspA, EspB, EspD) encoded in the LEE and secreted via the type III secretion system deliver Tir into the host cell. An EPEC Tir is tyrosine phosphorylated once delivered into the host (De Vinney et al. 2001). The complete role of the Esp proteins is still being defined (Donnenberg and Whittam 2001). Adherence results in the accumulation of polymerised actin filaments underneath the adhering bacteria and leads to the formation of a pedestal-like structure at the site of bacterial contact (Goffaux et al. 2001). Intracellular calcium increases and other changes follow which result in acute diarrhoea.

2.1.2 Enterotoxigenic E. coli (ETEC)

ETEC strains are causes of traveler’s diarrhoea and infantile diarrhoea, especially in developing countries, but they have also been linked with outbreaks of food-borne illness (Daniels et al. 2000). ETEC strains also cause diarrhoea also in neonatal pigs, calves, and lambs, and in weaning pigs (Mainil et al. 1993). Most diarrhoeic E. coli strains from cattle belong to the ETEC class. ETEC strains attach to the epithelial cells of jejunum and ileum by means of colonisation factor antigens (CFA) (Nataro and Kaper 1998). The adhesins of ETEC have host species specificity. The symptoms of ETEC infection are caused by the production of two types of enterotoxins, heat stable (ST) and heat labile (LT) enterotoxins. STs are small monomeric plasmid-mediated toxins that comprise two unrelated subgroups, STA and STB. The LT are large oligomeric toxins consisting of two major serogroups, LT-I and LT-II (Nataro and Kaper 1998). The LT-I is related to the cholera toxin. The ETEC strains can harbour ST or LT toxins, or both. STA increases the levels of cyclic guanylate monophoshate (cGMP) which leads to intestinal fluid secretion. STB causes loss of villous epithelial cells and partial villous atrophy. LT toxins also lead to a decrease in sodium absorption and osmotic diarrhoea. ETEC is characterised by watery diarrhoea.

2.1.3 Enteroinvasive E. coli (EIEC)

EIEC cause a dysentery-like, bloody, mucoid diarrhoea and share some of the virulence mechanisms associated with Shigella dysenteriae (Nataro and Kaper 1998). EIEC are a significant cause of diarrhoeal disease in young children, with high morbidity and mortality in the developing and underdeveloped world with low levels of sanitation and hygiene (Clarke 2001). A 120-140 MDa plasmid encodes genes necessary for attachment and invasion of colonic enterocytes by endocytosis and multiplication within the colonic cells.

2.1.4 Enteroaggregative E. coli (EAEC)

EAEC are a recently identified group of enterovirulent E. coli that have been associated primarily with persistent infantile diarrhoea particularly in underdeveloped countries but also in industrialised countries (Nataro et al. 1987; Bhan et al. 1989; Giron et al. 1991; Baquie et al. 1992; Paul et al. 1994; Nataro et al. 1998). Originally defined by the unique "stacked-brick" aggregative adherence to Hep-2 monolayers (Vial et al. 1988), EAEC are routinely detected by use of a gene probe derived from the 60-65 MDa plasmid responsible for the phenotype
(Baudry et al. 1990). Other putative virulence factors commonly associated with EAEC include aggregative adherence fimbriae (AAF/I, AAF/II or AAF/III) (Nataro et al. 1995; Czeczulin et al. 1997; Bernier et al. 2002). Other factors considered possible markers of virulence for EAEC include a cryptic secreted protein, a plasmid encoded toxin, a mucinase, an α-haemolysin and the cytotoxic distending toxin (CDT) (Suzart et al. 2001). EAEC strains adhere to cultured epithelial cells (Hep-2 cells) and do not secrete heat-labile or heat-stable enterotoxins. They often produce a toxin known as enteroaggregative heat-stable enterotoxin (EAST1) that activates through a guanylate cyclase receptor like StA (Savarino et al. 1993; Czeczulin et al. 1999). EAST1 is encoded by the astA gene localised in a plasmid. The adherence is mediated by bundle-forming fimbriae.

2.1.5 Diffusely adherent E. coli (DAEC)

DAEC or diarrhoea-associated haemolytic E. coli (DHEC) or cell-detaching E. coli have been described as a cause of diarrhoea (Clarke 2001). The virulence is mediated by α-haemolysin, cytotoxic necrotising factor 1 (CNF1) and diffuse adherence pattern to cultured epithelial cells. The pathogenic mechanisms are not fully understood. Strains with DAEC characteristics have also been isolated from diarrhoeic lambs (Blanco et al. 1996).

2.1.6 Cytotoxic distending toxin (CDT) -producing E. coli

CDT-producing E. coli are the latest class of diarrhoeagenic E. coli but they also have the virulence factors of EPEC or EAEC (Clarke 2001). The role of CDT-producing E. coli as a diarrhoeal pathogen is unclear.

2.2 Enterohaemorrhagic E. coli (EHEC), shiga toxin producing E. coli (STEC), vero cytotoxin producing E. coli (VTEC)

The term verotoxic or verocytotoxigenic or verocytotoxin producing E. coli (VTEC) is derived from the observation that these strains produced cytopathic effects on Vero (African green monkey kidney) cells (Konowalchuk et al. 1977). The name shiga toxin -producing or shigatoxigenic E. coli (STEC) (formerly shiga like toxin producing E. coli) comes from the fact that one of the cytotoxins produced is identical to the shiga toxins (Stx) produced by S. dysenteriae (O’Brien et al. 1983). The term enterohaemorrhagic E. coli (EHEC) denotes to a subset of STEC strains that are considered to be pathogens and have been associated with human illness (Nataro and Kaper 1998; Paton and Paton 1998). Typical EHEC refers to STEC strains that produce Stx and A/E lesions and possess the 60 MDa plasmid, while the term atypical denotes STEC strains that do not produce A/E lesions and/or do not possess the 60 MDa plasmid (Nataro and Kaper 1998). The serotypes included in typical EHEC are O157:H7/H-, O26:H11/H-, O111:H-, O113:H21 and O103:H2 (Mailil 1999). Serotype O26 can be defined as either EHEC or EPEC depending on the presence or absence of Stx. E. coli O157:H7 appears to be particularly virulent as it is responsible for the largest outbreaks and the most severe diseases, although non-O157 serotypes have also caused HUS (O’Brien and Kaper 1998; Griffin 1998).

A proposal to use the name shiga toxin to cover all these cytotoxins has been made (Calderwood et al. 1996) but the terms VTEC and STEC are interchangeable (Acheson 1998; O’Brien and Kaper 1998). EHEC cause disease in humans but unlike the other pathogenic E. coli that are mainly host specific, the reservoir of EHEC, at least of E. coli O157, is in the animal population.
2.2.1 Virulence factors of STEC

2.2.1.1 Shiga toxins (Stx), verocytotoxins (VT)

The major virulence factors of STEC are Stx, which are encoded on a lambda-like bacteriophage inserted into the chromosome (Nataro and Kaper 1998). The toxin genes can be transferred or lost by strains (Karch et al. 1992). The whole toxin is approximately 70 kDa, comprising one peptide A subunit (32 kDa) and five B units (7.7 kDa) (Nataro and Kaper 1998). The B subunits bind the toxin to a glycolipid receptor, globotriaosylceramide Gb$_2$, on the target cell. The toxin functions as follows: the toxin molecule is internalised, the A subunit is nicked by a furin protease and the active A1 subunit is released. The active A1 fragment moves to the Golgi apparatus and passes over to the endoplasmic reticulum, where it cleaves a specific N-glycosidic bond in the 28S ribosomal component of the 60S ribosomal unit resulting in the inhibition of protein synthesis and thus cell death.

STEC strains may express Stx1 or Stx2, or both, or multiple forms of Stx2. Stx1 differs from the Stx of S. dysenteriae by only one amino acid in the A polypeptide, whereas Stx2 shows only 55-60% homology (Paton and Paton 1998). Stx1 is highly conserved while Stx2 has antigenic variation. The variability primarily comes from the sequence diversity in the B subunit, which may alter receptor-binding preference. The Stx2 variants also differ in toxicity for tissue cultures and animals. The different variants presently known are Stx2, Stx2c, Stx2d, Stx2e and Stx2f (Schmidt et al. 2000). Human pathogenic STEC O157 strains usually contain stx$_2$ or both stx$_2$ and stx$_1$ genes (Karmali 1989; Paros et al. 1993). A large proportion of Australian E. coli O157 strains, however, produce Stx1 only, which might make them less virulent (Robins-Browne et al. 1998). Stx2c has been isolated from human strains but mostly from isolates that also contain stx$_1$, stx$_2$ or both genes (Furst et al. 2000). Stx2d has been isolated from asymptomatic carriers (Stephan and Hoezle 2000). Stx2e is mainly found with oedema disease in pigs (Gyles 1998). Stx2f has been isolated from pigeons (Dell'omo et al. 1998; Schmidt et al. 2000). Stx2 is more cytotoxic than Stx1 and more often isolated from severe cases (Ostroff et al. 1989a; Ostroff et al. 1989b).

The production of Stx was linked to HC and HUS in the mid 1980s (Karmali et al. 1985a). Although stx genes are considered important in the pathogenesis of EHEC, E. coli O157:H7 and E. coli O157:H- strains not possessing stx genes have been isolated from patients with diarrhoea and HUS (Schmidt et al. 1999). Stx toxins are important virulence factors in human disease but it is not clear whether they have a role in bovine infections (Rasmussen and Casey 2001).

2.2.1.2 Locus for enterocyte effacement (LEE)

The pathogenicity island LEE was originally discovered in EPEC strains. The LEE island of E. coli O157:H7 possesses all the genes found in EPEC LEE (Perna et al. 1998). The EHEC LEE is larger than that of EPEC. The LEE of E. coli O157 strain EDL933 consists of 54 genes. The type III secretion apparatus is fairly similar but the sequences coding for the secreted proteins of EPEC and EHEC are more diverse. Considerable variation in the Tir genes of EPEC and EHEC serogroups has been noted (Paton et al. 1998; De Vinney et al. 2001). EPEC and EHEC have different modes of pathogenesis and colonise different sites in the intestine, due to at least differences in the intimin or Tir type (Phillips et al. 2000). The initial colonisation site and site of A/E lesions of EHEC seems to be Peyer's patches in the terminal ileum (Phillips et al. 2000). In addition to Tir, there is evidence for the existence of one or more host cell intimin receptors (Smith et al. 2002). The EHEC LEE has an espF gene, which could be involved in cell death and loss of intestinal barrier function (Donnenberg and Whittam 2001). Eleven intimin types have been identified, but E. coli O157:H7/H- strains produce a γ type (Son et al. 2002). While intimin thus seems to be important in the
colonisation and the pathogenesis of EHEC, STEC strains lacking eae have caused HUS cases (Paton et al. 1999), which suggests that other colonisation factors must be present.

2.2.1.3 Other virulence factors

Virtually all *E. coli* O157:H7 isolates carry a large plasmid of approximately 90 kb, which is called EHEC plasmid (Karch et al. 1998). The EHEC plasmid carries genes for several putative virulence factors, including enterohaemolysin (encoded by *ehxA*), catalase-peroxidase (encoded by *katP*), lymphocyte inhibition factor (Lif), EspP (encoded by *espP*), *toxB* (encoded by *toxB*), *tagA*, and a predicted type II secretion system (*etp*). Enterohaemolysin is found in nearly all *E. coli* O157:H7 strains and widely among non-O157 strains. The role of enterohaemolysin in the pathogenesis is still unknown but serological response against enterohaemolysin has been detected from HUS patients, which indicates an association with virulence (Schmidt et al. 1995). The toxin lyses erythrocytes of human and bovine origin, and bovine but not human leukocytes. EspP cleaves pepsin and human coagulation factor V and may affect the normal coagulation cascade, increasing gastrointestinal haemorrhage (Brudet et al. 1997). *toxB* gene is needed in epithelial adherence (Tatsuno et al. 2001). Other smaller plasmids, like a 6.7 kb and 3.3 kb have been found in 57-62% of *E. coli* O157:H7 isolates (Paros et al. 1993; Karch et al. 1998).

EAST1 is often found in STEC strains. In one study all the 13 strains of *E. coli* O157:H7 examined possessed the *astA* gene (Paiva de Sousa and Dubreuil 2001). The significance of EAST1 in the pathogenesis of STEC is not known, but it could probably explain the nonbloody diarrhoea seen in infected individuals.

Quorum sensing is a widespread phenomenon in bacteria, first described in *Vibrio fischeri*. Through quorum sensing bacterial cells signal to each other by the production of hormonelike compounds called autoinducers (Sperandio et al. 2001). Gram-negative bacteria usually produce acyl-homoserine lactones as autoinducers. The LEE genes, as well as genes involved in the SOS response which induces *stx* genes, are regulated by quorum sensing (Sperandio et al. 2001; Sperandio et al. 2002). Gram-negative bacteria usually produce acyl-homoserine lactones as autoinducers. The LEE genes, as well as genes involved in the SOS response, which induces *stx* genes are regulated by quorum sensing. Thus, quorum sensing is a mechanism involved in basic physiological functions and virulence factors of *E. coli* O157:H7 (Sperandio et al. 2002).

2.2.2 Antimicrobial resistance of *E. coli* O157

The first studies showed that *E. coli* O157 strains were susceptible to antimicrobials commonly used in enteric infections (Ratnam et al. 1988). Later was observed that 24% of the 125 *E. coli* O157:H7/NM isolates were resistant to at least one antimicrobial and 19% to three or more (Meng et al. 1998). Cattle isolates had the highest rate (34%) of resistance. A low level of resistance was, however, observed among 210 US STEC *E. coli* O157:H7/NM isolates from humans, cattle, and food isolated in the period 1985-2000 using broth microdilution assay (Schroeder et al. 2002) as of the 13 antimicrobials tested, resistance to sulfamethoxazole (10%), and tetracycline (9%) were most common. Of the English and Welsh human isolates of STEC O157 approximately 20% were resistant to antimicrobial agents, predominantly streptomycin, sulphonamides and tetracycline (Willshaw et al. 2001a). Antimicrobial resistance cassettes were identified in US STEC O157 isolates (Zhao et al. 2001). Australian VTEC isolates were more susceptible to antimicrobials than non-VTEC isolates (Bettelheim et al. 2003), which might be explained by the fact that antimicrobials are not recommended in the treatment of human EHEC infections and animals are symptomfree carriers. Antimicrobial resistance is thus far not a major problem among *E. coli* O157 isolates.
2.2.3 Evolution of STEC and STEC *E. coli* O157:H7/H-

Population genetic analyses of EHEC strains have shown that these strains comprise two divergent lineages, termed EHEC1 and EHEC2, that are only distantly related but have experienced similar pathways of virulence gene acquisition (Whittam 1998). The EHEC1 lineage is comprised of a geographically disseminated cluster of strains with related genotypes bearing O157:H7 and O157:H- serotypes, while the EHEC2 lineage is serotypically and genotypically more diverse (Whittam 1998; Reid et al. 2000; Kim et al. 2001).

It has been suggested that EHEC O157 arose from transfer of the region (*gnd-rfb*) encoding the O157 antigen into an EPEC O55:H7 bearing the LEE island and a stx2 converting prophage (Feng et al. 1998). In subsequent steps, this ancestral population lost the ability to ferment sorbitol, was lysogenised by a stx1 converting phage, and acquired a mutation that inactivated the *uidA* gene, which resulted in a loss of β-glucuronidase activity (Whittam 1998; Feng et al. 1998).

The genome of *E. coli* O157:H7 is 5.5 Mb in size, 859 kb larger than that of the laboratory strain of *E. coli* K12 (Perna et al. 2001; Hayashi et al. 2001). The G-C content of the genome of *E. coli* O157:H7 is 50.5 mol% (Hayashi et al. 2001). The strains of *E. coli* O157:H7 and *E. coli* K12 share a common sequence of 4.1 Mb which is highly conserved. The remaining sequence consists of elements of horizontal transfer which contain already known virulence genes and possibly also other potential other virulence genes. The G-C content of the horizontal elements differs from the backbone G-C, which further suggests that they have come from other bacteria.

2.2.4 Sorbitol-fermenting (SF) *E. coli* O157:H-

Nonmotile STEC strains of serotype O157:H- which do ferment sorbitol (SF O157:H-) have rapidly emerged as important causes of human disease in continental Europe (Aleksic et al. 1992; Karch and Bielaszewska 2001). The reservoir of SF O157:H- is unclear, although they have sporadically been isolated from cattle (Bielaszewska et al. 2000) and from a pony (Karch and Bielaszewska 2001). SF O157:H- possesses the *fllic* gene encoding H7 antigen (Bielaszewska et al. 2000), but the gene is mutated (Reid et al. 1999). SF O157:H- show other characteristics that distinguish them from non-sorbitol fermenting *E. coli* O157:H7. SF O157:H- do not contain the pathogenicity island TAI conferring tellurite resistance, and are thus sensitive to tellurite. They do not usually express enterohaemolysin and they possess a plasmid encoded gene cluster, *sfp*, which mediates mannitol resistance, not detected in other STEC strains or in the family of *Enterobacteriaceae*. SF O157:H- represents a new, distinct clone within the O157 serogroup (Karch and Bielaszewska 2001).

2.3 Pathogenesis of STEC *E. coli* O157:H7/H-

Having survived the harsh conditions of stomach, STEC adheres to colon mucosal cells with proteins encoded by the LEE island leading to A/E formation and induction of inflammation (Paton and Paton 1998; Smith et al. 2002). Release of Stx from lytic bacterial cells follows, which leads to damage to mucosal endothelium causing HC (Smith et al. 2002). The classic pathological intestinal lesions characteristic of *E. coli* O157:H7 infections are haemorrhage and oedema in the lamina propria of the ascending and transverse colon (Riley et al. 1983; Remis et al. 1984; Griffin et al. 1990). Stx receptors are sited in endothelial cells. Stx2 binds to polymorphonuclear leucocytes (PMN) during the diarrhoeal phase, and PMN deliver toxin systematically (te Loo et al. 2000; te Loo et al. 2001). Gastrointestinal tract and kidney are the organs most commonly affected, but central nervous system, pancreatic, skeletal and myocardial involvement may also be present (Andreoli 1999). Increased numbers of PMN are found in the glomeruli of kidney autopsy samples from patients with diarrhoeal HUS. In experimental studies in rabbits, microvascular lesions appeared in the central nervous
system, colon and lungs where endothelial cells express Gb$_3$ receptors (Rondeau and Peraldi 1996). Renal glomerular microvascular endothelial cells are particularly sensitive to Stx (Melton-Celsa and O'Brien 1998). The Stx enter the systemic circulation causing microvascular damage at target organs by a process mediated by proinflammatory cytokines such as tumour necrosis factor α (TNF-α) and interleukins (IL) (Dundas et al. 1999). Pretreatment with TNF-α and IL-1 sensitized endothelial cells to cytotoxicity and induced an increase in the number of Gb$_3$ receptors on the glomerular endothelial cells (Andreoli 1999). Endothelial damage induces the formation of large von Willebrand multimers, which in turn may cause platelet aggregation with the formation of small-vessel thrombi in target organs, intravascular haemolysis and ischemia (Dundas et al. 1999). Fibrin but not von Willebrand factor is abundant in glomerular thrombi of _E. coli_ O157 -associated HUS (Tsai et al. 2001). Stx1 and Stx2 binding to renal tubular epithelial cells in a fatal human case has been demonstrated (Uchida et al. 1999). Stx binds to distal tubules and collecting ducts of the juxtaglomerular region in children and adults, but in children Stx also binds to glomerular capillaries (Pruimboom-Brees et al. 2000).

2.4 Clinical features in humans

The clinical manifestations of _E. coli_ O157 infection range from symptom-free carriage to non-bloody diarrhoea, HC, HUS and death (Mead and Griffin 1998). Infection of _E. coli_ O157 has a typical incubation period of 3-4 days as the bacterium colonizes the large bowel. The initial symptoms are abdominal cramps, fever (in less than 30%), vomiting (in 30-50%), and diarrhoea (Griffin et al. 1988; Griffin and Tauxe 1991). Within 1-2 days the diarrhoea becomes bloody and the abdominal pain increases. The proportion of persons acquiring bloody diarrhoea varies from 50-70% depending on several factors like age, immunologic status, and the virulence of the strain (Paton and Paton 1998). Benign courses of disease have also been described among young adults (Rodrique et al. 1995). In most patients the bloody diarrhoea resolves within a week, but 5% and up to 20% develop HUS (Karmali 1989; Mead and Griffin 1998).

HUS begins 1-14 days after the onset of diarrhoea and is heralded by increased pallor, mild jaundice, and oliguria (Andreoli 1999). HUS is characterised by haemolytic anaemia with fragmented erythrocytes, thrombocytopenia and renal failure, and was first described in 1955 by a Swiss haematologist (Gordjani et al. 1997). Occasionally, patients may develop thrombotic thrombocytopenic purpurae (TTP), a condition similar to HUS but with less severe renal injury and more prominent central nervous system abnormalities. Rare complications include pancreatitis, diabetes mellitus and pleural and pericardial effusions (Mead and Griffin 1998). Risk factors for the development of systemic complications are age of less than five or more than 65 years, bloody diarrhoea, fever, elevated number of leucocytes and treatment with intestinal antimitotic agents (Boyce et al. 1995; Mead and Griffin 1998; Andreoli 1999). In some studies female sex has also been reported as a risk factor of developing HUS (Mead and Griffin 1998).

HUS is the leading cause of acute renal failure in small children and _E. coli_ is a leading cause of HUS in children (Gordjani et al. 1997). The percentage of cases that progress to HUS ranged from 3 to 7% (Slutsker et al. 1998) in series of sporadic cases to about 20% or more in outbreaks involving elderly patients, young children or other immunocompromised patients (Carter et al. 1987), as occurred in a Scottish outbreak in 1996 involving elderly people: 34 of 512 patients developed HUS (Ahmed and Donaghy 1998). Up to 3 to 5% of patients with HUS die, 5% develop chronic sequelae, 30% proteinuria and 60% recover (Mead and Griffin 1998). The mortality from HUS is 3 to 17% but has been reported as high as 88% in elderly people treated conservatively (Carter et al. 1987). The mortality from HUS has been reported as high as 88% in elderly treated conservatively (Carter et al. 1987). In addition, survivors of HUS can
have impaired renal function and extrarenal problems including neurological defects, insulin-dependent diabetes mellitus, pancreatic insufficiency, or gastrointestinal complications (Andreoli 1999).

The treatment of patients with symptomatic *E. coli* O157 infection is mainly supportive. Antimotility agents are contraindicated (Neill 1998). Management of HUS requires attention to fluid and electrolyte balance, nutritional support, treatment of anaemia, and control of hypertension, seizures and azotaemia (Mead and Griffin 1998). About 50% of patients with HUS need dialysis (Mead and Griffin 1998), and 5-20% renal transplantation (Meyers et al. 1998). The benefits of plasma transfusions are inconclusive (Neill 1998; Meyers et al. 1998; Dundas et al. 1999; Tsai et al. 2001). A synthetic toxin receptor blockade may be used in the prevention of HUS (Armstrong et al. 1998; Takeda et al. 1999; Todd and Dundas 2001).

The use of antimicrobial drugs is controversial and not recommended since they may increase the risk for developing HUS, and the bloody diarrhoeal phase may last longer (Bell et al. 1997; Zimmerhackl 2000) although a recent, though debated, meta-analysis did not show a higher risk of HUS and antimicrobial therapy (Safdar et al. 2002). Subinhibitory concentrations of antimicrobials induce the release of Stx *in vitro* (Griff et al. 1998b; Kimmitt et al. 1999) and *in vivo* (Zhang et al. 2000) and might thus induce a more severe clinical disease. The response to antimicrobials seems to be strain-dependent (Griff et al. 1998b). In the Sakai outbreak in Japan, however, administration of fosfomycin in the early phase of the disease prevented HUS (Ikeda et al. 1999). In US prospective case-control and cohort studies the use of antibiotics in children younger than 13 and 10 years, respectively, was associated with the development of HUS (Slutsker et al. 1998; Wong et al. 2000).

Most patients recover spontaneously within a week (Mead and Griffin 1998), but the organism may be shed in the stools for several weeks. Children younger than five years shed *E. coli* for longer periods than older children or adults (Belongia et al. 1993), and children with severe disease shed longer (Karch et al. 1995). The mean duration of shedding in children with only diarrhoea was 13 days, versus 21 days in children with HUS, one of the latter shedding for 124 days. However, in another study, the severity of disease did not affect duration of shedding (Belongia et al. 1993). Although the bacterium may be shed for weeks, *E. coli* O157 could be cultured from only 33% of the stools of children with HUS ≥7 d after the onset of diarrhoea (Tarr et al. 1990).

### 2.5 Epidemiology of *E. coli* O157 infection in humans

#### 2.5.1 Incidence in humans

Incidence rates of *E. coli* O157 and other STEC infections in different geographic areas are difficult to compare because of variations in the surveillance systems, laboratory practices and other differences in the infrastructure. In North America, the British Isles, and Japan the most common STEC serotype in human infections is *E. coli* O157, but in Australia, Latin America and in some countries in continental Europe non-O157 STEC have more often been isolated from human illness (O'Brien and Kaper 1998). In the USA the frequency of non-O157 STEC infections is approximately 60% of that for *E. coli* O157 (Griffin et al. 2001). However, it should be noted that non-O157 are more under-diagnosed than *E. coli* O157 because not all clinical laboratories test for them. In Ontario, Canada, it has been estimated that 4-8 symptomatic cases of *E. coli* O157 per reported case are missed in the surveillance system (Michel et al. 2000), and in the USA the corresponding figure is estimated to be 13-27 (Mead et al. 1999). The under-reporting of non-O157 infections is most probably greater than those of *E. coli* O157. The incidence data mainly cover symptomatic infections, very little being known of the true incidence of asymptomatic carriage (Paton and Paton 1998).
Annual incidence rates of *E. coli* O157 infections of six per 100,000 have been reported in Scotland (MacDonald et al. 1996; Ogden et al. 2002) and in Canada (Spika et al. 1998). In the USA, *E. coli* O157 has been estimated to cause 73,480 illnesses, 2,168 hospitalisations, and 61 deaths annually (Griffin et al. 2001). Between 1995 and 1998, the incidence of *E. coli* O157 ranged from 1.28 to 2.10 per 100,000 in England and Wales (Willsaw et al. 2001a). The incidence of STEC in Finland was 0.8-1.4/100,000 (Keskimäki et al. 1998). Regional differences exist; the incidence in rural areas has been higher than in urban areas, as in the Grampian area of Scotland (8-9 per 100,000) (MacDonald et al. 1996; Coia et al. 1998) and rural parts of Ontario (Michel et al. 1999). Northern states of the USA has higher incidence of *E. coli* O157 infection than the southern (Mead et al. 1999).

High rates have also been reported from Argentina, which has the highest incidence of reported HUS in the world: 22/100,000 in children under five years of age (López et al. 1998). The incidence of HUS in continental Europe has been 0.2-1.9/100,000 (Caprioli and Tozzi 1998), in Italy 0.28/100,000 (Tozzi et al. 2003), in the UK 3.2/100,000 (Smith et al. 1998), and in Australia 1.35/100,000 (Elliott et al. 2001). It should be noted that not all countries report HUS.

### 2.5.2 Food and water-borne infections

In 1982, two outbreaks of bloody diarrhoea were associated with the consumption of hamburgers at restaurants in the US states of Oregon and Michigan (Riley et al. 1983). A previously rare serotype of *E. coli* O157:H7 was isolated from nine of 12 stools of the patients and from a meat lot in Michigan. Since then, several outbreaks have been linked to eating undercooked hamburgers (Pal et al. 1984; Bell et al. 1994; Cieslak et al. 1997; Tuttle et al. 1999). The biggest of these occurred in the USA in 1993, and involved 501 reported cases (Bell et al. 1994). Outbreaks have been caused by various meat products (Armstrong et al. 1996). A large Scottish outbreak in 1996 with 512 confirmed cases and 20 deaths was traced to a single food premises that had handled raw meat on the same preparation table as cooked meat heated at a low temperature (Cowden et al. 2001).

In the US outbreaks, the mode of transmission has most often been food (67%), followed by person-to-person spread (22%), swimming (8%) and drinking water (2%) (Griffin 1998). The source of infection may also be direct contact with animals or humans, or indirect contact. Most of the Canadian and US outbreaks have been associated with ground beef (Spika et al. 1998; Griffin et al. 2001). Ground beef has been an uncommon cause of infection in continental Europe (Caprioli and Tozzi 1998). Annually, 11-25 outbreaks are reported in England and Wales (Smith et al. 2001).

Unpasteurised milk was associated with cases of HUS in the US as far back as 1986 (Martin et al. 1986). Since then unpasteurised milk has been linked to several outbreaks. In Britain, yoghurt produced at a farm caused a local outbreak (Morgan et al. 1993). Post-pasteurisation contamination of milk caused an outbreak affecting more than 100 people in Scotland (Upton and Coia 1994) and in England (Goh et al. 2002). Other milk products, like unpasteurised cheese, have also been associated with non-O157 and *E. coli* O157 infection (Deschênes et al. 1996; MMWR 2000).

Acid products such as dry-cured salami and apple juice have caused illness, although dry fermented sausages in particular were thought to be safe products because of their low pH, low water activity (aw) and high salinity. Unpasteurised apple cider has been implicated in several outbreaks in the USA (Besser et al. 1993; Hilborn et al. 2000) and an outbreak has been associated with apple juice (Cody et al. 1999). Apple cider may become contaminated at several points. Drop apples are often used when making apple cider and they may come...
into contact with faeces on the ground; other possible sources are animal vectors, contaminated water, hands of workers or equipment (Hilborn et al. 2000). Cider has traditionally been made without pasteurisation and with no preservatives (Besser et al. 1993).

Dry-cured salami was implicated in outbreaks caused by serotype E. coli O157:H7 in the US states of Washington and California in 1994, where 17 persons fell ill after eating pre-sliced dry fermented salami (Tilden et al. 1996). In southern Ontario, Canada, 39 cases in the spring of 1998 were linked to eating Genoa salami that contained E. coli O157:H7 of the same phage type and PFGE genotype as the case patients (Williams et al. 2000). In October 2002, 25 cases in southern Sweden had eaten cold smoked sausage from a local producer (de Jong et al. 2003). Indistinguishable PFGE genotypes were isolated from the cases and two sausage samples. From December 1995 to March 1996, 28 Bavarian (German) children with HUS were identified, most shedding SF E. coli O157: H- (Ammon et al. 1999). This case-control study showed a relationship between the consumption of a heated sausage mortadella and a raw, spreadable fermented sausage. The somewhat confusing result might be biased or a cause of cross-contamination between raw and heated products. In 1995, 21 Australian HUS cases of E. coli O111:H- were linked to eating a locally produced fermented sausage that contained O111:H- (Paton et al. 1996).

The first reported outbreaks occurred at restaurants (Griffin et al. 2001). New types of outbreaks are wide-spread and have involved an extended spectrum of food items. Vegetables, other fresh produce and green products have arisen as new vehicles. Vegetables may have been cultivated using bovine manure as a fertiliser or washed using contaminated water. A vegetarian woman acquired E. coli O157:H7 infection after eating inadequately washed vegetables fertilised with bovine manure (Cieslak et al. 1993). An outbreak of E. coli O157:H7 infection in four families was associated with potatoes fertilised with cattle manure on the farm (Chapman et al. 1997). Alfalfa sprouts were the only common exposure in an outbreak in the states of Michigan and Virginia in 1997 (Breuer et al. 2001), and lettuce in other US outbreaks (Ackers et al. 1998; Hilborn et al. 1999). The largest outbreak so far occurred in Japan in 1996 involving 9451 cases but only 12 deaths (Michino et al. 1999). White radish sprouts were the most likely vehicle. Coleslaw (Jaeger and Acheson 2000) and cantaloupe (Feng 1995) have also been vehicles in E. coli O157 outbreaks.

Wild animals have been proposed a reservoir of STEC. However, only one outbreak of E. coli O157:H7 has been traced to wild animals, to jerky made from meat of black-tailed deer (Keene et al. 1997b), and one sporadic case of E. coli O157 occurred after eating grilled white-tailed deer (Rabatsky-Ehr et al. 2002). Epidemiological data do thus not support the hypothesis of wild animals being a major reservoir.

In outbreaks associated with swimming, however, the contamination has occurred mainly through human faeces (Keene et al. 1994; Cransberg et al. 1996; Ackman et al. 1997; Paunio et al. 1999; Samadpour et al. 2002a). In an outbreak in Washington state, same type of E. coli O157:H7 was isolated from humans, duck faeces and the water (Samadpour et al. 2002a). In most swimming-associated outbreaks the organism has not been detected in the water (Keene et al. 1994; Cransberg et al. 1996; Ackman et al. 1997; Paunio et al. 1999). The failure to recover E. coli O157 in water may be partially attributed to the limited sensitivity of microbiological methods and the possibility that E. coli O157:H7 could transform into a viable but nonculturable state (Wang and Doyle 1998). In outbreaks associated with swimming as well as drinking water, the number of faecal indicator organisms has exceeded the regulation level, which indicates an increased risk for acquisition of enteric illness (McQuigge et al. 2000; Olsen et al. 2002). In the swimming associated outbreak in Oregon, the peak counts of Enterococcus in the swimming area were 350 cfu/100 ml (Keene et al. 1994). In a waterborne outbreak of E. coli O157 in Scotland the tap water was heavily contaminated with E. coli (Dev
et al. 1991) and in a waterborne outbreak in rural Wyoming high coliform counts and Enterococcus faecium were detected in the storage tank (Olsen et al. 2002). Many of the swimming outbreaks have occurred in small lakes with shallow water and with high number of visitors. A children's paddling pool was the only common vehicle in a small outbreak in Britain (Brewster et al. 1994), and an improperly chlorinated swimming pool in an outbreak in Georgia, USA (Friedman et al. 1999). The low infective dose and survival of the organism in warm and cold water enable this mode of transmission.

Drinking water has been linked to outbreaks caused by E. coli O157 (Dev et al. 1991; Swerdlow et al. 1992; Isaacson et al. 1993). Outbreaks caused by contaminated drinking water are usually large. Drinking untreated water was associated with an outbreak of E. coli O157 in Swaziland (Isaacson et al. 1993). Drinking untreated well water was a risk factor for E. coli O157 infection (Slutsker et al. 1998), whereas drinking bottled water was protective (Locking et al. 2001). The largest water-borne outbreak occurred in Walkerton, Canada in 2000 and involved more than 1400 cases, resulting in the death of nine people (McQuigge et al. 2000). The Walkerton tragedy was caused by faecal contamination from a local cattle farm into a nearby town well with cracked casing. In a large water-borne outbreak in New York State, USA 775 suspected patients were identified after attending a county fair where unchlorinated water from shallow wells was used (Bopp et al. 2003).

2.5.3 Infections associated with farms

The first reported infections were traced to unpasteurised milk (Martin et al. 1986; Borczyk et al. 1987). The sources of infection at farms may be several: unpasteurised milk, other farm foods like vegetables fertilised with manure, contaminated drinking water, direct contact with cattle or other animals, or indirect contact with premises, although the various sources mainly have been caused by cattle faecal contamination. Outbreaks or sporadic infections have been identified after visiting open farms (Pritchard et al. 2000; Crump et al. 2002), farm visitor centres (Parry et al. 1995; Shukla et al. 1995; Milne et al. 1999), commercial farms (Trevena et al. 1999) and farm fairs (Bopp et al. 2003), and in persons living on a farm (Jackson et al. 1998; Trevena et al. 1999).

Visiting a farm or having a household member whose occupation involved contact with farm animals was a risk factor for sporadic E. coli O157 infection (Parry et al. 1998). However, persons working permanently with farm animals may produce protective LPS serum antibodies to E. coli O157 (Reymond et al. 1996; Evans et al. 2000). Contact and likely contact with animal faeces and exposure to the farming environment emerged as strong risk factors for E. coli O157 infection in a Scottish prospective matched case-control study (Locking et al. 2001). In an English prospective unmatched case-control study recreational or occupational visits were strongly associated with sporadic infection of E. coli O157 (O'Brien et al. 2001). Contact with calves and their environment was associated with an increased risk of E. coli O157:H7 infection, whereas hand washing was protective in a US case-control study (Crump et al. 2002). Although cattle are the most important animal reservoir of the organism, several animal species have been found shedding the agent, especially at farms with different animal species. However, the numbers of sheep and goats were not associated with human STEC infection, while swine were protective in a Canadian study (Valcour et al. 2002).

Infections have also occurred after contact with soil as E. coli O157 is able to survive in soil and manure for considerable periods. An outbreak of E. coli O157 infection occurred at a music festival held at a site previously used as a pasture for cattle, and the most likely vehicle was contaminated mud (Cramm et al. 1999). A Canadian epidemiological study showed that the application of manure to land was a potential risk factor of human STEC infection (Valcour et al. 2002).
2.5.4 Other epidemiological features

Most infections are thought to be sporadic. In England and Wales, sporadic cases account for approximately 80% of *E. coli* O157 infections (Smith et al. 2001). Infection with *E. coli* O157 is more common in the warm summer months in both the northern and southern hemispheres (Ostrowski et al. 1989a; Griffin and Tauxe 1991; Mead and Griffin 1998) although the association of summer and sporadic HUS was not clear in Australia (Elliott et al. 2001). Children under five years are more susceptible to severe disease than adults (Griffin and Tauxe 1991).

The infectious dose of EHEC is very low. In a salami-associated outbreak of 1994, the infectious dose was estimated to be fewer than 50 organisms (Tilden et al. 1995). In an Australian outbreak of O111:H- the salami was estimated to have fewer than one O111 per 10 g (Paton et al. 1996). In a hamburger-associated outbreak, fewer than 700 (from less than 13.5 to 675) organisms of *E. coli* O157:H7 per raw hamburger patty were detected and some children ate only a few bites (Tuttle et al. 1999). Because of the low infective dose, person-to-person spread is a problem, especially at institutions, such as day-care centres, elderly homes and hospitals (Kohli et al. 1994). The household transmission rate in Wales for sporadic *E. coli* O157 infection was calculated to be 4–15% (Parry and Salmon 1998).

Sporadic infections of *E. coli* O157 have been described in laboratory technicians (Booth and Rowe 1993) (Burnens et al. 1993) handling samples containing *E. coli* O157 (Rao et al. 1996). Infections have led to bloody diarrhoea and even to severe renal failure. *E. coli* O157 and other VTEC/STEC have now been categorised as severe pathogens and placed in the pathogen category II, which requires stricter control measures at the laboratory (STM 1998).

Outbreaks involving multiple pathogens have also occurred. In an Oregon swimming water outbreak *E. coli* O157:H7 and *Shigella sonnei* were isolated from the patients (Keene et al. 1994). In an outbreak of bloody diarrhoea of unknown aetiology in Cameroon, multiple pathogens were isolated from some of the patients: *S. dysenteriae*, *Entamoeba histolytica* and *E. coli* O157:H7 (Cunin et al. 1999). In the water-borne outbreaks in Walkerton and in New York State, *Campylobacter* and *E. coli* O157:H7 were isolated from the patients and water (McQuigge et al. 2000; Bopp et al. 2003). Patients may also excrete multiple STEC serotypes (Paton et al. 1996).

2.6 Epidemiology of *E. coli* O157 in animals

2.6.1 Cattle and sheep

2.6.1.1 Pathogenicity in cattle and sheep

*E. coli* O157 is not a primary cattle or sheep pathogen, although it has caused severe diarrhoea and A/E lesions in the large and small intestines of colostrum-deprived neonatal (<12 h) calves inoculated with a high dose ($10^{10}$ cfu) of *E. coli* O157:H7 (Dean-Nystrom et al. 1997). Systemic complications, such as HUS, have never been observed in cattle (Mainil 1999). Calves and sheep older than one week and adult cattle have remained healthy when challenged with $10^5-10^{10}$ cfu/ml *E. coli* O157:H7 (Cray and Moon 1995; Brown et al. 1997; Sanderson et al. 1999; Wray et al. 2000; Wales et al. 2001a). However, when weaned and fasted calves were inoculated with a high ($10^{10}$ cfu) dose of *E. coli* O157:H7 watery diarrhoea was developed in the calves and A/E lesions were observed in the rectum and caecum of calves with high levels of *E. coli* O157:H7 (Dean-Nystrom et al. 1999a). High levels of bacteria were suggested to be needed in order to produce A/E lesions (Dean-Nystrom et al. 1999a). On the contrary, when nontoxicogenic five-day old calves were inoculated with *E. coli* O157:H7, no clinical signs nor A/E lesions were observed and only a mild inflammatory
response in the intestine was noted, despite the numbers of the bacterium reached $10^{10}$ cfu/g in faeces within 24 h and remaining at this level for up to 24 days (Woodward et al. 1999). Small and sparse A/E lesions were observed in 6-day and 6-month-old sheep inoculated with a dose of $10^{6}$ cfu E. coli O157:H7 (Wales et al. 2001a; Wales et al. 2001b; Wales et al. 2002). The infectious dose for normal adult cattle was high ($>10^4$ and probably $>10^7$ cfu) (Cray and Moon 1995), but calves became colonized using a lower inoculation dose ($10^2$) (Besser et al. 2001). Calves shed E. coli O157:H7 already 24 h after inoculation (Shere et al. 1998). Not all individuals seem to become colonized and start shedding, even when they receive the same feed and inoculum level (Graue et al. 2002; Cookson et al. 2002). The reasons could be differences in the microbial flora of the gastrointestinal tract, immunological status and other unknown reasons.

Intimin seems to be needed for colonisation. A/E lesions and disease in cattle (Dean-Nystrom et al. 1998a; Cornick et al. 2002). The intestinal epithelial cells of cattle but not humans express the Stx receptor Gb3, which could be an explanation to the lack of systemic dissemination in cattle (Smith et al. 2002). Cattle lack systemic vascular receptors for Gb3, which could explain why they are tolerant to infection with E. coli O157:H7 (Pruimboom-Brees et al. 2000) and why previous infection with E. coli O157:H7 does not prevent from re-infection (Cray and Moon 1995; Kudva et al. 1997b; Wray et al. 2000). Cattle develop antibody response to E. coli O157 LPS, but it seems to have no correlation with shedding status or protection against re-infection with the same strain (Johnson et al. 1996b; Wray et al. 2000). Most cattle also developed neutralising antibodies to Stx1 but not to Stx2 (Johnson et al. 1996b).

2.6.1.2 Predilection sites

E. coli O157:H7 was most prevalent in the lower gastrointestinal digesta, specifically the caecum, colon and faeces (Cray and Moon 1995; Graue et al. 2002). A/E lesions have been detected in rectum and caecum of weaned calves after fasting, which suggests that these may be principal sites of colonisation during the carrier-state in cattle (Dean-Nystrom et al. 1998b; Baehler and Moxley 2000). E. coli O157:H7 attached in high numbers to mucosal epithelium within a region 5 cm proximally from the recto-anal junction (Naylor et al. 2003). Highest numbers were also found in the faeces with negative or significantly lower levels in other sites of the gastrointestinal tract: rumen, ileum, caecum and colon. In neonatal calves, however, A/E lesions were noted in large and small intestines (Dean-Nystrom et al. 1997; Dean-Nystrom et al. 1998b). On bovine in vitro organ culture, E. coli O157:H7 induced A/E lesions in the large intestine (Baehler and Moxley 2000). Peyer’s patches of terminal ileum were found a site of colonisation of explants of human and bovine intestine (Phillips et al. 2000). However, E. coli O157 was frequently isolated from rumen fluids and faecal samples of experimentally infected calves and the rumen environment was suggested to be a storage of E. coli O157 (Brown et al. 1997). But in another study E. coli O157:H7 rapidly eliminated from rumen fluids despite being isolated from faecal samples up to 67 days after inoculation and from mouth samples (Buchko et al. 2000). E. coli O157 has also been isolated from other parts of the gastrointestinal tract such as forestomachs and tonsils, but there is no evidence of spread to the liver, spleen or kidneys (Cray and Moon 1995; Brown et al. 1997). In conclusion, recent studies suggest that the principal site of colonisation of E. coli O157:H7 seems to be in the terminal hindgut, whereas E. coli serotypes O26, O111 and O5 do not share a tropism for the bovine terminal rectum (Naylor et al. 2003).

2.6.1.3 Cattle isolates, human pathogens?

Studies comparing bovine and human isolates using phage typing or genotyping methods such as RFLP, plasmid analysis, or PFGE have shown that indistinguishable subtypes are detected (Paros et al. 1993; Fegan and Desmarchelier 2002). When isolates from cattle and
humans have been compared more in detail, differences have been noted. Strains of human origin were more virulent in gnotobiotic pigs than strains of bovine origin (Baker et al. 1997; Moxley and Francis 1998). Using octamer-based scanning, most human and bovine isolates of *E. coli* O157:H7 were found in separate lineages, which suggest differences in the virulence (Kim et al. 1999). Significant differences were observed between human and bovine-derived *E. coli* O157 strains and their production of certain LEE-encoded virulence factors (McNally et al. 2001). These studies suggest that not all bovine *E. coli* O157 isolates are as virulent to humans, which could explain why *E. coli* O157 is more common in bovine animals than as the cause of human disease.

### 2.6.1.4 Prevalence of faecal shedding

Prevalence studies have been performed on farms and at slaughter. The results are somewhat conflicting and affected by several factors such as detection methods, sampling strategy, sample size, age of the animals and season. The prevalence values from different studies are very likely underestimated, due to insufficient sampling and insensitive laboratory procedures (Jordan et al. 1999; Meyer-Broseta et al. 2001). Most farm studies have not been designed to measure herd prevalence, which results in inaccurate estimates of herd prevalence (Jordan et al. 1999).

Figures obtained in older studies are lower because of the use of less sensitive detection methods like direct plating without enrichment (Wilson et al. 1992; Chapman et al. 1993; Hancock et al. 1994; Wilson et al. 1996) or without IMS (Wells et al. 1991; Hancock et al. 1994; Zhao et al. 1995; Faith et al. 1996; Rice et al. 1997; Hancock et al. 1998a). Also, tests based on faecal culture of only some of the animals of the herd give lower shedding prevalences (Jordan and McEwen 1998). Testing pat samples gives lower values than individual faecal samples as the numbers shed might be low and the bacterium could thus be diluted below the detection limit. It is also difficult to know how many individual faecal excretions the pat sample consists of. The sample size affects the study results, a bigger sample size resulting in more isolations of *E. coli* O157 (Kudva et al. 1995; Sanderson et al. 1995).

Herd prevalences of faecal shedding of *E. coli* O157:H7 in US dairy herds have ranged from 1 to 24% (Faith et al. 1996; Hancock et al. 1998a; Garber et al. 1999), and animal prevalences in US dairy herds and feedlots from 1.4 to 7.4% (Faith et al. 1996; Hancock et al. 1997b; Hancock et al. 1997c; Laegreid et al. 1999). As *E. coli* O157 is shed intermittently, the status of the herd cannot be assessed on only one sampling: longitudinal studies performed in the USA show that up to 75% of dairy herds (Hancock et al. 1997b) and 63% of feedlots have *E. coli* O157:H7 –positive cattle (Hancock et al. 1997c). During a 15-month study of a UK dairy herd, 74% of the animals excreted *E. coli* O157:H7 on only one occasion (Mechie et al. 1997), which makes prevalence estimating extremely difficult. Later studies using more sensitive methodology and larger samplings have given higher prevalence figures. When 2419 animals on 60 Danish dairy farms were examined, 17% of the farms and 3.6% of the cattle were positive for *E. coli* O157 (Nielsen et al. 2002). A large one-year survey consisting of 10,415 faecal samples of US feedlots revealed an animal prevalence of 11% (Sanchez et al. 2002). Another large cross-sectional survey on Scottish finishing beef cattle gave an animal prevalence of 7.9% (6.7-9.2%) (14849 samples), and 22.8% of groups had at least one animal shedding (Synge et al. 2001). *E. coli* O157 was present on almost half of British farms studied (Synge and Paiba 2000). However, a Norwegian study using enrichment and IMS detected only six *E. coli* O157:H- isolates (1970 samples from 197 farms) (Vold et al. 1998).

Studies targeting all STECs have often detected less *E. coli* O157 due to lower sensitivity or because multiple serotypes were being simultaneously excreted. The studies are therefore difficult to compare with those addressing only *E. coli* O157. In a study on Ontarion cattle
farms, 206 VTEC isolates were identified among 1790 cattle, none were *E. coli* O157:H7 (Wilson et al. 1992). In a French study on abattoirs, eight *E. coli* O157 isolates of 851 were detected, and none presented *stx* genes (Roberge et al. 2001). In another French abattoir study 70% (330/471) of cattle faecal samples were *stx* positive in PCR but only one *E. coli* O157:H7 was isolated (Pradel et al. 2000). Non-O157 STEC are commonly excreted by cattle; 18-42% of individual cattle excrete them at slaughter (Borie et al. 1997; Miyao et al. 1998; Pradel et al. 2000; Leung et al. 2001; Khan et al. 2002; Samadpour et al. 2002b).

Animal prevalences of faecal shedding in abattoir studies have ranged from less than 1% to 28% (Table 1). A high prevalence of 28% was observed in a US study of beef cattle slaughtered at a high-speed abattoir during the high-prevalence months of July and August (Elder et al. 2000). Some slaughterhouse study samples have been taken throughout the year (Chapman et al. 1997; Chapman et al. 2001a; Paiba et al. 2002). Studies performed in Belgium, Canada, Italy, the Netherlands and the UK have given prevalences of 6.3-15.7% (Heuvelink et al. 1998a; Bonardi et al. 1999; van Donkersgoed et al. 1999; Chapman et al. 2001a; Tutenen et al. 2002), whereas a Polish study at one abattoir revealed a low prevalence of 0.7%, despite the use of a sensitive method and a sample size of 20 g (Tutenen et al. 2002). This disparity could reflect geographical differences. Differences have also been detected within a country, as studies conducted at one UK abattoir (Chapman et al. 1997; Chapman et al. 2001a) gave higher prevalences than a nationwide UK study (Paiba et al. 2002). Cattle in eastern abattoirs had higher prevalence figures than in other parts of the UK (Paiba et al. 2002).

Prevalence figures in sheep seem to be lower than in cattle. In the British nationwide study mentioned above, 40/4171 (1.7% CI 95% 1.3-2.1) sheep shed *E. coli* O157:H7 in their faeces at slaughter (Paiba et al. 2002). In a one-year study at a Sheffield (UK) abattoir 2.2% (22/1000) of sheep (Chapman et al. 1997) and 7.4% (100/7200) of sheep shed *E. coli* O157 (Chapman et al. 2001a), compared to 4.1% (2/49) in the Netherlands. Although less frequently isolated from sheep faeces, *E. coli* O157 was more often isolated from lamb products (0.8%) than from beef (0.4%) products (Chapman et al. 2001a).

In conclusion, the herd prevalence of faecal shedding of *E. coli* O157 seems to be higher than the animal prevalence indicating that only a few animals might be excreting the pathogen in a herd. The organism is intermittently excreted in the faeces and the prevalence results are shedding prevalences rather than prevalences of infection or a carrier state. Differences in the shedding prevalence might exist between countries or regions due to farming practices, farm size, feeding regimes, climatic or other factors. Conclusions on the geographical differences should be drawn with caution as several variables may account for them.
Table 1. *E. coli* O157:H7/H− in faecal samples of adult cattle at slaughter. Only studies solely addressing *E. coli* O157 are included.

<table>
<thead>
<tr>
<th>Country</th>
<th>No. abattoirs</th>
<th>No. samples</th>
<th>No. positives (%)</th>
<th>Season</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>8</td>
<td>1281</td>
<td>81 (6.3%)</td>
<td>One year</td>
<td>Tuttenel et al. 2002</td>
</tr>
<tr>
<td>Canada</td>
<td>1</td>
<td>1247</td>
<td>93 (7.5%)</td>
<td>One year</td>
<td>van Donkersgoed et al. 1999</td>
</tr>
<tr>
<td>Italy</td>
<td>1</td>
<td>450</td>
<td>59 (13.1%)</td>
<td>One year</td>
<td>Bonardi et al. 1999</td>
</tr>
<tr>
<td>Italy</td>
<td>7</td>
<td>100</td>
<td>17 (17%)</td>
<td>Apr-Jan</td>
<td>Bonardi et al. 2001</td>
</tr>
<tr>
<td>Italy</td>
<td>18</td>
<td>484</td>
<td>15 (3.1%)</td>
<td>Feb-July</td>
<td>Conedera et al. 1997</td>
</tr>
<tr>
<td>Netherlands</td>
<td>5</td>
<td>540</td>
<td>57 (10.6%)</td>
<td>July-Nov</td>
<td>Heuvelink et al. 1998</td>
</tr>
<tr>
<td>Poland</td>
<td>1</td>
<td>551</td>
<td>4 (0.7%)</td>
<td>One year</td>
<td>Tuttenel et al. 2002</td>
</tr>
<tr>
<td>Turkey</td>
<td>1</td>
<td>75</td>
<td>1</td>
<td>Jan-Aug</td>
<td>Gun et al. 2001</td>
</tr>
<tr>
<td>Turkey</td>
<td>5</td>
<td>330</td>
<td>14 (4.2%)</td>
<td>Jan-April</td>
<td>Yilmaz et al. 2002</td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td>207</td>
<td>2 (1%)</td>
<td>*</td>
<td>Chapman et al. 1989</td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td>2103</td>
<td>84 (4%)</td>
<td>July-Sept</td>
<td>Chapman et al. 1993</td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td>4600</td>
<td>752 (15.7%)</td>
<td>One year</td>
<td>Chapman et al. 1997</td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td>4600</td>
<td>620 (12.9%)</td>
<td>One year</td>
<td>Chapman et al. 2001</td>
</tr>
<tr>
<td>UK</td>
<td>118</td>
<td>3939</td>
<td>186 (4.7%)</td>
<td>One year</td>
<td>Paiba et al. 2002</td>
</tr>
<tr>
<td>USA</td>
<td>1</td>
<td>103</td>
<td>4 (3.9%)</td>
<td>*</td>
<td>Rice et al. 1997</td>
</tr>
<tr>
<td>USA</td>
<td>4</td>
<td>327</td>
<td>91 (27.8%)</td>
<td>July-Aug</td>
<td>Elder et al. 2000</td>
</tr>
<tr>
<td>USA</td>
<td>1</td>
<td>200</td>
<td>11 (5.5%)</td>
<td>April</td>
<td>Barham et al. 2002</td>
</tr>
</tbody>
</table>

* Not mentioned

2.6.1.5 Carcass contamination

Carcass contamination by *E. coli* O157 has been studied more intensely during the recent years. Hides and pre-evisceration carcasses have been heavily contaminated with *E. coli* O157, possibly caused by dirty hides (Callaway et al. 2003b).

*E. coli* O157 was isolated from 30% (7/23) of chilled carcasses of rectal swab-positive cattle and from two (8%) of 25 chilled carcasses of rectally negative cattle by sampling neck trims and swabbing an adjacent area in May-June in the UK (Chapman et al. 1993), which suggests cross-contamination between carcasses. Cross-contamination was also observed in Italy where *E. coli* O157 was isolated from 7.3% of stool-negative cattle and totally from 12% of carcasses (Bonardi et al. 2001). A lower contamination with *E. coli* O157 was confirmed in 0.47% of the 4067 neck muscle samples of chilled carcasses from UK abattoirs (Richards et al. 1998). In a later study *E. coli* O157 were recovered from chilled carcasses from April to July: 1.4% (21 of 1500) beef carcasses and 0.7% (10 of 1500) lamb carcasses (Chapman et al. 2001a). Cattle carcasses at four US Midwestern high-speed abattoirs were sampled before dehiding, pre-evisceration, post-evisceration (after trimming) and post-processing in peak shedding months of July-August (Elder et al. 2000). Pre-evisceration contamination of the carcasses was high, at 43% (148/341), and greater than the hide (11%; 38 of 355), faecal (Table 1), or post-evisceration contamination (18% 59 of 332). However, no differences between the hide (4.5%) and faecal (5.5%) contamination percentages of Texas feedyard cattle were observed (Barham et al. 2002). Higher
contamination figures for brisket cattle hide were observed on carcasses at a UK abattoir: 22.2% (20 of 90) (Reid et al. 2002) and 32.9% (24 of 73) (Avery et al. 2002a). Brisket was the site of the hide most often contaminated with *E. coli* O157 (Reid et al. 2002). A high percentage of hides (74% of 50) in an Australian abattoir were contaminated with *stx* PCR-positive organisms (six *E. coli* O157 isolates), while only four carcasses were found *stx* PCR-positive (Midgley and Desmarchelier 2001).

Post processing samples taken in the cooler 2 h after antimicrobial intervention (steam pasteurization, hot water wash, organic acid wash, or combinations of these) showed a significantly reduced carcass contamination (2%; 6 of 330 post-processing), either due to the intervention methods, drying, cooling or a combination of these (Elder et al. 2000). On the contrary, washing did not reduce the incidence of *E. coli* O157:H7 on carcasses, whereas after 24 h chilling, the pathogen was not detected at carcass sites previously tested positive (McEvoy et al. 2001). In conclusion, carcass contamination seems to decline significantly during cold storage, but the efficacy of different intervention methods needs to be evaluated.

### 2.6.1.6 Duration of faecal shedding

The duration of shedding of *E. coli* O157 seems to fall into three categories according to one experimental study: less than one week, one month, or more than two months (Grauie et al. 2002), although studies performed in natural settings have not been able to demonstrate similar strict category. *E. coli* O157 seems to persist for extensive periods on farms (Shere et al. 1998), although the shedding in an individual animal often lasts less than two months (Besser et al. 1997). Cattle with slower rates of intestinal cell proliferation in the caecum and the distal colon were shedding longer than cattle with faster proliferation (Magnuson et al. 2000). In some studies long-term carriers have not been found (Garber et al. 1995; Hancock et al. 1997b; Hancock et al. 1997c) but persistence exceeding two months in clinically normal cattle and sheep has been detected (Kudva et al. 1995; Brown et al. 1997; Mechie et al. 1997; Shere et al. 1998; Hovde et al. 1999; Ohya and Itô 1999; Cornick et al. 2000). Inoculated calves shed *E. coli* O157:H7 in their faeces for a mean of 30 days, range of 20-43 days (Sanderson et al. 1999), or naturally infected and inoculated calves for 17-36 days (Shere et al. 1998). Long shedding periods were observed: in an experimental study *E. coli* O157:H7 was shed for over 20 weeks in two calves and 27 weeks in one calf originally inoculated with 10^10^ cfu (Cray and Moon 1995), while in another study one of the three inoculated calves shed *E. coli* O157:H7 for more than 31 weeks (Ohya and Itô 1999). The factors, which contribute to long-term carriage, are not well understood, but changes in livestock density, social stress, feed and withdrawal of milk might play a role in the post-weaning shedding (Gannon et al. 2002).

### 2.6.1.7 General aspects of shedding

Shedding seems to occur periodically. Most faecal shedding of *E. coli* O157:H7 is confined to peaks, when several animals are shedding, and lasts for periods of several days to several weeks (Hancock et al. 1997a; Hancock et al. 1997b). *E. coli* O157:H7 and other STEC are transient strains in the gastrointestinal tract of cattle, *E. coli* O157:H7 accounting for less than 1% of the *E. coli* isolates in two studied dairy herds (Hancock et al. 1998b). Anyway, herds might have a tendency to either a low or high prevalence of *E. coli* O157:H7, indicating that farm management practices could influence the occurrence of the pathogen (Garber et al. 1995; Hancock et al. 1997b). A herd can be negative for *E. coli* O157:H7 or have a very low incidence (Besser et al. 1997; Shere et al. 1998).

Horizontal transmission has been observed in both experimental and natural settings (Kudva et al. 1995; Shere et al. 1998; Wray et al. 2000; Shere et al. 2002; Cobbold and Desmarchelier 2002). An approximately threefold higher prevalence in cattle recently shipped
to feedlots was observed compared to randomly selected feedlot cattle or cattle on feed for several months (Hancock et al. 1997c).

The counts of *E. coli* O157 shed by cattle can be low. Few observational studies have provided the numbers shed by cattle. Using direct plating the counts of *E. coli* O157:H7 in faeces ranged from $2.0 \times 10^3$ to $8.7 \times 10^3$ cfu/g (Shere et al. 1998), or in naturally infected and inoculated calves from $6.0 \times 10^1$ cfu/g to $1.2 \times 10^3$ cfu/g (Shere et al. 2002). In another study using direct plating the numbers of *E. coli* O157 in the faeces of calves ranged from $10^3$ to $10^5$ cfu/g, and 48% (15/31) of faecal samples were positive with enrichment only, at populations <$10^2$ cfu/g (Zhao et al. 1995). Using MPN or direct plating 11 lambs and three ewes of 28 sheep shed less than 10 cfu/g *E. coli* O157 in faeces, while one lamb was shedding more than $10^5$ cfu/g (Strachan et al. 2001). The counts shed in experimental studies have been at similar levels: ranging from less than 30 to more than $10^5$ cfu/g (Cray and Moon 1995; Besser et al. 2001).

2.6.1.8 Effect of season on shedding of *E. coli* O157

The prevalence of *E. coli* O157 in cattle increases during the summer and early autumn (Hancock et al. 1994; Chapman et al. 1997; Mechie et al. 1997; Heuvelink et al. 1998; Garber et al. 1999; Conedera et al. 2001; van Donkersgoed et al. 2001; Chapman et al. 2001a). A seasonal effect has also been noted for sheep at farm and at slaughter (Kudva et al. 1996; Kudva et al. 1997a; Paiba et al. 2002). In some studies *E. coli* O157 has not been isolated at all in winter (Mechie et al. 1997), but other studies show a lower winter prevalence but that the organism is still being shed (Heuvelink et al. 1998). A seasonal effect was not observed in longitudinal studies of Wisconsin dairy herds (Shere et al. 1998) or Kansas cow-calf farms (Sargeant et al. 2000). Although abattoir studies have shown a clear seasonal effect the findings from farm studies are contradictory, which could suggest that the ecology on the farm is very complex.

2.6.1.9 Effect of age on shedding of *E. coli* O157

Faecal shedding is more common in young than adult cattle (Wells et al. 1991; Hancock et al. 1994). Weaned calves have the highest prevalence of faecal shedding (Garber et al. 1995; Zhao et al. 1995; Hancock et al. 1997a). Calves aged two to six months had the highest prevalence on Danish dairy farms (Nielsen et al. 2002). Veal calves rarely shed the organism (Conedera et al. 1997; Heuvelink et al. 1998a). Calves aged two weeks to three months had a significantly greater prevalence of STEC infection than younger calves (Wilson et al. 1993; Wilson et al. 1998). Of calves younger or older than eight weeks, 1.4% and 4.8%, respectively, shed the organism (Garber et al. 1995). Most beef calves (63-100%), weaned at six to seven months in US herds were serologically positive for *E. coli* O157 at weaning, and 13 of 15 herds had at least one positive calf shedding *E. coli* O157 (Laegreid et al. 1999). Beef calves seemed to acquire *E. coli* O157 infection from their dams (Gannon et al. 2002). In a study at one Canadian abattoir 12.4% of faecal samples from yearling cattle and 2.0% of cull cows shed *E. coli* O157:H7 (van Donkersgoed et al. 1999). A greater number of beef dams under five years than over five years were shedding *E. coli* O157 (Gannon et al. 2002). Cattle arriving at slaughter are usually 18-30 months old or cull cows, whereas different age categories are present on farms. This may explain how animal prevalences can be higher at farm compared to at slaughter.

2.6.1.10 Other management factors

Grouping and housing of dairy calves before weaning was associated with shedding of *E. coli* O157 (Garber et al. 1995; Shere et al. 1998). Shedding was higher among housed animals (Syngue et al. 2001). Herd size has not affected the status of shedding according to studies performed in the USA and Denmark (Garber et al. 1995; Hancock et al. 1998b; Sargeant et al. 2000).
but the herds classified as small (less than 100 cows) in these studies have had more cattle than the average dairy Finnish herds (no=15.8) (TIKE 1999).

The number of cattle introduced in the previous year appeared to be a risk factor of STEC infection (Wilson et al. 1998; Synge et al. 2001; Nielsen et al. 2002). One possible explanation for this is rapid transmission of the bacteria from a small number of patently shedding individual calves to a large number of naïve animals, possibly influenced by the stresses of weaning, transport, sorting and mixing (Kudva et al. 1996; Dargatz et al. 1997; Hancock et al. 1997c; Cray et al. 1998).

The use of nipple bottles was associated with reduced rate of calf infection (Wilson et al. 1993), while the use of open pails for feed calves was associated with increased infection among them (Wilson et al. 1998). Hygiene might play a role in transmission since herds that shared buckets and bottles among unweaned calves without rinsing or washing had a higher status of shedding (Garber et al. 1995). Similarly, water distributed in large tanks may become contaminated and introduce a source of infection compared to water cups or small water tanks (Shere et al. 1998).

Parturition, calving and weaning were important factors in maintaining E. coli O157:H7 infection in a Canadian beef herd (Gannon et al. 2002). In a UK dairy herd shedding rates of E. coli O157:H7 by dairy cattle were also highest after calving (Mechie et al. 1997).

Transportation stress has been suggested to increase shedding, but excretion of E. coli O157 during the transport of cattle from feedlot to packing plant decreased, although not significantly (Barham et al. 2002).

2.6.1.11 Effect of diet on shedding of E. coli O157

Cattle seem to shed E. coli O157 less at pasture compared to the pen environment, irrespective of age. In a Canadian beef herd more dams and their calves shed E. coli O157:H7 in their faeces in pens compared to the pasture environment (Gannon et al. 2002). Orally inoculated sheep also stopped shedding the organism when allowed to graze (Kudva et al. 1995). In a Swedish herd six calves kept on pasture were negative for E. coli O157 from April to August, whereas E. coli O157 was isolated during the same period from 1-4/6 calves of the same herd that stayed in indoor pens (Jonsson et al. 2001). Within-herd prevalence in weaned heifers fed in drylots was 1.8%, whereas 0.83% and 0.42% of them shed when allowed to graze on manured or non-manured pasture, although the differences were not significant over a 6-month period (Hancock et al. 1994).

The effect of grain- vs. hay-feeding on shedding of E. coli O157 in cattle is a debated item with contradictory results. A diet high in nutrients but low in fibre appeared to induce a lower incidence of transmission and shedding of E. coli O157:H7 but not to induce clearance of the organism from the intestine, whereas a low-energy and high-fibre diet induced shedding of larger numbers of E. coli O157:H7 but also promoted elimination of the organism (Kudva et al. 1995). This finding was further supported by a study in which sheep on a grass-hay diet shed E. coli O157:H7 for twice as long and in larger numbers than sheep fed a high protein and low-fibre diet (Kudva et al. 1997b). In contrast, cattle fed grain diets had larger numbers of acid-resistant total generic E. coli organisms in their faeces than cattle fed hay diets (Diez-Gonzalez et al. 1998). The authors suggested that feeding cattle hay diets five days prior to slaughter would reduce the risk of food-borne E. coli O157:H7 infections for humans (Diez-Gonzalez et al. 1998; Russell et al. 2000a; Russell et al. 2000b). Another study using different methods failed to support the hypothesis of hay-feeding since hay-fed animals shed E. coli O157:H7 for longer than the grain-fed animals, and acid-resistant E. coli O157:H7 was also
shed from cattle eating hay and grain (Hovde et al. 1999). No effect of diet was found on the duration of faecal shedding in calves, and neither differences in rumen populations of *E. coli* O157:H7 (Tkalcic et al. 2000). Cattle fed barley diet had increased faecal carriage compared to those fed a corn diet (Buchko et al. 2000). In addition, coumarins derived from plants inhibited growth of *E. coli* O157:H7 (Duncan et al. 1998; Duncan et al. 2000).

In a US study generic *E. coli* was widespread in moist cattle feeds (Lynn et al. 1998) and *E. coli* O157:H7 was able to proliferate to high populations in moist silage (Lynn et al. 1998; Sanchez et al. 2002). In a study of two Canadian feedlots *E. coli* O157:H7 was isolated from feedbunks (1.7%) but not from fresh total mixed rations (van Donkersgoed et al. 2001). *E. coli* O157:H7 did not survive a good silage fermentation process, indicating that properly ensiled and correctly stored grass is an unlikely source of infection (Byrne et al. 2002a).

Feed disruptions enhanced the shedding of *E. coli* O157:H7 (Kudva et al. 1995; Grauke et al. 2002) as did diet changes (Kudva et al. 1997b). *E. coli* O157:H7 grows well in rumen fluids of fasted cattle, while the growth was restricted in bovine rumen fluid of well-fed animals (Rasmussen et al. 1993). However, calves experimentally inoculated with *E. coli* O157:H7 did not show increased faecal shedding after feed had been withheld (Cray and Moon 1995; Harmon et al. 1999).

In conclusion, any feed change or feeding practice or stress could promote the transient colonisation of *E. coli* O157:H7 resulting in increased shedding. This could explain the association of increased shedding with the onset of lactation, weaning, and cattle recently placed on feed at feedlot (Mechie et al. 1997; Dargatz et al. 1997; Gannon et al. 2002; Sanchez et al. 2002).

### 2.6.1.12 Water as a source of transmission

Water troughs on farms have been found to contain *E. coli* O157:H7 (Faith et al. 1996; Hancock et al. 1998a; van Donkersgoed et al. 2001; LeJeune et al. 2001c) and in some studies the organism has been isolated from water (Faith et al. 1996; Sargeant et al. 2000; van Donkersgoed et al. 2001). The quality of drinking water offered to cattle was of poor quality: drinking water troughs located at 98 US dairy farms and one slaughterhouse had a mean log of coliforms 1.76/ml and *E. coli* 0.98/ml, and *E. coli* O157:H7 was isolated from 6/473 (1.3%) troughs (LeJeune et al. 2001c). In a simulated water trough microcosm *E. coli* O157 survived for at least 245 days (LeJeune et al. 2001b). Water containing $10^3$ cfu/ml was sufficient to transmit the organism and establish shedding in calves (Shere et al. 2002).

### 2.6.1.13 Genotyping results from farms

The sources of *E. coli* O157 on farms may be multiple or single. In some studies several PFGE subtypes have been isolated from one farm (Keene et al. 1997a; Rice et al. 1999), whereas in other studies a dominant subtype is usually detected (Faith et al. 1996). Feedlots had more subtypes than dairy farms (Rice et al. 1999). Indistinguishable subtypes can be detected on farms with no known contact with each other and at vast distances (Rice et al. 1999; Hancock et al. 2001). Multiple *E. coli* O157:H7 strains were isolated from one flock, a single animal shed simultaneously multiple *E. coli* O157:H7 strains, and strains shed by individuals changed over time (Kudva et al. 1997a). A shift of PFGE genotype in cattle and different PFGE subtypes from parallel colonies of the same animal were found in one study (Akiba et al. 2000). A shift of PFGE subtype and loss of *stx* gene were observed in human patients shedding long periods (Karch et al. 1995).

### 2.6.2. Other ruminants

Studies on ruminants other than cattle have been fewer, but *E. coli* O157 has been isolated
from goats, and goat milk has was a vehicle of an outbreak of SF O157:H7 (Bielaszewska et al. 1997; Heuvelink et al. 2002). *E. coli* O157 has been isolated from deer in the UK and the USA (Table 2). These results suggest that deer might be a reservoir of this pathogen. *E. coli* O157:H7 was not isolated from 268 faecal samples of Californian llamas (Rulofson et al. 2001). In a Norwegian study of 17 reindeer and 39 moose samples one moose calf sample was positive in a commercial cytotoxin test but no *E. coli* O157 was isolated. In this study the positive sample came from an area where moose occasionally share the same habitats as sheep or cattle (Wasteson et al. 1999). Non-O157 STEC have been isolated from deer samples in Japan (Asakura et al. 1998) and Belgium (Piérard et al. 1997).

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. samples</th>
<th>No. positives</th>
<th>Wild/farmed</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>32</td>
<td>3</td>
<td>Wild</td>
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<td>Keene et al. 1997b</td>
</tr>
<tr>
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<td>Ranch</td>
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<td>Rice et al. 1995</td>
</tr>
<tr>
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<td>3</td>
<td>Farmed</td>
<td>UK</td>
<td>Chapman and Ackroyd 1997</td>
</tr>
<tr>
<td>Deer</td>
<td>11</td>
<td>1</td>
<td>Wild</td>
<td>Western USA</td>
<td>Cody et al. 1999</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>212</td>
<td>5</td>
<td>Sharing rangeland with cattle</td>
<td>Kansas, USA</td>
<td>Sargeant et al. 1999</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>1608</td>
<td>4</td>
<td>Wild</td>
<td>Nebraska, USA</td>
<td>Renter et al. 2001</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>630</td>
<td>5</td>
<td>Wild</td>
<td>USA</td>
<td>Rice et al. 2003</td>
</tr>
</tbody>
</table>

2.6.3 Other animals

Studies of *E. coli* O157 in other animal species are sporadic and the numbers of samples analysed are smaller. *E. coli* O157:H7 is not species-specific; it is isolated from a wide range of animals. *E. coli* O157 is able to colonise chicken intestine (Stavric et al. 1993). It has been isolated from 0.9% of faecal samples from wild birds roosting near farmland in the UK (Wallace et al. 1997), and from birds on farms (Shere et al. 1998) and from wild rabbit faecal pellets from pasture used for cattle (Hancock et al. 1998a; Pritchard et al. 2001). Occasionally, *E. coli* O157 has been isolated from other animals, like horses (Trevena et al. 1996; Chalmers et al. 1997; Hancock et al. 1998a; Hancock et al. 1998a), dogs (Trevena et al. 1996; Hancock et al. 1998a; Hancock et al. 1998a), flies (Hancock et al. 1998a; Rahn et al. 1999; Iwasa et al. 1999), opossum (Renter et al. 2003), rats (Cizek et al. 1999), raccoons (Shere et al. 1998), and orang-utan (Beutin et al. 1996a).

Swine carry various STEC strains frequently, but seldom are such strains isolated from diseased human patients. In a Dutch study of swine, two rectal samples of 145 pigs were positive for *E. coli* O157 (Heuvelink et al. 1999). *E. coli* O157:H7 was able to colonize and persist in pigs for more than two months and might be or become a reservoir for *E. coli* O157:H7 (Booher et al. 2002). In Chile swine seem to be an important animal reservoir of STEC O157:H7 and other STEC strains, as indistinguishable PFGE genotypes have been isolated from humans and swine, and 69% of swine and 34% of cows from Santiago were colonized with EHEC strains (Borie et al. 1997; Rios et al. 1999). On the other hand, in an epidemiological Canadian study, swine appeared to be protective of human STEC infection (Valcour et al. 2002).
2.7 Control of *E. coli* O157 on farms, at slaughter and in food production

2.7.1 On-farm intervention strategies

Because *E. coli* O157 seems to be present on most cattle farms and occurs in several animal species, its eradication from live cattle and maintenance of herds free of infection seems not to be a realistic proposition (Hancock et al. 1998b; Jordan et al. 1999). A simulation model predicted that pre-harvest control measures that reduce *E. coli* O157 in faeces would lead to fewer human infections (Jordan et al. 1999). Pre-slaughter testing of cattle at herd level was considered to provide only a minor advantage, although testing of all animals might have some benefit.

Vaccines targeted against intimin or Stx have been suggested to reduce the susceptibility of cattle to colonization with *E. coli* O157 and other STEC (Dean-Nystrom et al. 1998b; Dean-Nystrom et al. 1999b; Cornick et al. 2002), but the theoretical feasibility has been questioned (Hancock et al. 1998b; Sanchez et al. 2002). Probiotic bacteria reduced the level of carriage of *E. coli* O157:H7 in cattle (Zhao et al. 1998; Ohya et al. 2000) but the effect has not yet been confirmed in natural settings. Specific bacteriophages lysed *E. coli* O157 cultures and were suggested to be used to eliminate the organism from the carrier animals (Kudva et al. 1999).

Manipulation of the feed content is a debated issue and has been discussed earlier in this review. Feed additives such as chlorate reduced the shedding of *E. coli* O157 in pigs (Anderson et al. 2001) and sheep (Callaway et al. 2003a). Hygiene control, minimizing faecal waste and hide contamination, proper water and feed hygiene including cleaning of water troughs, nipples and feed bunks, as well as limiting faecal contamination between high and low risk animals (calves vs. cows) have been proposed as control measures (Midgley and Desmarchelier 2001; Hancock et al. 2001; Sanchez et al. 2002).

2.7.2 Slaughter

The HACCP system should be applied for slaughter systems for the purpose of decreasing carcass contamination. Carcasses may be directly in contact with each other during processing, and contamination through personnel, knives, or other equipment may occur, as well as air- and water-borne contamination. Clean animals are of primary importance at slaughter because hides are a significant source of carcass contamination. Floors and other surfaces in cattle lairages and stunning boxes can be frequently contaminated with *E. coli* O157, at a rate of 7.7% in three UK cattle abattoirs (Small et al. 2002). Therefore, hide contamination at the abattoir lairage is considered of a great concern and hygienic measures are warranted (Avery et al. 2002a). Several countries have inspection protocols where slaughter animals are graded according to visual cleanliness (Ireland, UK, Finland and Sweden). Dirty animals should be excluded from slaughter as suggested (Heuvelink et al. 2001). However, van Donkersgoed et al. (1998) found little differences in generic *E. coli* counts on the carcasses from dirty cattle and visibly clean cattle. Much of the bacterial contamination of the carcass occurs during the removal of the hide (Gannon 1999). Good slaughtering practice is important, like cleaning knives and hands between carcasses.

Specific carcass interventions have been designed to reduce bacterial contamination. Visible contamination can be removed by knife trimming or washing (Gannon 1999). In some studies these treatments have reduced counts of *E. coli* O157 from carcasses, but they need time and skillful operators, and they do not remove invisible microbial contamination. Washing with cold (10–15°C) or warm (15–40°C) potable water may also redistribute bacteria from one area to another (Bolton et al. 2001). Whole-carcass contamination procedures have been
proposed as a solution. Carcasses can be washed with hot (75-85°C) water. Hot water washing has reduced the bacterial counts but the heat may also discolor the carcass surface (Dorsa et al. 1997b). Steam vacuum systems use hot water, steam and a vacuum to decontaminate small areas of the carcass. Decontamination effects have been demonstrated in this way, but there are associated problems: the method is suitable only for small areas of the carcass, faeces may be redistributed, and the temperature of the meat surface may reach only 34-49°C during treatment. In steam pasteurisation surface water is removed before the steam is applied, and afterwards the carcass surface is chilled with water. Steam pasteurisation may discolor and some sites may not receive complete decontamination.

Organic acid applications, such as lactic or acetic acids, are applied using a spray cabinet. There are varying reports on the decontaminating effect of organic acids. Acetic acid, lactic acid and trisodium phosphate on beef carcass surfaces can reduce contamination of E. coli O157:H7 but not eliminate it (Dorsa et al. 1997a; Dorsa et al. 1997b). A combination of hot water washing and organic acids showed a decontaminating effect (Castillo et al. 1998). E. coli O157 is unusually acid tolerant, hence the use of acids is problematic. A variety of chemical agents can be added to washing water.

Zero tolerance means that every carcass must be free from faecal contamination, ingesta and milk (Bastian and Sivelä 2000; Heuvelink et al. 2001). Each carcass is thoroughly inspected and any contamination found is removed by trimming with a knife. Trimming reduces carcass contamination when the knives and hooks are sterilised between carcases. In addition to continuous monitoring, at least three carcases an hour should be checked by a manager or supervisor (Bolton et al. 2001).

Carcasses are chilled immediately after carcass washing until the temperature of the deep round reaches 7°C or less. Data from the scientific literature are variable. Figures on carcass contamination with E. coli O157 have been lower after chilling (Elder et al. 2000). The other chilling parameters, such as air temperature, relative humidity, air speed and carcass spacing, have not been studied.

Irradiation has proven effective in the elimination of E. coli O157:H7, although resistance to gamma irradiation at subfreezing temperatures is significantly higher than at other temperatures (Thayer and Boyd 2001). Irradiation does not significantly alter the food when approved doses are used (Hollingsworth and Kaplan 1998), but it is an ethically controversial method for decontamination of pathogens.

2.7.3 Food production

The key point is that E. coli O157:H7 should not be present in ready-to-eat foods. Low levels of the organism could be accepted in raw material used for heated food products. Good hygiene should be maintained at all levels. HACCP analysis at food production plants is essential, and good manufacturing practices in general should be followed. Vegetables and salads should be washed with water of potable quality, and raw and cooked foods should be treated and stored separately. Pasteurisation of milk and juice, the use of pasteurised milk or milk of equivalent heat treatment in the manufacture of dairy products (Bastian and Sivelä 2000), and adequate heat treatment of meat, especially for ground beef, are necessary.

2.7.3.1 Modified atmosphere

The growth of E. coli O157:H7 is relatively tolerant for CO₂, but growth could be inhibited at 10°C at high CO₂ concentration and pH<6.0 (Sutherland et al. 1997). E. coli O157:H7 multiplied without modified atmosphere at 10°C, but in a high CO₂/low CO mixture and in a high O₂ mixture growth was markedly reduced (Nissen et al. 2000).
2.7.3.2 Background microflora

Background microflora have been demonstrated to inhibit the growth of *E. coli* O157:H7 in ground beef (Palumbo et al. 1997; Vold et al. 2000). *E. coli* O157:H7 strains grew in irradiated ground beef held at 8, 12, and 15°C, but their growth was inhibited in fresh ground beef. Although the growth of *E. coli* O157 was inhibited the populations in the fresh ground beef remained stable. Background flora (10^5-10^6 cfu/g), consisting mainly of lactic acid bacteria added to ground beef spiked with *E. coli* O157:H7 (10^6 cfu/g) and stored either aerobically or anaerobically at 12°C, inhibited the growth of *E. coli* O157:H7 under aerobic conditions, but under anaerobic conditions the growth was inhibited regardless of the background flora (Vold et al. 2000). However, beef microflora delayed the onset of growth of *E. coli* O157:H7 on beef carcass tissue at 12°C (Berry and Koohmaraei 2001). Although endogenous microflora inhibit the growth of *E. coli* O157, they do not decrease the original levels or destroy the pathogen.

2.8 Survival of *E. coli* O157

2.8.1 Survival of *E. coli* O157 in fermented products

Pathogenic *E. coli* O157 seem to be more acid-tolerant than non-pathogenic strains (Arnold and Kaspar 1995). In 1992, an outbreak caused by apple cider showed that *E. coli* O157:H7 could be transmitted via a food with a pH level < 4.0 (Besser et al. 1993). *E. coli* O157 survives during the fermentation and drying of fermented sausage, although the numbers decrease by approximately 1-2 log_{10} (Glass et al. 1992; Hinkens et al. 1996; Calicioglu et al. 1997; Faith et al. 1997; Riordan et al. 1998; Doores et al. 1998; Faith et al. 1998b). The pH of dry fermented sausage is usually 4.4-4.8 at the lowest, the aw less than 0.90 and the levels of salt and sodium nitrite do not provide a reduction of *E. coli* O157 by 5 log_{10} as recommended by the US Food Safety and Inspection Service. A 5-log_{10} reduction was caused by fermentation at a higher temperature (43.3°C) (Getty et al. 1999) or by post-fermentation heating to 60°C instantaneously or to 55°C for 60 min (Hinkens et al. 1996; Calicioglu et al. 2002). Storage at ambient temperature for at least two weeks was recommended in order to result in a 5-log_{10} reduction (Faith et al. 1997). A mild heating step in fermented meat production has been suggested in order to yield a greater reduction in the counts (Riordan et al. 1996). Heating changed the appearance and odour of the product (Hinkens et al. 1996). The effect of different starter cultures has not been studied in more detail, but fermenting sausage without a starter culture decreased the counts of *E. coli* O157 less than using a starter (Calicioglu et al. 2002).

The counts of *E. coli* O157 during fermentation of other products decrease but are not eliminated. Cheddar cheese (pH 4.95-5.2) made of milk containing *E. coli* O157:H7 10^6 cfu/ml showed a 2 log_{10} unit reduction after 60 days of ripening, with viable *E. coli* O157:H7 still detected in 25 g of cheese after 158 days, whereas cheese made with 1 cfu/ml showed a reduction in *E. coli* O157:H7 to 1 or less than 1 cfu/g in 60 days, with no *E. coli* O157 detected in 25 g of cheese at 158 days (Reitsma and Henning 1996). In yoghurt (pH 4.5-4.6 in the final product) the numbers of *E. coli* O157:H7 decreased from 3.49-3.52 log_{10} to 2.72-2.73 log_{10} or from 7.08-7.38 to 5.32-5.41 log_{10} during a fermentation of 5 h at 42°C and thereafter storage at 4°C up to seven days (Massa et al. 1997).

2.8.2 Survival of *E. coli* O157 in water

*E. coli* O157 may survive particularly in cold water for long periods. After an outbreak associated with municipal drinking water the organism was noted to survive for at least 35 days in water maintained at 5°C and 20°C (Swerdlow et al. 1992). *E. coli* O157 inoculated into pond water from a farm survived for 21 days at 13°C (Porter et al. 1997), and using inoculation levels of 10^7 and 10^9/ml *E. coli* O157:H7 survived in farm water stored in a field for
14 and 24 days, respectively (McGee et al. 2002). When \(10^3\text{cfu/ml}\) of \(E.\ coli\ O157:H7\) was inoculated, the pathogen survived best in pure water at 8°C, decreasing by 1-2 \(\log_{10}\) by 91 days, and worst in lake water at 25°C, which suggests that \(E.\ coli\ O157:H7\) is not a good competitor (Wang and Doyle 1998). \(E.\ coli\ O157\) decreased more rapidly at 20°C than at 5°C in well water (Rice et al. 1992), but in bottled water held at 22°C it was able to persist for more than 300 days (Warburton et al. 1998).

2.8.3 Survival of \(E.\ coli\ O157\) in faeces

\(E.\ coli\ O157:H7\) can persist in cattle faeces for extended periods, which makes faeces a potential vehicle of transmission. During the first three days of incubation at 22 and 37°C \(E.\ coli\ O157:H7\) increased by 1.5 to 2 \(\log_{10}\) (Wang et al. 1996; Kudva et al. 1998). After initial increase, the counts start to decrease. When a five-strain mixture of \(E.\ coli\ O157:H7\) \((10^3\text{ and }10^5\text{ cfu/g})\) was added into bovine faeces the pathogen survived for 42 and 49 days at 37°C, for 49 and 56 days at 22°C, and for 63 and 70 days at 5°C, respectively (Wang et al. 1996). \(E.\ coli\ O157\) also survived at 4 and 20°C for 34 weeks in faeces of rats infected with \(10^5\text{ cfu/g}\) (Clizek et al. 2000). A decrease of 5 \(\log_{10}\) from the inoculated concentration of \(10^8\) was noted during 99 days at 10°C (Bolton et al. 1999), and a similar \((3.5-5.5\ \log_{10})\) reduction was observed in slurry samples inoculated with \(6\ \log_{10}\) after a storage of 12 weeks at 10°C (McGee et al. 2001). However, a rapid decline was observed within nine days in slurry inoculated with \(7\ \log_{10}\) and stored at 18-20°C, to levels less than \(100\ \text{cfu/ml}\) (Maule 2000). \(E.\ coli\ O157:H7\) could survive in bovine, aerated ovine, and nonaerated ovine manure piles stored in the US state of Idaho exposed to climatic conditions for up to 47 days, 4 months and 21 months, respectively (Kudva et al. 1998). It should be noted that the pathogen was never recovered from the dry top layer of the manure piles. \(E.\ coli\ O157:H7\) also survived best in faeces incubated without aeration at temperatures below 23°C (10°C, 4°C or -20°C), but was not well recovered at temperatures of 37°C, 45°C, or 70°C, which suggests that the bacterium is eliminated by composting process. \(E.\ coli\ O157:H7\) was not detected after 72 h composting at 45°C (Lung et al. 2001). Cattle manure should be held for 105 days at 4°C or 45 days at 37°C to achieve a 5 \(\log_{10}\) units reduction of \(E.\ coli\ O157:H7\) (Himathongkham et al. 1999).

2.8.4 Survival of \(E.\ coli\ O157\) in soil and environment

\(E.\ coli\ O157:H7\) survived for 105 days in soil naturally contaminated by grazing sheep (Ogden et al. 2002). In an experimental study \(E.\ coli\ O157\) survived for 130 days at 18°C (Maule et al. 1997). \(E.\ coli\ O157\) was isolated from contaminated materials like wood and straw for up to 38 weeks after inoculation (Randall et al. 1999). Survival of \(E.\ coli\ O157:H7\) was dependent on soil type, with 5 \(\log_{10}\) reductions in sandy soils within eight weeks and in loam and clay soils within 35 weeks (Fenlon et al. 2000). High numbers of the pathogen have been inoculated in the experimental studies, which might suggest that the survival of the bacterium of natural levels is lower. When \(E.\ coli\ O157\) cells \((3 \times 10^{10} \text{ cfu/100 ml})\) were inoculated in cattle slurry to arable and grass plots on a clay loam soil, the organism was only detected in the soil and the grass during the first week after land application (Fenlon et al. 2000). \(E.\ coli\ O157\) was inactivated more rapidly in unautoclaved soil than in autoclaved soil at temperatures of 5°C, 15°C and 21°C (Jiang et al. 2002). In unautoclaved soil small numbers survived for periods of more than 226 days at 15°C and more than 231 days at 21°C. Micro-organisms naturally occurring in soil appear to have a major influence on rates of inactivation of \(E.\ coli\ O157\) at 15°C and 21°C. \(E.\ coli\ O157\) is able to survive and replicate in a protozoan (Acanthamoeba polyphaga) (Barker et al. 1999), which suggests that the bacterium could survive for periods in the environment.

2.8.5 Effect of pH on \(E.\ coli\ O157\)

\(E.\ coli\ O157:H7\) is unusually resistant to inactivation at low pH (Benjamin and Datta 1995; Lin et al. 1996). Strain to strain variation exists (Arnold and Kaspar 1996; Waterman and Small
Several mechanisms enable *E. coli* to resist acidic conditions: an acid-induced oxidative system, an acid-induced arginine-dependent system and a glutamate-dependent system (Lin et al. 1996). The oxidative system is active in the absence of glucose and the glutamate- and arginine-dependent systems are active during fermentation. All three systems are active during stationary-phase growth, which suggests the involvement of the alternate, stationary-phase sigma factor σ^7, which is encoded by *rpoS* (Arnold and Kaspar 1995; Price et al. 2000). Mutations in the *rpoS* gene may account for differences in acid resistance (Waterman and Small 1996).

Acid resistance enhances the survival in acid products and enables the colonisation of *E. coli* O157 in the human gut. The low infectious dose of *E. coli* O157:H7 is thought to correlate with acid resistance (Lin et al. 1996; Law 2000). An *rpoS*-lacking strain was significantly less shed from the calves than an *rpoS*-containing strain, which indicates that *rpoS* plays a role in *E. coli* O157:H7 shedding in calves, possibly by inducing resistance to gastrointestinal stress (Price et al. 2000). Exposure to low pH and low temperature induces an acid tolerance response (Weagant et al. 1994; Leyer et al. 1995; Lin et al. 1996; Duffy et al. 2000). Once induced, acid resistance is stable at 4°C (Law 2000). *E. coli* O157:H7 declined to unmeasurable numbers by day seven when held at 25°C, but at refrigeration temperature the organism was still detected at day 20 (Besser et al. 1993). *E. coli* O157:H7 inoculated at levels of 10^5 cfu/g into mayonnaise-based sauces (pH 3.68-4.44) and stored at 25°C became undetectable after 3 days, whereas when stored at 7°C it was detectable up to 35 days (Weagant et al. 1994).

Stationary phase bacteria are more acid resistant than exponentially growing organisms and do not need prior exposure to low pH to exhibit resistance (Arnold and Kaspar 1995). pH-dependent acid resistance provides *E. coli* O157:H7 with cross-protection against heat treatments (Buchanan and Edelson 1999). When subjected to sublethal stress conditions, e.g. pH 4.0, strains of *E. coli* O157:H7 exhibited significantly greater resistance to low pH, high salt (20% w/v) and heat (56°C) for 80 min than did controls maintained at pH 7.0 (Rowe and Kirk 1999).

No loss of viability was observed in an *E. coli* O157:H7 strain at pH levels of 3.0 and 2.5 for at least five hours at 37°C (Benjamin and Datta 1995), but no growth was observed at 8°C in acidic environment (Betts 2000). The type and level of organic acid had a significant effect on the survival of *E. coli* O157; the effectiveness of acidulants in inhibiting growth in beef slurry was in the order: acetic acid, lactic acid, citric acid (Abdul-Raouf et al. 1993; Diez-Gonzalez and Russell 1999; Betts 2000). No growth was observed when acetic acid was used, whereas using citric acid growth occurred in pH 3.75 at 15°C, and in using lactic acid in pH 3.78 at 12 and 15°C. *E. coli* O157 could survive for extended periods of time in products with a pH of 2.8-3.3 and an acetic acid concentration of 1% (Betts 2000). Acid-adapted *E. coli* O157:H7 had an increased resistance to lactic acid and survived better in shredded dry salami and apple cider (Leyer et al. 1995).

2.8.6 Effect of water activity (a_w) on *E. coli* O157

*E. coli* O157 can grow at a water activity of 0.95 (equivalent to 8% NaCl) (Betts 2000). *E. coli* O157:H7 survived the fermentation and drying process of a dry fermented meat product that reached water activity of 0.87 and pH 4.74 at the end of the process (Hinkens et al. 1996).

2.8.7 Effect of temperature on *E. coli* O157

*E. coli* O157 is not unusually heat-resistant and heat treatment of 70°C for two min reduces the numbers of *E. coli* O157:H7 by 6 log_{10} units (Stringer et al. 2000). Many factors can
influence heat resistance, including growth conditions, such as growth phase of the cells, composition, pH and water activity of the growth medium, growth temperature, holding period before heat treatment, heat shock, the heating method used (e.g. open heating system), rate of heating, media (including its composition), pH, \( a_w \) and choice of humectant, the recovery conditions, and attachment to solid surfaces. The mean of \( D_{90C} \) values of \textit{E. coli} O157:H7 in meat was 1.8 min and the \( z \) value 5.5°C, (range 0.3-10.0 min) (Stringer et al. 2000). The ability of \textit{E. coli} O157:H7 to tolerate heat is strain-dependent. \( D_{90C} \) of 17 \textit{E. coli} O157:H7 strains in brain heart infusion (BHI) broth ranged from 2.6 to 21.5 min and the \( D_{90C} \) from 0.69 to 2.13 min (Whiting and Golden 2002). Heating medium has significant effect on heat resistance. Heat resistance was different in beef than in tryptone soya broth (Williams and Ingham 1997). D values of burgers containing 30% non-meat ingredients were higher than those of whole-meat burgers (Byrne et al. 2002b). The burgers with less meat had a significantly lower \( a_w \) (mean 0.978) than the whole-meat burgers (mean 0.993). A decrease in \( a_w \) (from 0.98 to 0.96) resulted in an increase in the heat resistance of \textit{E. coli} O157 in salt and sucrose solutions (Kaur et al. 1996).

Cold shocking in milk, whole egg or sausage resulted in significant enhancement in survival of \textit{E. coli} O157:H7, but not in frozen ground beef or ground pork (Bollman et al. 2001). Heat resistance was greater when cells were in stationary phase and when they were grown at 37°C rather than at lower temperatures, and cultures stored frozen (-18°C) were more resistant than those stored under refrigeration (3°C) or at 15°C (Jackson et al. 1996). On the contrary, \textit{E. coli} O157:H7 was less heat resistant when two strains were held in apple juice (pH 3.4) at 4°C for 24 h, while storage at 21°C for two hours decreased the heat tolerance of only one of the strains (Ingham and Uljas 1998). However, freezing and tempering steps of beef trimmings decreased the heat resistance (Byrne et al. 2002b). The increase of heat resistance by heat shock depends on environmental conditions.

Heat shock increased the heat resistance of cells in TSB but not in beef slurry (Williams and Ingham 1997), heat shock at 42, 45, 48 or 50°C had little effect on exponential-phase cells grown in nutrient broth (Kaur et al. 1998), and heat shock at 42°C for five min induced larger increases in heat resistance in cells grown aerobically than grown anaerobically (Stringer et al. 2000). Although a heat shock at 45°C for 30 min in TSB and ground beef slurry increased D values the effect was lost upon subsequent chilling and re-warming (Williams and Ingham 1997). Acid-adaptation cross-protects the cells to heat (Cheville et al. 1996). \textit{E. coli} O157 is more resistant to heat in frozen patties than in patties stored at 15°C (Jackson et al. 1996).

\textit{E. coli} O157 is able to survive at refrigeration temperatures better than at room temperatures (Conner and Kotrofa 1995). No growth was observed at 8°C in acidic environment (pH 3.56-4.65) (Betts 2000), whereas the lowest temperatures of growth in milk have been observed at 6.5°C (Kauppi et al. 1996), at 7°C (Heuvelink et al. 1998b), and at 8°C (Wang et al. 1997). \textit{E. coli} O157:H7 does not grow under the refrigeration temperatures (2-5°C) but is able to form long filaments under such conditions (Bell 2002). \textit{E. coli} O157:H7 decreased only slightly in ground beef stored over nine months at -20°C (Meng and Doyle 1998).

At least some strains of \textit{E. coli} O157:H7 are heat sensitive, which demands accurate incubators in the laboratory performing analyses. \textit{E. coli} O157:H7 grew well in TSB between 30 and 42°C and poorly in the range 44-45.5°C (Meng and Doyle 1998). The temperature range for turbidity and gas for a single \textit{E. coli} O157:H7 isolate was 24.3-41.0°C (Raghubeer and Matches 1990). The ability to grow at 45.5°C is dependent on the medium, the inoculum size and the strain (Ferenc et al. 2000). Most strains of \textit{E. coli} O157:H7 studied grew from 8-10°C to 45°C in BHI, but not all in the selective broth of EC at 42 or 45°C (Palumbo et al. 1995). The optimal growth temperature of 20 \textit{E. coli} O157 strains was 40.2°C (Gonthier et al. 2001).
2.8.8 Effect of food additives and disinfection on survival of *E. coli* O157

*E. coli* O157:H7 was found able to grow in NaCl concentrations as high as 6.5%, but the doubling time was about threefold longer at 4.5% than at 2.5% (Glass et al. 1992). The bacterium in this study was destroyed at 8.5%. In another study, *E. coli* O157:H7 cells at pH 6.5 grew in salt concentration of 7, 8 or 9%, but the growth rate was decreased (Jordan and Davies 2001). NaCl concentration of 3.3%, a sodium nitrite concentration of 300 ppm and low pH (4.4) reduced the counts of *E. coli* O157:H7 more (4.79 log_{10} units) than did 2.5% salt, 100 ppm nitrite and a pH of 4.8 (0.84 log unit) in the manufacture of fermented sausage (Riordan et al. 1998). The addition of 0.1% sodium benzoate prevented growth at 8°C and reduced the counts to undetectable levels within 7 days (Besser et al. 1993). *E. coli* O157 is not particularly resistant to disinfectants (Taylor et al. 1999; Oie et al. 1999). Most strains of *E. coli* O157:H7 have no unusual tolerance to chlorine (Rice et al. 1999; Zhao et al. 2001).

2.9 Isolation, identification and typing of *E. coli* O157

As the infectious dose of *E. coli* O157 is low, sensitive methods are needed to find low numbers of the bacteria. *E. coli* O157 can be analysed using traditional culture methods, immunological methods or DNA based methods, or a combination of several methods. Several factors affect the sensitivity of the method, e.g. laboratory techniques, handling of the samples and sample size. Samples should be analysed fresh. Even if *E. coli* O157 tolerates freezing, better results have been obtained using fresh samples (McDonough et al. 2000). *E. coli* O157 was recovered from 22% of previously positive samples that had been stored frozen (-70°C) between 19 and 58 months (Chapman et al. 2001c). Several culture methods have been used but comparative studies testing all the different combinations (enrichment broths, enrichment conditions, plating media) have not been performed. Most comparisons have been made using small numbers of samples and artificially inoculated samples.

2.9.1 Sample size

In food analysis, a sample size of 25 g or ml is used most commonly, but faecal samples have been analysed using 50 mg, swab (0.1g), 1 g, 2 g, 5 g, 10 g, 20 g, or 25 g samples (Table 3). As animals may shed only small numbers of the bacteria bigger sample sizes detect more positives. A sample size of 10 g was more sensitive than 1 g or swab samples (Kudva et al. 1995; Sanderson et al. 1995) but the other sample sizes have not been compared.
Table 3. Sample sizes (g) of faecal material used in different studies

<table>
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<th>50 mg</th>
<th>Swab (0.1 g)</th>
<th>1 g</th>
<th>2 g</th>
<th>5 g</th>
<th>10 g</th>
<th>20 g</th>
<th>25 g</th>
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<td>Cobbold and Desmarquiller 2001</td>
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<td>Martin et al. 1994</td>
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<td>van Donkergoed et al. 2001</td>
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2.9.2 Enrichment

The use of enrichment is preferable to direct plating (de Boer and Heuvelink 2000). *E. coli* O157:H7 was not recovered from ovine faeces inoculated with 2.4 cfu/g using direct plating onto SMAC-MUG (Kudva et al. 1995). Several enrichment broths (Doyle and Schoeni 1987; Okrend et al. 1990b; Padhye and Doyle 1991; Kim and Doyle 1992; Fratamico et al. 1992; Chapman et al. 1994; Kudva et al. 1995; Weagant et al. 1995; Restaino et al. 1996; Jiang et al. 1998; McDonough et al. 2000; Chapman et al. 2001c) and procedures have been described (Table 4), but comparative studies testing different enrichment broths, temperatures and other conditions using several matrices have not been performed. In a review article the use of MTSBn or mECn were recommended as the most appropriate enrichment broths (de Boer and Heuvelink 2000). However, somewhat conflicting results have been obtained in comparative studies. IMS with mECn resulted in more isolations than BPW-VCC (Bennett et al. 1996). The performances of mECn, EEB and GNn were comparable when bovine faecal samples were incubated at 37°C for 18 to 24 h (McDonough et al. 2000). The most effective enrichment for ground beef was incubation in MTSBn or in mECn at 42°C for 18 h followed by IMS, and for radish sprouts mECn at 42°C for 18 h (Hara-Kudo et al. 1999). MTSBn detected more positive bovine faecal samples than BPW after 6 h enrichment, whereas after 24 h BPW was superior to MTSBn (Tutenel et al. 2003). Enrichment in MTSBn or a two-stage enrichment in BHI and then in tryptone phosphate medium at 37°C for 10 h was superior to MTSBca or mECn for recovering heat-stressed *E. coli* O157 from ground beef, whereas a commercial enrichment broth, EZ Coli, performed poorly (Taormina et al. 1998). However, acid/salt stressed *E. coli* O157 was better recovered from enrichment broths (TSB or BPW) without bile salts or VCC antibiotics (Stephens and Joyson 1998). In the ISO 16654/2001 method MTSBn is used as the enrichment broth (ISO 2001). The ISO method showed a low limit of detection (1-2 cfu/25g) when tested on artificially contaminated dairy products (Voitoux et al. 2002). Enrichment at 41.5°C for 24 h in a broth, sTSB, to which bile salts were added after 6 h of incubation, gave more positives than enrichment in BPW-VCC at 37°C for 6 h when analysing raw meat products (Chapman et al. 2001c). sTSB contains Oxysol™, a product of *E. coli* plasma membrane shown to enhance growth of *E. coli* O157 (Jiang et al. 1998). BPW without antimicrobials recovered more *E. coli* O157 than BPW-VCC (Synge et al. 1998). Universal pre-enrichment broth performed equally well as BPW or TSB in supporting the growth of uninjured *E. coli* O157:H7 (Jiang et al. 1998).
Table 4. Selective enrichment broths used for E. coli O157

<table>
<thead>
<tr>
<th>Enrichment broth</th>
<th>Supplement</th>
<th>Short form</th>
<th>Reference</th>
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<tr>
<td>Modified tryptone soya broth</td>
<td>Acriflavin</td>
<td>MTSBa</td>
<td>Kim and Doyle 1992</td>
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<td></td>
<td>Casa-amino acids</td>
<td>MTSBa</td>
<td>Padyhe and Doyle 1991</td>
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<td></td>
<td>Novobiocin</td>
<td>MTSBn</td>
<td>Doyle and Schoeni 1987</td>
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<td></td>
<td>Oxyrase, novobiocin</td>
<td>sTSB</td>
<td>Chapman et al. 2001</td>
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<td></td>
<td>Vancomycin, cefsulodin, cefixime</td>
<td>EEB</td>
<td>Wézagant et al. 1995</td>
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<td></td>
<td>Vancomycin, cefixime, tellurite</td>
<td>TSB-CTV</td>
<td>Kudva et al. 1995</td>
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<tr>
<td>Buffered peptone water</td>
<td>Casa-aminoacids</td>
<td>mBPW</td>
<td>Restalno et al. 1996</td>
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<td></td>
<td>Vancomycin, cefsulodin, cefixime</td>
<td>BPW-VCC</td>
<td>Chapman et al. 1994</td>
</tr>
<tr>
<td>Gram negative broth</td>
<td>Novobiocin</td>
<td>GNn</td>
<td>McDonough et al. 2000</td>
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<td>Lauryl tryptose broth</td>
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<td>Fratamico et al. 1992</td>
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<tr>
<td>Modified E. coli broth</td>
<td>Novobiocin</td>
<td>mECn</td>
<td>Okrend et al. 1990</td>
</tr>
<tr>
<td>Universal pre-enrichment broth</td>
<td>Novobiocin</td>
<td>UPB</td>
<td>Jiang et al. 1998</td>
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The ratio between sample and enrichment broth varies from 1:10 (ISO 2001) to 1:5 (Grauke et al. 2002), 1:20 (Kudva et al. 1995), or 1:50 (Kudva et al. 1995; Fegan and Desmarchelier 1999). Comparisons on the optimal ratio are scarce. E. coli O157:H7 was recovered when 1 g of faeces was enriched in 50 ml or 10 g in 200 ml TSB-CTV, but not when 0.1 or 1 g was enriched in 7 ml TSB-CTV (McDonough et al. 2000).

Enrichment temperatures and lengths may also vary. The optimal duration of enrichment depends on the competing microflora, the matrix and whether the cells are injured or not. Stressed, heat-, freeze- or acid-injured cells need a longer enrichment. Samples subjected to cold stress at 4°C for 14 days required about 3.5 h longer enrichment than samples that were enriched immediately after inoculation (Uyttendaele et al. 1998). Following 6-h enrichment in mECn at 37°C, PCR products were visible in samples inoculated with 100 cfu/g, after 8-h enrichment in samples with 10 cfu/g and after 12-h in those inoculated with 1 cfu/g (Fratamico et al. 2000). Heat-injured cells often required 3 to 4 h for recovery before growth was initiated in UPB (Zhao and Doyle 2001).

Conflicting results have been obtained using enrichment temperatures of 37, 41.5, 42, and 44.5°C. Enrichment in MTSBn at 42°C was more selective than 37°C (Bolton et al. 1996), whereas the growth of microflora on raw ground beef was markedly suppressed at 42°C (Blais et al. 1997). An enrichment temperature of 42°C gave more positives than 37°C when analysing soil samples (Hepburn et al. 2002). On the other hand, starved cells of E. coli O157:H7 grew markedly less in MTSBn and especially in mECn at 42°C, whereas the growth suppression was seldom observed in non-selective broths (TSB and BPW) at 37°C (Sata et al. 1999). The upper temperature is preferable, especially when analysing samples containing competing microflora, because many of these have optimal at 37°C. However, it should be noted that the upper temperature is critical, too, because some E. coli O157 strains grow poorly at temperatures above 42°C, especially in selective media (Raghubeer and Matches 1990; Doyle 1991). Eighteen studied E. coli O157 strains were able to grow in non-selective media at 45.5°C, whereas most of the strains did not grow in EC broth at the same temperature (Ferenc et al. 2000). Unsupplemented TSB at 44.5°C for 24 h and IMS gave
most positive isolations from water and sediment samples (LeJeune et al. 2001a). Other enrichment conditions, such as shaking versus static, are not well documented. Incubation at 42°C without shaking effectively suppressed the ground beef microflora while allowing good growth of \textit{E. coli} O157 (Blais et al. 1997).

2.9.3 Plating media

Typical strains of \textit{E. coli} O157 do not ferment sorbitol within 24 h and are β-glucuronidase-negative (Ratnam et al. 1988). However, sorbitol-fermenting and β-glucuronidase-producing \textit{E. coli} O157:H7/H- strains have also been described (Okrend et al. 1990a; Hayes et al. 1995). The most commonly used agar media for detection of \textit{E. coli} O157 is based on Sorbitol MacConkey agar (SMAC) (Ratnam et al. 1988). The problem with plating media is often the overgrowth with other organisms. \textit{Enterobacteriacea} grow on SMAC, therefore the selectivity of SMAC has been improved by the addition of cefixime and rhamnose (CR-SMAC) because \textit{E. coli} O157 does not ferment rhamnose unlike most non-sorbitol-fermenting organisms (Chapman et al. 1991). Cefixime-tellurite supplemented SMAC (CT-SMAC) was more sensitive than CR-SMAC (Zadik et al. 1993) and has now mostly replaced CR-SMAC. Cefixime-tellurite supplements inhibit many nonsorbitol-fermenting organisms including \textit{Proteus} spp., \textit{Morganella morganii}, \textit{Providencia} spp., \textit{Aeromonas} spp. and \textit{Plesiomonas} spp. (Zadik et al. 1993) and \textit{Hafnia alvei} (Weagent et al. 1995). SF O157:H- are tellurite resistant and do not grow on CT-SMAC (Karch et al. 1996). CT-SMAC has been modified by adding salicin but this media has not yet been widely evaluated (Fujisawa et al. 2000; Fujisawa et al. 2002). MTSB incubated at 42°C for 6 h followed by IMS and subculture to CT-SMAC was the most sensitive and selective procedure (Bolton et al. 1996). Plating media using β-glucuronidase-negativity, the fluorogenic compound 4-methylumbelliferyl-β-D-glucuronide (MUG), or the chromogenic compound 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) have been developed (Okrend et al. 1990a). Haemorrhagic colitis (HC) agar contains sorbitol, bile salts and MUG as well as an indicator dye (Szabo et al. 1986). SMAC has also been supplemented with MUG (Kudva et al. 1995). Commercial chromogenic media that use β-glucuronidase have been developed, like Biosynth Culture Media O157:H7 (BCM), CHROMagar O157, Fluorocult HC (HC) and Rainbow agar O157 (RB) (Manafi and Kremsmaier 2003). CHROMagar was sensitive and specific for \textit{E. coli} O157 (Wallace and Jones 1996), whereas CT-SMAC was more selective than the chromogenic medium (CHROMagar) when analysing dairy products (Onoue et al. 1999; Voitoux et al. 2002). EHEC agar based on enterohaemolysin production was sensitive with milk samples, but with meat samples the background flora outnumbered the spiked strain (Hudson et al. 2000). The recovery of \textit{E. coli} O157:H7 from salami, a product with few problems of background flora, was best on TSA, then on modified eosin methylene blue (MEMB) agar, and worst on SMAC supplemented with MUG (Clavero and Beuchat 1996). MEMB and Rainbow agar supported the recovery of heat-stressed cells better than CHROMagar, CT-SMAC or CR-SMAC (Taormina et al. 1998). The use of CT-SMAC and a media not based on sorbitol fermentation was recommended in order to catch even sorbitol-positive isolates (de Boer and Heuvelink 2000).

2.9.4 Resuscitation

Heat-, freeze-, acid- or salt-stressed \textit{E. coli} O157 may be present in foods and in environment, and as the bacterium has a low infectious dose and injured cells usually are able to retain pathogenicity it is important to be able to detect cells in a stressed state. The use of resuscitation improves the detection, especially of freeze-, acid-, salt- or heat-injured cells. Improved recoveries were found after resuscitation (2 h at 25°C) on TSA before overlay with SMAC supplemented with MUG (McCleery and Rowe 1995), and after a 4-h incubation on a membrane on TSA prior to transfer onto SMAC (Blackburn and McCarthy 2000). Allowing food samples to stand for three hours at room temperature in non-selective broth
prior to enrichment in mECn and IMS enhanced the detection of freeze-injured cells of *E. coli* O157 (Nakagawa et al. 2000). Freeze-injured *E. coli* O157 were most efficiently recovered in mEC broth without bile salts at 25°C for 2 h, and then selectively enriched at 42°C for 18 h by adding bile salts and novobiocin (Hara-Kudo et al. 2000).

2.9.5 Vero cell toxicity

Shiga toxins produce a cytotoxic effect on Vero cells *in vitro* (Konовалchuk et al. 1977). HeLa cells have also been used but are less sensitive to Stx2e (Paton and Paton 1998). The vero cell test is sensitive, but it needs trained personnel, is difficult to standardize and takes approximately one week to perform (Paton and Paton 1998; Karch et al. 1999). Vero cell assay was formerly used in the analysis of faecal extracts, and now mainly in the characterisation of isolates and evaluation of new Stx-detection assays. The sensitivity of the test can be enhanced by supplementing cultures with mitomycin C, polymyxin B or quinolones to increase production and release of Stx (Griffin and Gemski 1983; Karmali et al. 1985b). Cattle can often harbour strains that do not produce stx (Chapman et al. 1993).

2.9.6 Immunological tests

The use of immunomagnetic separation, IMS, para-magnetic beads coated with O157 antibodies, sensitizes the detection 10-100-fold from samples of minced beef, milk, sprout and faeces (Chapman et al. 1994; Wright et al. 1994; Bolton et al. 1995; Bolton et al. 1996; Karch et al. 1996; Chapman and Siddons 1996; Bennett et al. 1996). Non-specific binding of other organisms to the beads is a problem in foods with high background flora, but the use of a low-ionic strength solution decreased it (Tomoyasu 1998). IMS is labour-intensive and the procedure must be carried out carefully to prevent cross-contamination of the samples and the laboratory personnel. Several commercial systems are available, as well as beads for some other serotypes.

Since culture methods are time-consuming and laborious, rapid immunological techniques have been developed. Although a preliminary result might be obtained quicker with rapid techniques, the results must be confirmed with other methods and the organism isolated (de Boer and Heuvelink 2000). Several of the rapid tests have been evaluated and found sensitive after enrichment. Typical sensitivities of immunoassays are approximately $10^2$-10$^6$ cfu/ml, thus the performance of the test is highly dependent on enrichment (Blackburn and McCarthy 2000; Pruett et al. 2002). Rapid tests may be based on Stx production or they may also use polyclonal antibodies or monoclonal antibodies to *E. coli* O157. It must be noted that not all strains produce toxin, or the production needs to be enhanced. The commercial toxin production tests Verotox F and VTEC-RPLA were sensitive and reliable when compared with vero cell assay (Beutin et al. 1996b; Karmali et al. 1999). The sensitivity of the Premier-EHEC test on human stool specimens has been 89-100%, and specificity 99.7% (Kehl et al. 1997; Mackenzie et al. 1998). Rapid commercial ELISA methods (Reveal 8, VIDAS, EHEC-Tek) had poor performances, but the sensitivity was improved with incorporation of the resuscitation stage (Blackburn and McCarthy 2000).

2.9.7 Polymerase chain reaction (PCR) methods

PCR is the most widely used molecular method to screen STEC. PCR offers the advantage of detecting *E. coli* O157 regardless of its ability to ferment sorbitol, as well as the possibility of detecting other STEC. PCR is considered a sensitive diagnostic test, in theory able to detect the genome of one bacterium. The application of PCR for direct detection is hampered by the presence of inhibitors in the samples. Best results were obtained when testing extracts of broth cultures, because inhibitors are diluted during enrichment and bacterial cells multiplied (Paton and Paton 1998). Commercial DNA extraction methods and other procedures, including IMS, have been developed to overcome the problem with inhibitors (Lindqvist 1997;
The sensitivity of PCR is dependent on the initial number of target pathogen cells in the sample, the DNA extraction method, the enrichment and the state of cells (Paton and Paton 1998). When using enrichment, a multiplex PCR was able to detect ≤1 cfu/g (Fratamico et al. 2000). PCR assays have been designed using primers targeted to stx genes: stx1, stx2, and stx2 variants (Karch and Meyer 1989; Olsvik and Stockbline 1993), eae (Gannon et al. 1993; Heuvelink et al. 1995), uilA (Cebula et al. 1995), flIC (Gannon et al. 1997), O157 rfb (Paton and Paton 1998; Desmarchelier et al. 1998), and ehxA (Schmidt et al. 1995). When 14 different PCR systems for detection and subtyping of stx genes were tested, only one system (Lin et al. 1993) was able to detect stx1, stx2 and the variant genes in all studied strains (Bastian et al. 1998). Multiplex PCR methods targeting several genes have also been described (Paton and Paton 1998) described a multiplex PCR assay targeting for stx1, stx2, eaeA, hlyA, and rfb of E. coli O157 and E. coli O111. A multiplex PCR targeting for stx1, stx2, eaeA, hlyA, and flIC has been developed (Fratamico et al. 2000). PCR with primers targeting rfbO157 after enrichment in STSB at 41.5°C for 24 h and IMS was more sensitive than culture in CT-CMAC (Chapman et al. 2001). A commercial PCR assay produced substantially larger number of presumptive and culture-confirmed E. coli O157:H7 than two immunoassays (Pruett et al. 2002). PCR methods might be a solution to the problem of competing micro-organisms in the samples. Traditional PCR methods are labour-intensive, but real-time PCR assays can be automated; they are sensitive and rapid and can quantify the number of bacterial cells. Comparative studies testing different PCR assays in natural samples have not been published.

2.9.8 Identification

Typical strains of E. coli O157:H7 ferment sorbitol slowly and are β-glucuronidase negative (Ratnam et al. 1988). E. coli O157 do not ferment rhamnose on agar plates (Smith and Scotland 1993). E. coli O157 strains are usually urease negative but some Italian cattle isolates (Conedera et al. 1997; Bonardi et al. 1999), and up to 27% (22/81) of Belgian cattle isolates were urease positive (Tutenel et al. 2002). Urease gene was common among Japanese clinical EHEC strains (Nakano et al. 2001). Lactose negative (Tutenel et al. 2002) as well as indole negative isolates have been detected (Bopp et al. 1987).

2.9.9 Typing of E. coli O157

Because of the clonal nature of E. coli O157:H7 and H+, a variety of pheno- and genotyping methods have been used and E. coli O157 isolates should not be classified as epidemiologically related or unrelated on the basis of a single typing method alone (Nataro and Kaper 1998; Grif et al. 1998a).

2.9.9.1. Phenotyping

Some other bacteria, such as Citrobacter freundii, E. hermannii, Yersinia enterocolitica serotype O9 and Salmonella O group N, may cross-react with O157 antiserum or latex reagents (Nataro and Kaper 1998; Stockbline et al. 1998). Most E. coli O157 isolates are motile, but approximately 12% are nonmotile (Feng et al. 1996). Nonmotile isolates either lack the flagellar antigen or the gene might be mutated (Reid et al. 1999). Isolates may need multiple passages in motility medium before the flagellar antigen is detectable (Feng et al. 1996). About 3% of the E. coli O157 isolates at the Centers for Disease Control and Prevention (CDC) carry flagellar antigens other than H7; H4, H12, H16, H29, H39, H42 and H45 (Stockbline et al. 1998). Up till now, isolates with other flagellar antigens than H7 have not been associated with human EHEC infections.

Phage typing relies on the characterization of strains by their patterns of resistance or susceptibility to a standard set of phages. Phage typing is simple and rapid and good for first level strain discrimination (Stockbline et al. 1998). However, it is only available at reference
laboratories because of the need to maintain biologically active phages and control strains. The reference laboratory of phage typing for *E. coli* O157 is situated in Ottawa, Canada, where the current phage typing system was and continues to be developed (Ahmed et al. 1987; Khakhria et al. 1990). The scheme comprises 16 bacteriophages and recognises 88 phage types (Griff et al. 1998a). Phage typing has been efficient in differentiating *E. coli* O157:H7 strains. As the sole subtyping method it is robust; it produced a value of 0.786 for the diversity index in a Scottish study of 124 *E. coli* O157:H7 isolates (Krause et al. 1996), and a value of 0.85 when used to study 51 Canadian *E. coli* O157:H7 isolates (Louie et al. 1999). In combination with PFGE, phage typing gives optimal discrimination (Preston et al. 2000).

Antimicrobial susceptibility testing is a simple, robust and cheap method, which might in some instances give additional information (Nataro and Kaper 1998). Formerly, *E. coli* O157 isolates were almost universally susceptible, but antimicrobial resistance has in recent years become more common and can sometimes be an epidemiological marker (Strockbine et al. 1998; Willshaw et al. 2001b).

2.9.9.2 Genotyping

Plasmid profile analysis was used when the first outbreaks were investigated, but it provides inadequate discrimination because most isolates harbour plasmids 4.5 and 60 MDa (Ostroff et al. 1989a; Louie et al. 1999). Hybridisation of restriction enzyme digested DNA with bacteriophage λ (λ-RFLP) has been used in some studies (Grimm et al. 1995). The technique is discriminatory but laborious (Samadpour 1995; Nataro and Kaper 1998; Strockbine et al. 1998). Hybridisation with stx probes gives less complex but sensitive RFLP patterns (Samadpour 1995). Random amplification of polymorphic DNA (RAPD) has been used by some researchers because it is less costly, rapid and differentiates adequately (Birch et al. 1996; Galland et al. 2001), but the RAPD subtyping methods are in-house and not as discriminatory as PFGE.

Ribotyping uses restriction fragments of ribosomal RNA genes for characterisation of organisms. Ribosomal genes are phylogenetically highly conserved and *E. coli* O157:H7 is a phylogenetically young organism (Nataro and Kaper 1998). Ribotyping was found unsuitable for detection of epidemiological relatedness in *E. coli* O157:H7 strains, because it was unable to differentiate epidemiologically unrelated strains of *E. coli* O157:H7 (Strockbine et al. 1998; Griff et al. 1998b). However, combination of PFGE and ribotyping provided superior discriminatory performance (Strockbine et al. 1998; Avery et al. 2002b).

Discrimination between stx2 and stx2 variant genes has been developed using restriction fragment-length polymorphism (RFLP) (Piérard et al. 1998), which can be used in conjunction with other typing methods (Willshaw et al. 2001b).

PFGE has been proven an efficient tool in outbreak investigations (Barrett et al. 1994; Swaminathan et al. 2001) and is widely used as a typing method. PFGE allows the generation of simplified chromosomal restriction fragment patterns. The organisms are embedded in agarose and then lysed, and the chromosomal DNA is digested with restriction endonucleases that cut infrequently. (Barrett et al. 1994) recommended the use of XbaI as a restriction enzyme, and the use of AvrII for further discrimination if needed. The standardisation of the method has been initiated in the US where PulseNet, the national molecular subtyping network for foodborne disease surveillance was established by the CDC in 1996 (Swaminathan et al. 2001). A national network has been established in Finland between the Public Health Institute (KTL) and the National Veterinary and Food Research Institute (EELA) using the same protocol as Pulsenet (Ranta et al. 2000). The standard
PulseNet method specifies the use of enzyme XbaI (Proctor et al. 2002), but enzymes BlnI and/or SpeI have also been used (Breuer et al. 2001). However, due to the clonal nature of E. coli O157:H7 serotype, PFGE may fail to discriminate between epidemiologically unrelated isolates (Grim et al. 1998a). Patterns may be difficult to interpret when the profiles do not match exactly. The degradation of the genomic DNA of some strains is a problem (Meng et al. 1995). Interpretation of PFGE genotypes remains subjective and the proposed criteria (Tenover et al. 1995) may not be appropriate for E. coli O157 (Strockbine et al. 1998; Willshaw et al. 2001b).

Amplified-fragment length polymorphism (AFLP) is based on selective amplification of restriction enzyme digested genomic fragments by radioactively or fluorescently labelled primers (FAFLP) (Savelkoul et al. 1999). The fragments can be visualised with the laser detection system of an automated sequencer. When a rare cutter is used in PFGE analysis, a rare and a frequent cutter are used in FAFLP. The power of discrimination can be increased or decreased by the use of different selective primers. FAFLP and PFGE exhibited equivalent discriminatory powers when 71 isolates of E. coli O157:H7 strains were analysed (Smith et al. 2000). However, in one study PFGE had a higher discriminatory power than FAFLP with the primer combination EcoRI+0 and MseI+C (Heir et al. 2001).

The development of a novel typing method, polymorphic amplified typing sequences (PATS), was based on the presence or absence of genomic DNA (Kudva et al. 2002). The method uses primer pairs amplifying DNA around E. coli O157 XbaI sites and virulence genes. The method awaits evaluation.
3 AIMS OF THE PRESENT STUDY

The purpose of this study was to investigate whether Finnish animal production and animal products are a source of *E. coli* O157. Investigations were carried to:

1. investigate and compare the survival of *E. coli* O157:H7 in dry sausage fermented by two commercial starter cultures (I)

2. study the prevalence of *E. coli* O157 in Finnish cattle and level of carcass contamination at slaughter (II, IV)

3. describe faecal shedding of *E. coli* O157 at farm level and identify sources of infection at a cattle finishing unit (IV, V)

4. identify the possible transmission routes of *E. coli* O157 from cattle to humans using epidemiological data and genotyping and phenotyping (V)

5. study the prevalence of *E. coli* O157 in reindeer (III)

6. optimise isolation methods in the analysis of *E. coli* O157 from food, faecal and surface samples (I, IV)
4 MATERIALS AND METHODS

4.1 Sausage manufacture (I)

4.1.1 Strains

Two different commercial starter cultures, FloraCarn LC (Chr. Hansen, Hershholm, Denmark) and Müller RM 52 (Rudolf Müller, Pohlheim, Germany), were used for the fermentation of sausage. FloraCarn LC contained *Staphylococcus xylosus* DD-34, *Pediococcus acidilactici* PA-2 and *Lactobacillus bavaricus* MI-401, while Müller RM 52 contained *Staphylococcus carnosus* MIII and *Lactobacillus curvatus* Lb3. The sausage batter was inoculated with a strain of *E. coli* O157:H7 (EELA367), isolated from a human case of HUS in 1992 in Finland.

4.1.2 Raw material

The fermented sausage used in study I was manufactured from frozen meat and fat (35% pork, 35% beef, 30% fat) obtained from a wholesale market. The commercial additives used were: spices (0.175%), ascorbic acid (0.025%), NaNO₂ (0.0120%), NaCl (3%), and glucose (0.7%).

4.1.3 Manufacturing process

The meat used for sausage preparation was stored frozen at -20°C. It was first chopped in a Seydelmann cutter K41 (Seydelmann, Stuttgart, Germany), and then a starter culture - FloraCarn LC or Müller RM 52 – was added along with spices, ascorbic acid, NaNO₂, NaCl, and glucose, and the mass was mixed in the cutter.

The sausage mass was divided into four portions, each weighing 5 kg. In the first experiment the portions were inoculated with 3.78-5.68 log₁₀ units of *E. coli* O157:H7, and in the second experiment 2.00-3.88 log₁₀ units of *E. coli* O157:H7 were used (Table 1, paper I). From each portion sausages weighing 600-700 g were stuffed into fibrous casings using a hand stuffer.

The sausages were fermented and dried in a smoke chamber for 15 days: 2 days at 23°C (4 h 80% relative humidity (rh), 20 h 92% rh, 24 h 90% rh), 24 h at 22°C and 88% rh, 24 h at 21°C and 85% rh, 24 h at 20°C and 80% rh, and 10 days at 17°C (6 days 75% rh and 4 days 74% rh). The sausages were smoked for 30 min during days 2-5. After fermentation the sausages were stored at 15-17°C 70-75% rh for 34 days.

4.2 Sampling

4.2.1 Faecal samples (II-V)

The rectal faecal samples in study II were collected from 1448 clinically healthy cattle (one sample per animal) at 14 largest abattoirs during June - December 1997. The sample size was based on estimates of a predicted prevalence of 0.2% (with a confidence level of 95%) in the total number of cattle slaughtered. The animals sampled at the slaughterhouses were chosen randomly.

The rectal faecal samples in study III were taken from 1387 animals at ten reindeer slaughterhouses and at two slaughtering sites during October 1998 – March 1999. Systematic random sampling was used in the main reindeer-raising area and random sampling in the other parts of the reindeer-raising area. The animals sampled at the slaughterhouses were
chosen randomly.

In study IV individual rectal faecal samples were taken from bull calves entering the farm in May, July, September and December 1999. All groups except the one arriving in July were sampled in a livestock transporter before entering the barn. The July group was sampled in the barn one day after arrival. Individual rectal samples were taken from the bulls four to seven times at one-monthly intervals. At the abattoir, faecal samples from animals that had arrived in May, July and September were taken.

In study V faecal samples from five humans with bloody diarrhoea and from their close contacts were taken by medical personnel and analysed at human clinical laboratories. At five suspected farms the local veterinarian collected individual rectal samples from cows, heifers, and calves, and group faecal samples from bulls over one year of age in pens. The animals were monitored by means of repeated sampling; one farm was sampled four times, three farms five times, and one farm eight times.

4.2.2 Surface samples (IV-V)

Carcass surfaces (IV, V) were swabbed after stunning, dehiding, and evisceration of cattle using three sterile 7.5×7.5 cm gauze pads wetted with sterile BPW. The sampling area comprised the brisket, the inner and outer sides of the thigh, and the pelvic cavity.

Environmental samples (IV, V) were taken from barn surfaces (feed passages, water bowls, and floors of pens) using similar sterile pads wetted with BPW as used for taking caecass surface samples. The whole feed passage outside the respective pens was swabbed, the inside of the water bowl and 2-3 10×20 cm areas of the pen floor.

4.2.3 Meat, milk, feed and slurry samples (IV-V)

A total of 421 reindeer meat samples were taken at reindeer slaughterhouses at the same time as the faecal samples (paper III). A sample from a milk tank was taken from four farms (paper V). Samples from feed and slurry were taken in studies IV and V.

4.3 Description of the farms (IV, V)

4.3.1 Finishing unit (IV)

The farm studied was situated in southern Finland, and the herd comprised about 300 cattle. Approximately three-month-old uncastrated bull calves arrived on the farm after weaning, mainly in lots from dairy farms. The pens in the calf department were routinely cleaned with a high pressure cleaner the day before arrival of a new lot. The bull calves in this study were delivered by the slaughterhouse organisation and first housed in a separate calf department containing pens for four animals before they were moved later to bigger pens for seven to eight animals. When the new calves arrived there were always some animals from the previous group still in the calf department. There were no empty periods between the groups. The animals were housed indoors throughout the year and were slaughtered at the age of 14 to 17 months. The pens had a slatted concrete floor, and manure and urine were removed by mechanical scrapes. Slurry was stored in tanks outdoors, treated by aeration and applied to fields with a slurry injector in the spring. The animals received concentrated feed twice daily, and the maximum portion was 2.8 kg. The concentrated feed comprised barley (2 kg), turnip rape (0.6 kg) and dried mash (0.2 kg). Calcium, minerals and salt were the only feed additives given. The animals were treated with antimicrobial agents only if there was a veterinary medical indication. No other medications were given. Predried grass silage (1,000 kg) was given to the herd at each feeding, along with concentrated feed fresh mash (1,000 kg).
4.3.2 Dairy farms (V)

The farms studied had dairy cows, calves and young cattle as the sole farm animal species, and no new animals were introduced during the follow-up period after each human case. All animals at each farm were housed in a single building.

4.4 Microbiological methods (I-V)

4.4.1 Isolation and identification of *E. coli* O157 using enrichment (I-V)

Samples were analysed according previously published methods (Bolton et al. 1995; ISO 2001). Sausage samples of 25 g were added to 225 ml prewarmed (37°C) modified tryptone soya broth (MTSBn) (tryptone soya broth, Oxoid CM129B; Oxoid, Basingstoke, UK) with 20 mg/ml Na-novobiocin (Sigma N1628, St. Louis, USA), and incubated at 41.5°C for 6 h. Sausage samples that had been frozen (-70°C) were thawed and analyzed by enrichment at 41.5°C for 6 and 24 h. Immunomagnetic separation (IMS) was performed using Dynabeads® anti-*E. coli* O157 (Dynabeads anti-*E. coli* O157, Dynal A.S., Oslo, Norway) according to the manufacturer's instructions. Fifty μl of the IMS-complex was spread onto sorbitol MacConkey agar plates without (SMAC; Lab161, LabM) or with cefixime-tellurite supplement (CT-SMAC; Supplement X161, LabM). Plates were incubated overnight at 37°C. Sorbitol-negative colonies were tested for the production of β-glucuronidase and indole, and the fermentation of raffinose and dulcitol by standard techniques. The colonies were further identified as *E. coli* using API 20E (bioMérieux, Marcy l'Etoile, France). Isolation of *E. coli* O157 from cattle (study II, IV, V) and reindeer faeces (study III) was performed as described above, except that a sample size of one gram was used. Cattle faecal samples taken at abattoir in study IV were also analysed using a sample size of 10 g. Rumen (study IV), feed (study IV) and milk (study V) samples were analysed using a sample size of 25 g.

*E. coli* O157 isolates from human mixed faecal cultures were received from hospital laboratories. All cultures were investigated for *stx*₁, *stx*₂, and *aeae* genes at the National Public Health Institute (KTL) as described by (Keskimäki et al. 1998). The O157 isolates were identified biochemically by API20E (bioMérieux, France).

The isolation method based on ISO 16654:2001 has been validated using spiked minced meat, milk and faecal samples and accredited by the Finnish accreditation body FINAS.

Study III: Spiked faecal and meat samples with a control *E. coli* O157:H7 strain (ATCC 43888) were analysed once a month to control the procedure. The method used could detect less than 10 cfu/g in faeces and 10 cfu per meat sample in the validation analyses (data not shown). The validation analyses were performed as part of the quality control of the accredited method ISO16654:2001. A control *E. coli* O157:H7 strain (ATCC 43888) was used each time solid agar plates were used.

4.4.2 Enumeration of *E. coli* O157 (I, IV)

For enumeration of *E. coli* O157:H7 from sausage (study I), a sample of 10 g was homogenised in a stomacher with 90 ml of a diluent containing 0.1% peptone in 0.85% saline. Tenfold dilutions of the samples were further serially diluted with peptone saline solution. *E. coli* O157:H7 was enumerated according to NMKL method No. 125 (NMKL 1996), modified by using an incubation temperature of 41.5°C. In a poured plate technique TSA was used as a base layer which was pre-incubated at 22-24°C for 1 h before addition of violet-red-bile agar medium (VRB; LAB 31, LabM) as a top layer.

Study IV: Quantitative determination by the most-probable-number (MPN) method was
performed for 14 faecal samples that yielded *E. coli* O157 after enrichment in 1-g samples from the abattoir and for 25 randomly chosen faecal samples taken at farm in July, September, and October. One gram of faecal material was added into each of five tubes containing 9 ml of MTSBn. Each sample tube was diluted 1 to $10^8$, then 1-ml portions from each dilution level were added to five tubes with MTSBn, and the analysis was continued as described above for faecal samples. The MPN was determined using a protocol involving the sums of positive and negative tubes (Peeler et al. 1992).

4.4.3 Isolation and enumeration of lactic acid bacteria, staphylococci and *E. coli* (I)

Lactic acid bacteria were quantitated on de Man-Rogosa-Sharp agar (MRS; Lab 93, LabM) with sorbic acid (97% sorbic acid; Fluka 85510, Buchs, Switzerland) according to NMKL Method No. 140 (NMKL 1991), and staphylococci on blood agar overnight at 37°C. Enumeration of *E. coli* from meat raw material was performed as described for enumeration of *E. coli* O157.

4.4.4 Detection of Stx production and the enterohaemolytic phenotype

Study II, V: Stx production in the isolates was determined by the reverse passive latex agglutination test (VTec-RPLA, Oxoid) with and without polymyxin extraction. In study V the isolates were only tested without polymyxin extraction: the human isolates with the Denka Seiken test kit (Denka Seiken Co. Ltd, Tokyo, Japan) and the animal isolates with VTec-RPLA test.

The polymyxin extraction was performed as described (Karmali et al. 1985a). Briefly, the isolates were inoculated into 20 ml of Penassay broth (Antibiotic Medium 3, Difco 0243-17-8, Difco, Detroit, Michigan, USA) and incubated at 37°C for 4-5 h. The broth culture was centrifuged at 10,000 g for 5 min, and the bacterial pellet washed twice in phosphate buffered saline (PBS; Dulbecco A, Oxoid BR14a). The pellet was resuspended in 1 ml of PBS containing 0.1 mg/ml of polymyxin B sulphate (Sigma P-1004) and incubated in a waterbath at 37°C for 30 min. After centrifugation at 10,000 g for 10 min the supernatant was filtered through a 0.22 μm membrane filter (Millipore Corp., Molsheim, France). When testing without polymyxin B extraction the isolates were inoculated into CA-YE broth as described (Evans et al. 1973) and incubated at 37°C for 18-20 h with shaking, then centrifuged at 4,000 rpm at 4°C for 20 min. The filtrates were analysed according to manufacturer’s instructions in the RPLA, which enabled detection of toxin titres to a dilution of 1:128. *E. coli* ATCC 43894 served as positive and *E. coli* ATCC 43888 as negative control.

The enterohaemolytic phenotype was detected on plates containing defibrinated, washed sheep blood and CaCl₂ as described previously (Beutin et al. 1989). After overnight incubation at 37°C, the enterohaemolytic phenotype was detected by the occurrence of small, turbid zones of haemolysis around the colonies. A blood agar with unwashed sheep blood was used as a comparison plate.

4.5 Measurement of pH and a$_w$ (I)

pH values were measured using a WTW pH 537 meter (WTW, Weilheim, Germany) equipped with an Ingold LoT 406-M 6-DXK-ST/25 electrode. a$_w$ values were determined using a Rotronic Hygrokop (Rotronic, Bassersdorf, Switzerland).

4.6 Serotyping (I-V)

Serotyping of the O157 and H7 antigens was performed using slide agglutination tests (Denka Seiken, Tokyo, Japan). To exclude false positive O157 agglutination, every isolate showing a positive agglutination was suspended in saline and boiled at 100°C for one hour.
Thereafter the agglutination test was repeated. Strains, which were nonmotile after three passages through semi-solid agar, were defined as nonmotile (NM).

4.7 Phage typing (V)

Phage typing of the animal and human isolates of *E. coli* O157 was performed as described previously (Ahmed et al. 1987; Khakhria et al. 1990; Saari et al. 2001) at KTL.

4.8 PCR (II, IV, V)

For PCR, a loopful of *E. coli* O157 culture was taken from the agar medium (CT-SMAC), suspended in 0.5 ml of sterile distilled water and boiled for 20 minutes. The *stx₁* and *stx₂* sequences were detected using a primer pair and amplification procedure with the modification of 25 cycles (Olsvik and Strockbine 1993). The *eae* gene was amplified using a specific primer pair (Heuvelink et al. 1995). The PCR reaction was performed using the following program: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min, and then final extension at 72°C for 10 min. The oligonucleotides used as primers were purchased from Pharmacia Biotech (Uppsala, Sweden).

4.9 PFGE (II, IV, V)

The PFGE were grown essentially as described (Maslow et al. 1993). Briefly, cells were grown in Trypticase Soy Broth (TSB; BBL Cat No.11768, Becton Dickinson and Company, Cockeysville, Maryland, USA) overnight at 37°C and washed cells were embedded in 1.6% (w/v) low melting temperature agarose (In Cert agarose, FMC Bioproducts, BioWhittaker Molecular Applications, Rockland, Maine, USA). Cell-lysis was carried out overnight in ESP (0.5 M EDTA, 10% sodium lauryl sarcosine, 0.5 mg/ml proteinase K). Agarose plugs were carefully washed with 1x TE (10 mM Tris, 1 mM EDTA), and DNA in agarose was cleaved with 10 U of the restriction endonuclease XbaI (New England Biolabs, Beverly, Massachusetts, USA) at 37°C for 4 h. The CHEF-DR III Variable Angle Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Hercules, California, USA) was used to perform electrophoresis. The samples were electrophoresed through 1.2% (w/v) agarose gel (SeaKem Gold, FMC Bioproducts) in 0.5 x TBE buffer (89 mM Tris-borate 2.0 mM EDTA, pH 8.3). The separation parameters were as follows: 200 V, 120°, 14°C, switch times 5-40 s and running time 20 h. Low-Range PFGE marker (New England Biolabs) was used as the fragment size marker. The gels were stained for 45 min with ethidium bromide (0.5 μg/ml in water), destained for at least 2 h in water, and photographed under UV illumination. Patterns with at least a single band difference over the 48 kb area were classified as different subtypes. Each PFGE pattern was given a number within the serotype, e.g. O157:H7/H⁻: 1.1, 1.2, 1.3 etc. Analysis of the PFGE patterns was performed with the BioNumerics program, version 1.01 (Applied Maths, Kortrijk, Belgium). The band patterns were compared using Dice's index (Dice 1945). The optimisation was 0.50% and tolerance 1.0-1.1%. The PFGE profiles were compared using a national standard protocol based on a shared computer-based network (Rantala et al. 2000).

4.10 Statistical methods (I-IV)

Study I: The rate of decrease in numbers of *E. coli* O157:H7 and the difference of rates in different treatments were estimated using a linear regression model of log₁₀ cfu on the number of days of fermentation and drying. The probability of finding *E. coli* O157:H7 after freezing was modelled using logistic regression on the log₁₀ cfu concentration of the sample. The enrichment times of 6 h and 24 h were compared. The analyses were made using the R statistical program (Ihaka and Gentleman 1996).
Study II: The probability of an occurrence in each group was estimated using observed frequencies in the groups. The 95% confidence intervals for each probability were calculated using the likelihood function obtained from the binomial probability distribution and its asymptotic chi square distribution (so-called profile likelihood method) (Aitkin et al. 1989). The statistical significance of the difference in the probabilities was investigated using a logistic regression model. Odds ratios (OR) and their confidence intervals (CI) were also obtained from the logistic regression analysis (Hosmer and Lemeshow 1989).

Study IV: Enrichment times of 6 h and 24 h were compared, as were the 1-g and 10-g sample sizes, using McNemar’s test. The quantitative counts of *E. coli* O157 for the faecal samples originating at abattoir or farm were compared. The analyses were performed with the SAS statistical program version 8.0 (SAS Institute, Cary, NC, USA).
5 RESULTS

5.1 Modification of the isolation method for E. coli O157 (I, IV)

When sausage sub-samples containing low numbers (< 3.2 log_{10} units/g) of E. coli O157:H7 were analysed by the enrichment procedure after freezing and thawing, E. coli O157:H7 was more often detected after the 24-hour than after the 6-hour enrichment period (OR = 5.105, CI 95% 2.71-7.50; P = 0.0044) (Table 2, paper I). The sub-samples were stored in the freezer for several months; E. coli O157 was isolated after 24 h enrichment from 20/23 sub-samples containing less than 10 cfu/g. On the contrary, when farm surface samples taken from the finishing unit were analysed, E. coli O157 was more often isolated after the 6-h than the 24-h enrichment (p<0.0001), except in two instances (study IV).

When samples from faecal material taken at the abattoir were analysed using sample sizes of both one and ten grams, E. coli O157 was more often isolated from the 10 g than the 1 g faecal samples (p<0.0001).

5.2 Survival of E. coli O157:H7 during the manufacture, storage and freezing of fermented sausage (I)

The counts of E. coli O157:H7 decreased more using a starter culture containing S. carnosus and L. curvatus (Müller RM 52) than a starter culture containing S. xylosus, P. acidilactici and L. bavaricus (Flora Carn LC) at all the inoculation levels (first experiment P < 0.0015, second experiment P < 0.0002). With Flora Carn LC, fermentation and storage for 49 days reduced the count of E. coli O157:H7 in sausages with an inoculum of log_{10} 5.4-6.58 cfu/g by log_{10} 2.79 cfu/g (SD 0.48). The reduction with Müller RM 52, however, was log_{10} 4.84 cfu/g (SD 0.71), resulting in a concentration of log_{10} 1.0 cfu/g. E. coli O157:H7 was still detected by enrichment from the sausages throughout the 49 days of manufacture, except on day 21. The pH values of the sausages were 4.39-4.63 in the two experiments. E. coli O157:H7 was isolated from all except two samples after freezing.

5.3 E. coli O157 in cattle faeces, rumen contents and on carcass surfaces at slaughter (II, IV, V)

E. coli O157 was isolated from 19 (1.31%; 95% CI, 0.73-1.90%) of 1448 1-g cattle faecal samples. The isolates originated from 16 farms and eight abattoirs, and three of the farms had two positive samples. The percentage of positive isolates per abattoir varied from 0 to 6.93%. Of the sampled 1448 animals, 1258 (86.9%) could be traced to 1108 farms. The animals excreting E. coli O157 were located mainly in southern areas of the country. No isolates were found from cattle from the far north (Province of Lapland). Thus, the prevalence of E. coli O157 in southern Finland (Provinces of Western, Eastern and Southern Finland) was higher than in the north (Provinces of Oulu and Lapland) (OR=3.6; 95% CI, 0.86-15.3; 90% CI, 1.08-12.11; p=0.08). More positive isolations were obtained during the warm months of June to September (18 of 19 findings) (OR=14.2; 95% CI 2.0-98.9; p=0.007) than in October to December.

Samples from faecal material, rumen contents and carcass surfaces were taken from 38 bulls of group 1, 18 of group 2 and from all 31 animals of group 3 (study V). E. coli O157 was found most often from faecal samples in group 2 (38.9%) and least often in group 3 (9.7%). E. coli O157 was isolated from nine of 11 faecal samples taken in August and from 10 of 41 samples taken in June, whereas only one of the 27 samples taken from October to January was positive. Two rumen samples were positive, one in May and the other in August. Both animals
with positive rumen samples also had positive faecal samples. Only one carcass surface sample was positive (group 2, August). In the study of the association of human cases to dairy farms, *E. coli* O157 was not isolated from any of the carcass surface samples (n=64) taken from cattle of two farms (farm 2 and 4, V).

**5.4 *E. coli* O157 in reindeer faeces and meat at slaughter (III)**

*E. coli* O157 was not detected from the 1387 faecal one-gram and 421 meat 25-gram samples. A total of 925 (66.7%) of the faecal and 164 (61.0%) of the meat samples were stored in the freezer before analysis. The freezing of faecal samples did not weaken the isolation of *E. coli* O157:H7 according to the freezing tolerance test. The prevalence of *E. coli* O157 in Finnish reindeer faeces was thus less than 0.22% (95% CI).

**5.5 Cattle as a source of transmission of *E. coli* O157 for humans (V)**

Five human patients (age 2 to 17 y, median 3 y) were hospitalised with bloody diarrhoea; two of them developed HUS. Two of the patients were dairy farm residents; three lived in the neighbourhood of a dairy farm or often visited a dairy farm. *E. coli* O157:H7 was isolated from the stools of the five hospitalised cases and four family members of the third case. In the other families no-one else developed the disease, or had *E. coli* O157 isolated from their faecal specimens. None of the persons who mainly took care of the animals developed disease, and the stools of the farmers sampled cultured negative for *E. coli* O157. The origin of the infection could be traced to five dairy farms. *E. coli* O157 was isolated from all five farms five to 16 days after the first human faecal specimen was taken.

At dairy farms 2, 3, and 5 several cattle (14 of 18, 11 of 68 and 17 of 45, respectively) were excreting *E. coli* O157 during the first sampling, whereas only one eight-month-old bull calf of the 31 cattle tested on farm 1 yielded the pathogen. On farm 4, *E. coli* O157:H7 was isolated from two pooled faecal samples taken soon after the human case. On three farms *E. coli* O157:H7 was also isolated during the subsequent samplings: from farm 1 eleven months after the first sampling, from farm 2 one month after, and from farm 3 two months and twelve months after the first sampling. On farm 5, SF *E. coli* O157:H- was isolated from two cattle 10 months after the human case. *E. coli* O157:H7 of human and corresponding cattle isolates from the farms were indistinguishable using phage typing and PFGE genotyping.

**5.6 *E. coli* O157 at a cattle finishing unit (IV)**

Of the 688 faecal samples taken at the cattle finishing unit during the study period of May 1999 to May 2000, 110 (16%) were positive for *E. coli* O157. All bull calves in groups 1, 3 and 4 sampled in the livestock transporter were negative for *E. coli* O157 on arrival at the farm, whereas six bull calves (28.6%) in group 2 sampled one day after their arrival were positive. During the fattening period the *E. coli* O157 infection rate in groups 1, 3 and 4 varied from 10.3 to 38.5%, from 9.68 to 25.81% and from 0 to 23.81%, respectively. No seasonal variation in the shedding of *E. coli* O157 was found at farm among any of the groups studied. At the times of the peak shedding rates the mean ages of the bulls was 8.24 months (SD 1.18) for group 1, 6.01 months (SD 0.77) and 9.17 months (SD 0.77) for group 3, and 6.86 months (SD 0.56) for group 4. At seven, six and four sampling times for bulls in group 1, 3, and 4, respectively, 76.9, 58.1 and 26.2% of the animals, respectively, were shedding *E. coli* O157 at least once.

**5.7 Detection of *E. coli* O157 from barn floor, feed passage, water cups and water nipples (IV, V)**

Of the 203 barn surface samples taken at the finishing unit (IV), 49 (24.6%) yielded *E. coli* O157. Of the 203 barn surface samples taken at the finishing unit (IV), 49 (24.6%) yielded *E.
coli O157. Nine to 21 surface samples were taken during each session. *E. coli* O157 was isolated from samples taken from water cups (18 of 74 samples; 24.3%), floor (8/10; 80%) and feed passage (24/65; 36.9%), but not from water nipples (0/51). In December 13/20 samples were positive, and the percentage of positive barn surface samples varied from 0 to 55% for the different sampling times (Fig. 3., paper IV). In study V, by contrast, none of the barn surface (n=164), feed (n=15) or milk (n=4) samples taken at the dairy farms yielded *E. coli* O157 (V).

5.8 Enumeration of *E. coli* O157 from faecal samples using the MPN method (IV)

Five abattoir and two farm samples had MPN below the detection limit (<0.2/g), while the highest MPN exceeded 1.6×10^6 (Table 2, paper IV). Low counts (≤0.4 MPN/g) were more often encountered in the samples from the abattoir than the farm (OR=7.00, CI 95% 1.13-50.76, p=0.02).

5.9 Characteristics of *E. coli* O157 isolates from cattle and humans (II, IV, V)

Most *E. coli* O157 isolates in our studies carried stx2 only: 16/19 isolates in study II, all nine human and 55/58 cattle isolates in study V, and all but two cattle isolates in study IV. One isolate in study II and two cattle faecal isolates in study IV carried both the stx1 and stx2. Two isolates in study II, and three cattle isolates in study IV did not harbour stx genes. All of the isolates (study II, IV) and all but two cattle isolates (IV) harboured the eae gene. Enterohaemolysin was expressed by all isolates in study II and IV and all but three cattle isolates (V).

Of the 17 isolates harbouring stx, 16 were Stx-positive in RPLA when tested by polymyxin extraction, but only eight (47.1%) isolates were Stx positive when tested without extraction. In all strains the toxin titres were raised with polymyxin extraction. All the isolates possessing stx2 also produced it.

Four of the isolates did not produce indole despite repeated tests by two methods. Eighteen of the 19 isolates in the prevalence study and all of the isolates associated with the human illnesses were motile and agglutinated by H7 antiserum (study II, V). Six of the 134 (4.5%) faecal isolates, three of 25 (12%) barn surface isolates and one of the two rumen isolates were nonmotile. SF O157:H- were isolated from two cattle from farm 5, and they lacked the analysed virulence genes (stx, eae) and did not produce enterohaemolysin (paper V).

5.10 Phage (PT) and PFGE types of *E. coli* O157 isolates (II, IV, V)

A combination of phage typing and PFGE genotyping was used in the comparison of human and cattle strains in study V. The phage types (PTs) and PFGE genotypes of all the human and cattle isolates from the dairy farms suspected to be the sources of the infections were indistinguishable, regardless of when the specimens were taken from the animals (V). At one of the farms (farm 5, study V), the human isolate and 16 of 17 cattle isolates collected temporally close to the time human case was identified all belonged to PT4 and PFGE genotype 1.15, except one of the PT4 cattle isolates, which was of a closely related PFGE genotype 1.37. PFGE analysis of two later isolates from farm 5 was not successful because their DNA was degraded even on repeat analysis. Their PT was rdnc8 (rdnc=reacts but does not conform), unlike the other isolates.

PFGE was used as a genotyping method in studies II, IV and V. A total of 18 PFGE genotypes were found in the studies II, IV and V: 1.1, 1.1*, 1.2, 1.3, 1.6, 1.7, 1.12, 1.14, 1.15,
1.24, 1.37, 1.42, 1.43, 1.44, 1.45, 1.62, 1.63, and 1.65. In the prevalence study of slaughter cattle (II) ten different PFGE genotypes were found among 19 E. coli O157 isolates, the genotype 1.42 being the most prevalent. Identical or closely related PFGE genotypes were detected from three farms with two positive samples (farm G 1.42; farm I 1.42 and 1.43; farm M 1.1 and 1.6). The indole negative isolates (n=4) belonged to two closely related PFGE banding patterns (1.1 and 1.6).

Of the 113 faecal isolates from calves at the finishing unit (study IV); 90 (79.6%) belonged to PFGE genotype 1.1, 17 (14.9%) to PFGE genotype 1.1*, 5 (4.4%) belonged to PFGE genotype 1.45 and 1 (0.88%) belonged to PFGE genotype 1.65. All of the PFGE genotypes of the farm faecal isolates were closely related. A PFGE genotype 1.62 isolate clearly differing from the other genotypes was isolated from the rumen contents and faecal sample of the same bull. A PFGE genotype 1.63 isolate was detected in a sample from the rumen contents of a bull whose faecal isolate was a genotype 1.1 isolate. Bulls that excreted E. coli O157 more than once tended to have isolates with indistinguishable PFGE genotypes from repeated samplings, although a closely related genotype was detected at least once for eight of 30 bulls with more than one positive isolate. The farm surface isolates were PFGE genotypes 1.1, 1.1*, and 1.45. A mean of 3.02 colonies (SD 1.09) was analysed from each positive farm surface sample. In six of 49 cases (12.2%) two different PFGE genotypes were identified from the farm surface sample.

In the study V, PFGE genotype 1.1 was detected on three of the five farms, PFGE genotype 1.3 on one farm and PFGE genotypes 1.15 and 1.37 on one farm. PFGE genotype 1.37 was detected in only one animal.
6 DISCUSSION

6.1 Modification of the isolation method of *E. coli* O157 (I, IV)

The earliest studies used direct culture without enrichment on a selective medium (Hancock et al. 1994), and some studies have not used IMS (van Donkersgoed et al. 2001). Using 10 g samples, enrichment and IMS before selective culture have been shown to increase the sensitivity of the method several-fold (Chapman et al. 1994; Zhao et al. 1995; Sanderson et al. 1995; Besser et al. 1997; de Boer and Heuvelink 2000). Our findings also revealed that a sample size of 10 grams gave significantly more positives than 1 g (study IV), but bigger sizes such as 20 or 25 g might be more sensitive. Sample sizes of 20-25 g might be difficult to obtain from small calves but not from older animals. In our other studies a sample size of 1 g was used, and other researchers have used sample sizes of swab (0.1 g) to 1, 2, 5, 10, 20 or 25 g.

Freezing samples at -20°C before isolation decreases the numbers of viable cells (Chapman et al. 2001c; Raccach et al. 2002). Freezing faecal samples at -70°C resulted in 80% and 58% recovery of *E. coli* O157:H7 (McDonough et al. 2000). However, only small losses were observed in cell numbers of *E. coli* O157:H7 inoculated in ground beef stored at -20°C and 1°C and occurred within the first day of storage (Barkocy-Gallagher et al. 2002). In our study *E. coli* O157:H7 was detected from frozen fermented sausage, but an enrichment time longer than 6 h was needed to detect the stressed cells (paper I). The optimal length of enrichment depends on the competing microflora, the matrix and whether the cells are injured or not. Stressed, heat-, freeze- or acid-injured cells need a longer enrichment. Samples subjected to cold stress at 4°C for 14 days required about 3.5 h longer enrichment than those enriched immediately after inoculation (Uyttendaele et al. 1998). In highly contaminated samples the shorter enrichment time finds more positives because the bacterium grows rapidly and contaminants are suppressed. When we analyzed faecal samples, more positives have been obtained using 6 h enrichment (data not shown). The enrichment time of 6 h was also superior to 24 h in the analysis of barn surface samples (paper IV). Consistent with our results, enrichment for 6 h was superior to 24 h when analysing bovine faecal samples (Tutu nel et al. 2003). We analysed the samples according to the ISO method but other isolation procedures have also been described. MTSBn incubated at 42°C for 6 h followed by IMS and subculture to CT-SMAC was the most sensitive and selective procedure (Bolton et al. 1996).

6.2 Survival of *E. coli* O157:H7 during the manufacture, storage and freezing of fermented sausage (I)

The counts of *E. coli* O157:H7, an isolate from a patient with HUS, decreased during the manufacture but the pathogen was not totally eliminated. The only recommended regulation for sausage manufacture related to *E. coli* O157 is the one given by the US Food Safety and Inspection Service (FSIS), which states that sausage manufacturing processes should lead to a 5 log_{10} unit reduction in populations of *E. coli* O157:H7. The European Union has no regulations related to processing requirements and STEC.

A large variety in the manufacturing processes of dry sausage exists. Our process included a five-day fermentation at 20-23°C. In our study 5 log_{10} unit reduction in counts of *E. coli* O157:H7 was obtained only using a starter that contained *S. chamuscis* and *L. curvatus*, and with fermentation and drying for 49 days. Fermentation and two days of drying reduced the counts of *E. coli* O157:H7 by 1.25-3.75 log_{10} units. Survival of *E. coli* O157:H7 has also been studied in dry sausages manufactured using a shorter fermentation period (about 12 h) at a
higher temperature (37-38°C), before a drying period of 12-21 days at 13°C (Glass et al. 1992; Hinkens et al. 1996; Faith et al. 1997; Riordan et al. 1998; Faith et al. 1998a). In other inoculation studies, reductions of 1-2 log_{10} units have been achieved (Glass et al. 1992; Hinkens et al. 1996; Caliçoglu et al. 1997; Faith et al. 1997; Riordan et al. 1998; Doores et al. 1998; Faith et al. 1998b). When 3.56-4.54 log_{10} units of _E. coli_ O157:H7 were inoculated to sliced salami, the product was stored at 5 and 20°C for 32 days, the counts were reduced by 2-5 log_{10} units during the storage (Clavero and Beuchat 1996). In these studies the pathogen counts decreased during fermentation by 0.5-1.5 log_{10} units using a standard sausage formulation (Faith et al. 1998b) fermented salami for 60-64 h at 24°C and dried it for 21 days at 13°C, which reduced the counts by 1.1-2.1 log_{10} units. In a bioluminescent model system representing fermented sausage production, _E. coli_ O157:H7 was eliminated more rapidly at a lower temperature (22°C) and with longer fermentation (3 days) than at higher temperature (37°C) with shorter (1 day) fermentation (Tomicka et al. 1997).

The results of our study suggest that significant differences exist in the reduction rates of pathogens when different starter cultures are used. A starter containing _S. carnosus_ and _L. curvatus_ induced a greater reduction in the counts of _E. coli_ O157:H7 than starter containing _S. xylosus, P. acidilactici_ and _L. bavaricus_. The reduction was at least twofold than reported in previous studies in which _P. acidilactici_ or a pediococcal starter culture was used. The reasons for the disparity between the starters remain unclear, although the starter containing _S. carnosus_ and _L. curvatus_ caused a slightly greater pH reduction than the other starter. Further studies would be needed to elucidate the true difference between different starters.

In Finland, dry sausage manufacturers ferment and dry the sausage during at least 21 days before releasing the product to the market. In our study _E. coli_ O157:H7 was detectable in sausages after 21 days, either in direct culture or by enrichment. According to these results a ripening period longer than 21 days would be needed to ensure consumer safety.

The pathogen counts inoculated in our study as well as in most other experiments have been higher than those found in naturally contaminated products (Tilden et al. 1996; Paton et al. 1996; Tuttle et al. 1999). Lower bacterial counts might, however, be completely eliminated from the sausage if the manufacturing time is long enough to ensure sufficient decrease in pH and aw. However, the infectious dose of _E. coli_ O157:H7 may be less than 50 cells, the pathogen may be distributed unevenly in the sausage, and cross-contamination may occur during manufacturing thus increasing the need for a high safety margin. In conclusion, _E. coli_ O157:H7 should not be present either in meat, in the processing environment, or in sausage, which places high demands on the HACCP procedure of the fermentation process.

Several meat treatment processes, which decrease the initial contamination, have been proposed, such as carcass washing and irradiation. However, these unfortunately increase costs and provide no protection against cross-contamination. Freezing, thawing and refreezing reduced the counts of _E. coli_ O157:H7 by 1-2 log_{10} units (Faith et al. 1998b), and storage at ambient temperature was more effective than storage at a refrigeration temperature of 4°C (Faith et al. 1997; Faith et al. 1998a; Caliçoglu et al. 2001; Caliçoglu et al. 2002). In our study, _E. coli_ O157:H7 was isolated from all except two samples after freezing. Quantitative analysis should have been performed after freezing in order to obtain some data on the reduction rate during storage in freezer. The drying and storage temperature in our study (15-17°C) was somewhat lower than room temperature, and may be one of the reasons for the relative rapid death rate found in our studies. However, long-term storage at ambient temperatures may cause undesirable sensory and quality changes in the sausage. Post-fermentation heating has reduced the counts of _E. coli_ O157:H7 by more than 5 log_{10} units (Hinkens et al. 1996; Caliçoglu et al. 1997; Doores et al. 1998; Getty et al. 1999). Heating may also cause undesirable changes in the texture and taste of the sausage and can
thus not be considered as a suitable method for pathogen control. Key factors are still good hygiene at the farm level and during the slaughtering process in order to prevent pathogens from entering the meat (Duffy et al. 2000).

6.3 *E. coli* O157 in cattle faeces, rumen contents and carcass surfaces at slaughter (II, IV, V)

In the prevalence study (paper II), *E. coli* O157:H7/NM was isolated from 19 of 1448 (1.31%) cattle faecal samples at eight of the 14 investigated abattoirs. The highest occurrence (6.93%) was observed at an abattoir in the Province of Southern Finland (Table 1, study II). Most studies have been performed at one abattoir, but studies performed in Belgium, the Netherlands, the UK and in Turkey have given national prevalences of 4.2-11% (Heuvelink et al. 1998a; Gun et al. 2001; Tutenel et al. 2002; Paiba et al. 2002; Yilmaz et al. 2002). A faecal shedding prevalence of up to 28% was observed at four Midwestern abattoirs in peak shedding season (Elder et al. 2000), but a lower prevalence than in our study was observed in Poland (Tutenel et al. 2002). The prevalence in our study would probably have been higher if a bigger than 1-g sample size had been used, since a high percentage of the animals from the finishing unit were found shedding at slaughter: 31.6% with the 1-gram sample size and 55.3% with the 10-gram size (paper IV). Results from different studies are difficult to compare because of differences in study design, sampling site (farm/abattoir), number of farms, geographical representativeness, age of animals (adult/young), sampling season and microbiological methods. These studies suggest that contamination rate of cattle vary between countries, which might be caused by differences in farm management strategies, feeding regimes, climate or other factors.

In the prevalence study, the 19 isolates from cattle faeces originated from 16 farms, mostly located in southern Finland. None were detected at the abattoir located in the northern-most region in Finland (Province of Lapland), and only two positive samples were found in the southern region of the Province of Oulu (Fig. 1, paper II). The percentage of positive faecal samples per abattoir varied from zero to 6.93, which suggests an uneven distribution of *E. coli* O157 across the country. Northern parts of the country have a shorter summer period, colder climate and lower animal densities, which might be reasons for the possible difference. In a Dutch study, cattle originating from farms located in the western region seemed less often infected than cattle from elsewhere, which was thought to be influenced by the smaller number of animals originating from the west delivered for slaughter (Heuvelink et al. 1998a). The total number of the faecal samples in the Dutch study was rather low, which might make it difficult to analyse the result. The prevalence of faecal carriage of *E. coli* O157 in the eastern region of the UK was significantly higher than in the other regions, which was speculated to be caused by longer transportation journeys (Paiba et al. 2002). However, since the number of positive isolates in our study was small, conclusions from the potential differences in the geographical distribution need further studies, although the reasons might be related to fewer animals or to climatic factors.

Shedding of *E. coli* O157:H7 is affected at least by age, diet and feeding, stress and seasonal effects. At slaughter most positive *E. coli* O157 samples were detected in July (3.85%) and September (2.46%) in the prevalence study (paper II), and in August in the study of cattle finishing unit (paper IV). Although our data is limited on annuality as the study was performed in June-December, it gives some knowledge on the difference in shedding between summer and autumn. In the studies II and IV the shedding rate at slaughter seemed to be higher during the warmer months. In other abattoir studies the prevalence has been higher during summer than winter (Chapman et al. 1997; van Donkersgoed et al. 1999). The peak in human infections has also been reported to be in the summer period (Griffin and Tauxe 1991). The reason for seasonality in shedding at slaughter remains to be clarified.
E. coli O157:H7 is more frequently carried by calves and heifers than by adult animals (Wells et al. 1991; Zhao et al. 1995). In vitro studies have shown that the growth of E. coli was more inhibited by rumen fluid collected from well-fed animals than from cattle fasted for 24-48 h (Rasmussen et al. 1993). However, fasting may have little impact on faecal shedding once the colon is colonised (Harmon et al. 1999). Withholding feed prior to inoculation increased the shedding of E. coli O157:H7 in bovine calves (Cray et al. 1998). In the present study the adult animals were studied, and they may have been subjected to dietary stress before slaughter, thus possibly increasing the faecal excretion of E. coli O157. In a US study shedding at slaughter was not higher than on farm two weeks earlier (Barham et al. 2002).

Carcass surfaces were seldom found to be contaminated; only one positive carcass swab sample was detected. The animals were very clean on visible ante-mortem inspection and their hides were already dry and clean at the farm. In a US study much higher percentages of carcasses were positive for E. coli O157, and they seemed to be contaminated by neighbouring carcasses (Elder et al. 2000). Cross-contamination was also observed in a British and Italian study (Bonardi et al. 2001; Small et al. 2002). Differences in slaughter hygiene and farm management, as well as sampling sites and techniques, may explain such disparities. The animals in the present study were housed indoors and not washed before slaughter; carcasses are minimally washed and not sprayed at Finnish abattoirs. These procedures might decrease cross-contamination between carcasses and survival of the bacterium in the farm environment.

6.4 E. coli O157 in reindeer faeces and meat at slaughter (III)

Wild animals, especially wild ruminants, have been proposed as a possible reservoir of STEC. The Finnish reindeer population appears not to be a reservoir of E. coli O157 since the prevalence of E. coli O157 was low (less than 0.22%). E. coli O157 has been isolated from 0.25-30% of deer faeces in studies performed mainly in the USA and the UK (Rice et al. 1995; Chapman and Ackroyd 1997; Keene et al. 1997b; Cody et al. 1999; Sargeant et al. 1999; Renter et al. 2001; Rice et al. 2003). E. coli O157:H7 was also isolated from a Pennsylvanian water reservoir around which deer were abundant and cattle absent (McGowan et al. 1989). Many of the studies on STEC in deer have been performed in cattle-raising areas, but some of them lack information on possible interspecies transmission. The isolates of deer and cattle sharing the same habitat on a Texas ranch were indistinguishable (Rice et al. 1995).

The prevalence of E. coli O157:H7 in cattle is highest in summer (Hancock et al. 1994) and in young animals (Wells et al. 1991), but it is not known whether this is also the case for wild animals. Our study was carried out in late autumn – winter, because this is the slaughtering period and thus the only practical time for taking larger numbers of samples from semi-domesticated reindeer. Reindeer are slaughtered seasonally from September to the beginning of March. The majority (66.7 per cent) of slaughtered reindeer are less than one year old (Paliskunnat 1999). Before entering the abattoir, reindeer have been transported over considerable distances and may thus have been subjected to dietary and other stress that could have increased the shedding of E. coli O157 and the possibility of detecting the organism in our study.

The reason that E. coli O157 was not isolated from reindeer could have been associated with low animal population densities or with less intensive feeding or other management factors compared to cattle farming. High cattle density has been associated with an elevated risk of STEC infection in a rural population (Michel et al. 1999). We did not isolate any strains of E. coli O157 from cattle raised and slaughtered in the reindeer-raising area in our prevalence
study (paper II). In 1997 the number of cattle in the major reindeer-raising area was 5538 animals, and in the whole area there were 60,250 animals (TIKE 1999). The number of live reindeer in the reindeer-raising area during the study period was 224,900 animals (Paliskunnat 1999). Densities of both cattle and reindeer are rather low in the whole reindeer-raising area. As reindeer are semi-domesticated animals and can move over large areas it is difficult to take samples from other animals sharing the same habitats in order to elucidate their possible role as reservoir. In summer and autumn, the diet of reindeer consists mainly of natural feedstuffs (sedge, grass, birch leaves, mushrooms). In winter, most reindeer receive supplementary feed (commercial feed, hay, silage) in addition to lichen.

6.5 Cattle as a source of transmission of *E. coli* O157 for humans (V)

Five children living on or regularly visiting dairy farms fell ill with bloody diarrhoea caused by *E. coli* O157:H7. Isolation of *E. coli* O157:H7 with indistinguishable phage and genotypes between children and the farm cattle, together with recent direct or indirect contact with bovines and consumption of unpasteurised milk from these farms, indicated that the infections in the children originated from the farms. *E. coli* O157:H7 was isolated from the faecal samples of cattle on farms even when the specimens were taken one to two weeks after the first positive human specimen. This indicates that *E. coli* O157:H7 may persist on farms for several weeks. A year later, however, *E. coli* O157 was only sporadically isolated from two of the cattle farms. On three of the farms, several animals were shedding the pathogen concurrently with the human cases, which might have increased the risk of exposure leading to infection. Our findings were consistent with a Canadian case report in which *E. coli* O157:H7 was isolated from 62% of cattle on a farm with a child with bloody diarrhoea (Jackson et al. 1998). In other reports the number of cattle shedding the pathogen at the time of human cases has varied from 1/180 cows (Crampin et al. 1999) or 4/132 cattle (Keene et al. 1997a) to higher numbers (33/216) of animals (Crump et al. 2002). In the latter study the true number of cattle shedding might have been higher because a small sample size (swab) was used and no enrichment or IMS. Comparing results between studies is complicated by differences in detection methods and sampling strategies.

All the farms in our study were ordinary, commercial dairy farms with a herd size close to the Finnish average. *E. coli* O157:H7 was isolated from the stools of dairy cattle on all five farms. The milk samples, negative for *E. coli* O157:H7, were taken once, 1-2 weeks after the onset of symptoms in the human cases. *E. coli* O157:H7 may have been present in milk only transiently. Most Finnish dairy farms are family managed and the consumption of unpasteurised milk is common among dairy farm dwellers and neighbouring households, as in case 1.

In general, most EHEC cases are sporadic, as were four of our cases without links to any other cases. In our family outbreak, the three-year-old boy probably acquired the infection from the barn and transmitted it by direct contact with his siblings and mother, although transmission via contaminated clothing, shoes or unpasteurised milk cannot be excluded. Except for one teenager (17 y), our cases were young children (2-6 y), compatible with children under five years old being the most susceptible to complicated disease (Griffin and Tauxe 1991).

None of the persons caring for the animals developed the disease or excreted the pathogen, which might be explained by protective acquired immunity. Childhood exposure to bovine Stx1 strains (Wilson et al. 1996) seems to protect from STEC infections, and farm residents have higher frequencies of O157 LPS and Stx1 antibodies than urban inhabitants (Reymond et al. 1996). In a cluster associated with consumption of unpasteurised goat milk the farm residents were asymptomatic (Bielaszewska et al. 1997), as in a family outbreak caused by
Stx1-producing O111:H-; in the latter case the persons had high levels of Stx1 antibodies (Karmali et al. 1994).

EHEC cases have been linked to visits to commercial (Borczyk et al. 1987; Trevena et al. 1996) and open farms (Renwick et al. 1993; Parry et al. 1995; Shukla et al. 1995; Milne et al. 1999; Pritchard et al. 2000). Contact and likely contact with animal faeces and exposure to the farming environment emerged as strong risk factors for sporadic E. coli O157 infections in prospective case-control studies in the UK (Locking et al. 2001; O'Brien et al. 2001). Living in rural communities and contact with animals emerged as risk factors for STEC in other studies performed in the UK, Canada, Germany and Austria (MacDonald et al. 1996; Parry and Salmon 1998; Michel et al. 1999; Verweyen et al. 1999). Farmers in a British study were not found to be at increased risk, but the risk among farmers’ children could not be assessed (O'Brien et al. 2001). Children living on farms or nearby risk acquiring EHEC infections, as indicated by our study. A recommendation not to give unpasteurised dairy products to children under five years old was made in France after a cluster of HUS cases due to unpasteurised cheese (Deschênes et al. 1996). Some countries, such as Sweden (Andersson et al. 2000), UK (HSE 1998) and USA (MMWR 2001), have produced guidelines for farm visits. However, our study suggests that guidelines may also need to be developed for children consuming farm products and living on farms.

6.6 E. coli O157 on meat-producing cattle farms

In our study the calves most probably acquired E. coli O157 infection at the farm, although the possibility of lorry surfaces being the source of the infection could not be ruled out as they were not sampled. In contrast to our findings, 3.8% of the calves entering an Italian heifer raising operation (13 of 341) were positive for E. coli O157 on arrival (Conedera et al. 2001), and 6.9% of calves in a US range beef herd were shedding E. coli O157 at weaning (Laegreid et al. 1999). The calves in our study were already weaned on arrival, and higher prevalences have been detected in calves after weaning which should have increased the faecal carriage (Hancock et al. 1994; Garber et al. 1995; Zhao et al. 1995). Most bull calves entering cattle finishing units in Finland arrive from dairy herds, with only a few new animals entering the farm, which might explain why the calves were not shedding on arrival. In a cohort study on dairy farms the animals first tested positive when moved from individual to group housing (Shere et al. 1998).

A total of 59 (52.2%) of all the bulls studied shed E. coli O157 at least once during the fattening period. Other longitudinal studies have also found high percentages of animals excreting the pathogen, e.g. a Czech study found 19.7% (72/365) (Cizek et al. 1999) and an Italian study 10.7% (138/1293) (Conedera et al. 2001) of cattle sampled with individual faecal samples to be positive for E. coli O157. In studies using pat samples a much lower percentage of positive samples has been found, ranging from 0.26 to 3.6% (Hancock et al. 1994; Hancock et al. 1997c; Herriott et al. 1998; Galland et al. 2001; van Donkersgoed et al. 2001). Pat sampling might underestimate the true herd prevalence because negative samples dilute the numbers of E. coli O157, and the survival of E. coli O157 may also decrease in pat samples. Comparison of results from different prevalence studies is also complicated by the variety of microbiological methods used and the differences in sample sizes.

A high percentage of the bulls were shedding at least once: 26-77% in groups 1, 3 and 4. The barn surfaces were continuously contaminated, and spreading of the infection was possible. Horizontal transmission has been observed in both experimental and natural settings (Kudva et al. 1995; Wray et al. 2000; Shere et al. 2002; Cobbold and DesMarchelier 2002). Although the other groups were not sampled as many times as group 1, it is likely that a higher percentage of shedders would have been noted in them, too, with more sampling. Thus, the
status of shedding in an individual animal cannot be estimated from just one sampling. Persistent shedders were not common. Four of 113 (3.53%) animals were shedding *E. coli* O157 at four samplings on the farm and 13 (11.5%) at three. This might reflect continuous exposure or carrier status of the animals. The duration of shedding of *E. coli* O157 seems to fall in three categories: less than one week, one month, or more than two months (Grauke et al. 2002).

We found that the animals were also shedders in the colder months. The outside temperature during the sampling in October was +3°C, and in November 0°C. The shedding increased in the calves during the study period, being lowest one month after arrival and highest at the age of 6-9 months. The animals were not on pasture but were fed grass during the summer months of June to August. In a longitudinal study of Wisconsin dairy herds, seasonal shedding was not observed (Shere et al. 1998), whereas in a British longitudinal study no excretion was detected in a dairy herd between November and May (Mechie et al. 1997). Higher prevalences were found in other herds tested during the summer months compared with herds tested during the spring (Garber et al. 1999), and the same was found in a heifer raising operation in Italy (Conedera et al. 2001). More shedders were detected in Washington dairy herds in summer (Hancock et al. 1994).

6.7 Detection of *E. coli* O157 from barn floor, feed passage, water cups and water nipples (IV, V)

The swab samples taken from the barn surfaces before introduction of new animals showed that the washing procedures had failed to remove *E. coli* O157 (IV). The pens were emptied and washed using a high pressure cleaner, but not disinfected the day before arrival of the new calves. So the washing procedure, and especially the empty period of approximately one day, were not sufficient to destroy *E. coli* O157; the floor was still visibly wet when the new calves arrived. In an abattoir study *E. coli* O157 was detected in the lairage in spite of routine cleaning (Small et al. 2002). In addition, the calf department in our study was not completely vacated between the different calf lots; some from the former lot remained, even though calves from different lots were not mixed in the same pens. In order to decrease the numbers of *E. coli* O157 at the farm, proper cleaning and disinfection and a longer empty period between different lots would be needed. The surfaces should be dry before introduction of new animals and the production system would preferably be all-in-all out.

The farm surface samples, especially from water cups and feed passages, were positive. *E. coli* O157 was not isolated from water nipples but the number of samples was too small for conclusions. In other studies the use of nipple bottles was associated with reduced rate of calf infection (Wilson et al. 1993), and the use of open pails to feed calves was associated with increased infection among calves (Wilson et al. 1998). Hygiene might play a role in transmission since herds that shared buckets and bottles among unweaned calves without rinsing or washing had a higher status of shedding (Garber et al. 1995). Water troughs have been contaminated with *E. coli* O157 (Faith et al. 1996; Shere et al. 1998). In an experimental study *E. coli* O157 was isolated from tonsils (Cray and Moon 1995) and from mouth samples (Buchko et al. 2000). *E. coli* O157 was isolated from a high percentage (74.8%) of oral cavities of fed cattle (Keen and Elder 2002), which could introduce a source of contamination. Pen test devices, such as ropes tied to pen rails, were shown effective catching oral contamination of *E. coli* O157 (Fegan et al. 2003).

*E. coli* O157 has been isolated from drinking water used for cattle (Shere et al. 1998; Hancock et al. 1998a; van Donkersgoed et al. 2001), and water has been suggested as a vehicle of transmission on farms. The bacterium can survive in water and in simulate water trough microcosm for extended periods (LeJeune et al. 2001a; LeJeune et al. 2001b). In our
study the drinking water of bulls and farmers came from the same source, although the farm family had not experienced episodes of diarrhoea. Although we did not analyze the water for the presence of *E. coli* O157 it is unlikely that water was the source.

6.8 Enumeration of *E. coli* O157 from faecal samples using the MPN method (IV)

The MPN in faecal samples taken at abattoirs were lower than in those taken on the farm. In an experimental study, calves shed higher numbers (cfu/g) of *E. coli* O157 than adult animals (Cray and Moon 1995), which could explain why the bulls in our study shed lower numbers at abattoir than farm. In a study on a beef farm more calves were positive on direct plating than cows (63.5% vs. 24%) (Gannon et al. 2002), which also strengthens the hypothesis. MPN is the only possible method for quantitative analysis when using enrichment and immunomagnetic separation. The MPN method used in this study detected low counts of >0.2/g. In previous studies counts have been detected using direct plating of the diluted sample. Direct plating detects samples with counts >10^2/g, so it would have detected only 30% (13/37) of the samples in our study. Differences in methods mean that counts obtained in other studies cannot be directly compared to our results. In another study using direct plating the numbers of *E. coli* O157 in the faeces of calves ranged from 10^3 to 10^6 cfu/g, and 48% (15/31) of faecal samples were positive with enrichment only, at populations <10^5 cfu/g (Zhao et al. 1995). Our MPN counts were possibly lower than the true values because the samples were stored refrigerated for 8-157 days (mean 45.6, SD 46.3) and only count analysed when the qualitative results were obtained. This might explain why 19% (7/37) of the samples were below the detection limit even when they were positive in the qualitative analysis.

6.9 Characteristics of *E. coli* O157:H7/H- isolates (II, IV, V)

Most of the isolates carried the stx2 and eae genes. Other studies performed in the northern hemisphere have also observed that cattle isolates more often harbour stx2 gene than stx2, or the combination of the genes (Chapman et al. 1997; Nielsen and Scheutz 2002). Nonmotile, non-sorbitol fermenting isolates were detected in the prevalence study (1/19) and in the finishing unit (although they were in minority; studies II, IV). Nonmotile isolates of *E. coli* O157 have often carried the flaC gene, even though the gene has not been expressed (Schmidt et al. 1999). The prevalence of *E. coli* O157:H- than the motile variant is higher in Australian cattle (Fegan and Desmarchelier 2002).

Using polymyxin extraction more isolates produced shigatoxin (paper II, V). In other studies a high number of cattle isolates have not produced Stx without induction (Chapman et al. 1993; Nielsen and Scheutz 2002; Avery et al. 2002a). However, the cattle isolates associated with human infections were Stx-producing without polymyxin extraction. The Stx production of HUS-associated STEC was significantly higher than that of bovine-associated STEC (Ritchie et al. 2003).

Unusual biochemical characteristics were observed, like the inability of some strains to produce indole (paper II, study IV, data not shown). Indole-negative *E. coli* O157 strains were repeatedly isolated from bull faecal samples from the finishing unit (study IV). The occurrence of indole negative strains has been reported earlier (Bopp et al. 1987). The indole-negative strains in our study often possessed the virulence factors analysed, but these isolates could be studied in more detail.

During the follow-up, SF O157:H- was isolated from two dairy cows on farm 5 (paper V). The isolates were found when fairly light colonies were picked from the agar media and confirmed. These isolates were not found in the human case and were potentially less virulent because
they lacked the virulence genes \( stx_1, stx_2, eae \) and were not enterohaemolytic. Sorbitol-fermenting O157:H- has been isolated from a dairy cow in the Czech Republic, where it was associated with two human cases (Bielaszewska et al. 2000). However, although the Czech isolate harboured \( stx_2 \) and \( eae \) it did not produce enterohaemolysin. (Midgley and Desmarchelier 2001) isolated from an Australian cattle feedlot SF \( E. \) coli O157:H- that lacked the virulence genes \( stx, eae, eltXA \), like the isolates in our study. Non-STEC O157 are relatively commonly isolated from other animal species, pigs in particular (Schroeder et al. 2002).

6.10 Phage (PT) and PFGE types of \( E. \) coli O157 isolates (II, IV, V)

PFGE has been established as a reliable and highly discriminating technique for the genotypic differentiation of bacterial strains (Barrett et al. 1994). The combination of phage and PFGE typing is considered to provide optimal discrimination. The PFGE profiles of the isolates in this study were compared using a computer-based network, a system that has proved a powerful tool in the USA (PulseNet) (Swaminathan et al. 2001), and now in Finland.

Consistent results were obtained combining phage typing and a more discriminating typing method, PFGE (paper V). The phage types 2 and 4 and found on four of the five farms are common in Finland: they constituted 67% of \( E. \) coli O157 strains isolated from humans in the 1990s (Saari et al. 2001). PT2 has also been detected in cattle-associated outbreaks in the UK (Shukla et al. 1995; Trevena et al. 1996; Clark et al. 1997; Trevena et al. 1999; Crampin et al. 1995; Pritchard et al. 2000) and in a goat-associated outbreak in the Czech Republic (Bielaszewska et al. 1997). Human and cattle isolates from farm 1 and two cattle isolates from farm 5 were designated as rdcn in the phage typing. So far, these are the only isolates belonging to rdcn2 and 8 categories found in Finland. The PT and PFGE types of \( E. \) coli O157:H7 isolated from the animals during the follow-up were identical to those detected earlier.

The PFGE type 1.1 was detected in the prevalence study of slaughter cattle (paper II), in the finishing unit (paper IV), and on four of the farms associated with human illness (paper V). It was the most common among Finnish human isolates in the 1990s (Saari et al. 2001). PFGE genotypes 1.3, 1.12, 1.15 and 1.37 have been only sporadically encountered in humans in Finland and the other PFGE genotypes not thus far. Most isolates on the farms were of a single PFGE genotype or a closely related one. Compatible with our findings, a single PFGE type comprised a majority of the isolates on Wisconsin farms (Shere et al. 1998; Heuvelink et al. 1998c; Rice et al. 1999; Nielsen and Scheutz 2002) or very similar (Faith et al. 1998; Gannon et al. 2002) types was found. In a Pennsylvanian dairy and petting farm 28/33 (85%) of \( E. \) coli O157 isolates were of a single PFGE type (Crump et al. 2002). However, in a longitudinal study in a dairy herd different PFGE types were present simultaneously (Besser et al. 1997). This might suggest that sources on farms could be either single or multiple.

In our studies only one colony from each positive faecal sample was analysed because during previous samplings at the finishing unit a mean of 4.5 colonies from 44 positive faecal samples were analysed and the PFGE genotype was similar. In 12.2% of cases at least one parallel colony from the farm surfaces had a different PFGE genotype, but on most occasions the genotype of the parallel colonies was indistinguishable. Multiple colonies analysed from single faecal enrichment have usually been of a single PFGE genotype (Besser et al. 1997). In the tracing of a ground beef associated outbreak, six of seven colonies from one enrichment broth were indistinguishable, whereas the seventh was different (Proctor et al. 2002). However, other genotypes closely related or completely different could have been isolated from parallel enrichments of the same sample, as more than one genotype, even though closely related, was isolated from repeated samplings of some bulls in our study. We
did not test multiple colonies of faecal material. Clonal turnover or shift in *E. coli* O157:H7 type has been noted in individual humans and cattle that shed the organism for long periods (Karch et al. 1995; Faith et al. 1996; Akiba et al. 2000).

One PFGE genotype (1.62) isolated from faeces and rumen contents of a bull at slaughter differed considerably from the other genotypes, and was never isolated from any of the sampling sites in the present study (paper IV). The strain with this genotype could have been acquired during the transfer or at the abattoir. Different genotype of *E. coli* O157:H7 was recovered from the hide at slaughter than from the feedlot (Midgley and Desmarchelier 2001).
7 CONCLUSIONS

1. *E. coli* O157:H7 was able to survive during the 49-day long fermentation, drying and storage of dry sausage. Differences in the decrease rate of *E. coli* O157:H7 between the two commercial starter cultures were detected. The reasons for the disparity could not be elucidated.

2. The prevalence of *E. coli* O157 in Finnish slaughter cattle was 1.31% (95% CI 0.73-1.90%) during a half-year study. The figure was lower than in recent studies performed in Europe and the USA, but a bigger sample size would probably have increased the number of positives. The prevalence of *E. coli* O157 was higher during June-September than October-December. *E. coli* O157 was not recovered from faecal samples from the northernmost region where cattle density is low.

3. Reindeer seem not to be an important reservoir of *E. coli* O157 infection: the prevalence was less than 0.22%. The study was performed in winter and a higher prevalence might have been detected in summer. Also, storage of 66.7% of the faecal samples in freezer might have decreased the sensitivity of the analysis.

4. Shedding at dairy farms associated with human infections of *E. coli* O157 was confined to peaks with several animals shedding. At other sampling times *E. coli* O157 was isolated only sporadically. In the cattle finishing unit with new animals arriving *E. coli* O157 was shed at all sampling times, which suggests that the sources of infection might be continuous or periodic. *E. coli* O157 was not recovered from any of the calves arriving at the finishing unit and sampled at the lorry before arrival, whereas calves sampled first in the barn the day after arrival were excreting. During the fattening period the infection rate of *E. coli* O157 varied between 0 and 38.5%. Seasonal variation in shedding was detected in bulls at slaughter, but no absence in shedding at the farm was observed during the cold months. *E. coli* O157 was detected from 9.7-38.9% of the faecal samples taken at slaughter, while only two rumen and one carcass surface samples were found positive. Faecal samples taken at the abattoir had lower counts (<0.4/g) than those at the farm (p<0.05).

5. *E. coli* O157 seems to persist in farm for extended periods and to resist routine cleaning procedures. Indistinguishable isolates of *E. coli* O157 were continuously detected from the surfaces on the farm.

6. The optimal isolation method for *E. coli* O157 depends on the background flora, the state of cells, and the matrix. A 24-h enrichment detected more *E. coli* O157 from frozen sausage samples, whereas a 6-h enrichment was more efficient when analysing barn surface samples.

7. Indistinguishable PFGE genotypes were detected in human and cattle isolates epidemiologically associated. Using PFGE, most isolates on the farms belonged to one genotype. 32% of the isolates from the abattoir survey belonged to PFGE genotypes isolated from humans.

8. The proportion of cattle excreting the type that caused the human infection varied from 3.2-66.7% when sampled soon after the human cases. Although the infection could be traced back to the farms, it could not be established whether the infection was unpasteurised milk or direct or indirect contact with cattle.
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