VEROCYTOTOXIN PRODUCING *E. COLI* O157 ON FARMS

Prevalences, risk factors and transmission

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Prevalences, risk factors and transmission

Jannigje Maria Schouten

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Verocytotoxinen producerende E. coli O157 op veebedrijven: prevalenties, risicofactoren en transmissie

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Abstract

Infection with verocytotoxin producing *Escherichia coli* O157 in humans can lead to mild or bloody diarrhoea, with e.g. the haemolytic uraemic syndrome (HUS) as possible complication. Cattle appear to be important reservoirs of O157 VTEC. The main objectives of research described in this thesis are investigating prevalences, risk factors, and transmission of O157 VTEC to understand the dynamics of O157 VTEC in Dutch cattle. Data from a monitoring program in Dutch animal herds indicated that O157 VTEC is endemic in The Netherlands, with higher prevalences during summer and early fall. Risk factors for infection were identified. Within-herd prevalence, potential environmental reservoirs, intermediate hosts and DNA types of O157 VTEC isolates were determined in a longitudinal study of a positive dairy farm. DNA clusters indicated persistence on the farm during winter and spring. Quantification of transmission using data from this dairy herd and from an experiment with calves showed that transmission was higher in calves. Transmission of O157 VTEC during summer might differ from transmission during winter. Two other experiments indicated that both previously infected heifers and previously contaminated pastures did not function as reservoir of O157 VTEC between shedding seasons. A retrospective cohort study established that positive farms from the Dutch monitoring program were only slightly more likely to be found positive in a next shedding season than previously negative farms, so between-herd transmission seems to occur. Factors associated with a positive test at second sampling implied (re-)introduction rather than long-term persistence of infection. Results of this thesis suggest that several types of O157 VTEC might persist on a farm for some time, with possibly different strains as the most prevailing types in subsequent shedding seasons. Ultimately, the within-herd infection might become extinct due to limiting numbers of susceptible cattle. Between-herd transmission of O157 VTEC can lead to persistence in a larger region, e.g. The Netherlands, maintaining the endemic status in Dutch cattle populations. Because of this, exposure of humans to O157 VTEC cannot be ruled out in The Netherlands. Humans might become infected through food- and waterborne transmission and by transmission directly from animals to humans. When aiming at reducing risks for humans by interventions at farm-level, it is of importance to reduce the number of positive animals and farms. For this, more specific research for the effect of intervention measures on introduction, transmission and survival of O157 VTEC on farms, and economic (cost-benefit) analysis, should be performed.

Life is what happens to you
while you are busy making other plans
(John Lennon)

Voorwoord

September, 1997. Bij de diploma-uitreiking schuift Prof. Noordhuizen tot ieders hilariteit omzichtig een haastig volgekrabbeld papiertje onder mijn diploma mee over de tafel. "*E. coli* O157, hamburger disease, aio-project met het RIVM. Iets voor jou?". Daarmee is de eerste steen voor mijn aio-loopbaan gelegd.

Januari, 1998. De start van het promotieonderzoek. Op het RIVM word ik in een paar maanden ingewijd in de VTEC-laboratoriumgeheimen. Cécile, dank voor je geduld met een microbiologisch onervaren "Veeteler" en al je hulp op het lab. Annet, met jouw proefschrift heb jij de basis gelegd voor mijn onderzoek èn een nog voortdurend gezellig contact.

Koninginnedag, 1998. Na een bewogen periode moet ik door ziekte een pas op de plaats maken. De wereld staat even op zijn kop...
Een jaar later dan gepland begint het eerste echte onderzoek.

Gezien de aanloop is het werk beschreven in dit proefschrift niet vanzelfsprekend tot stand gekomen. Ook na mijn herstel bleef het, zoals voor zovele aio's, een traject met "hindernissen", zoals een val van een paard en een MKZ uitbraak die veel praktisch werk in het water deed vallen. De steun en het enthousiasme van mensen om me heen zijn de afgelopen jaren voor mij heel belangrijk geweest. Al deze mensen wil ik dan ook van harte bedanken, een aantal in het bijzonder.

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Wageningen, augustus 2005

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List of abbreviations

Eae-gene	*E. coli* attaching-and-effacing gene
EHEC	Enterohaemorrhagic *Escherichia coli*
CFU	Colony forming units
HC	Haemorrhagic Colitis
HUS	Haemolytic Uremic Syndrome
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
OR	Odds ratio
R_0	Reproduction ratio
SIR	Susceptible-Infectious-Recovered
STEC	Shigatoxin producing *Escherichia coli*
VT	Vero(cyto)toxin(s)
VTEC	Verocytotoxin producing *Escherichia coli*

Chapter 1

General introduction

Based on:

Schouten, J.M., Kramer, J., 2002. Kennisoverzicht VTEC en de Nederlandse Rundveesector. Rapport Productschap Vee, Vlees en Eieren, 49 pp (in Dutch)

1.1. Verocytotoxin producing Escherichia coli (VTEC)

The majority of *Escherichia coli* bacteria is harmless for both humans and animals and play an important role in maintaining the physiological balance of the gastro-intestinal tract. The gram-negative rod shaped bacteria can grow both under aerobic and anaerobic conditions. Depending on, among others, temperature and medium, *E. coli* can grow at a pH varying from 4.4 to 9.0 (Varnam & Evans, 1991). Multiple acid resistant mechanisms contribute to the survival capacity of *E. coli* in acid environments (Diez-Gonzalez *et al.*, 1998).

E. coli strains are classified by O (somatic; O-specific LPS), H (flagellar) and K (capsular) surface antigens (Kauffmann, 1947). Each O-antigen forms a separate serogroup. Until now, 167 different serogroups are identified. Within a serogroup various serotypes are distinguished by the H-antigen, which is formed by proteins at the surface of an *E. coli* strain. By now, 55 different H-antigens are identified. Within one serogroup multiple H-antigens can be identified (e.g. *E. coli* O157:H7, *E. coli* O26:H11 and *E. coli* O26:H32). The K-antigen is identified by the chemical composition of the capsular antigen. Almost 80 different K-antigens are known (Lior, 1996).

Some *E. coli* strains can cause clinical symptoms in humans, among which enteritis. Based on pathogenesis, epidemiology, diagnostics, clinical symptoms and various O:H serogroups and types, human pathogenic *E. coli* strains are classified in six groups; enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC or EAggEC), and diffusely adherent *E. coli* (DAEC) (Doyle, 1990; Nataro & Kaper, 1998).

The serotype that can be considered one of the most important ones among the EHEC group because of the severity of the symptoms caused by infection in humans, is *E. coli* O157:H7 (Doyle, 1990). This EHEC belongs to the verocytotoxin producing *E. coli* (VTEC). VTEC are defined as the complete group of *E. coli* that are producing verocytotoxins (or verotoxins). VTEC do not always induce clinical signs and are not enterohaemorrhagic until additional virulence factors are present (Figure 1.1). This thesis is focused on potentially human pathogenic O157 VTEC. Increasingly other VTEC serotypes have been related to human infections (*e.g.* O26:H11, O103:H2, O111:NM (nonmotile) and O113:H21). Whether this reflects increasing prevalences or a change in methods of screening remains to be investigated.

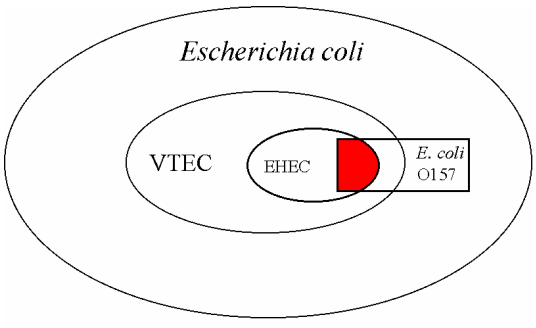

Figure 1.1 Schematic overview of types of *Escherichia coli*. The coloured part shows the human pathogenic verocytotoxin producing *E. coli* O157 (O157 VTEC).

1.2. Virulence factors

The O157 VTEC strains identified in the studies of this thesis, are diagnosed as O157 serogroups that *(I)* have genes for the production of one or both of the most frequently occurring vero(cyto)toxins, verotoxin 1 (VT1) and verotoxin 2 (VT2), and *(II)* possess the gene that may cause attaching-effacing (AE) laesions in the colon of humans.

(I) Verotoxins are proteins, existing of an enzymatic active subunit A and five receptor binding subunits B (Melton-Celsa and O'Brien, 1998). The amino acid sequence of VT1 only differs in one amino acid from the toxin produced by *Shigella dysenteriae* type 1, and cannot be distinguished in antigenicity. Therefore, verocytotoxines are also called Shiga-like toxins (SLT), and VTEC is also mentioned as STEC (Shiga toxin-producing *E. coli*) or SLTEC (Shigalike toxin-producing *E. coli)*.

(II) With an EHEC infection the bacteria attaches to the enterocyte membrane, after which microvilli disappear and rearrangement of the cytoskeleton occurs. Genes encoding for forming of these AE-laesions (*eae*-gene) are on the chromosomal locus for enterocyte effacement (LEE) and are encoding production of the membrane protein intimin (Kaper *et al.*, 1998). With EHEC infection, intimin plays a role in the specific binding of the bacteria to the epithelium of the colon. In combination with verotoxin production of EHEC, this can lead to severe tissue damage and diverse clinical signs in humans. Still, indications exist that the occurrence of AE laesions as a result of EHEC infection is not necessarily always associated with intimin (Dytoc *et al.*, 1994).

Clinically, more (potential) virulence factors of VTEC are described. For example, of VT2 several subtypes are mentioned, among which VT2c, VT2d and VT2e (Melton-Celsa & O'Brien, 1998) and another virulence factor associated with human pathogenic *E. coli* O157 can be the production of haemolysin (Hly) (Bauer & Welch, 1996). Enterohaemolysin is found to be associated with VT production in EHEC (Beutin *et al.*, 1989). Besides, enterohaemolysin seems to play a role in ion permeability of membranes (Heuvelink, 2000a).

1.3. Clinical disease in humans; public health

Infection with VTEC can lead to asymptomatic infection, mild diarrhoea, bloody diarrhoea or haemorrhagic colitis (HC), with as possible complications the haemolytic uremic syndrome (HUS) or trombocytic thrombocytopenic purpura (TTP) (Griffin 1995; Kar *et al.,* 1996). Especially children younger than five and elderly are susceptible for this. A severe infection can already be caused by only 100 to 200 bacteria (Nataro & Kaper, 1998).

In case of symptomatic VTEC infection, clinical symptoms show after about three days (varying from 1 to 9 days) and most patients recover in about ten days. In Europe, about 7% of the confirmed cases develop HUS (WHO, 1997; Kar *et al.*, 1996; Heuvelink *et al.*, 1999). HUS occurs when verotoxins enter the bloodstream and bind with B-subunits to the verotoxin receptor, glycolipid-globotriaosylceramide (Gb3), on the kidney. Gb3 is located on the epithelium of renal cells, glomerular endothelium cells and in the glomeruli of kidneys in children younger than three years old (Lingwood, 1994). After binding, the toxin is internalized and the A-subunit of the verotoxin is activated. With this, the A-subunit is released and inhibits protein synthesis of the cell and enhances clinical complications (Endo *et al.*, 1988). Characteristic clinical complications for HUS are haemolytic anaemia, trombocytopenia and acute kidney insufficiency; an outcome of HUS can be (direct or late) end stage renal disease (ESRD), indicating irreversible failure of the renal function (Siegler, 1995). Treatment with antibiotics is not recommended, as some studies have reported an increase in episodes of haemolytic uremic syndrome following use of antibiotics for the VTEC infection (Dundas *et al.*, 2001; Wong *et al.*, 2000), but most patients recover spontaneously after hospital submission. In the acute phase of the disease 5-10% of the patients die and in another 5-10% kidney damage remains after infection (Bitzan *et al.*, 1993; Kar *et al.,* 1996).

Since 1982, in several countries VTEC outbreaks are reported. Besides sporadic cases, varying incidences were reported in countries in all continents from 0.1-9.85/100,000 population (VTEC) or 2.0-7.8/100,000 children younger than 5 years (HUS) (WHO, 1997; Kaper and O'Brien, 1998; Kar *et al.*, 1996), with large outbreaks[1] in North-America (USA, Canada), Asia (Japan), and Europe (Scotland, England, and Wales). Potential sources of (often foodborne) infection varied from animal products to vegetables and other, not always consumption related, sources of infection (Table 1.1).

The first confirmed HUS outbreak in The Netherlands, which involved four children, was described in 1996. Source of infection was most probably swimming water. In two of the four cases, *E. coli* O157 was isolated from the faeces (WHO, 1997; Cransberg *et al.*, 1996). In April 1998, an outbreak of *E. coli* O157 occurred in a family living on a farm in the centre of The Netherlands. One of the parents and four of six children were infected. A one-year-old was hospitalised and developed HUS. Probable source of infection were veal calves of their farm. Besides, person-to-person spread seems to have taken place (Heuvelink *et al.*, 1998a). At the end of 1999 and in 2002, children were infected with *E. coli* O157 after visiting their grandfather's farm. Research on the farm indicated that the cattle excreted *E. coli* O157

[1] Outbreak = cluster of infections in space and time, defined by disease (e.g. HUS) or presence of a pathogen (e.g. O157 VTEC)

(Anonymous, 2001; Heuvelink *et al.*, 2002). Also after a visit to a petting zoo, at the end of July 2000, a 1.5-year-old boy was hospitalised and was diagnosed having HUS. Research showed that some sheep and a goat at the petting zoo were positive for *E. coli* O157. DNA type of isolates from the animals was identical to that of the human isolate (Heuvelink *et al.*, 2000b).

Table 1.1 Sources of infection associated with O157 VTEC outbreaks reported in literature

Sources	Reference	Country
Animal products		
Meat		
- (rendered) beef	- Allison *et al.* (2000); Chapman *et al.* (1993); Griffin (1995); Rodrigue *et al.* (1995); Belongia *et al.* (1991); Johnson *et al.* (1995); Rajpura *et al.* (2003); Vogt and Dippold (2005); Willshaw *et al.* (1994)	- UK (Scotland); Canada; USA
- hamburgers	- Barrett *et al.*, 1994; Brandt *et al.*, 1994; Bell *et al.*, 1994; Cieslak *et al.*, 1997	- USA; Canada
- (unpasteurised) sausage, salami	- Tilden *et al.* (1996); WHO (1997) ; Williams *et al.* (2000)	- USA; Spain; Canada
- poultry, wild, lamb	- Buchanan & Doyle (1997) ; Chapman *et al.*(1999)	- USA; England
Milk products		
- raw milk	- Allison *et al.* (2000); Chapman (1999); Trevena *et al.* (1996); Keene *et al.* (1997); Liptakova *et al.* (2004)	- UK (Scotland, England); USA; Slovakia
- yoghurt	- Morgan *et al.* (1993)	- USA
- raw cheese	- Honish *et al.* (2005)	- Canada
Vegetables		
- radish sprouts	- Watanabe *et al.* (1999), Michino *et al.* (1999)	- Japan
- lettuce and salads	- Ackers *et al.* (1998); Hilborn *et al.* (1999); Duffell *et al.* (2003)	- USA, UK, France
- alfalfa sprouts	- Breuer *et al.* (2001); Mohle-Boetani *et al.* (2001)	- USA
- pickles	- Ozeki *et al.* (2003)	- Japan
- potatoes	- Buchanan & Doyle (1997); Chapman *et al.* (1997)	- USA, UK
Other		
- person-to-person contact	- Al-Jader *et al.* (1999); Belongia *et al.* (1993); Reida *et al.* (1994); Parry and Salmon (1998); Pritchard *et al.* (2000)	- UK; Canada; Germany; France
- animal-to-person contact	- Heuvelink *et al.* (2000b); Heuvelink *et al.* (1998a); Chapman *et al.* (1999); Milne *et al.* (1999); Trevena *et al.* (1996); Renwick *et al.* (1993)	- The Netherlands; UK (England, Wales); USA; Canada
- swimming water	- WHO (1997); Keene *et al.* (1994); Brewster *et al.* (1994); Bruce *et al.* (2003); Cransberg *et al.* (1996); Friedman *et al.* (1999); Harrison and Kinra (2004); Samadpour *et al.* (2002)	- USA; The Netherlands; UK
- drinking water	- Akashi *et al.* (1994); Bopp *et al.* (2003); WHO (1997); Hrudey *et al.* (2003); Licence *et al.* (2001); Swerdlow *et al.* (1992)	- Japan; USA; Spain; Canada; UK (Scotland)
- (unpasteurised) apple juice	- Buchanan & Doyle (1997); Cody *et al.* (1999); Besser *et al.* (1993); Hilborn *et al.* (2000); Tamblyn *et al.* (1999)	- USA; Canada
- mayonnaise	- Keene *et al.* (1993)	- USA
- sauces for sea fish	- WHO (1997)	- Spain

In The Netherlands, each year about 30 individual cases of HUS are reported, with two-third of the cases in children younger than five years old (WHO, 1997; Heuvelink *et al.*, 1999a, Havelaar *et al.*, 2003). The incidence of HUS is 2.0 per 100.000 children younger than 5 years (Kar *et al.*, 1996). Although not all cases of HUS are associated with VTEC, O157 VTEC is the bacteria most frequently isolated from HUS patients (Kar, 1996). Between January 1999 and June 2001, 93 cases of symptomatic O157 VTEC were found, with individual isolates showing 17 clusters of DNA types (Duynhoven *et al.,* 2002). It was concluded that with these incidences, O157 VTEC in The Netherlands is a limited public health problem, although selective testing policy and the low sensitivity of culture techniques probably caused underestimating of incidences.

From a model of Havelaar *et al.* (2003) based on epidemiological surveys carried out between 1990 and 2000, estimated incidences of O157 VTEC related disease in The Netherlands were low, with a median of 1250 and a mean of 2100 cases of gastroenteritis, 590 cases of HC and 22 cases of HUS per year. Still, the mean disease burden of O157 VTEC in The Netherlands, was 116 DALY (Disability Adjusted Life Year) per year (90%CI 85-160), although highly variable. DALY's are a public health indicator and are the sum of years lost by premature mortality and life years spent in illness, weighted for severity of illness, integrating different clinical manifestations of the infection. Mortality due to HUS (58 DALY), mortality due to ESRD (21 DALY) and renal dialysis due to ESRD (21 DALY) were the main constituents of the disease burden. When compared with campylobacteriosis, another foodborne disease (disease burden of 1400 DALY/year), the burden of O157 VTEC is lower in absolute sense, but substantially higher per primary case (55 vs. 4.4 Daly's per 1000 cases) (Havelaar *et al.*, 2003).

1.4. O157 VTEC in food production animals

Many human VTEC infections can be traced back to faecally contaminated (animal) food products. Especially cattle products play a frequent role. From literature, cattle appear to be important reservoirs of O157 VTEC. Prevalences of O157 VTEC in cattle populations vary considerably between countries (0-13% positive animals, 1-87% positive herds, 0-68% positive animals within herds; Table 1.2), partly because of the way of sampling and sensitivity of the method of isolation and partly due to the differing groups of animals. Also other animals, *e.g.* sheep, pigs, horses and chickens, were found to be carriers of O157 VTEC (Armstrong *et al.*, 1996; Beutin *et al.*, 1993; Chapman *et al.*, 2001; Hancock *et al.*, 1998; Heuvelink *et al.*, 1998b; Schoeni & Doyle, 1994).

Table 1.2 Overview of reported prevalences of O157 VTEC in cattle populations

	Prevalences			
Country	**% Positive animals**	**% Positive animals within herds**	**% Positive herds**	**Reference**
Australia				
Australia	1.9%			Cobbold and Desmarchelier, 2000
	10% (grass fed beef cattle at slaughter) 15% (lot fed beef cattle at slaughter)			Fegan *et al.*, 2004
Asia				
Japan	1.5%			Fukushima and Seki, 2004
		46.3% (heifers, 1998) 36.8% (heifers, 1999) 31.7% (heifers, 2000)		Ezawa *et al.*, 2004b
	13.0%	33.7%	75%	Ezawa *et al.*, 2004a
	3.5%			Widiasih *et al.*, 2004
Taiwan	0.13%		2.6%	Lin *et al.*, 2001
North-America				
USA	6.5%	3-34.6%	38.5%	Dunn *et al.*, 2004
		7.4%	87%	Laegreid *et al.*, 1999
	1.0%			Hancock *et al.*, 1997a
	1.4%	0% -5.5%	75%	Hancock *et al.*, 1997b
		1.1%-6.1%		Hancock *et al.*, 1998
			7.1 %	Faith *et al.*, 1996
	1.5-2.9% (pre-weaning calves) 4.9-5.3% (weaned calves)			Zhao *et al.*, 1995
			24.2%	Garber *et al.*, 1999
			63% (feedlots)	Dargatz *et al.*, 1997
	3.4% (sampled on farm) 3.9 (sampled at slaughter)			Rice *et al.*, 1997
Canada	2.4% (barley fed cattle) 1.3% (corn fed cattle)			Berg *et al.*, 2004
South-America				
Argentina	0.5%			Meichtri *et al.*, 2004
Brazil	1.5%		21.4%	Cerquira *et al.*, 1999

Table 1.2 Overview of reported prevalences of O157 VTEC in cattle populations (continued)

	Prevalences			
Country	**% Positive animals**	**% Positive animals within herds**	**% Positive herds**	**Reference**
Europe				
Sweden			8.9%	Eriksson *et al.*, 2005
	1.2% (at slaughter)			Albihn *et al.*, 2003
Denmark	1.5%			WHO, 1997
	3.6%	21%	17%	Nielsen *et al.*, 2002
Norway			1%	Vold *et al.*, 1998
		5.5% (calves)		Cizek *et al.*, 1999
		31.2% (fattening bulls)		
Finland	1.3% (at slaughter)			Lahti *et al.*, 2001
Spain	0.3%		3.1%	Blanco *et al.*, 1996a
				Blanco *et al.*, 1996b
Germany	0-0.8%			Beutin, 1999
Switzerland	4.6%		17% (conventional farms)	Kuhnert *et al.*, 2005
			25% (organic farms)	
Belgium	6.3%			Tutenel *et al.*, 2002
Poland	0.7%			Tutenel *et al.*, 2002
Italy	13.1% (at slaughter)			Bonardi *et al.*, 1999
UK; England/ Wales	0.8%			Richards *et al.*, 1998
			34.5% (dairy)	Synge and Paiba, 2000
			48.4% (veal)	
	12.9%			Chapman *et al.*, 2001
	4.2%	1.1-51.4%	38.7%	Paiba *et al.*, 2003
	10.3% (among animals form infected herds)			
UK; Scotland		0-14% (lactating cows on a repeatedly sampled dairy herd)		Mechie *et al.*, 1997
		0-40% (non-lactating cows)		
		0-68% (heifers)		
		0-56% (calves)		
	8.6% (veal; 12-30 months)			Synge and Paiba, 2000

Table 1.2 Overview of reported prevalences of O157 VTEC in cattle populations (continued)

	Prevalences			
Country	**% Positive animals**	**% Positive animals within herds**	**% Positive herds**	**Reference**
UK; Scotland	11.2% (housed beef cattle in winter months, at slaughter)		33.7% (beef finishing groups in winter months)	Ogden *et al.* 2004
	7.5% (beef cattle on pastures in summer months, at slaughter)		40.4% (beef finishing groups in summer)	
Turkey	4.2%			Yilmaz *et al.*, 2002
The Netherlands		10.9 % (0.8%-22.4% for positive dairy farms)		Heuvelink *et al.*, 1998c
	10.6% (adult cattle at slaughter) 0.5% (veal calves at slaughter)			Heuvelink *et al.*, 1998b

Cattle are only one segment in the transmission of O157 VTEC (Figure 1.2). Literature indicates that the bacteria can be found at several locations on and in the vicinity of the farm, including other animals, water, soil, feed etc. (Cobbold & Desmarchelier, 2000; Rahn *et al.*, 1997; Mechie *et al.*, 1997; Hancock *et al.*, 1998). From the moment O157 VTEC colonises in the gastro-intestinal tract of cattle, intermittent periods of faecal excretion occur. Strong evidence exists for seasonal excretion and transmission, with periods of maximum numbers of shedding coinciding with peaks in human infection (Garber *et al.*, 1999; Mechie *et al.*, 1997; Synge & Paiba, 2000).

To understand the dynamics of O157 VTEC in cattle populations, it is important to have insight into the influence of animal factors and management factors on prevalence, transmission and persistence of O157 VTEC in cattle systems. These have been investigated world-wide (Table 1.3).

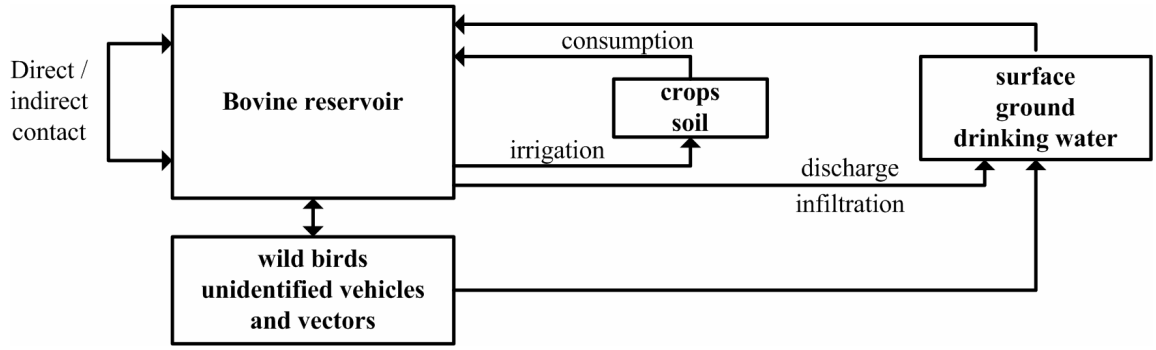

Figure 1.2 Transmission routes of *E. coli* O157:H7 on cattle farms (modified from Wallace, 1999)

In general, because VTEC is excreted in faeces, hygiene seems very important within farm management. In persistence of VTEC on a farm, faeces might play an important role. *E. coli* O157 can survive in faeces for considerable time. Experiments on the survival of *E. coli* O157:H7 indicated that in faeces of sheep, the bacteria are able to survive for at least 4-12 months. In mixed faeces of cattle this was approximately 2 months (Kudva *et al.*, 1998). Kudva *et al.* (1998) showed that in non-mixed faeces *E. coli* O157:H7 survives best at temperatures below 23^{o}C, so survival of *E. coli* O157 in faeces depends on temperature. VTEC was able to survive in cattle faeces at least 42, 49 and 63 days at 37, 22 and 5oC respectively (Wang *et al.*, 1996). Fukushima *et al.* (1999) also found similar results regarding the relationship between survival of VTEC and temperature. This research also showed that the bacteria can survive at least 100 days in frozen faeces of -20^{o}C. Conclusively, faeces can act as a reservoir and vehicle for *E. coli* O157. Possibly, frequent removal of faeces from the stables might reduce transmission. However, Garber *et al.* (1999) showed that farms that remove faeces by flushing are found positive more often than those that use other methods. Possibly, flushing transmits the bacteria or creates an environment favourable to survive. Also, in (faecally contaminated) water trough sediments VTEC might persist (Hancock *et al.*, 2001), resulting in transmission when more than one animal drinks from it (e.g. in group housing). Finally, on dairy farms, manure is being used for manuring the pastures, which might induce transmission of O157 (see also Figure 1.2).

Table 1.3 Overview of factors associated with O157 VTEC on farms.

Factor	Association	Reference
Animal factors		
- young age (2-6 months compared to adults or unweaned calves)	positive	- Cobbold & Desmarchelier (2000); Cray & Moon (1995); Hancock *et al.* (1997a); Kuhnert *et al.* (2005); Nielsen *et al.* (2002); Wallace (1999); Wilson *et al.* (1993);
Management factors		
- calves getting colostrum from the mother	inverse	- Rugbjerg *et al.* (2003)
- weaning	positive	- Bach *et al.* (2004); Rugbjerg *et al.* (2003)
- calf movement within herd	positive	- Rugbjerg *et al.* (2003)
- type of feed	positive/ inverse	- Brown *et al.* (1997); Church (1975); Cray *et al.* (1998); Dargatz *et al.* (1997); Diez-Gonzalez *et al.* (1998); Garber *et al.* (1999); Herriott *et al.* (1998); Hovde *et al.* (1999); Kudva *et al.* (1995); Lynn *et al.* (1998); Rugbjerg *et al.* (2003); Synge & Paiba (2000); Wilson *et al.* (1998); Zhao *et al.* (1998)
- final milking	positive	- Kuhnert *et al.* (2005)
- cows in pasture	positive	- Kuhnert *et al.* (2005)
- group housing	positive	- Cobold & Desmarchelier (2000); Garber *et al.* (1999); Hancock *et al.* (1997b); Wilson *et al.* (1993)
- water (sediments in troughs, surface water)	positive	- Davies *et al.* (1995); Hancock *et al.* (1998); Donkersgoed *et al.* (2001); Faith *et al.* (1996); LeJeune *et al.* (2001); Hancock *et al.* (2001)
- faeces / hygiene	positive/ inverse	- Garber *et al.* (1999); Fukushima *et al.* (1999); Hancock *et al.* (1997b); Kudva *et al.* (1998); Wang *et al.* (1996)
- introduction new animals	positive	- Nielsen *et al.* (2002); Wilson *et al.* (1993); Wilson *et al.* (1998)
- removal infected animals	inverse	- Hancock *et al.* (1997a); Mechie *et al.* (1997)
- transport	positive	-Bach *et al.* (2004)
Other factors		
- presence of other (farm) animals on the farm	positive	- Fischer *et al.* (2001); Hancock *et al.* (1998)
- presence of birds / vermin	positive	- Cìzek *et al.* (1999); Hancock *et al.* (1998); Rahn *et al.* (1997)

1.5. Overview of the cattle population in The Netherlands

In The Netherlands, 43,000 cattle farms contain about 3.7 million animals (CBS, 2005). The main goal of cattle production is to produce milk. Besides producing milk, dairy cattle form the basis of the beef supply chain. About 35% of the female calves on dairy farms are raised for dairy cattle replacement. The remaining part of female calves is either exported as breeding animal or sold as veal calf. Of the male calves, only a very low percentage is kept for breeding, the majority is sold within one or two weeks to a veal or beef farm. Dairy cattle that are culled usually go to the slaughterhouse.

For dairy farms, Heuvelink *et al.* (1998c) reported seven of ten sampled farms positive for O157 VTEC. Within those farms, prevalences varied from 0.8 to 22.4%. VTEC contamination of milk can occur when udder and/or tits are covered with faeces. In this way, VTEC might get to the milk tank. As from March until November 1997, milk samples were taken from milk tanks from 1011 Dutch dairy farms; no O157 VTEC was found (Heuvelink *et al.*, 1998d).

Besides dairy, veal production is an important sector in The Netherlands. The majority (90%) of Dutch veal farmers work on a contract basis for an integration. This means that, in return for housing and growing the calves, the farmer receives a fixed payment. The integration, which is producer of calf-milk replacer, delivers both the calves and the milk, and arranges the sale or slaughter of the fattened calves. Two categories of veal production in The Netherlands are ‘white veal’ and ‘pink veal’. Calves fattened for white meat are mainly fed with milk replacer. Generally, white veal farms work with the “all-in, all-out” principle for each unit of their farm. Ninety percent of white veal is exported. Pink veal is a typical Dutch product, with growing interest on the international markets (PVE, 2005). Approximately 35% of the veal farms have pink veal herds, but this is still increasing. Calves of pink veal herds are fed roughage besides the milk replacer. At these farms, mostly no all-in-all-out system is adopted.

Prevalences of O157 VTEC among veal farms in The Netherlands have not been investigated until the studies described in this thesis. Usually, weaned calves show higher prevalences and longer shedding duration than unweaned calves and adult cattle (Hancock, *et al.*, 1997a; Cobbold & Desmarchelier, 2000).

1.6. Aim and outline of the thesis

Because of the severe consequences of human infection, the large range of products that can be contaminated, the easy spread and the low amount of bacteria necessary for infecting

people, O157 VTEC is a realistic threat for public health. Therefore, it is important to get insight into the situation of VTEC in The Netherlands. In the human health sector, several groups in the Netherlands performed both clinical and epidemiological VTEC studies (University Hospital Nijmegen, National Institute for Public Health and the Environment (RIVM)), emphasising O157 VTEC as serotype most often associated with (sporadic) human infections. *E. coli* O157 in animal products and the dairy supply chain have been investigated and described by Heuvelink (2000a). Although this research confirmed the idea that cattle play an important role as reservoir of O157 VTEC, little was known on the occurrence and behaviour of O157 VTEC in the Dutch primary animal production sector at the start of the research described in this thesis. Cattle herd prevalences and prevalences in other types of animals were not determined, risk factors for infection with O157 VTEC in Dutch cattle herds were not known, and no knowledge existed on whether and how the bacteria spread between animals and farms. This thesis principally aims at increasing this insight into O157 VTEC in the primary sector, with emphasis on dairy and veal, using a number of (epidemiological) methods; literature research, observational epidemiology (field research), and transmission experiments.

During the project, quantitative data were collected on prevalences in dairy cattle, veal calves, pigs, layer hens and broilers in a monitoring program. This monitoring program for zoonotic agents in farm animals has started in The Netherlands in 1996 by the National Institute for Human Health and the Environment (RIVM), to comply with the Directive for Zoönoses (92/117/EEG) of the EU and in commission of The Food and Consumer Product Safety Authority (VWA) (Giessen & Visser, 2000). Using these data, analyses of risk factors for O157 VTEC infection of dairy and veal cattle farms could be performed (Chapters 2 and 3).

To gain insight into the dynamics of O157 VTEC on a farm, several studies were carried out. Within-herd prevalence, potential environmental reservoirs, intermediate hosts and DNA types of O157 VTEC isolates were investigated in a longitudinal field study of a dairy farm that was found positive in the monitoring program (Chapter 4). These longitudinal data were also used to quantify transmission, together with the results of a transmission experiment with calves. Also, the role of previously infected calves and pastures in the transmission of O157 VTEC was tested in experiments (Chapter 5). Both positive and negative farms from the monitoring program in the period 1997-2000, were sampled again to learn more about on-farm persistence or (re-)introduction of O157 VTEC (Chapter 6).

In the General Discussion (Chapter 7), results and methodological approaches presented in the different chapters are discussed, regarding the epidemiology and dynamics of O157 VTEC as objective of this thesis. Remaining questions considering the dynamics of O157 VTEC, resulting in potential aspects for further research, are discussed. Finally, implications

for the Dutch cattle sector are discussed and indications for intervention strategies are presented in order to reduce the prevalence of O157 VTEC in animals that will end up in the food chain or that are in contact with humans, eventually lowering the risk for public health.

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Chapter 2

Prevalence estimation and risk factors for *Escherichia coli* O157 on Dutch dairy farms

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Abstract

To estimate the prevalence of *E. coli* O157 on Dutch dairy herds, faecal samples were collected once from 678 randomly selected dairy farms in the period October 1996 through December 2000. Samples were cultured for *E. coli* O157. Thirty-eight isolates were tested for virulence genes (eae, VT1 and VT2). A questionnaire about farm characteristics was taken from the farm manager, resulting in variables that could be analysed to identify and quantify factors associated with presence of *E. coli* O157

In total, 49 of the 678 herds (7.2%) showed at least one positive pooled sample. *E. coli* O157 was not isolated from herds sampled in December through April in consecutive years (except for one isolate found in March, 2000). VT- and eae-genes were found in 37 and 38 isolates, respectively. Logistic regression was performed on variables obtained from the questionnaire, comparing *E. coli* O157 positive herds to negative herds. To account for season, a sine function was included in logistic regression as offset variable. In the final model, the presence of at least one pig at the farm (OR=3.4), purchase of animals within the last 2 years before sampling (OR=1.9), supply of maize (OR=0.29) to the cows, and sampling a herd in the year 1999 or 2000 (compared to sampling in 1998; OR=2.1 and 2.9, respectively) had associations with the presence of *E. coli* O157.

2.1 Introduction

Human pathogenic strains of *E. coli* O157 produce verocytotoxins (VT) and contain the *E. coli* attaching and effacing (*eae*) gene that enhances the possibility of the bacteria attaching to the human intestinal mucosa. This may result in a variety of human diseases, including non-bloody to severe bloody diarrhoea, hemorrhagic colitis (HC) and the potentially lethal hemolytic-uremic syndrome (HUS) (Tesh and O'Brien, 1991).

Consumption of undercooked ground-beef and raw (i.e. unpasteurized) milk were considered the main causes of *E. coli* O157 infections in humans (Griffin and Tauxe, 1991; Meng and Doyle, 1998). Recently, direct contact with infected animals (Renwick *et al.*, 1993; Armstrong *et al.*, 1996) has also been reported to cause infection. Cattle are the main reservoir host for *E. coli* O157 and other verocytotoxin producing *E. coli* (VTEC) in the developed world (Armstrong *et al.*, 1996), although domestic animals in general can transmit *E. coli* O157 (Beutin *et al.*, 1993). Faecal shedding of the bacteria can lead to contamination of *e.g.*milk, crops, surface water and infection of rodents and insects (with bacteria subsequently entering the human population). Furthermore, cross-contamination with *E. coli* O157 in slaughterhouses and butcher shops cannot yet be prevented, resulting in possible contamination of meat-products from cattle, lamb, pigs and poultry in retail outlets (Heuvelink *et al.*, 1999a).

Little was known about presence of VT-producing *E. coli* O157 on cattle farms in The Netherlands, or factors inhibiting or facilitating infection with this bacterium. In 1996, a monitoring program was started in The Netherlands to study prevalence and risk factors of zoonotic pathogens on Dutch farms. We analysed data from Dutch dairy herds obtained within this monitoring program to identify potential risk factors for testing positive to *E. coli* O157.

2.2 Materials and methods

2.2.1. Data

As part of the national monitoring program in The Netherlands, faecal samples are collected once on randomly selected farms throughout the year by an inspector of the Inspectorate for Health Protection and Veterinary Public Health (KvW). To estimate prevalence, a statistically adequate sample size has been calculated annually, based on the actual population size of dairy herds in The Netherlands and the estimated prevalence in the monitoring program in the previous year(s). In the formula for prevalence estimation (Noordhuizen *et al*., 2001), a 90% confidence level and an accuracy of 3% has been used.

Because the KvW is divided in regions, stratification of the study population into these regions was applied. Afterwards, it was checked whether regions were distributed as expected with a Chi-square test. The Dutch Animal Health Service was assigned to compile the study population. Farmers were requested to have their herd participate in the monitoring program. Fourteen percent of the addressed farmers refused to participate in the study. Specific details of the monitoring program can be found in the report of Bouwknegt *et al.* (2003).

From a herd of lactating cows, depending on herd size, a number of faecal samples were collected from the stable floor in a plastic bag. Samples were pooled to a maximum of 13 different samples per pool, with a maximum of five pooled samples per farm (Table 2.1, Bouwknegt *et al.*, 2003). Pooled samples were transported in a cooled container to the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. From October 1996 through December 2000, in total 23,000 samples were collected in 2,907 pooled samples on 678 dairy farms.

Table 2.1 Number of (pooled) samples per herd, based on herd size

Herd size (No. of animals)	**No. of samples**	**No. of pooled samples**
1 – 24	equals herd size (max. 20)	2
25 – 29	20	2
30 – 39	25	2
40 – 49	30	3
50 – 59	35	3
60 – 89	40	4
90 – 199	50	4
200 – 499	55	5
≥ 500	60	5

Table 2.2 Variables about farm characteristics and management derived from the questionnaire, with frequencies and prevalences of *E. coli* O157 (n=678) in the Netherlands in 1996-2000.

Variables	**Frequency (No.)**	**Prevalence (%)**
Farm characteristics		
Farmyard paving (no, yes) [a], also subdivided in:	53; 610	13.2; 6.7
Presence of asphalt (no, yes)	84; 79	7.2; 7.6
Presence of bricks (no, yes)	413; 250	7.0; 7.6
Presence of concrete (no, yes)	331; 332	7.0; 7.5
Type of stable (tying stall, loose housing with cubicles, other)	89; 469; 16	11.2; 6.4; 6.3
Presence of other ruminants (no, sheep/goat, bulls, other)	371; 180; 60; 23	7.6; 5.6; 6.7; 13.0
Presence of other farm animals (no, yes), also subdivided in:	337; 323	6.5; 8.1
At least one animal belonging to poultry species (no, yes)	548; 106	7.7; 5.7
At least one pig (no, yes) [a]	540; 114	6.1; 13.2
At least one horse (no, yes)	518; 136	7.1; 8.1
Other (no, yes)	643; 20	7.4; 5.0
Grazing of dairy cattle during summer (no, yes)	70; 504	5.7; 7.3
Grazing of d. cattle separately from other ruminants (no, yes)	81; 414	6.2; 7.3
Common grazing of young stock	469; 99	7.7; 5.1
Other farms within 1 km (no, yes), also subdivided in:	36; 625	5.6; 7.2
At least one farm with cattle (no, yes)	106; 539	7.6; 7.2
At least on farm with poultry (no, yes)	504; 131	7.3; 6.9
At least one farm with pigs (no, yes) [a]	352; 283	6.0; 8.8
At least one farm with horses (no, yes)	537; 98	7.6; 5.1
Other (no, yes)	488; 147	7.4; 6.8
Animal purchase within two years before sampling (no, yes) [a]	317; 255	6.0; 8.6
Breed of the herd (HF/FH, MRY, other) [a, b]	451; 37; 168	7.5; 0.0; 7.7
Dairy herd size (0-50; 50-100; >100)	51; 95; 34	3.9; 9.7; 14.7
Purchase of roughage (no, yes)	104; 36	8.7; 13.9
Roughage supplied (no, yes), also subdivided in:	161; 380	6.8; 7.6
Sugar beet pulp supplied (no, yes) [a]	385; 156	6.5; 9.6
Soy supplied (no, yes)	448; 93	7.4; 8.6
Brewers' grain supplied (no, yes)	437; 104	7.6; 6.7
Potato products supplied (no, yes)	435; 106	7.8; 5.7
Maize supplied (no, yes) [a]	442; 99	8.4; 3.0
Other roughage supplied (no, yes) [a]	460; 81	8.0; 3.7
Concentrates supplier (6 largest in the country, other, more than 1 supplier per herd) [a, b]	83; 79; 87; 51; 46; 58; 197; 54	14.5; 6.3; 10.3; 5.9; 0.0; 5.2; 7.1; 1.9
Tap water used in the stable (no, yes)	277; 380	7.9; 6.6
Surface water used in the stable (no, yes)	619; 38	7.3; 5.3
Spring water used in the stable (no, yes)	399; 258	6.8; 7.8

Table 2.2 Continued

Variables	Frequency (No.)	Prevalence (%)
Farm characteristics		
Tap water used on pasture (no, yes)	486; 157	6.8; 7.6
Surface water used on pasture (no, yes)	387; 256	7.5; 6.3
Spring water used on pasture (no, yes)	367; 276	7.4; 6.5
Purchase of slurry	109; 31	10.1; 9.7
Hygienic measures		
Removal of manure from loose stall (0; 1; 2; >2 times/day)	10; 53; 374; 61	10.0; 9.4; 6.7; 1.6
Entrance room of stable (absent, same as bulk milk tank room (BMR), separated from BMR without hygiene barrier or with unused hygiene barrier, separated from BMR with used hygiene barrier)	362; 96; 94; 46	6.6; 6.3; 9.6; 4.4
Use of disinfection tub (no, yes)	371; 287	6.7; 7.7
Refreshing of disinfection tub (0; 1-2; 3-5; >5 times/week)	55; 169; 74; 26	9.1; 8.3; 9.5; 3.9
Boot disinfection (0-30, 31-60, >60 times per month) [c]	147; 60; 14	8.8; 3.3; 7.1
Overall washes (0-4, 5-8, 9-12, >12 times per month) [c]	129; 114; 76; 111	10.1; 4.4; 4.0; 9.9
Hygienic measures regarding visitors (no, yes)	246; 402	7.3; 6.7
Cleaning of externally acquired equipment (no, yes)	332; 129	5.7; 8.5
Other pathogens		
Treatment against a *Fasciola hepatica* infection within the year before sampling (no, yes) [a,b]	528; 45	7.8; 0.0
Salmonella spp. infection at sampling (absent, present) [a,b]	659; 13	7.4; 0.0
Campylobacter spp. infection at sampling (absent, present)	243; 50	9.1; 10.0
Other factors		
Children entering the stable (no, yes)	179; 475	7.8; 7.0
Non-farm animals on the farmyard, (outside the stable) [a] (no, yes), also subdivided in:	56; 605	14.3; 6.5
Rodents present on farmyard (no, yes)	390; 271	6.9; 7.4
Dog(s) present on farmyard (no, yes) [a]	190; 471	10.0; 5.9
Cat(s) present on farmyard (no, yes)	175; 486	7.4; 7.0
Non-farm animals in the stable (no. yes), also subdivided in rodents, birds, dogs, cats, other	74; 582	5.4; 7.4
Region in which farm is located (north, mid/east, west, south) [a]	172; 210; 87; 193	6.4; 7.6; 2.3; 9.8
Year of sampling (1996+1997, 1998, 1999, 2000) [a]	90; 267; 163; 158	7.8; 5.2; 8.6; 8.9

[a] Variables with a P <0.30 (-2LL) in univariable analysis

[b] Variables with a prevalence of zero in one of their categories could not be calculated and are excluded for multivariable analysis

[c] Variables are excluded from multivariable analysis due to >25% missing values in the database

Together with the samples, information about farm characteristics and management was collected by means of a questionnaire taken from the farm manager, filled out by the inspector. A limited number of questionnaires were used as pilot-forms to examine its functionality before the start of the program. Adjustments were made and the questionnaires were thereafter used routinely. Unfortunately, alterations to the questionnaires had to be made in later years to enhance data quality or to collect additional information, disabling some variables to be properly analysed. Pre-fixed answers were supplied for the majority of questions; answers to open-ended questions were categorized afterwards, based on frequency of occurrence. For more details regarding the questionnaire, we refer to Bouwknegt *et al*. (2003). Table 2.2 shows the variables derived from the questionnaire.

2.2.2. Case definition and sample processing

Microbiological analysis of the faecal samples was started within 48 hours after sampling at the RIVM. Until 2000, all pooled samples per herd were mixed thoroughly on the lab before taking one portion for microbiological analysis. Since January 2000, each pooled sample per herd was tested for the presence of *E. coli* O157. A farm was considered positive if *E. coli* O157 was cultured from at least one of the pooled samples. An outline of the method of microbiological analysis is presented below (See also Tilburg and Van de Giessen, 1996).

A portion of 10 ± 0.5 grams of a pooled sample was added to 90 ml modified Trypton soya broth (Oxoid Ltd., Basingstoke, England) with acriflavine (10 mg/l; mTSB+A) and cultured for 6-8 hours at 37 ^{o}C (± 1 ^{o}C). One ml of the culture was added to 20 µl dynabeads (Dynal, Oslo, Norway) anti-*E.coli* O157 in a tube and incubated for 30 minutes at room temperature on a rotary shaker. Subsequently, Immuno Magnetic Separation (IMS) was done. After IMS, the solution was inoculated onto sorbitol MacConkey agar (SMAC) (Oxoid), enriched with 0.05 mg/l cefixime and 2.5 mg/l tellurite (CT-SMAC). The plate was incubated at 37 ^{o}C (± 1 ^{o}C) for 18-20 hours and screened for sorbitol-negative colonies. If present, 12 sorbitol-negative colonies per sample were inoculated onto both SMAC supplemented with 0.1 g 4-methylbelliferyl-B-D-glucuronide (MUG) (Sigma Chemical Co., St. Louis, MO, USA) and eosin methylene blue agar (37,5 g/l EMB) (Oxoid) and incubated at 37 ^{o}C (± 1 ^{o}C) for 18-20 hours. Achromatic colonies nn-fluorescent to UV light on SMAC + MUG and showing the distinct *E. coli* O157 pink-mauve colour on EMB were considered suspect. Suspected *E. coli* O157 colonies were tested with the Wellcolex agglutination latex kit (Murex; Kent, UK) in order to ascertain the authenticity of the colonies**.** Colonies that were affirmed by agglutination were serotyped.

E. coli O157 isolates subsequently were screened for possession of genes encoding for the most-common verocytotoxins (VTI and VTII) and the eae-gene; a polymerase chain-reaction (PCR) with the use of established primers was used (Tilburg and van de Giessen, 1996).

2.2.3. Statistical analysis

In this cross-sectional study, *E. coli* O157-positive herds were compared to *E. coli* O157-negative herds regarding exposure to potentially associated factors by logistic regression. The strength of association between a factor and the presence of *E. coli* O157 in a herd is presented in terms of odds ratios (OR) (Noordhuizen *et al.*, 2001).

Because of the seasonal effect on prevalence (Figure 2.1), a basic probability (*p*) of being found positive based on month of sampling, was calculated *a priori* for each herd. We fit a sine function (1) on monthly prevalences of the years 1997 to 2000 (months in which herds were sampled, were excluded). The log of $\underline{p}$ for each herd was included in logistic regression as offset variable, to account for season when testing the other factors. By doing this, herds sampled in winter could be retained for analysis, despite zero-prevalences in these months. The ability to use more records increased the power of the statistical analysis.

$$\underline{p} = \mu_1 + \mu_2 * \mathrm{SIN}((2\pi t / \mu_3) + \mu_4) + 0.01 \quad (1)$$

$$\mathrm{OFFSET} = \mathrm{Log}\ \underline{p} \quad (2)$$

where

μ_1 = intercept (0.068)

μ_2, μ_3, μ_4 = constants (respectively -0.077, 12.278 and 0.492)

t = month of sampling within a year (1 to 12)

The logistic regression was performed according to the method described by Hosmer and Lemeshow (1989). First, all variables derived from the questionnaire (Table 2.2) were subjected to univariable analysis (PROC LOGISTIC, SAS, 1996). Because of the specification of an offset in regression analysis, regression parameters for variables with a zero-prevalence in one or more of the categories could not be calculated using exact logistic regression (Derr, 2000). All variables with a *P*-value<0.30 based on –2 log likelihood (-2LL) (other than variables with a zero-prevalence or with >25% missing values) entered multivariable analysis. Monitoring the change in -2 LL ($P<0.10$) of the model, variables were excluded one-by-one from the multivariable analysis by descending *P*, until all variables had a $P<0.10$ (based on Wald's statistic). Within this backward-elimination procedure, exclusion of

variables was checked for confounding by monitoring the change in regression parameters (b; confounding was considered to be present if $\Delta b>25\%$ or $\Delta b>0.1$ if $-0.4<b<0.4$). Two-way interaction terms were tested for significance by monitoring the change in -2 LL ($P<0.10$) of the model with and without interaction. For the final model the Hosmer-Lemeshow Goodness-of-Fit Statistic was computed (Hosmer and Lemeshow, 1989).

2.3. Results

2.3.1. Descriptive statistics

Forty-nine out of 678 dairy herds were *E. coli* O157 positive (7.2%; 90CI: 5.6-8.8%) (Figure 2.1). From 11 isolates, PCR results were missing. From the remaining 38 isolates, 10 isolates (26%; 90CI 14-38%) contained both the VT1 and the VT2 gene. Twenty-seven isolates (71%; 90CI 59-91%) exclusively contained the VT2 gene and 1 isolate showed no VT-genes. All 38 (100%) isolates contained the *eae*-gene.

The sampled herds represent about 2.2% of all Dutch dairy herds (CBS, 1999). Although herd size categories according to number of pooled samples were known, unfortunately for only 180 (26.5%) of the herds the exact herd size was recorded by the sampling inspectors. The median number of animals (milk and offspring) on these observed farms was 65 (range: 8–300).

Farms in the mid/eastern parts of The Netherlands (Figure 2.2) are underrepresented in the database, while farms in the southern parts are overrepresented ($P_{\chi 2}<0.01$; Table 2.3).

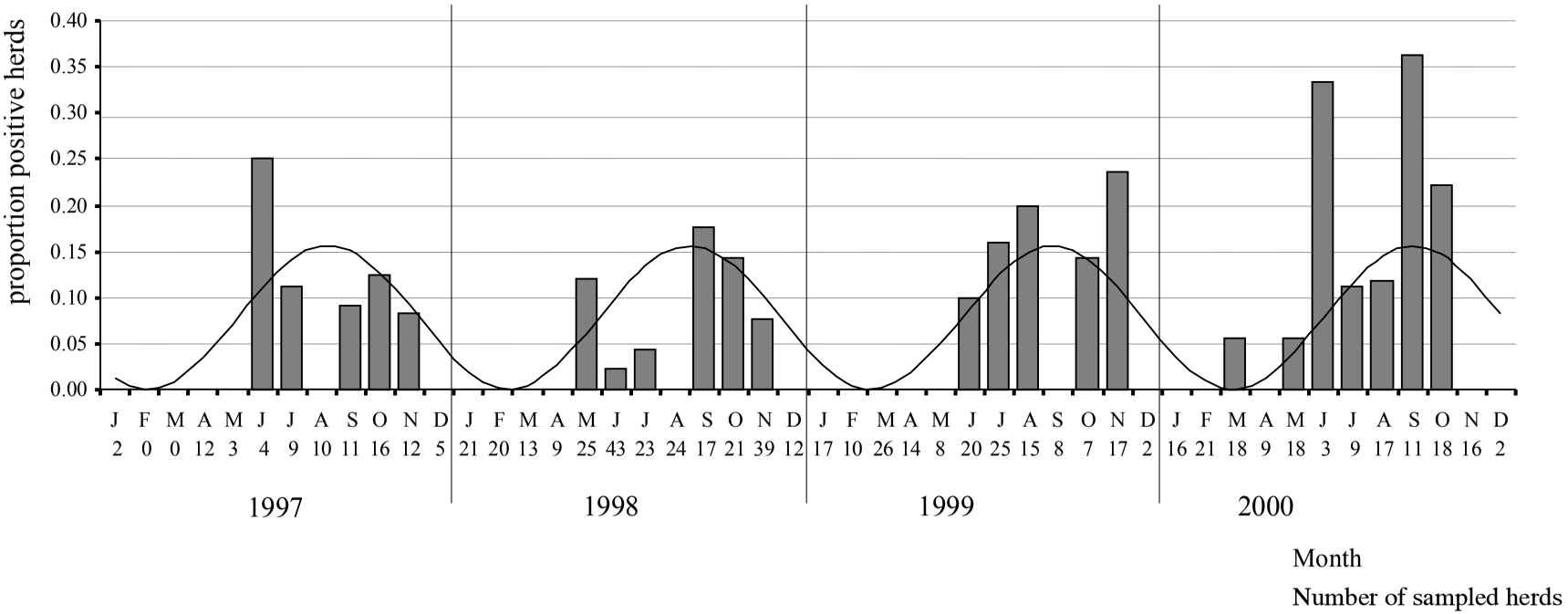

Figure 2.1 Monthly prevalences of *E. coli* O157-positive herds per month (#positive / #sampled) in The Netherlands (January 1997 to December 2000), and fitted sine function ($p = 0.068 - 0.077 * SIN((2\pi t / 12.278) + 0.492) + 0.01$)

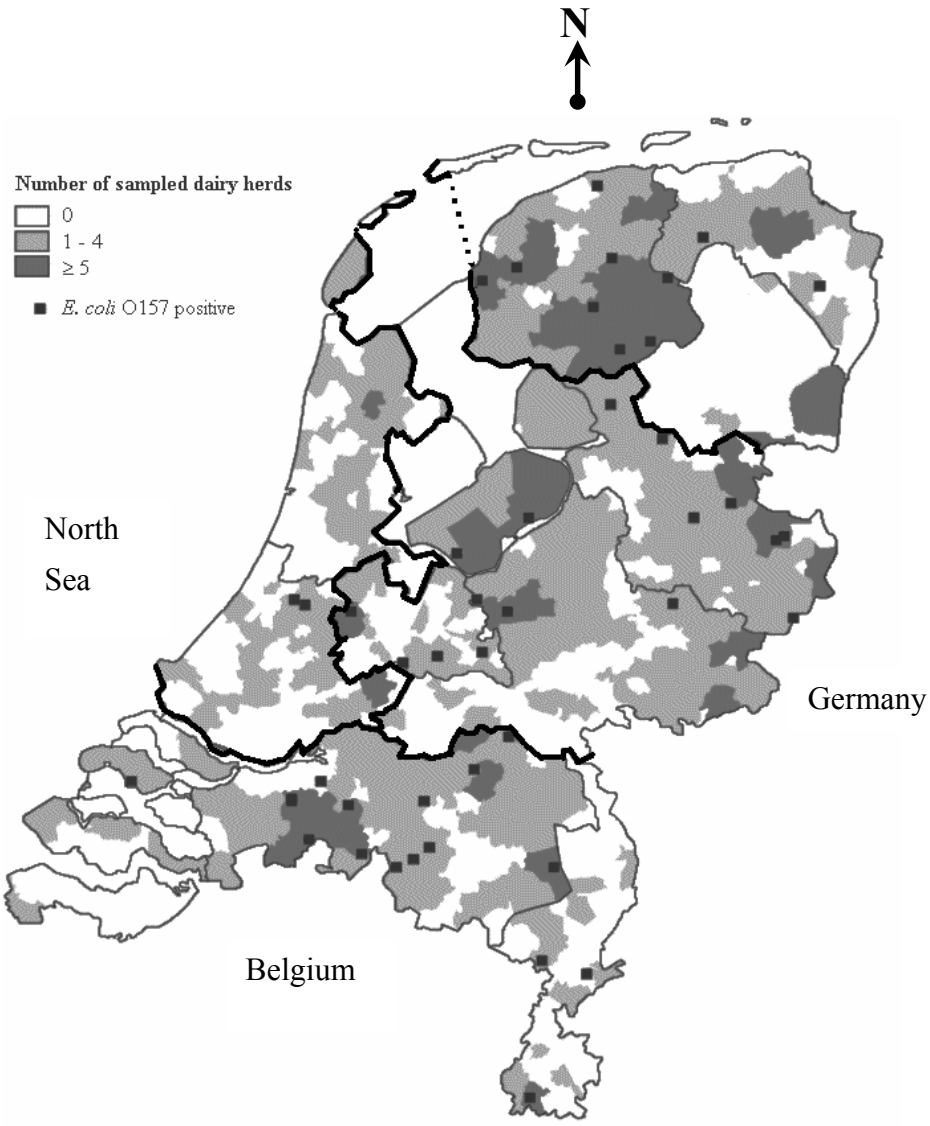

Figure 2.2 Geographic distribution of sampled dairy herds and positive tested dairy herds in The Netherlands (GIS). Borders of geographical regions North, Mid/East, West and South are indicated by bold lines. Note: not all 49 O157-positive detected herds can be distinguished, due to analogous zip codes

Table 2.3 Number and percentage of dairy farms per region in The Netherlands (based on data of C.B.S. (1999)) and in the database

	The Netherlands		**Database**	
Region (Province)	**No.**	**%**	**No.**	**%**
North (Groningen, Friesland, Drenthe)	7,569	25	172	26
Mid/East (Overijssel, Flevoland, Gelderland, Utrecht)	13,129	43	210	32
West (Noord-Holland, Zuid-Holland)	3,869	13	87	13
South (Zeeland, Noord Brabant, Limburg)	5,733	19	193	29
Total	30,300	100	662	100

[a] Of 16 dairy farms in the database, region was unknown

2.3.2. Statistical analysis

Univariable analysis resulted in 17 variables for multivariable analysis (Table 2.2). Four variables had a prevalence of zero in at least one of the categories and could not be analysed in the model. These were treatment of *Fasciola hepatica* infection within the last year before sampling, breed of the animals, concentrates supplier and presence of a *Salmonella spp*. infection at time of sampling. These variables, except *Salmonella* infection, were significantly (P<0.05) associated with *E. coli* O157 in the bivariate Fisher's exact test.

Two variables with a *P*<0.30 in the univariable analysis were omitted from multivariable analysis, because these had more than 25% missing values: boot disinfections (n=221) and overall washings (n=430).

After backward elimination, 4 variables remained in the final model of this study (Table 2.4). Due to missing values, this final model included data of 528 herds, of which 40 were case herds (7.6%). Interactions were not significant (*P*>0.10). The Hosmer-Lemeshow Goodness-of-Fit Statistic was 6.75 (*P*=0.56; df=8). There was no evidence of a lack of fit in the selected model (Hosmer and Lemeshow, 1989).

Table 2.4 Variables and their categories in the final logistic regression model, frequencies (Freq), prevalence (Prev), Odds Ratio (OR), 90%-confidence interval and Wald's *P*-value (n=528)

Variable	**Category**	**Freq (No.)**	**Prev (%)**	**OR**	**90%-CI**	***P*-value (Wald's)**
Farm characteristics						
At least one pig on the farm	Absent	436	6.2	1.00		
	Present	92	14.1	3.35	1.74-6.45	0.002
Purchase of animals	No	293	6.5	1.00		
	Yes	235	8.9	1.89	1.06-3.76	0.069
Maize supplied	No	431	8.6	1.00		
	Yes	97	3.1	0.29	0.10-0.80	0.046
Other factors						
Year of sampling[a]	1998	235	5.5	1.00		
	1999	141	9.2	2.06	1.02-4.15	0.089
	2000	152	9.2	2.91	1.42-5.94	0.014

[a] The category '1996+1997' has been omitted because of containing only one record in the final model

2.4. Discussion

The prevalence of 7.2% might be biased due to the voluntary basis of the study, because herds with (other) suspected health problems might be over- or underrepresented depending on the attitude of the manager about those health problems (Thrusfield, 1995).

Faecal samples were taken from the stable floor. It is therefore possible that collected faecal pats were outside the host for a certain amount of time. However, *E. coli* O157 still can be detected from inoculated faeces outside the host after 1 to 18 weeks (depending on temperature and number of inoculated bacteria) (Fukushima *et al.*, 1999 Himathongkham *et al.*, 1999). Survival of *E. coli* O157 is higher in manure held in the environment than held in laboratory conditions (Kudva *et al.*, 1998). Because in our study apparently fresh faeces were sampled (and not *e.g.* dried-out lumps), there are no indications that false-negative results occurred due to this. Yet, the sampling method implies that faeces of only part of the cows in a herd were sampled. Within-herd prevalences vary between 0.8% and 22.4% in Dutch dairy herds infected with *E. coli* O157, sampling all cows in a herd individually (Heuvelink *et al.*, 1998). Our sampling method gives a larger probability of missing an infected animal (especially in low-prevalence herds). Consequently, underestimating the prevalence of infected herds might have occurred.

Furthermore, a farm was only visited once for collecting faecal samples. *E. coli* O157 are shed intermittently (Hancock *et al.*, 1997; Mechie *et al.*, 1997) -- indicating that strong fluctuations in apparent prevalence can occur. Through this misclassification bias, prevalence might be underestimated and the effect of exposure factors present on a farm might incorrectly appear to be preventive on that farm, affecting calculated OR's. Sequential sampling with a monthly interval to deal with intermittent shedding (Hancock *et al.*, 1997) was not within the program scope.

The seasonal pattern we found agrees with results from Mechie *et al.* (1997), Hancock *et al.* (1997), Heuvelink *et al.* (1998) and Garber *et al.* (1999). Mechie *et al.* (1997) assumed that conditions in the winter are not sufficient for *E. coli* O157 to survive on the farm in relatively large numbers. For only the period May till November, our study demonstrated a mean prevalence of 10.6% (48/453). Because few *E. coli* O157 isolates were found on dairy farms in the period December to April from 1996 to 2000, potential risk factors are not properly determinable for herds sampled in this period. Including the seasonal pattern in the model as a categorical variable (*e.g.* months or seasons) -- instead of incorporating the seasonal prevalence distribution in an offset in logistic regression analysis -- would have excluded herds sampled in the period December through April from the analysis. (We tried and got nearly identical outcomes; data not presented.) However, with exclusion, the probability of being found positive for *E. coli* O157 for all the herds sampled in the excluded

months is estimated to be zero -- which is biologically unlikely. By fitting a sine function on monthly prevalences and implementing this specified seasonal effect in logistic regression as an offset, this unreasonable assumption was avoided. Moreover, power for statistical analysis was gained, because all herds then were available.

Several variables with a prevalence of zero could not be analysed in the multivariable models. Some of these variables, like *Fasciola hepatica* infection, cattle breed or concentrates supplier, might be associated with a negative *E. coli* O157 status, and should therefore be the subject of future research.

Positively associated with the presence of *E. coli* O157 was purchase of animals within 2 years before sampling -- consistent with Wilson *et al.* (1993) and Nielsen *et al.* (2002), for Canadian and Danish dairy farms, respectively.

Feeding corn silage was negatively associated with *E. coli* O157 presence, although Herriott *et al*. (1998) found the contradictory (but not significant) indication that feeding corn silage to heifers is associated with a higher prevalence of *E. coli* O157. Previous reports showed an association between low pH (from volatile fatty acid concentration in the intestines) and *E. coli* infection (Kudva *et al*., 1997; Diez-Gonzalez *et al*., 1998; Russel *et al*., 2000). Considering the carbohydrate content of maize, corn silage is presumed to decrease pH. Based on this, higher prevalence is expected among herds to which maize is supplied, so other mechanisms than feeding regimen might be involved here.

Although pigs are a potential reservoir host of VT-producing *E. coli* (Booher *et al.*, 2002), prevalences of O157 VTEC in pigs in The Netherlands are generally low (Heuvelink *et al.*, 1999b). An explanation for the observed association might be unidentified management routines on mixed farms. Manure types and storage conditions, for instance, influence the survival of O157 VTEC (Kudva *et al.*, 1998). Additionally, because the stomach pH in monogastric animals usually is lower than in ruminants, VTEC that colonises the pig gut might be more acid resistant and therefore more persistent in cattle. Furthermore, mixed systems with pigs might be less closed as result of regular purchase of animals.

The significant (P<0.10) year effect presumably results from changes in lab techniques in the course of time (see §2.2.); the probability of O157 detection in pool samples with immunomagnetic separation is high (Dam-Deisz and Evers, 2001), but the earlier method might have had only 55% sensitivity (unpublished data). However, table 2.4 seems to show a continuous increase in prevalence from 1996 to 1999 and an apparent prevalence remaining unchanged between 1999 and 2000. Thus, a change in prevalence might have taken place independently of the change of processing technique.

In The Netherlands, the annual incidence of recognised human VTEC infections (0.25 cases per 100,000 inhabitants) is relatively low compared to other countries like Germany, The United Kingdom, and Canada (Duynhoven *et al.*, 2002). However, O157 VTEC is

common in Dutch dairy herds during May through November (the shedding season). The herd-level prevalence appears to be similar to (or just slightly lower than) that of other countries (Garber *et al.*, 1999; Nielsen *et al.*, 2002).

We caution that variables can be positively associated with faecal shedding of *E. coli* O157 and interpreted as risk factors, though they are the result of actions taken by the farm manager to correct health problems in general. Therefore, and due to the low prevalence of *E. coli* O157, most results presented in this study should be considered as indications for further (experimental) research rather than the revelation of causal factors associated with an *E. coli* O157 infection of dairy farms.

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Chapter 3

Escherichia coli O157 prevalence in Dutch poultry, pig finishing and veal herds and risk factors on Dutch veal herds

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Abstract

In the period October 1996 through December 2000, a total of 7163 pooled faecal samples of laying hen and broiler flocks, finishing-pig herds and veal herds were examined for the presence of *Salmonella* spp., *Campylobacter* spp. and verocytotoxin-producing *E. coli* O157 as part of a national monitoring programme in The Netherlands. Isolates were tested for *eae* and VT genes. Risk factors for Dutch veal herds were quantified. For all herd/flock types, faecal samples were cultured for *E. coli* O157. Of broiler flocks, laying flocks and finishing pig herds, respectively, 1.7%, 0.5% and 0.4% were *E. coli* O157 positive. In total, 42 of the 454 veal herds (9.3%) showed at least one positive pooled sample. *E. coli* O157-positive herds were compared (with logistic regression) to negative herds, regarding variables obtained from the questionnaire taken from the farm manager. To account for season, a sine function was included in logistic regression as offset variable. In the final model, 'pink-veal production' (compared to white-veal production), 'group housing of the sampled herd' (compared to individual housing), 'More than one stable present' (compared to one stable present), 'hygienic measures regarding visitors' (compared to no hygienic measures), 'interval arrival-sampling of a herd of >20 weeks' (compared to < 10 wks), and 'presence of other farms within 1 km distance' (compared to no presence of farms < 1 km) showed associations ($P<0.05$) with the presence of *E. coli* O157. These results need careful interpretation; they should be considered as indications for further (experimental or cohort-based) research rather then causal associations.

3.1. Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC), and especially *E. coli* O157, has emerged in the past 10-15 years as an important zoonotic agent causing non-bloody diarrhoea or haemorrhagic colitis (HC), with potential complications such as haemolytic uraemic syndrome (HUS) (Bell, 2002). Young children (0-4 years of age) and the elderly are particular risk groups (Karmali *et al*., 1985). In The Netherlands, surveillance of laboratory-confirmed VTEC infections yielded an annual incidence of 0.25 cases per 100,000 inhabitants, with ~40% of the patients being hospitalised (Duijnhoven *et al*., 2002).

Some virulence of VTEC arises from toxin production, from verocytotoxin genes (VTI and VTII). Also, the presence of the *E. coli* attaching and effacing gene (*eae*-gene; that enhances the ability of the pathogen to attach to and efface the intestine wall), has been associated with pathogenicity (Tesh and O'Brien, 1991).

Consumption of undercooked ground-beef and unpasteurized milk, direct contact with an infected animal or person, and consumption of faecally contaminated crops or surface water are causes of *E. coli* O157 infections in humans (Griffin and Tauxe, 1991; Meng and Doyle, 1998; Armstrong *et al*., 1996; Ackers *et al*., 1998; Keene *et al*., 1994). Cattle are regarded as the main reservoir for *E. coli* O157 and other verocytotoxin producing *E. coli* (VTEC) in the developed world (Armstrong *et al*., 1996), but domestic animals in general can harbour *E. coli* O157 (Beutin *et al*., 1993).

Managerial and environmental factors are assumed to play a role in the emergence of *E. coli* O157 on cattle farms (Wilson *et al.*, 1993; Dargatz *et al*., 1997; Herriott *et al*., 1998; Garber *et al*., 1999; Nielsen *et al*., 2002). In a previous study (Schouten *et al*., 2004), factors possibly inhibiting or facilitating infection with VTEC O157 of dairy farms in The Netherlands were identified and quantified. As part of the Dutch monitoring program for zoonotic bacteria in farm animals (Bouwknegt *et al.*, 2003), VTEC O157 infections also were surveyed for herds of veal calves, poultry flocks (broilers and laying hens) and finishing-pig herds. In this paper, prevalences from October 1996 through 2000 are presented for those categories of farm animals. Additionally, questionnaire data from Dutch veal herds are analysed for factors associated with *E. coli* O157 contamination.

3.2. Materials and methods

3.2.1. Data

As part of the national Dutch monitoring program in farms, faecal samples were collected once on randomly selected farms with veal calves, broilers, laying hens or finishing pigs

throughout the year by employees of the Inspectorate for Health Protection and Veterinary Public Health (KvW). To estimate prevalence, a statistically adequate sample size has been calculated annually, based on the actual population size of dairy herds in The Netherlands and the estimated prevalence in the monitoring program in the previous year(s). In the formula for prevalence estimation (Noordhuizen *et al*., 2001), a 90% confidence level and an accuracy of 3% has been used. The Dutch Foundation for Quality Guarantee of Veal (SKV) assisted in selection and stratification for geographical region and farm size of the veal-herd study population. Farmers were requested to have their herd participate in the monitoring program. Because the SKV is a farmer's foundation that guarantees privacy of the farmers, the percentage of the addressed farmers refusing to participate was not revealed. Specific details of the monitoring program can be found in the report of Bouwknegt *et al*. (2003). Afterwards, it was checked whether regions and farm sizes were distributed as expected with a Chi-square test.

The unit of observation and analysis throughout the entire program was a herd or flock of animals. For layers, broilers and veal calves, a flock or herd included all animals of similar age housed within one building. Depending on herd or flock size, 12 to 60 faecal samples were collected from the stable floor or the running manure conveyor belt. Individual samples were pooled to a maximum of 13 samples per pool, with a maximum of five pooled samples per farm (Table 3.1). The method of faecal pooling (up to 13 samples) is extrapolated from a study for Salmonella executed at the RIVM (Giessen *et al.*, 1991).

Table 3.1 Number of (pooled) faecal samples per herd, based on herd/flock size, for *E. coli* O157 detection

Herd size (No. of animals)	**No. of samples**	**No. of pooled samples**
1 – 24	equals herd size (max. 20)	2
25 – 29	20	2
30 – 39	25	2
40 – 49	30	3
50 – 59	35	3
60 – 89	40	4
90 – 199	50	4
200 – 499	55	5
≥ 500	60	5

In addition to sampling, information about farm characteristics and management was collected with a questionnaire, filled out by the KvW-associate with assistance of the herd manager. A limited number of questionnaires were used as pilot-forms to examine its functionality before the start of the program. Adjustments were made and the questionnaires were thereafter used routinely. Unfortunately, alterations to the questionnaires had to be

made in later years to enhance data quality or to collect additional information, disabling some variables to be properly analyzed. Pre-fixed answers were supplied for the majority of questions; answers to open-ended questions were categorized afterwards. For more details regarding the questionnaire, we refer to Bouwknegt *et al*. (2003). Table 3.2 shows the variables derived from the questionnaire for veal farms.

3.2.2. Case definition and sample processing

Microbiological analysis of the faecal samples was performed within 48 hours after sampling at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. Until 2000, all collected pool samples per herd were mixed thoroughly on the lab before taking one portion of 10 g for microbiological analysis. From January 2000 onwards, each pooled sample per herd was tested separately microbiologically. A farm was considered positive if *E. coli* O157 was cultured from at least one of the pooled samples. Isolation methods, using Immuno Magnetic Separation (IMS), were described in detail by Schouten *et al*. (2004). Diagnostic sensitivity of IMS is as low as 0,49 cfu/g feces detected (Dam-Deisz and Evers, 2001).

In brief, samples were cultured by incubating in modified Trypton soya broth (Oxoid Ltd., Basingstoke, England) with acriflavine (mTSB+a). Subsequently, IMS was done**.** After IMS, the solution was inoculated onto sorbitol MacConkey agar (SMAC) (Oxoid), enriched with cefixime and tellurite (CT-SMAC), incubated, and screened for sorbitol-negative colonies. Sorbitol-negative colonies were inoculated onto both SMAC supplemented with 4-methylbelliferyl-B-D-glucuronide (MUG) (Sigma Chemical Co., St. Louis, MO) and eosin methylene blue agar (EMB) (Oxoid) and incubated. Suspected *E. coli* O157 colonies were tested by agglutination for confirmation. Finally, one isolate per flock or herd that was confirmed by agglutination, was serotyped.

E. coli O157 isolates subsequently were screened for possession of genes encoding for the VTI and VTII and for the *eae*-gene; a polymerase chain-reaction (PCR) with the use of established primers was used (Tilburg and van de Giessen, 1996).

Table 3.2 Variables concerning farm characteristics and management derived from the questionnaire of veal herds (n=455) in 1996-2000, with frequencies (Freq) and prevalences (Prev) of variables with P <0.25 (-2LL) in univariable analysis

Variables	**Freq (n)**	**Prev (%)**
Farm characteristics		
Farmyard paving (no, yes), also subdivided in:	83; 364	3.6; 10.4
Type of housing of sampled herd (group: no, yes)	217; 177	2.3; 19.2
Number of stables (1, >1)	148; 279	4.1; 11.8
Ventilation system (natural: no, yes)	170; 221	2.4; 15.4
Presence of at least 1 other ruminant on the farm (no, yes)	231; 215	10.4; 7.9
Other farms within 1 km (no, yes), also subdivided in:	16; 434	25.0; 8.3
At least on farm with poultry (no, yes)	267; 177	10.3; 6.7
Size of sampled herd(0-200, >200 calves)	255; 187	12.9; 3.2
Pink meat produced (instead of white) (no, yes)	333; 102	2.1; 33.3
Other feed than milk supplied (no, yes), also subdivided in:	63; 337	3.2; 10.7
Maize supplied (no, yes)	198; 200	3.0; 16.0
Concentrates supplied (no, yes)	303; 95	6.6; 19.0
Barley supplied (no, yes)	344; 54	10.8; 1.9
Producing for an integration , subdivided in: no, 6 largest in the country, other	17; 106; 61; 77; 46; 25; 21; 80	52.9; 4.7; 3.2; 6.5; 4.4; 0.0; 0.0; 21.3[a]
Water used in the stable (spring no, yes)	282; 160	6.4; 14.4
Medicines supplied (no, yes), also subdivided in:	61; 383	21.3; 7.3
Oxytetracycline and/or Starters' mix supplied (no, yes)	168; 263	16.1; 4.9
Hygienic measures		
Cleaning of stable between production (no, only manure removal, manure removal and wet cleaning	46; 30; 325	28.3; 13.3; 6.8
Entrance room of stable (absent or same as feed preparing room (FPR), separated from FPR)	339; 44	8.6; 22.5
Presence of hand washing facility (no, yes)	55; 130[b]	3.6; 12.3
Use of separate boots for stables (no, yes)	206; 225	11.2; 7.1
Hygienic measures regarding visitors (no, yes)	173; 256	4.6; 12.1
Other factors		
Children entering the stable (no, yes)	228; 213	7.5; 11.3
Birds present in the stable (no, yes)	252; 195	7.5; 11.3
Dogs present in the stable (no, yes)	328; 119	6.7; 16.0
Interval (in weeks) between arrival of veal herd on the farm and date of sampling (0-10, 10-20, >20, unknown)	131; 125; 113; 85	3.1; 7.2; 15.9; 12.9
Year of sampling (1996+1997, 1998, 1999, 2000)	116; 148; 59; 131	2.6; 5.4; 13.6; 17.6

[a] Variables with a prevalence of zero in one of their categories could not be calculated and are excluded for multivariable analysis

[b] Variables are excluded from multivariable analysis due to >25% missing values in the database

3.2.3. Statistical analysis

For veal herds, *E. coli* O157-positive herds were compared to *E. coli* O157-negative herds for risk factors by logistic regression (Thrusfield, 1995).

Because we expected seasonal effect on prevalence, a basic probability (p) to be found positive, based on month of sampling, was calculated *a priori* for each herd. This was achieved by fitting a sine-function (1) on monthly prevalences of the years 1997 to 2000 (months in which no herds were sampled, were excluded). The log of p for each herd was included in logistic regression analysis as offset value (2).

$$\underline{p} = \mu_1 + \mu_2 * \text{SIN}((2\pi t / \mu_3) + \mu_4) \quad (1)$$

$$\text{OFFSET} = \text{Log } \underline{p} \quad (2)$$

where

μ_1 = intercept (0.115)

μ_2, μ_3, μ_4 = constants (respectively -0.085, 12.910 and 0.687)

t = month of sampling within a year (1 to 12)

The logistic regression was performed according to the method described by Hosmer and Lemeshow (1989). First, univariable logistic regressions were performed for each variable derived from the questionnaire (PROC LOGISTIC, SAS, 1996). Because of the specification of an offset, regression parameters for variables with a zero-prevalence in one or more of the categories could not be calculated (SAS, 1996). All variables associated with *E. coli* O157 positive herds at a $P<0.25$ based on –2 log likelihood (-2LL), other than variables with a zero-prevalence or variables with a large number (>25%) of missing values, entered multivariable analysis. Collinearity was checked using Fisher's Exact Test. Variables that showed possible collinearity, were checked in a bivariate model to establish whether their standard errors were much larger than expected from the univariate models – which affirms collinearity (Hosmer and Lemeshow, 1989).

Due to sparseness of observations, variables were assigned to four clusters, containing general farm characteristics, hygiene factors, feeding aspects and specific characteristics for the sampled herd. Six different sub-models, each containing 2 clusters, were created to enable comparison of all variables. The variable 'year of sampling' was expected to have influence on all other variables and was added to every sub-model. In the multivariable models, variables were excluded one-by-one by descending *P*-values, until all variables had a $P<0.10$ (based on Wald's statistic). Within this backward-elimination procedure, exclusion of variables was checked for confounding by monitoring the change in regression parameters (b). If the change in parameter estimates exceeded 25% (or $\Delta b>0.1$, if $-0.4<b<0.4$), the

deleted variable was considered a potential confounder and included in the model again. Conclusively, a final model was created based on the outcome of the six previous models. Two-way interaction terms were tested for significance by monitoring the change in -2 log likelihood (P<0.10) of the final multivariable logistic model with and without interaction. Goodness-of-fit of the final model was computed by using the Hosmer-Lemeshow goodness-of-fit statistic (Hosmer and Lemeshow, 1989).

3.3. Results

3.3.1. Descriptive statistics

In total, about 80,000 samples (aggregated into 7,163 pooled samples) were collected on 1,469 farms from October 1996 through December 2000 (Table 3.3).

From broiler flocks, laying flocks and finishing pig herds, respectively 1.7% (6/349), 0.5% (2/429) and 0.4% (1/229) were found *E. coli* O157 positive in the period from October 1996 through 19999 (Table 3.3). Because of the low prevalences, sampling on these types of farm was not continued in 2000. In 1997, pig farms were not investigated due to the classical swine fever (CSF) outbreak in The Netherlands. For one of the 463 sampled veal herds, microbiological results for *E. coli* O157 were missing. Of the remaining 462 herds, 42 were found positive for *E. coli* O157 (9.1%). From these 462 herds (or records), 66 (14.3%) appeared to be sampled more than once in the period from October 1996 through 2000 and therefore were found twice or more in the database. Of these 66 herds, 59 had sampled different groups of animals in different years, so we decided to keep these in the database to prevent loss of power of the analysis. For farms where the same group of animals was sampled more than once (6 herds twice, 1 herd three times), results from the second and third sampling were excluded from the analysis. These 8 observations were negative for O157. In total, 42 out of 454 veal herds were found *E. coli* O157 positive (9.3%). Mean monthly prevalences in the study period varied from 0% to 35% (Figure 3.1). Considering only the period May till November, our study demonstrated a mean prevalence over the study period of 17.6% (31/176) among veal herds**,** with mean monthly prevalences varying from 8.8% in May to 35.0% in September. In the period November through April, a mean prevalence of 4.0% was calculated, varying from 2.0% in January to 8.6% in April. Average prevalences per year were significantly increasing from 2.6% to 17.6% (χ^2=20.8, df=3, $P_{\chi 2}$ <0.001; Table 3.3).

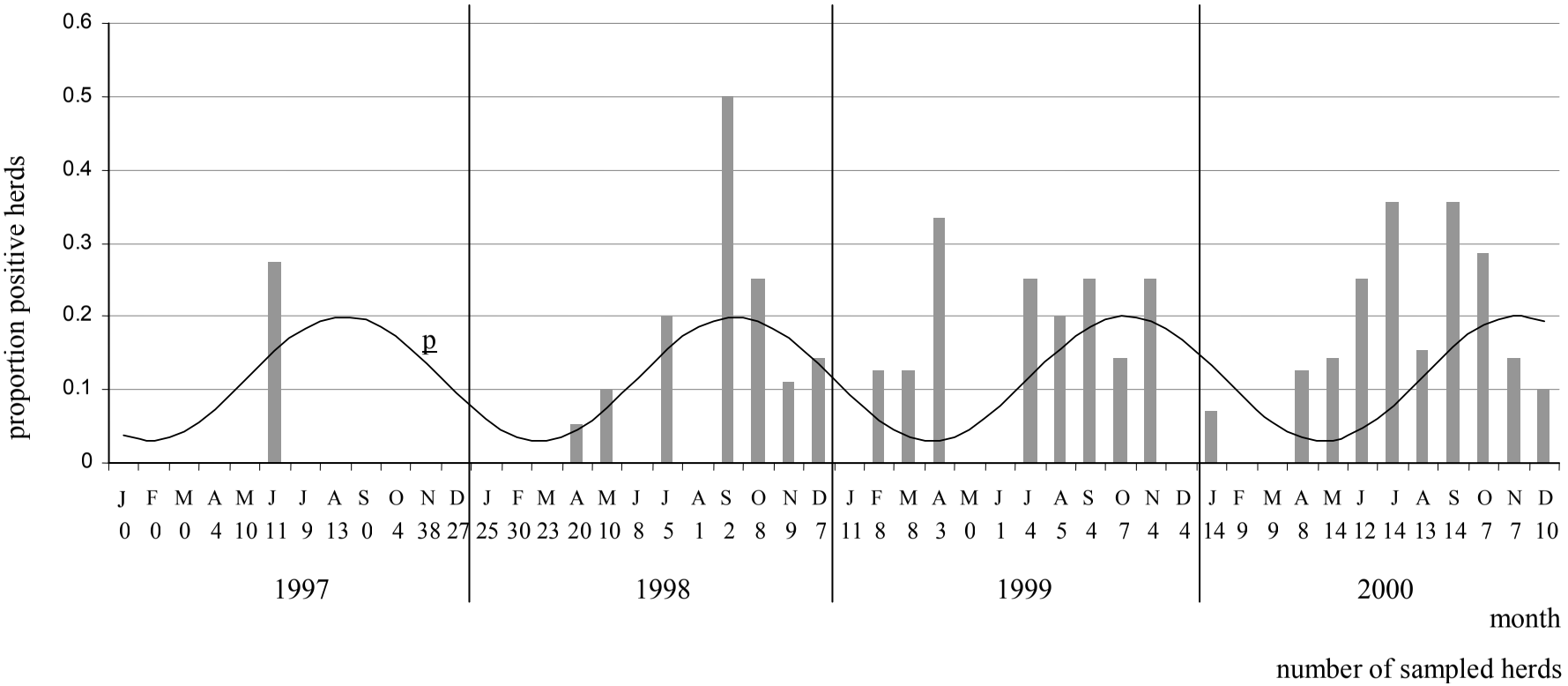

Figure 3.1 Number of sampled herds and proportions of *E. coli* O157 positive herds per month (#positive / #sampled) for the period January 1997 up to December 2000, and fit sine function ($\underline{p} = 0.115 - 0.085 * SIN((2\pi t / 12.910) + 0.687)$; $R^2=0.21$)

Table 3.3 Number of sampled herds or flocks, number of (pooled) samples taken, number of *E. coli* O157 positive tested herds/flocks, and prevalences (Prev) in herds/flocks of veal calves, broilers, laying hens, and finishing pigs.

Type of herd/flock	Year of sampling	No. herds or flocks sampled	No. pooled samples	No. positive tested herds or flocks	Prev (%)	95%CI
Veal calves	1996+1997	117	552	3	2.6	0.5 - 7.3
	1998	152	687	8	5.3	2.3 - 10.1
	1999	60	281	8	13.3	5.9 - 24.6
	2000	133	621	23	17.3	11.3 - 24.8
	Total	462	2,141	42	9.1	6.6 - 12.1
Broilers	1996+1997	63	336	0	0.0	0 - 5.7[c]
	1998	186	925	2	1.1	0.1 - 3.8
	1999	100	496	4	4.0	1.1 - 9.9
	2000	n.i.[a]	-	-	-	-
	Total	349	1,757	6	1.7	0.6 - 3.7
Laying hens	1996+1997	114	587	1	0.9	0.0 - 4.8
	1998	202	1,003	0	0.0	0 - 1.8[c]
	1999	113	553	1	0.9	0.0 - 4.8
	2000	n.i.[a]	-	-	-	-
	Total	429	2,143	2	0.5	0.0 - 1.7
Finishing pigs	1996[b]	7	35	0	0.0	0 - 41.0[*]
	1998	41	191	1	2.4	0.0 - 12.9
	1999	188	896	0	0.0	0 - 1.9[c]
	2000	n.i.[a]	-	-	-	-
	Total	229	1,122	1	0.4	0.0 - 2.4

[a] not investigated

[b] herds not sampled in 1997, due to CSF outbreak in The Netherlands

[c] one-sided 97.5%CI

The herds used for statistical analyses represent about 16.0% of all Dutch veal herds in 2000 (CBS, 2001). From 176 sampled herds (38.7%) herd size was recorded. The average number of animals on these observed farms was 528 (median=417, range: 5–9000), compared to an average of 271 in the Netherlands in 2000 (CBS, 2001). Compared to all veal herds in The Netherlands, small farms (<100 calves) appeared to be underrepresented, while large farms (>300 calves) are overrepresented in the database (χ^2=87.7, df=6, $P_{\chi 2}$ <0.001; Figure 3.2).

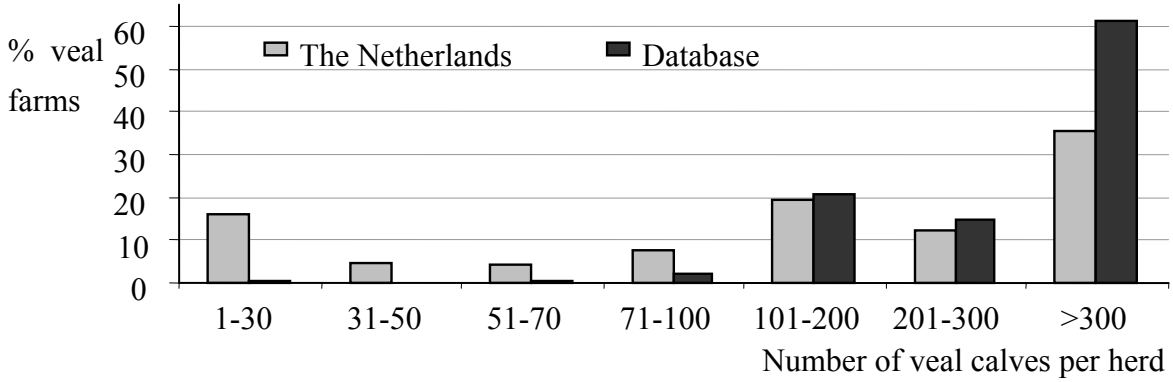

Figure 3.2 Distribution of farm size of veal herds in The Netherlands and in the database (CBS, 2001)

Although stratification for geographical region of the veal-herd study population was striven for, the distribution of regions in the database was not completely representative for The Netherlands; farms in the Northern and mid-part of The Netherlands were underrepresented, as farms in the South turned out to be overrepresented (χ^2=21.0=, df=2, $P_{\chi 2}$ =0.001, Table 3.4, Figure 3.3).

For veal herds, PCR results of five isolates were missing. Of the remaining 37 isolates, 10 isolates (26%) contained both the VTI and the VTII gene. Twenty-seven isolates (71%) exclusively contained the VTII gene and all 37 isolates contained the *eae*-gene. The single isolate from a finishing-pig herd and three of the four PCR-tested isolates from broiler flocks contained both VTI and VTII genes. One isolate from a broiler flock held no VT gene at all. All tested isolates from these types of herds or flocks possessed the *eae*-gene. Isolates from laying hen flocks were not tested with PCR.

Table 3.4 Number and percentage of veal farms per region in The Netherlands (based on data of CBS (2001)) and in the database

	The Netherlands		**Database**	
Region (Province)	**N**	**%**	**n**	**%**
North (Groningen, Friesland, Drenthe, Noord-Holland)	357	13.2	34	7.6
Mid (Overijssel, Flevoland, Gelderland, Utrecht, Zuid-Holland)	1872	65.3	279	62.7
South (Zeeland, Noord-Brabant, Limburg)	656	21.5	132	29.7
Total	2885	100	445[a]	100

[a] Of 10 veal farms in the database, region was unknown

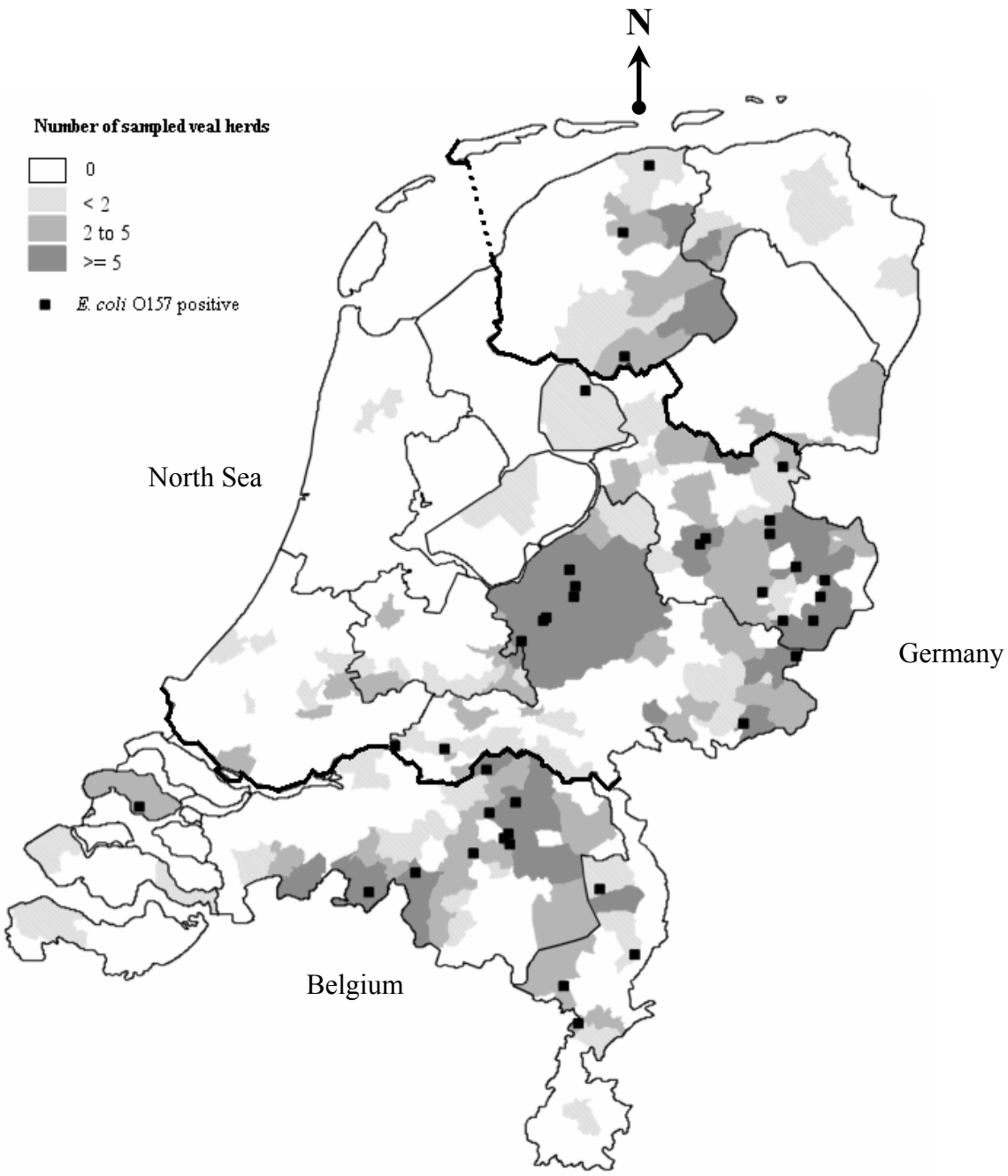

Figure 3.3 Geographic distribution of sampled veal herds and positive tested veal herds in The Netherlands (GIS). Borders of the geographical regions North, Mid and South are indicated by bold lines. Note: not all of the 42 O157 positive detected herds can be distinguished, due to identical postal codes

3.3.2. Statistical analysis

Univariable analysis resulted in 30 out of 65 variables remaining for multivariable analysis (Table 3.3).

Fisher's exact test indicated collinearity between the variables 'pink veal production' and 'Oxytetracycline (OTC) and/or Starters' mix (including OTC) supplied', 'group housing', 'natural ventilation system', 'number of calves', 'maize supplemented', 'barley supplemented', 'concentrates supplemented', and 'dog(s) entering the stable' ($p<0.01$). Comparing standard errors of estimates from univariate and bivariate (with 'pink veal production') models, collinearity was affirmed for all variables, except 'group housing'. Therefore, we decided to include 'pink veal production' and 'group housing' in the backward-elimination procedure, while all other correlated variables were eliminated from the analysis.

The variable 'producing for an integration' had a $P<0.25$ in univariable analysis, but had a prevalence of zero in two of the categories and could therefore not be included in the model. However, this variable was significantly ($P<0.05$) associated with *E. coli* O157 in the bivariate Fisher's exact test. In the "original" final model, the variables 'farmyard paving' and 'year of sampling' also contained one category with a prevalence of zero, which made the validity of the model fit questionable. Therefore, these variables were omitted from the final model. After using the backward-elimination procedure, 9 variables (Table 3.5) remained in the true final multivariable regression model. Due to missing values, this final model had data from 311 herds (10.0% cases). Risk factors were 'group housing of the sampled herd', 'more than one stable present' , 'pink veal production', 'hygienic measures regarding visitors', and 'interval arrival-sampling of a herd >20 weeks, or unknown'. One risk-reducing factor was 'presence of other farms within one km distance'. The variables 'presence of an entrance room (separated from feed preparing room)' and 'use of separate boots for stables' have $P>0.10$, but were confounders for 'interval arrival-sampling of a herd', 'hygienic measures regarding visitors' and 'more than one stable present' and therefore were forced into the model. Most interactions could not be tested in the final model due to the low prevalence. Interactions that could be calculated did not show statistical significance ($P>0.10$).

The value of the Hosmer-Lemeshow goodness-of-fit statistic was 3.74 ($P=0.88$; df=8). There is no evidence of a lack of fit of the final model (Hosmer and Lemeshow, 1989).

Table 3.5 Variables and their categories from veal herds (sampled in 1996-2000) in the final logistic regression model, frequencies (Freq), prevalence (Prev), Odds Ratio (OR), 90%-confidence interval and Wald's *P*-value (n=311 herds)

Variable	**Category**	**Freq (n)**	**Prev (%)**	**OR**	**90%-CI**	***P*-value (Wald's)**
Group housing of sampled herd	No	157	2.5	1.0		
	Yes	154	17.5	8.9	2.0-39.1	0.015
More than one stable on the farm	No	98	3.1	1.0		
	Yes	213	13.2	5.0	1.4-18.6	0.042
Pink-veal production	No	231	2.6	1.0		
	Yes	80	31.3	6.1	2.4-15.3	0.001
Entrance room of stable present (separated from feed preparing room)	No	275	8.0	1.0		
	Yes	36	25.0	2.3	0.8-6.55	0.193[a]
Other farms within 1 km distance	No	12	25.0	1.0		
	Yes	299	9.4	0.1	0.01-0.27	0.003
Use of separate boots for stables	No	136	14.0	1.0		
	Yes	175	6.9	0.4	0.2-1.00	0.101[a]
Hygienic measures regarding visitors	No	113	4.4	1.0		
	Yes	198	13.1	4.3	1.4-13.5	0.037
Interval arrival-sampling of veal herd (in weeks)	< 10	105	1.9	1.0		
	10-20	91	6.6	3.0	0.6-16.7	0.285
	>20	96	17.7	8.5	1.7-41.4	0.027
	Unknown	19	31.6	30.6	4.0-237.0	0.006

[a] Potential confounder for 'interval arrival-sampling of a herd', 'hygienic measures regarding visitors' and 'more than one stable present'

3.4. Discussion

Selection bias might have occurred, due to the voluntary basis of the study (Schouten *et al*., 2004). This might be an explanation for the dissimilar distribution of farm sizes in the database than in The Netherlands (Figure 3.1).

Faecal samples were taken from the stable floor or the running manure conveyer belt. It is therefore possible that collected faecal materials were outside the host for some time. However, visually fresh faeces were collected, so the effect of this sampling method on the microbiological results is likely to be small (Schouten *et al*., 2004). Yet, the sampling method implies that faeces of only part of the animals in a flock or herd are sampled, so individual shedders might have been missed. Therefore, underestimation of the prevalence among herds or flocks might have occurred.

Furthermore, herds only were sampled once. Because *E. coli* O157 are shed intermittently by cattle, strong fluctuations in point prevalences can occur (Hancock *et al*., 1997; Mechie *et al*., 1997). A herd harbouring *E. coli* O157 could therefore be tested negative. Therefore, an underestimation of the prevalence within the sampled population of veal herds might have occurred. Also, through this misclassification bias, the effect of exposure factors present on a farm might be falsely assumed to be negatively associated. Sequential sampling with a monthly interval partly prevents such bias (Hancock *et al*., 1997).

Within poultry flocks and finishing pig herds, we found O157 VTEC occasionally. In literature, information about prevalences of O157 VTEC within these types of herds or flocks is rare. Incidentally, enterohemorrhagic *E. coli* have been found in slaughter pigs (Borie *et al*., 1997; Chapman *et al*., 1997). In the Netherlands, Heuvelink *et al*. (1999) isolated *E. coli* O157 strains from pig and turkey flocks at slaughter -- but not from chicken flocks. However, in both poultry and pigs, persistent colonization of *E. coli* O157:H7 can occur, leading to excretion of the organism in the faeces (Stavric *et al*., 1993; Schoeni and Doyle, 1994; Booher *et al*., 2002); pigs and poultry therefore have the potential to be reservoir hosts for O157 VTEC.

In Dutch veal herds, O157 VTEC appears to be more regular than in other farm animals and prevalences seem to be increasing in time. The sample examination procedure during the first 3 years of the study might have underestimated the prevalences. Additional research at the laboratory of the RIVM indicated that the method of pooling the pool samples on the lab before 2000 might have given 45% false negatives (relative SE=55%; unpublished data). However, prevalences of O157 VTEC in veal herds still seem to have increased in the years 2001 and 2002, compared to the year 2000 (Bouwknegt *et al*., 2003*)*. The relative sensitivity of 55% of the laboratory's pooling method until 2000 (compared to the method applied as from 2000) might have also led to misclassification bias; the effect of exposure factors

present on a farm might be falsely assumed to be negatively associated, which entails levelling out of risk factors in the final model.

In veal herds, a seasonal pattern in infection with *E. coli* O157 was found. Unlike in dairy, positive veal herds were also detected in winter, though at a lower level than in summer (Schouten *et al*., 2004). The observed seasonal effect coincides with literature (Chapman *et al*., 1997; Hancock *et al*., 1997; Mechie *et al*., 1997) and should be allowed for in the risk analysis. Incorporating the seasonal prevalence distribution as offset in logistic regression analysis instead of including the season of sampling in the model as a categorical variable (*e.g.* months or seasons), will have increased the power of the analysis. This is because herds sampled in months/seasons where no O157 VTEC is isolated (prevalence of zero), do not have to be excluded from the analysis because of the calculated basic probability to be tested positive in a certain month. Besides, the offset only took 1df in the model, while a categorical seasonal variable would have taken more (which would have lowered the power). In general, herds being sampled in the winter have a lower basic probability to be found positive for O157 VTEC. By specifying this probability as offset, managerial and environmental circumstances of herds found positive during the winter were weighted more heavily in the model than herds found positive in the more-typical shedding season. In the fitted sine-function, the phase is 12.9 months, while biologically it should be 12. Also, the increasing trend of the yearly prevalences has not been incorporated in the function. Although this has been taken into account by including the variable "year of sampling" in all sub-models, a more-complex function might better fit the monthly prevalences. However, the effect on the final multivariable model outcomes was negligible. So although the fit of the function appears to be low (R^2=0.21), our method seemed to level out any categorical seasonal variable and was preferable because of the higher power.

Prevalences in the shedding season among veal herds (17.6% in May-November) were significantly higher ($P_{\chi 2}$ <0.01) than among dairy farms (10.6% in May-November; Schouten *et al*., 2004). This might partly be explained by the age-difference; calves shed larger amounts of *E. coli* O157 and for longer periods compared with adult cattle (Cray and Moon, 1995). Also, veal farms are not closed production systems (unlike most dairy farms). Former studies affirm a positive association of cattle entry in a herd and (O157) VTEC contamination (Wilson *et al.*, 1993; Nielsen *et al.*, 2002). Moreover, veal calves originate from many herds, which increases the probability of introduction of VTEC O157.

The factor 'presence of other farm(s) within 1 km distance of the sampled herd' was negatively associated with the presence of O157 VTEC on veal farms. An explanation for this intuitively illogical finding could not be found. Because this effect was measured based on only 12 observations in one of the categories, it should be interpreted with caution.

The factor 'interval between arrival and sampling of a herd' indicated that a longer interval is associated with an increased probability of O157 VTEC contamination -- perhaps associated with age of the calves. Calves older than 2 months are found positive more often than younger calves (Nielsen *et al*., 2002). The effect of the longer interval might also be explained by the larger period of time that calves are housed together in a herd and transmission could have taken place - with an increased probability of detection of the group's infection - or the administering of medications (antibiotics) at arrival on the farm. The category "unknown" of this variable shows a very high odds ratio, but this was based on few observations and demands interpretation with care. This category is included in the analysis, instead of classifying the variable for those herds as "missing", because removal resulted in a loss of positive detected herds in the analysis - leading to more variables with a prevalence of zero in one of its categories.

Mainly herds kept for pink-veal production were found positive for O157 VTEC (31.3% vs. 2.6% of white veal herds). This is biologically very likely; pink-veal production systems differ considerably from white-veal systems. Calves for pink veal usually stay longer on the farm (35 wk vs. 25 wk, respectively), which increases the time that transmission can occur. Pink-veal production has a very distinct feeding strategy, with for example the supply of roughage and concentrates, which might influence the O157 VTEC status. Feeding regimes seem to have an effect on the presence on O157 VTEC infections in ruminants (Schouten *et al*., 2004l; Dargatz *et al*., 1997; Diez-Gonzalez *et al*., 1998; Herriott *et al*., 1998). However, whether the described effects are comparable in calves is not known. Furthermore, pink-veal production systems are presumed to apply less-strict biosecurity measures than white-veal systems. Collineairity was found between "pink veal production" and 'OTC and/or Starters' mix (including OTC) supplied', 'group housing', 'ventilation system', 'number of calves', maize supplemented', 'barley supplemented', 'concentrates supplemented', and 'dog(s) entering the stable'. In univariable analysis, these were found positively associated with O157 VTEC contamination of veal herds in general. However, when entering "pink veal production" to the model, the correlated variables consequently were omitted from the model. Possibly, some of these variables might be underlying explaining factors for the larger prevalence of O157 VTEC among pink veal herds than among white veal herds.

The variable "group housing of sampled herds" was retained in the final model and showed a significant effect. Housing calves in groups appeared to be a considerable risk factor in this study as compared to individual housing – regardless of pink- or white-veal systems; this could be rationally explained by the limited contact structure of individual housed calves and related lower expected rate of transmission (Bouma *et al.*, 2004).

Farms with more than one stable had higher risk. This might involve biosecurity as well, which should be applied very strictly to every stable, to prevent introduction of the pathogen to one of the stables and also transmission from the pathogen from one stable to another.

Associations between variables and presence of *E. coli* O157 on veal farms in this study should be interpreted carefully. The variable 'hygienic measures regarding visitors' was positively associated with faecal shedding of *E. coli* O157 and therefore considered to be a risk factor. Yet, it might the result of actions taken by the farm manager due to the recognition of another pathogen-associated problem or to the fact that the farm is visited more often than average. Therefore, and due to the relatively low prevalence of *E. coli* O157, most results presented in this study should rather be considered as indications for further (experimental or cohort-based) research.

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Chapter 4

A longitudinal study of *Escherichia coli* O157 in cattle of a Dutch dairy farm and in the farm environment

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Abstract

From July 1999 till November 2000, a longitudinal study was conducted on a dairy farm in The Netherlands to study within herd prevalence and types of verocytotoxin producing *Escherichia coli* (VTEC) of serogroup O157 over time, and determine environmental reservoirs and possible transmission routes.

Faeces, blood, milk and environmental samples were collected 14 times with intervals varying from 4-10 weeks during the study period. Faecal samples were selectively cultured for *Escherichia coli* O157. Isolates were tested by PCR for the most common virulence genes, VTI, VTII and eae, and typed by pulsed field gel electrophoresis. In total, 71 isolates were obtained, of which 49 from dairy cows, eight from young stock, five from other animals and nine from the environment. Positive samples were all detected in summer and early fall. VT- and *eae*-genes were found in all tested isolates, except in one. DNA typing showed that three clusters of O157 isolates could be identified. One of these clusters contained samples of two shedding seasons, indicating persistence on the farm during winter and spring.

Repeated measures analysis of variance showed that cows with O157 VTEC infection had higher daily milk production in the period preceding sampling ($p=0.0055$). There was no significant association between the results of the LPS-ELISA on serum samples from dairy cows and their O157 status.

4.1. Introduction

The strain of *Escherichia coli* expressing somatic O-antigen 157 was first described as a food borne human pathogen in 1982 (Riley *et al.,* 1983). Human illness associated with *E. coli* O157 has been reported with increasing frequency since. The strain typically contains genes that encode verocytotoxins (VT1 and/or VT2), attaching and effacing proteins (*eaeA*), and hemolysins (*hly*). Symptoms caused by verocytotoxin producing *E. coli* (VTEC) of serogroup O157, range from non-bloody diarrhoea to haemorrhagic colitis (HC) (Tesh and O'Brien, 1991) and, particularly in children and elderly, to the potentially lethal hemolytic-uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP) (Karmali *et al*., 1989; O'Brien and Kaper, 1998).

O157 VTEC has been demonstrated to be transmitted to humans by a variety of foods, water, person-to-person or animal-to-person contact (Dev *et al.,* 1991; Renwick *et al*., 1993; Keene *et al*., 1994; Armstrong *et al*., 1996; Ackers *et al*., 1998; Heuvelink *et al.,* 1999). The majority of outbreaks, however, were related to cattle or cattle products (Griffin and Tauxe, 1991; Meng and Doyle, 1998). Epidemiological studies have identified cattle as main reservoir host for *E. coli* O157 and other VTEC (Chapman *et al*., 1993; Armstrong *et al.,* 1996; Heuvelink *et al*., 1998b), but domestic animals in general have been shown to excrete *E. coli* O157 (Beutin *et al*., 1993; Hancock *et al.,* 1998).

The presence of O157 VTEC in cattle and their environment has been observed frequently (Hancock *et al*., 1997; Mechie *et al*., 1997; Garber *et al*., 1999). Little is known about within farm dynamics of O157 VTEC on cattle farms in The Netherlands, though.

From a monitoring program (Bouwknegt *et al*., 2003), an O157 VTEC positively tested dairy farm was selected for a longitudinal study. The purpose of this study was to estimate prevalence's of O157 VTEC in time, identify possible environmental reservoirs and intermediate hosts for O157 VTEC and determine types of O157 VTEC within a farm. From this, more could be learned about within-farm transmission. Furthermore, it was tested whether or not O157 infection of individual cows is associated with milk production level. Additionally, the association between antibody levels and bacteriological culturing was studied.

4.2. Materials and methods

4.2.1 Dairy farm characteristics

A dairy farm, situated in the central part of The Netherlands, was randomly selected from a group of dairy farms that recently tested positive for O157 VTEC in a monitoring program. In this monitoring program, which was conducted by the National Institute for Human Health and the Environment (RIVM), herds are randomly selected from the population of registered dairy herds and are sampled once (Schouten *et al*., 2004). The farm in the current study was sampled and tested O157 VTEC positive in October 1998. The longitudinal study started July 2, 1999 and ended November 13, 2000. During this period, animals and the environment of the farm were sampled 14 times, with intervals of 4 to 10 weeks.

During the study period, the farm had an average herd size of 75 Holstein/Friesian cows and 50 young stock, and about 50,000 m^2 of pasture. Approximately 20% of the dairy cows were non-lactating at sampling moments. Lactating cows were held in a loose housing system with slatted floor and cubicles from November to March, and were on pastures during the day from April through October. Non-lactating cows, heifers and calves were housed separately from lactating cows. During winter, they were in three adjacent barns. In this study, non-lactating cows were assigned to the same group as young stock, since they were housed in the same barn. In summer, heifers and calves older than 3 months were on pastures at 1.5 km distance, and were therefore not sampled. Non-lactating cows and a small number of heifers and calves stayed in pastures beside the farm premises. The number of sampled calves, heifers and non-lactating cows on the farm premises varied therefore with season from 6 to 76.

No animals were purchased since 1998. For breeding, mainly artificial insemination was used. For servicing heifers, a young bull, which was bred on the farm, was used. Calving took place all year round, with clustering in August. Calves were separated from their dams immediately after birth and fed colostrum, preferably from their own mother. Pre-weaning calves were housed individually in separate calf boxes or in groups of three or four in straw bedded pens and were fed fresh cow milk and calf concentrate until weaning at approx. 3 months of age. Male calves, however, were sold for veal production at two weeks of age, except three per year that were retained on the farm. After weaning, calves were reared in groups of 4 to 8 animals and housed in pens with slatted floors during winter or transported to pasture in summer. In winter, calves six months of age were moved in groups of approximately 15 animals to the barn with non-lactating cows.

Dairy cattle were provided an amount of dairy concentrates adjusted to milk yield. Young stock was fed calf concentrates during their stay outside. In winter, dairy cattle, young

stock and weaned calves were fed various forms of supplemental feed, including grass silage, corn silage, hay and potatoes.

Slurry and manure was injected into the soil on owned land intended for grazing and harvesting. Occasionally, pig manure was purchased for fertilizing land.

4.2.2 Sample collection

Dairy cows, non-lactating cows and young stock

In winter, all animals were sampled individually. Per cow, minimally 10 grams of faeces was collected directly in plastic bags by rectal palpation. From calves younger than 6 months, rectal swabs were taken. Swabs were put in a tube or jar with 2-5 ml of a peptone solution. For dairy cows, all samples were taken in the stable directly after morning milking, with the cows tied at the feed bunk. During summer months, fresh-looking faecal samples from non-lactating dairy cows and young stock on the pastures adjacent to the farm premises were taken randomly from the ground and were pooled per six samples.

Blood samples were drawn from cattle present on the farm premises, by tail vein puncture (heifers and adult cows) or from the vena jugularis (calves <6 months), using a 10 ml vacutainer (Venoject).

Fore-milk was sampled and pooled per group of eight simultaneously milked cows at morning milking. Of each cow, five foremilk streams per quarter were pooled in a jar, directly after pre-treating the teats with a dry cloth. Per pool, individual animal numbers were registered. After milking, teats were sprayed with active iodine solution with lanoline and glycerine. A sample of the bulk milk tank and the milk filter of the morning milking were taken for analysis.

Dairy characteristics for each lactating cow were obtained from the milk testing reports of the Dutch Cattle Association (NRS). These variables were age, lactation number, calving date, cumulative milk production, estimated 305-day milk production, and somatic cell count for each dairy cow per sampling.

Other animals

When handling of the animals was possible, rectal swabs were taken individually from sheep present on the farm or on adjacent pasture. From pony's, optically fresh faeces was sampled from the stable floor. Rectal swabs were taken from dogs, manageable cats and a pet rabbit. From the chickens present in a run, fresh droppings were collected and pooled. Also droppings likely originating from wild birds were collected.

Seven days before sampling, mouse/rat traps were placed on specified sites on the farm premises, appointed with help of the farm manager. When rodents were caught before the day

of sampling, they were put in a closed box and cooled until the moment of transport. A day before sampling, sticky fly traps were put in specified places in the stables. At sampling, fly traps were collected and subsequently transported in jars.

Environment

Environmental samples included a variety of feed, hay, straw, wood shavings, grass and corn silage (in jars); water from water troughs, pools, puddles and ditches (in bottles); and swabs from the environment of cattle (feeding troughs, feed bunk, slatted floor, milking parlour, silo's, tractor tyres, manure injector etc.), using large sterile cotton swabs. About forty environmental samples were taken per sampling.

4.2.3 Sample processing

All samples were cooled and transported in Styrofoam boxes. Samples other than blood were taken to the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands. Samples were, if necessary, prepared for microbiological analysis, *e.g.* feed was stomached, water was filtered with cellulose nitrate membrane filters (pore size 0.45 µm, Sartorius, Germany), and gastro-intestinal tract of rodents were prepared from the body. Blood samples were taken to the Animal Sciences Group in Lelystad for serological analysis.

Microbiological analysis

Microbiological analysis of the samples was performed within 48 hours after sampling to determine whether *E. coli* O157 was present. Isolation methods are described in detail by Schouten *et al*. (2004). In brief, portions of maximally 10 ± 0.5 grams of a (pooled) faecal or milk sample, entire water/milk filters, or swabs with peptone solution, were cultured in 90 ml modified Trypton soya broth (Oxoid Ltd., Basingstoke, England) with acriflavine (10 mg/l; mTSB+A) for 6-8 hours at 37 ^{o}C (± 1 ^{o}C). Subsequently, Immuno Magnetic Separation (IMS) was done on one ml of the culture**.** After IMS, the solution was inoculated onto sorbitol MacConkey agar (SMAC) (Oxoid), enriched with 0.05 mg/l cefixime and 2.5 mg/l tellurite (CT-SMAC), incubated at 37 ^{o}C (± 1 ^{o}C) for 18-20 hours, and screened for sorbitol-negative colonies. If present, 12 sorbitol-negative colonies per sample were inoculated onto both SMAC supplemented with 0.1 g 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma Chemical Co., St. Louis, MO) and eosin methylene blue agar (37,5 g/l EMB) (Oxoid) and incubated at 37 ^{o}C (± 1 ^{o}C) for 18-20 hours. Suspected *E. coli* O157 colonies were tested using the Wellcolex agglutination latex kit (Murex; Kent, UK) in order to ascertain the authenticity of the colonies. Finally, one isolate per sample that was confirmed by

agglutination, was serotyped. A sample was considered positive when serotyping identified the isolate as *E. coli* O157.

E. coli O157 isolates were tested for possession of genes encoding for the most relevant verocytotoxins, VTI and VTII, and the eae-gene, applying a polymerase chain reaction (PCR) technique. Established oligonucleotide primers were used (Tilburg and van de Giessen, 1996; Bouwknegt *et al*., 2003)

Pulsed Field Gel Electrophoresis

Additionally, DNA fingerprints were made from the isolates using pulsed field gel electrophoresis (PFGE) based on Barrett *et al*. (1994), with *Xba* I as restriction enzyme. Cluster analysis of fingerprints was done with Bionumerics® (Dendrogram type=UPGMA, Similarity coefficient=Dice). Isolates with at least 95% matching DNA-fragments are considered 'highly related'. Isolates are considered indistinguishable when 100% of the DNA-fragments is analogous.

Serological analysis

Serum was prepared by centrifugation and stored at -20 ^{o}C until analysis. All samples were tested for antibodies to lipopolysaccharide (LPS) of O157 VTEC using an indirect ELISA. Briefly, each well of micro dilution plates was coated with 5 µg of LPS, prepared from strain CIDC-1 by the hot water-phenol method as described by Zijderveld *et al*. (1992). The plates were subsequently washed three times with tap water containing 0.05% Tween 80. Next, 100 µl of serial twofold dilutions of sera with phosphate buffered saline pH 7.2, containing 0.05% Tween 80 (1:20, 1:40, 1:80, etc.) were added and plates were incubated for 1h at 37 ^{o}C. After washing again, 100 µl of HRPO-labeled mouse monoclonal anti-bovine IgG1 was added and plates were incubated for 1h at 37 ^{o}C. After that the plates were washed, 100 µl of the substrate solution (containing 0.1% (wt/vol) 5-aminosalicylic acid and 0.005% (wt/vol) hydrogen peroxide) was added and optical densities were read at 450 nm after 2 to 2.5h of incubation at room temperature. Positive and negative control sera were included in each test plate. These were obtained from animals used in a transmission experiment. Negative control sera were gained from 10 animals, which were repeatedly tested and found negative for *E. coli* O157, just before inoculation with 10^{9} cfu of *E. coli* O157. Positive sera were gained from the same animals, which were still shedding, 5 days after inoculation.

Titers of sera were expressed as the reciprocal of the dilution yielding an absorbance that was 50% of the maximum obtainable absorbance value.

4.2.4 Statistical analysis

Monthly milk testing reports present, per cow, cumulative milk production during lactation. From these data, per cow daily milk production (DMP in kg) was calculated for each period preceding sampling. Since multiple measurements per animal cannot be regarded as independent units of observation, the association of *E. coli* O157 infection at each sampling with DMP in the interval preceding that sampling, was analysed by using repeated measures analysis of variance (PROC MIXED (Little *et al*., 1996) of SAS VERSION 8.00), with O157 infection, lactation number (class variable), number of days in milk at time of sampling (DIM) and shedding season (May-October vs. November-April) included as fixed effects. A first-order autoregressive covariance structure (AR(1)) fitted best and was used to account for within-cow variation. Satterthwaite's method was used to calculate the denominator degrees of freedom for F-tests on the fixed effects in the model. Model assumptions were evaluated by examining the distribution of residuals.

Logistic regression was performed to model the effect of level of the highest titer measured per animal ($titer_{max}$) on the probability of O157 VTEC infection during the study (titer ≤80 vs. titer ≥160).

4.3. Results

4.3.1 Descriptive statistics

The study comprised data of 94 dairy cows. The mean number of lactating dairy cows per sampling was 59 (median 60; range 55-62). Dairy cows were on average sampled 9 times (median=10; range 1-14). The age of the sampled dairy cattle ranged from 2-9 years, with a mean of 3.6 years and a median of 3.0. The average estimated 305-day milk production was 8,189 kilograms (SD= 1,299, median=8,122, range: 3,886–11,821 kg).

In total, 2,164 samples were tested for O157 VTEC during the study. These included 157 milk related samples, 1,262 faecal samples of cattle (830 dairy, 422 young stock and non-lactating cows, 10 pool samples), 148 faecal samples of other animals, 3 faecal samples of the farmer and 594 environmental samples. Seventy-one samples tested positive for O157 VTEC.

O157 VTEC was neither isolated from any of the raw milk samples (n=140), nor from milk filters (n=4) and bulk milk samples (n=13). Of the 71 isolates of O157 VTEC, 49 originated from faecal samples of dairy cows. In total, 39 of 94 (41.4%) individually sampled dairy cows were found O157 VTEC positive at least once during the study. Nine dairy cows

tested positive in more than one sampling. From these animals, four were shedding two samplings in a row and one three in a row. The other four cows were found positive at two samplings with one negative sampling in-between. All isolates from dairy cows were found in the months May-October (Table 4.1), with a prevalence up to 29.5%. No cows were found positive in both shedding seasons.

The dataset available for statistical analysis of daily milk production (DMP) comprised data of 93 dairy cows. Due to missing data on milk production or change in lactation number, only of 534 of 830 records (64.3%) daily milk production between samplings could be calculated (mean 26.1 kg; SD=7.1; median 25.6; range: 3.3-58.4). DMP in the period preceding testing O157 positive was higher (lsmeans=30.2 kg; SEM=1.06; n=25) than when tested negative (lsmeans=27.5, SEM=0.56; n=509) (p=0.0055). DMP's for first and second lactating cows were lowest, respectively 23.1 and 27.2 kg; DMP's for lactation numbers 3-8 were respectively 30.9, 30.0, 31.3, 27.7, 27.9 and 32.3 kg (P<0.0001). Increasing DIM was associated with decreasing DMP (P<0.0001). No significant effect of shedding season on DMP was found and was therefore left out of the model. The interaction between O157 and lactation number could not be estimated. The interaction between O157 and DIM was not significant (p=0.84) and therefore deleted from the model. Additionally, the statistical model was also run only for cows sampled in the shedding season (May-October) (252 DMP out of 475 records) and yielded the same conclusion about the association between O157 positive and DMP (P=0.006). Also least square means of DMP were similar, respectively 30.1 and 27.5 kg for positive and negative tested animals.

From young stock and non-lactating cows 422 individual and 10 pooled faecal samples were taken. From the individually sampled young stock, O157 VTEC was isolated from six samples (1.4%). One heifer was found positive twice, at samplings in August and November 2000. Also two pooled faecal samples from a pasture with a group of young stock were found O157 VTEC positive, in June and August 2000 (Table 4.1).

From other animals on the farm, 148 samples were taken (Table 4.2). O157 VTEC was isolated from two of the 39 swabs taken from 2 dogs, from one of 18 faecal samples of the pony's and from the chickens in the run (2/12 pool samples). Samples from sheep and goats were not found positive. At sampling six, twelve and thirteen, faecal samples were obtained from the farmer, which were all negative.

Table 4.1 Number of faecal samples (including rectal swabs) and percentage of O157 VTEC isolates in cattle on a Dutch dairy farm within the period July 1999-November 2000.

	Year	**1999**					**2000**								
	Week of study	0	4	8	13	19	26	31	36	42	46	51	56	60	70
Sample	**Total**	**2/7**	**30/7**	**30/8**	**4/10**	**12/11**	**5/1**	**8/2**	**15/3**	**26/4**	**22/5**	**28/6**	**2/8**	**31/8**	**13/11**
Lactating cows															
Faeces individually															
Tested	830[a]	61	59	56	58	58	61	59	60	62	60	60	62	59	55
No. pos	49	18	12	7	1	0	0	0	0	0	3	5	3	0	0
%	5.9	29.5	20.3	12.5	1.7	0.0	0.0	0.0	0.0	0.0	5.0	8.3	4.8	0.0	0.0
Non-lactating cows and young stock															
Faeces individually															
Tested	422	12	6	6	6	18	58	76	74	61	10	17	10	16	52
No. pos	6	2	0	0	0	0	0	0	0	0	0	1	2	0	1
%	1.4	16.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.9	20.0	0.0	1.9
Faecal pools															
Tested	10	3	0	1	0	0	1	0	0	1	0	2	1	1	0
No. pos	2	0	0	0	0	0	0	0	0	0	0	1	1	0	0
%	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0	100	0.0	0.0

[a]Samples were taken from 94 different dairy cows, of which 39 (41.4%) were found positive at least once during the study

Table 4.2 Number of samples and percentage of O157 VTEC isolates from other animals than cattle on a Dutch dairy farm within the period July 1999-November 2000.

Samples from		**Tested**	**No. positive**	**%**	**Date of isolation**
Sheep/goats (n=6)	*individual*	51	0	0	
	pools	6	0	0	
Horses (n=2)*	*individual*	18	1	5.6	2-8-2000
Dogs (n=2)**	*individual*	39	2	5.1	2-7-1999; 2-8-2000
Chickens (n≈10)	*pools*	12	2	16.7	28-6-2000; 2-8-2000
Birds	*pools*	5	0	0	
Flies/maggots	*pools*	4	0	0	
Cat	*individual*	1	0	0	
Mouse	*individual*	1	0	0	
Rabbit	*individual*	10	0	0	
Hedgehog	*individual*	1	0	0	
Farmer	*individual*	3	0	0	

* One pony was present at the farm for 3 sampling rounds

** At sampling one, 10 pups were present on the farm. One extra sample was taken from dog faeces found in the stable at sampling one.

From 594 environmental samples, 1.5% (9/594) was tested positive for O157 VTEC. One isolate was found in a puddle on the farm premises and two on the manure injector (both the filling tube at the front and the injectors at the back) (sampling 1), one in swabs from the feed bunk (sampling 4) and five on the slatted floor in the dairy stable (samplings 1 and 11).

No blood samples were taken at samplings 2, 6, 12 and 14. A total of 579 blood samples were obtained from 136 animals (dairy cows and young stock). From dairy cattle, 397 blood samples were drawn of 85 cows. The serological response in cattle was low, with titers ranging from 20 to 1280 (median: 80). Maximum measured titers during the study ($titer_{max}$) of animals that were tested O157 VTEC positive at least once did not significantly differ from maximum titers of animals that were tested negative during the whole study. Of dairy cows with $titer_{max} \geq 160$, 34.5% (10/29) were found O157 positive during the study. Of the animals with $titer_{max} \leq 80$, 46.4% (26/56) were found O157 positive ($p=0.29$; likelihood ratio test).

PCR results from one dairy cow and one calf were missing. All isolates from cattle exclusively contained the VTII gene and the *eae*-gene except one isolate from a heifer in

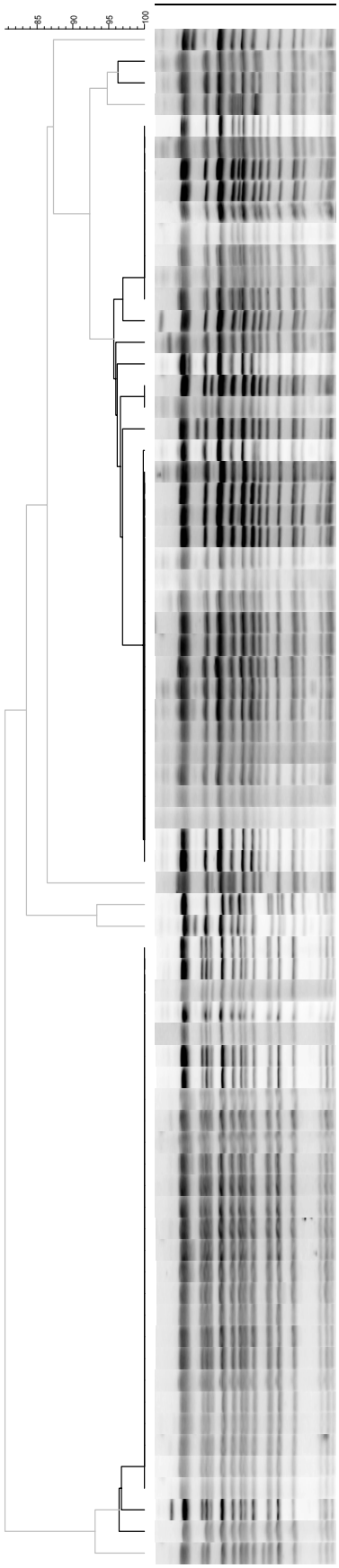
which no *eae*-gene was found. Isolates from the dogs, chickens, the pony, and from the environment contained the VTII gene as well. Two isolates found in swabs of the housing of lactating cows also contained VTI encoding genes. All tested isolates from these samples possessed the *eae*-gene.

4.3.2 PFGE

PFGE cluster analyses showed three clusters of isolates A, B and C, which had at least 95% of fragments in common, containing 2, 35 and 28 isolates, respectively (Figure 4.1). Besides those clusters, six isolates with less than 95% corresponding DNA fragments were found in individual dairy cows, a pool sample from the dairy stable, the feed bunk and a calf individually held in a box. These isolates were found at samplings one, two, four and twelve.

The smallest cluster, A, consists of two isolates from two different dairy cows at the first and the second sampling, both in summer 1999. Within cluster B, three groups of indistinguishable isolates can be distinguished, with 9 (b1), 2 (b2) and 20 (b3) isolates respectively. These originated from individual dairy cows, individual young stock, the manure injector, a dog and a puddle, all sampled at the first, second and third sampling. Four other isolates of cows were highly related. Cluster C contained one group of 26 (c1) indistinguishable isolates (100% equal), and two highly related isolates. Cluster C, however, covers a period from July 1999 (first sampling) to August 2000 (sampling 12) and therefore incorporates two shedding seasons. This strain was found in four cows in the first shedding season and was the most frequently shed strain in animals in the subsequent shedding season in 2000. Cluster C included isolates from individual dairy cows, a dog and a pony, and pooled faecal samples from the slatted floor, a pasture with young stock and from the chicken run.

From the nine dairy cows that were found positive for O157 VTEC more than once during the study, four dairy cows (2, 3, 6, 7; Figure 4.1) only shed highly related or indistinguishable strains belonging to cluster B and one dairy cow (9; Figure 4.1) shed indistinguishable strains from cluster C. In three animals (4, 5, 8; Figure 4.1), strains from both clusters B and C were found at different samplings (Figure 4.1). From one cow, a strain belonging to both clusters A and C was isolated.



PFGE cluster[a]		Source	Date of isolation
		dairy cow	2-7-99
A		dairy cow	2-7-99
A		dairy cow[1]	30-7-99
		dairy cow	30-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow[2]	2-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow[3]	2-7-99
B	b1	dairy cow	2-7-99
B	b1	dairy cow	2-7-99
B		dairy cow	2-7-99
B		dairy cow[4]	30-7-99
B		dairy cow[5]	30-8-99
B	b2	dairy cow	2-7-99
B	b2	dairy cow	30-8-99
B		manure injector, front	2-7-99
B	b3	dairy cow[6]	30-8-99
B	b3	Dog	2-7-99
B	b3	manure injector, back	2-7-99
B	b3	Puddle	2-7-99
B	b3	dairy cow[6]	2-7-99
B	b3	Heifer	2-7-99
B	b3	Heifer	2-7-99
B	b3	dairy cow	2-7-99
B	b3	dairy cow[7]	30-7-99
B	b3	dairy cow	30-7-99
B	b3	dairy cow[2]	30-7-99
B	b3	dairy cow[3]	30-7-99
B	b3	dairy cow	30-7-99
B	b3	dairy cow	30-7-99
B	b3	dairy cow	30-7-99
B	b3	dairy cow	30-7-99
B	b3	dairy cow[2]	30-8-99
B	b3	dairy cow	30-8-99
B	b3	dairy cow[8]	30-8-99
B	b3	dairy cow[7]	30-8-99
		dairy cow	30-7-99
		dairy stable floor	2-7-99
		feed bunk	4-10-99
C	c1	dairy cow[1]	2-7-99
C	c1	dairy cow[5]	2-7-99
C	c1	dairy cow[8]	2-7-99
C	c1	dairy cow[4]	4-10-99
C	c1	heifer	13-11-00
C	c1	dairy cow	22-5-00
C	c1	dairy cow	22-5-00
C	c1	dairy cow	28-6-00
C	c1	dairy cow[9]	28-6-00
C	c1	dairy cow	28-6-00
C	c1	dairy stable floor	28-6-00
C	c1	dairy stable floor	28-6-00
C	c1	dairy stable floor	28-6-00
C	c1	chickens	28-6-00
C	c1	dairy cow	28-6-00
C	c1	calf	28-6-00
C	c1	pool heifers	28-6-00
C	c1	dairy stable floor	2-8-00
C	c1	dog	2-8-00
C	c1	chickens	2-8-00
C	c1	dairy cow[9]	2-8-00
C	c1	pool heifers	2-8-00
C	c1	heifer	2-8-00
C	c1	dairy cow	2-8-00
C	c1	dairy cow	2-8-00
C	c1	horse	2-8-00
C		dairy cow	22-5-00
C		dairy cow	28-6-00
		calf	2-8-00

[a] Closely related isolates (A, B, C) and indistinguishable isolates (b1, b2, b3, c1) are defined as >95% and 100% fragments in common, respectively; [1, 2, 3, …] Dairy cows that were found positive more than one sampling

Figure 4.1 Results of pulsed-field gel electrophoresis of O157 VTEC isolates from a Dutch dairy farm in 07/1999-11/2000. Clusters A, B and C are identified with at least 95% common fragments.

4.4. Discussion

The purpose of this longitudinal study was to examine within-herd prevalence's in time and determine possible environmental reservoirs, intermediate hosts and PFGE types of O157 VTEC. With this information, more could be learned about within farm transmission. Also, it was tested whether O157 VTEC infection of individual cows was associated with level of daily milk production in the period preceding sampling. LPS-ELISA as a diagnostic tool for O157 VTEC detection was explored.

From non-lactating dairy cows and young stock on the pastures faecal samples were taken from the ground and were pooled (per 6 samples). It is possible that collected faecal materials were outside the host for some time. However, visually fresh faeces were collected, so the effect of this sampling method on the microbiological results is likely to be small (Schouten *et al*., 2004). Additionally, diagnostic sensitivity of Immunomagnetic separation (IMS) is rather high (as low as 0.49 cfu/g feces detected), and therewith the probability of detection in pool samples (Dam-Deisz and Evers, 2001).

Considering on-farm presence and transmission of O157 VTEC in this study, three groups were distinguished: cattle, other animals and the environment. Transmission within and between these groups might contribute to the persistence of O157 VTEC infection on a farm. PFGE is known to have a high discriminatory power, which is of great value for determining cluster contamination. PFGE results of this study confirm that O157 VTEC of certain types were distributed in cattle, the environment of cattle and in other animals on the infected farm. In former studies, dogs, horses and chickens were indicated as potential shedders of O157 VTEC (Armstrong *et al*., 1996; Beutin *et al*., 1993; Chapman *et al*., 1997; Hancock *et al*., 1998). Results of this study support these findings. In this study, however, it is unclear in which direction transmission has taken place and whether O157 VTEC was able to multiply in the environment or animals other than cattle.

Regarding cattle, this study provides additional evidence on seasonal variation in O157 VTEC shedding in The Netherlands. In a monitoring program, a prevalence of 7.2% (49/678) among dairy herds was found in the period November 1996 through December 2000 (Schouten *et al*., accepted). In that program, only one dairy herd was found positive from December to April), which is consistent with the results of the current study. The seasonal pattern in this study is also in agreement with results of other longitudinal or cross-sectional studies (Hancock *et al*., 1997; Garber *et al*., 1999; Mechie *et al*., 1997). Heuvelink *et al*. (1998a) already found a similar seasonal pattern on dairy farms in The Netherlands, though they detected O157 VTEC in calves < 4 months old during winter. In our study, no calves were found positive during winter.

Given the seasonal effect, the question is whether repeated infection of a farm is a result of introduction of new bacteria on the farm or a matter of persistence in cattle, intermediate hosts or the environment. Results suggest that, although a lower percentage of cattle was found positive in 2000 compared to 1999, the most occurring type of O157 of the second season was already present in a low prevalence in the first season. This finding might indicate certain extent of survival of O157 VTEC over seasons. The persisting type seemed in 2000 to be less prevailing in cattle, but more present in the environment or other animals. Possibly, this strain might be more adapted to circumstances outside the presumed main host and therefore able to persist on the farm for a longer period of time, including the winter period.

Heuvelink *et al*. (1998b) presented within-herd prevalence's varying between 0.8% and 22.4% in seven positively tested Dutch dairy herds in September-November. From these herds, all cattle (including young stock) were sampled individually once and O157 VTEC was merely isolated from young stock <12 months. Within our study, mainly dairy cows appeared to be shedding O157 VTEC, with monthly prevalence's up to 29.5%. Although weaned calves are known to shed higher numbers of *E. coli* O157:H7 and for a longer period of time than older animals (Cray and Moon, 1995), only few isolates were found in young stock. However, young animals were mainly absent from the farm (and therefore not tested) during the shedding season, which might explain these findings.

E. coli O157 are known to be shed intermittently by infected animals, indicating that strong fluctuations in prevalence can occur (Hancock *et al*., 1997; Mechie *et al*., 1997). An infected, but not shedding animal could thus be tested negative. Due to this mis-classification bias, the prevalence of infected animals might be underestimated (and the effect of dairy production factors might be under- or overestimated in the analysis). However, sequential sampling with a monthly interval has shown to be a good way to deal with the intermittent shedding of *E. coli* O157 and to detect infected animals (Hancock *et al*., 1997).

In contrast to results of Mechie and Chapman (1997) and Rahn *et al*. (1997), fore stream milk samples, bulk milk tank samples and milk filters, were not tested positive for O157 VTEC. This might be due to a good hygienic milking management, which might prevent contamination of milk, or due to a too low density of bacteria for detection with the method applied. Although this method has been shown to detect low amounts of bacteria in faeces (10^1 cfu/g sample; Dam-Deisz and Evers, 2001), results might differ in milk.

Environmental sites with which cattle are in contact, *e.g.* the feed bunk, might play a role in transmission of O157 VTEC within a dairy farm. Although Faith *et al*. (1996), Hancock *et al*. (1998), Donkersgoed *et al*. (2001) and Lejeune *et al*. (2001) indicated water troughs as reservoirs for O157 VTEC, in our study no isolates were found in water samples from troughs and pools on the pasture. Wang and Doyle (1998) demonstrated that *E. coli* O157:H7 can enter a viable but non-culturable (VBNC) state in water. This state in the natural

environment poses a concern, since the source of contamination can be overlooked when conventional recovery methods are used. Water troughs might therefore play a more important role in transmission of O157 VTEC within herds than can be concluded from this study.

Considering the high prevalence found in faecal samples, not only from individual animals but also from the environment, the assumption of feces playing a crucial role in transmission is supported. The isolation of O157 VTEC from samples of both the filling tube and the injectors at the back of the manure injector indicates spread on pastures, possibly maintaining the infection on the farm or even introducing the infection on other farms.

In the mixed general linear regression model, the effect of O157 VTEC infection on daily milk production preceding sampling was significant. Infected animals had higher DMP in the period before testing than animals that were found negative. Possibly, a higher milk production makes an animal more susceptible to infection with O157 VTEC, like this seems to apply to other common environmental pathogens (Burvenich *et al.*, 2003). However, high producing cows, having a larger feed and water intake, might also have an increased probability of infection.

No dairy cows that were shedding in the first season, were found positive in the second season. This might indicate that a certain level of immunity was acquired, induced by the infection in the first season. Potter *et al*. (2004) established that cattle are able to develop immunity for some virulence factors of O157:H7 i.e. secreted proteins that are assumed to play a role in colonization of host epithelial cells. Although not significant, in our study 46% of cows with $titer_{max}<80$ (LPS-ELISA) were found positive, whereas the prevalence in animals with higher titers was 34%. Peripheral blood antibodies are very likely not associated with protective immunity. However, higher titers might reflect earlier infection (Johnson *et al.*, 1996), in which protective cellular immunity was developed. Unfortunately, we did not do any cellular immunity test, nor measure local IgA production.

Laegreid *et al*. (1999) suggested that most calves are exposed to *E. coli* O157 early in their life. Furthermore, *E. coli* O157 LPS antigen shares structural elements with LPS antigens of other bacterial species, e.g. *Brucella abortus* and *Yersinia enterocolitica*. This also complicates the interpretation of assays for anti-O157 antibodies (Laegreid *et al*., 1998). All together, serology of this type appears to be no suitable tool for detection of O157 VTEC infection in individual cattle in a dairy herd, but methods based on other epitopes might give different results.

Results of this study should be interpreted realizing that only one colony per positive tested sample was serotyped and typed by PFGE. PFGE analysis of multiple isolates from one animal revealed that some cows indeed harbour O157:H7 strains that have different *XbaI*

restriction endonuclease digestion profiles (Faith *et al.*, 1996). So, selecting only one colony per isolate for further analysis might result in missing less prevailing strains of O157 VTEC.

To gain more insight into persistence of O157 VTEC on dairy herds (also during winter), currently farms which were tested for *E. coli* O157 before in a monitoring program until 2000 (Schouten *et al.*, 2004), are sampled again. Results might give an indication whether previously infected farms have a higher probability to be found positive than farms that were previously found negative. PFGE then might indicate strains that persisted on these farms.

Transmission of O157 VTEC might have occurred within the three groups (cattle, other animals, and environment) and between those groups. From data derived from this study on a single farm, it is not clear in which direction transmission takes place. Most results presented in this study should therefore be considered as indications for further research. Using the O157 VTEC status of individual cows tested in this study, transmission within a dairy herd will be further modeled.

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Chapter 5

Transmission and its quantification of verocytotoxin producing *Escherichia coli* O157 in calves and dairy cattle

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Abstract

Data from a longitudinal study of fourteen months in a dairy herd and data from an experiment with calves were used to quantify transmission of O157 VTEC in cattle. For the latter, two groups of ten calves were placed on two pastures. Of each group, five were inoculated with 10^9 CFU O157 VTEC and, when considered infectious, placed back into their group. All contact calves became positive within six days after reunification. The estimate of the basic reproduction ratio $R_{0,experiment}$ was 7.3 (95% CI 3.9-11.5), indicating that each infectious calf will infect on average seven other calves during an assumed infectious period of 28 days in a fully susceptible population. $R_{0,dairy\ herd}$ appeared to be about 10 times lower (0.71; 95% CI 0.53-0.99), possibly indicating an age-effect.

After the transmission experiment, six contact-infected animals that were shedding continuously during the experiment were housed in a tying stall during winter. After forty days, all six were negative for O157 VTEC. In June, after a period of 34 weeks in which the heifers stayed negative, they were placed on a clean and isolated pasture to observe whether they started shedding again. On each pasture that was infected with high doses of O157 VTEC during the transmission experiment the previous summer, newly purchased susceptible calves were placed. None of the heifers or calves started shedding during the 14 weeks from the start of these experiments, indicating that both the heifers and the previously contaminated pasture did not function as reservoir of O157 VTEC.

5.1. Introduction

Verocytotoxin producing *Escherichia coli* (VTEC), especially those of the serotype O157:H7, are causally related to diarrhoea, haemorrhagic colitis (HC) and the potentially lethal haemolytic-uremic syndrome (HUS) in humans (Tesh and O'Brien, 1991; Karmali, 1989; O'Brien and Kaper, 1998). Humans most often become infected with VTEC following the consumption of contaminated foods (meat, milk or raw vegetables), or by direct transmission from patients or infected animals (Dev *et al.,* 1991; Renwick *et al.*, 1993; Keene *et al.*, 1994; Armstrong *et al.*, 1996; Ackers *et al.*, 1998; Heuvelink *et al.,* 1999). The majority of outbreaks are generally related to cattle or products of bovine origin (Griffin and Tauxe, 1991; Meng and Doyle, 1998). Epidemiological studies have identified cattle as main host for *E. coli* O157 and other VTEC (Chapman *et al.*, 1993; Armstrong *et al.,* 1996; Heuvelink *et al.*, 1998; Schouten *et al.*, 2004; Schouten *et al.*, 2005a; Schouten *et al.*, 2005b).

The presence of O157 VTEC in cattle and their environment has been investigated frequently (Hancock *et al.*, 1997; Mechie *et al.*, 1997; Garber *et al.*, 1999; Schouten *et al.*, 2004; Schouten *et al.*, 2005a, b). Several studies showed a seasonal effect in prevalence of O157 VTEC in cattle: the shedding season appears to be summer and early fall. Also the duration and magnitude of *E. coli* O157:H7 shedding in cattle faeces and localization in the gastro-intestinal tract of experimentally infected cattle has been studied (Brown *et al.*, 1997; Cray and Moon, 1995; Wells *et al.*, 1991; Zhao *et al.*, 1995). Despite that, little is known about dynamics of O157 VTEC within cattle farms. In only few papers transmission within a cattle population was quantified (Laegreid *et al.*, 2004; Turner *et al.*, 2003). Transmission can quantitatively be assessed by using experimental or field data and can be expressed by the basic reproduction ratio (R_0), which is the average number of secondary cases caused by one typical infectious individual during its entire infectious period in a fully susceptible population. If R_0 is larger than one, each infected animal will at average infect 1 or more susceptibles, which may lead to a major outbreak (Kermack and McKendrick, 1991; Jong and Kimman, 1994).

Another important question regarding transmission dynamics of O157 VTEC, is whether and how previously positive animals or herds become infectious again in the next shedding season, after a period of non-shedding. Stress or dietary changes, specifically in the summer months might trigger shedding of potentially latent O157 VTEC carriers. Secondly, water, soil and manure can be long-term reservoirs for *E. coli* O157 (Faith *et al.*, 1996) and re-infection of animals may occur. However, no studies have been done to assess infectivity of soil and manure.

The aim of the studies presented in this paper is to calculate reproduction for O157 VTEC from a transmission experiment with calves and from a longitudinally sampled dairy

herd that was known to be positive for O157 VTEC. Additionally, we investigated the role of previously positive animals and pastures in initiating the infection in the next shedding season.

5.2. Material and methods

5.2.1. Experimental transmission

In September 2000, twenty 12-15 weeks old susceptible Holstein Friesian heifer calves were purchased from a single farm. The calves all tested microbiologically negative for O157 VTEC three times during three weeks before arriving at the experimental facilities. Calves were randomly assigned to two groups (group 1 and group 2) of ten animals each and put on two paddocks of 2,000 m^2 each. The soil of these paddocks was tested 3 times with a one-week interval, and O157 VTEC was not detected.

After an adaptation period of fourteen days, in which calves were microbiologically tested twice for O157 VTEC, 5 randomly selected calves of each group were housed in two separate climate-controlled units with group housing and orally inoculated with 10^9 CFU (Colony Forming Units) of a doxytetracycline-resistant strain of *Escherichia coli* O157, containing VTI, VTII and eae-encoding genes (strain 20G8), that was isolated from cattle faeces in a study of Heuvelink *et al.* (1998). The units had separate floor drains and faeces were removed once a day. When considered infectious (i.e. microbiologically positive, followed by shedding for three consecutive days, section 2.4.1), inoculated calves were joined with the group of contact calves they originated from. All calves were clinically normal at the time of inoculation and at the time of reunification with their group mates that were considered susceptible contact calves.

Calves were fed concentrates and, to make sure that the calves had sufficient roughage (both at pasture and in the climate-controlled units), grass pelleted meals in an amount appropriate for their age was provided. Water was provided *ad libitum*. All concentrates and grass pelleted meals used were irradiated (γ-irradiation: 9 kGrey) in order to prevent foodborne infections.

As from day 0 (day of experimental inoculation), both inoculated and contact calves were examined daily for 14 days for any clinical abnormalities like diarrhoea, pyrexia, and anorexia. Rectal faecal samples were taken daily and cultured to detect O157 VTEC.

At day 40, fourteen calves were euthanized with sodium pentobarbital and examined by necroscopy and histologically for the presence of attaching and effacing lesions in the gastrointestinal tract. The remaining six (contact infected) calves, which almost continuously

shed O157 VTEC during the experiment, were housed indoors and tied individually, so that the probability of infecting each other was minimized. From that moment (October 2002), faecal and blood samples were collected three times a week for 8 weeks, then twice a week for 4 weeks. Next, these calves were tested weekly for another six months, until June 2003.

The Ethical Committee for Experimentation with Animals (Lelystad, the Netherlands) approved the experimental protocol.

5.2.2. Transmission by previously infected calves and pastures

A second experiment was performed to investigate the possibility of formerly contact-infected calves and pastures to function as reservoirs for O157 VTEC between shedding seasons. Three pastures were used in this experiment that were lying fallow during winter and from which one grass cut was removed in spring.

In June 2003, six previously contact-infected heifers from the transmission experiment that were found negative since day 81 of the experiment were placed on a pasture on which no ruminants grazed for at least one year and that tested negative for O157 VTEC for 4 times during the months April and May 2003 ("clean pasture"). Per sampling, 4 pooled soil samples, each consisting of 10 random samples of approx. 20 grams taken in a bag at appointed sites in the paddocks (0-5 cm deep), were analyzed for O157 VTEC.

Also in June 2003, ten purchased bull calves that microbiologically tested negative 3 times were divided in two groups of five and put on two pastures that were highly contaminated with O157 VTEC during the transmission experiment the season before.

During these two experiments, faeces and blood samples were collected weekly from all animals for 14 successive weeks. Besides that, from all three pastures soil samples were taken twice a month as described above.

5.2.3. Field transmission

A dairy farm situated in the centre of The Netherlands was selected from a group of dairy farms that was tested positive for O157 VTEC in a monitoring program. The longitudinal study started 2 July 1999 and ended 13 November 2000. During this period, animals and the environment of the herd were sampled 14 times, with intervals of 4 to 10 weeks. Animal population, housing and farm management are described in Schouten *et al.* (2005b). During the study period, the herd had an average size of 75 Holstein Friesian cows, of which on average 20% was non-lactating at each sampling. Non-lactating cows were housed separately from lactating cows. From all dairy cows, faeces were collected in plastic bags by rectal palpation. Because the O157 VTEC status of dairy cows on this farm was assessed repeatedly, transmission between dairy cows can be quantified.

5.2.4. Sample processing and analysis

Faecal samples were transported in cool boxes and analysis started within 24 hours. Outlines of the microbiological techniques are presented below.

5.2.5. Microbiological analysis

Isolation methods are described in detail by Schouten *et al.* (2004). In brief, isolation was performed using enrichment in mTSB+A, subsequent immuno magnetic separation (IMS), incubation on sorbitol MacConkey agar (SMAC) (Oxoid) with cefixime and tellurite (CT-SMAC), and screening for sorbitol-negative colonies. Colonies were incubated on both SMAC supplemented with 4-methylbelliferyl-B-D-glucuronide (MUG) (Sigma Chemical Co., St. Louis, MO) and eosin methylene blue agar (EMB) (Oxoid); suspected *E. coli* O157 colonies were tested by agglutination in order to ascertain the authenticity. Within the transmission experiment, a sample was considered positive based on the outcome of the agglutination test. Isolates from the longitudinal study that were confirmed by agglutination, were also serotyped. A sample was considered positive when serotyping identified the isolate as *E. coli* O157. The isolates were subsequently screened for possession of genes encoding for the most-common verocytotoxins (VTI and VTII) and the eae-gene by polymerase chain-reaction (Schouten *et al.*, 2005b).

5.2.6. Pulsed Field Gel Electrophoresis

DNA fingerprints were made from isolates found during the longitudinal study using pulsed field gel electrophoresis (PFGE), with *Xba* I as restriction enzyme. Cluster analysis of fingerprints was done with Bionumerics® (Dendrogram type=UPGMA, Similarity coefficient=Dice). Isolates are considered indistinguishable when 100% of the DNA-fragments is analogous. Isolates with at least 95% matching DNA-fragments are considered highly related and for transmission modelling considered to be part of the same infection episode.

5.2.7. Statistical analysis

For proper analysis of the data, it is essential to determine the infection status – susceptible or infectious - of the individual animals during the trials. This was done for each animal at each sampling moment in each of the studies. For this, different assumptions regarding infectivity were made. In the longitudinal study, the O157 VTEC status for each lactating cow was determined for any of the fourteen sampling moments, assuming a positively tested animal to be infectious (I) and a negatively tested animal to be susceptible

(S). For the transmission experiment, the status for each calf was determined for each sampling moment, assuming a calf to be infectious (I) from any day of shedding followed by three consecutive positive samplings and to be susceptible (S) when found negative for three consecutive samplings. These assumptions are based on the intermittent excretion of *E. coli* O157 infected animals (Brown *et al.*, 1997; Faith *et al.*, 1996; Zhao *et al.*, 1995) and the fact that in the experiment daily sampling was performed, while in the study on the dairy farm sampling took place approximately every 4 to 6 weeks.

A series of events, i.e. infectious contacts or incidents of new infections (cases) on consecutive time points (i.e. samplings) can be considered a stochastic process. It was assumed that infectious animals stop shedding after a while and become susceptible again, which means that the infection gives no immunity. Therefore, a susceptible-infectious-susceptible model (SIS-model) can be used to describe the transmission of O157 VTEC both in the experiment and in the longitudinal study. The SIS model can be represented as:

$$S \xrightarrow{\beta * S * I / N} I \xrightarrow{\alpha * I} S$$

The number of animals that are susceptible and become infectious per time interval depend on the infection rate β, the number of susceptible (S) and number of infectious animals (I). Infectious animals become susceptible each time interval with a rate α. This implies that the mean length of the infectious period is 1/α. R_0 is then defined as β/α (Jong and Kimman, 1994).

The transmission parameter β can be estimated using a function of I, S, C, N and Δt, defining the stochastic process based on a binomial distribution. For this, we assumed that all animals were randomly in contact, that susceptible and infectious animals were homogeneous groups, the infection rate was constant during the whole infectious period, and the duration of the infectious period was exponentially distributed.

The number of new cases at the end of each time interval can be described with the following model:

$$C \cong \beta * (S * I) / N * \Delta t \qquad (1)$$

Where

C = number of new cases at the end of a time interval

S = number of susceptible animals (S(t)) at the start of the interval

I = average number of infectious animals (I(t)) during the interval

N = population size (=S+I for dairy herd; =10 for experiment)

Δt = sampling interval

Taking the log of this model results in:

$$\log C = \log(ß) + \log(S*I/N*\Delta t) \quad (2)$$

The data can be statistically analyzed using generalized linear models (GLM; Becker, 1989). Applying GLM, the whole course of the (experimental) infection chain is used to estimate the transmission parameter β. General linear regression (STATA® 8) with a complementary-log-log link function and log(I/N*Δt) as an offset was used, while S gives the number of trials for the binomial distribution.

The estimated parameter is log (β); exponentiation gives β. If the length of the total infectious period for O157 VTEC is known, the reproduction ratio R_0 can be calculated by simply multiplying β with the length of the infectious period (1/α).

5.3. Results

5.3.1. Descriptive results of transmission experiment

The first days following inoculation with 10^9 CFU of *E. coli* O157:H7, no clinical disease was observed. After reunification of the calves, all maintained a normal alertness and appetite. Contact-infected calves remained clinically healthy throughout the whole experiment. Two inoculated calves, however, started coughing in week 2 and 3 of the experiment. Necropsy at the end of the experiment showed that these animals had suffered slight pneumonia.

All ten inoculated animals shed the bacterium in their faeces starting day 1 post inoculation (p.i.) for more than 3 days. Therefore, they were all considered infectious from the day they started shedding. Due to the duration of the applied test (2 days), the infectious animals could not be reunited with the contact animals before day 5 p.i.. One respectively 2 days after reunification contact calves were found positive (group 1 at day 7 p.i; group 2 at day 6 p.i.). All contact animals were tested positive within 4 and 6 days (i.e at day 9 and at day 11 p.i), for groups 1 and 2 respectively. In group 1, four of five inoculated animals were found positive at each sampling after that, except for the last sampling (day 39 p.i). In group 2, inoculated animals shed more intermittently; every inoculated animal was found negative in at least one sample. The number of positively and negatively tested calves at each sampling moment in both groups is presented in Table 5.1.

Table 5.1 Number of O157 VTEC positive inoculated and contact calves per sampling (days post inoculation, p.i.) and input for the transmission model for group 1 and 2 of the experiment. At day 5, inoculated calves were joined with the susceptible calves.

		Group 1 (n=10)						Group 2 (n=10)					
		O157 positive		Input model				O157 positive		Input model			
Sampling	Days p.i.	Inoculated calves	Contact calves	I[a]	S[b]	C[c]	Δt[d]	Inoculated calves	Contact calves	I[a]	S[b]	C[c]	Δt[d]
0	0	5/5	-	0	-	-	-	5/5	-	0	-	-	-
1	1	5/5	-	5	-	-	-	5/5	-	5	-	-	-
2	2	5/5	-	5	-	-	-	5/5	-	5	-	-	-
3	3	5/5	-	5	-	-	-	5/5	-	5	-	-	-
4	4	5/5	-	5	-	-	-	5/5	-	5	-	-	-
5	5	5/5	0/5	5	5	0	-	5/5	0/5	5	5	0	-
6	6	5/5	0/5	5	5	0	1	5/5	1/5	5.5	5	1	1
7	7	5/5	1/5	5.5	5	1	1	5/5	3/5	7	4	2	1
8	8	5/5	3/5	7	4	2	1	5/5	3/5	8	2	0	1
9	9	5/5	5/5	9	2	2	1	5/5	3/5	8	2	0	1
10	10	5/5	5/5	10	0	0	1	5/5	4/5	8	2	0	1
11	11	5/5	5/5	10	0	0	1	5/5	5/5	8.5	2	1	1
12	12	5/5	5/5	10	0	0	1	5/5	4/5	9	1	0	1
13	13	5/5	5/5	10	0	0	1	5/5	4/5	9	1	0	1
14	14	5/5	4/5	9.5	0	0	1	5/5	3/5	8.5	1	0	1
15	16	5/5	4/5	9	1	0	2	4/5	3/5	7.5	2	0	2
16	18	5/5	4/5	9	1	0	2	4/5	4/5	7.5	3	1	2
17	21	4/5	4/5	8.5	1	0	3	4/5	4/5	8	2	0	3
18	23	4/5	4/5	8	2	0	2	1/5	3/5	6	2	0	2
19	25	5/5	5/5	9	2	2	2	0/5	3/5	3.5	6	0	2
20	29	5/5	5/5	10	0	0	4	1/5	3/5	4	7	2	4
21	32	5/5	5/5	10	0	0	3	4/5	4/5	6.5	5	4	3
22	36	5/5	5/5	10	0	0	4	4/5	5/5	9	2	2	4
23	39	1/5	3/5	7.5	0	0	3	2/5	4/5	8.5	3	0	3

[a] Average number of infectious calves during the time interval preceding sampling. New cases are assumed to be infected on average halfway the time interval.

[b] Number of susceptible calves at the beginning of the time interval preceding sampling.

[c] Number of new cases per interval of sampling

[d] Interval between samplings (in days)

At the end of the experiment (day 40), necropsy and histological examination of the rumen, reticulum, omasum, ceacum, colon, ileum and duodenum of the euthanized calves (10 inoculated, 4 contact) did not show any attaching-and-effacing lesions.

On day 40, the six most continuously shedding contact-infected calves of the experiment, with also a positive culture outcome at day 36 p.i., were housed in a tying stall. In the subsequent weeks, the number of calves shedding O157 VTEC declined. Until day 81, four of the six calves were shedding intermittently, with three or less animals shedding at the same sampling moment. As from day 81 p.i., all animals tested negative for O157 VTEC until the last day of sampling (day 100; Figure 5.1).

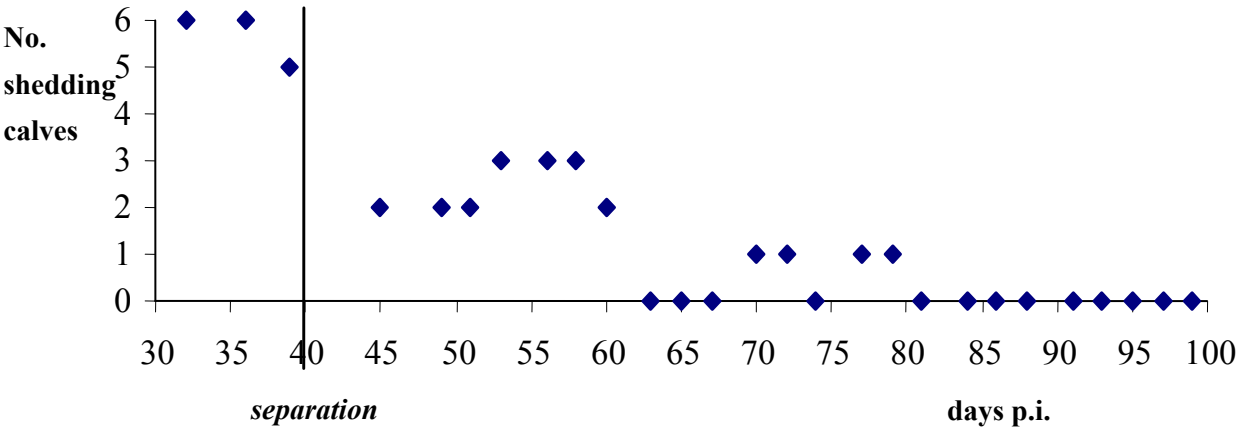

Figure 5.1 Number of shedders of six contact infected calves after separation of the group and individual tied housing

5.3.2. Descriptive results of experiments on pastures and previously infected calves

O157 VTEC has not been detected in the six previously infected animals while grazing on the "clean" pasture. Also, the 10 new susceptible calves on the previously infected pastures of the transmission experiment did not start shedding. O157 VTEC has not been detected in any of the soil samples.

5.3.3. Descriptive results of the longitudinal study

Descriptive statistics and result of PFGE typing of this study are presented in detail in Schouten *et al.* (2005b). Per sampling, 0-29.5% of the cattle was found positive for *E. coli* O157, with prevalences of 0% during winter. Nine cows were found positive in more than one sampling. At the end of the sampling period O157 VTEC was isolated at least once from 41.4% of the herd.

5.3.4. Estimation of O157 VTEC transmission by statistical modelling

For both the transmission experiment and the longitudinal study in the dairy herd, the numbers of susceptible (S), infectious (I) and contact infected calves (C) per time interval were counted (Table 5.1, Table 5.2) and used as transmission model input.

Table 5.2 Number of sampled and O157 VTEC positive dairy cows per sampling of the longitudinal study, and input for the transmission model for the dairy cow population

			Input model				
Sampling[a]	**Week of study**	**No. O157 pos./ No. sampled**	**I**[b]	**S**[c]	**C**[d]	**N**[e]	**Δt**[f]
0	0	18/61	-	-	-	-	-
1	4	12/59	14.5	43	8	57.5	28
2	8	7/56	9	47	5	56	31
3	13	1/58	4	49	1	53	35
4	19	0/58	0.5	57	0	57.5	39
5	26	0/61	0	58	0	58	54
6	31	0/59	0	61	0	61	34
7	36	0/60	0	59	0	59	35
8	42	0/62	0	60	0	60	42
9	46	3/60	1.5	62	3	63.5	25
10	51	5/60	3.5	57	5	60.5	37
11	56	3/62	4	55	2	59	35
12	60	0/59	1.5	59	0	60.5	29
13	70	0/55	0	59	0	59	74

[a] Samplings 1, 2, 3, 4, 9, 10, 11, 12, and 13 were in the shedding season (5, 6, 7, 8 were December, January, February, March)

[b] Average number of infectious calves during the time interval preceding sampling. New cases are assumed to be infected on average halfway the time interval.

[c] Number of susceptible calves at the beginning of the time interval preceding sampling.

[d] Number of new cases per interval of sampling

[e] N = I + S per time interval

[f] Time interval between samplings (in days)

To calculate R_0, the calculated beta's were multiplied with 28 days, assuming this to be the length of the infectious period. This was based on the average length of shedding in the experiment, the interval of sampling in the longitudinal study (which was at least a month) and literature (Brown *et al.*, 1997, Cray and Moon, 1995). Reproduction ratio's (and 95%CI's) for group 1 resp. group 2 are R_0=10.3 (5.0-21.9) and R_0=6.4 (3.6-10.9), and overall R_0=7.3 (3.9-11.5; Table 5.3). When assuming that a positive calf is infectious immediately and is susceptible immediately when tested negative, the estimate for group 2 and the overall

estimate would increase to 7.0 (4.2-12.0) and 7.9 (5.3-12.3), respectively. For group 1, no changes were observed.

Table 5.3 Transmission coefficients and Reproduction ratios with their 95% confidence intervals estimated from data of a transmission experiment and a longitudinal field study

	# obs[a]	B	95% CI	R_0[b]	95% CI
Experiments					
Group 1	10	0.37	0.18-0.78	10.3	5.04-21.9
Group 2	19	0.23	0.13-0.39	6.4	3.64-10.9
All	29	0.26	0.14-0.41	7.3	3.92-11.5
Longitudinal study					
Shedding season 1	4	0.022	0.017-0.058	0.62	0.48-1.62
Shedding season 2	4	0.031	0.017 -0.037	0.87	0.48-1.04
Total study duration	8	0.025	0.017-0.037	0.70	0.48-1.04
Total study duration[c]	13	0.026	0.019-0.035	0.71	0.53-0.99

[a] Number of observations (time intervals) that could be used in GLM-model

[b] Calculated using an assumed mean shedding duration of 28 days

[c] Assumption: certain extent of infectivity remains during the non-shedding season; I+1, S-1 for each time interval

In the longitudinal study, R_0 was 0.62 (0.48-1.62) for the first shedding season, 0.87 (0.48-1.04) for the second shedding season and 0.70 (0.48-1.04) for the total study period (Table 5.3). When the assumption was made that a certain amount of infectivity remained during winter by considering an animal infectious instead of susceptible (I+1; S-1) at each sampling interval, the overall estimate became 0.71 (0.53-0.99).

5.4. Discussion

The aim of this paper was to quantify transmission of O157 VTEC based on the results of a longitudinally sampled dairy farm that was known to be positive for O157 VTEC and an experiment with calves. Additionally, we investigated the role of previously positive animals and pastures in initiating the infection in the next shedding season.

The estimate of the basic reproduction ratio from the experiment, $R_{0,experiment}$, was 7.3 (i.e. larger than 1), indicating that each infectious calf can infect on average seven other calves during an assumed infectious period of 28 days in a fully susceptible population, very likely leading to a major outbreak. In contrast, $R_{0,dairy\ herd}$ was 0.73 (i.e. smaller than 1, although not

significant because the 95%CI included 1). Because some assumptions underlying transmission models are violated in our study, i.e. homogeneous groups that are randomly in contact, and an exponentially distributed duration of the infectious period, these estimates should be considered a first rough quantification of transmission in cattle. Laegreid and Keen (2004) estimated R_0 for O157 VTEC to be 5.23 (3.87-6.64) in calves of a beef-cow herd using on the final size of the infected population based on serology. However, previous research of the same group (Laegreid *et al.*, 1999) indicated that the proportion of animals shedding O157 VTEC in faeces was substantially lower (7.4%, range 2-30%) than the proportion of animals showing a positive antibody response (83%, range 63-100%). It is not clear whether calves in which O157 VTEC passes the gastro-intestinal tract, but do not necessarily become infectious shedders, might also show seroconversion. If that is the case, they can be misclassified, leading to overestimating R_0 when antibody titres are used for estimating transmission rates.

An important assumption for interpreting the results is when to consider an animal to be infectious. In the experiment, we observed continuous shedders and intermittent shedders. For the transmission experiment, we therefore assumed that a calf was infectious as from any sampling it is tested positive, followed by three consecutive positive samplings (and susceptible when it is tested negative for three consecutive samplings). In an alternative analysis we considered a calf being infectious when it is tested positive and susceptible when it is tested negative in the faeces. Hardly any difference was found in the reproduction ratios, however.

Because there is seasonal variation in O157 VTEC shedding in cattle (Heuvelink *et al.*, 1998; Schouten *et al.*, 2004; Schouten *et al.*, 2005a, b; Mechie *et al.*, 1997; Hancock *et al.*, 1997; Garber *et al.*, 1999), transmission rates might differ between seasons. Modelling a seasonal effect in calculating the transmission rate, might better mimic reality (i.e. $C \approx \beta * (1 + \eta\sin(2\pi t)/365) * (S*I) / N*\Delta t$ (with $|\eta| \leq 1$)). However, we had insufficient data to do this. The beta calculated from the experiment might be considered as a transmission rate for summer/early fall (in which the experiment was carried out) in calves and might differ substantially from the beta that would be found during winter. For the dairy herd, we also modelled transmission assuming a certain number of infectious animals, although not detected, present in the population during winter, implying that the infection continued during winter. By adding one (+1) to numbers for I and extracting one (-1) of the numbers for S for each interval, 5 extra intervals could be used in the analysis and thus the power of the analysis increased. Estimates were similar, however.

Because the length of the infectious period is unknown and the excretion pattern varies widely between animals (Cray and Moon, 1995; Brown *et al.*, 1997; Sanderson *et al.*, 1999; Wells *et al.*, 1991; Zhao *et al.*, 1995), it is difficult to determine when the infection chain has

ended. For calculating R_0 in this study, we assumed the infectious period for a calf or cow to be on average 28 days. However, individual adult cows were reported to be able to shed up to 100 days and calves even up to 189 days (Steward and Flint, 1999). After the transmission experiment, when shedding calves were housed individually, O157 VTEC was detected for approximately 40 days. When the average infectious period for dairy cows would exceed 38 days, R_0 would become larger than one, enabling major outbreaks to occur. This indicates that more precise information is needed about the length of the infectious period for dairy cattle in order to correctly estimate R_0.

Estimated infection rates for the experiments were about ten times higher than those for the longitudinal study which may have several causes. First of all, calves were experimentally infected with rather high doses and therefore excretion in these calves is expected to be considerably higher than in the naturally infected dairy cows of the longitudinal study. Secondly, weaned calves are known to shed larger number of O157 VTEC and for a longer period than older animals, even when calves and cows are infected with identical doses (Cray and Moon, 1995). In the longitudinal study only data of adult animals were used as model input, since young stock was only sampled during winter. This probably resulted in an overall lower transmission rate as young stock represent the better shedders. Thirdly, the circumstances in the transmission experiment could be much more controlled than those at the dairy farm, resulting in a smaller role of e.g. the environment. PFGE results of the longitudinal study confirmed that O157 VTEC is able to survive in the environment of cattle and in other animals (Schouten *et al.*, 2005b). On the farm, a combination of several factors (cattle, other animals, and environment) might play a role in the dynamics of O157 VTEC, which was not considered in the transmission model. Furthermore, when sampling of the dairy herd started (summer), the O157 VTEC outbreak appeared to be at its peak and from that moment on was declining. As a result a low R_0 was calculated for the first shedding season, although the outbreak in this season seemed to have affected relatively more animals than in the second. When starting sampling at the beginning of the shedding season, possibly more contact infections would have been detected. Finally, calves in the experiment were additionally fed concentrates in a feeding trough on the ground, where calves frequently put their claws in while eating. Faecal contamination of feed could easily have occurred, leading to (indirect) transmission. Dairy cows were fed roughage from a feeding bunk and concentrates from a feeding dispenser, which both are less easily contaminated with faeces.

Some calves tested positive in the experiment seemed to start shedding again after a period of being tested negative, and some cattle in the longitudinal study also were found positive more than once during a shedding season. However, cattle that were infected in the first shedding season were not found positive for O157 VTEC in the second shedding season

(Schouten *et al.*, 2005b). So, the shedding of O157 VTEC seemed to have limited itself to one shedding season. Possibly, a certain level of immunity has been acquired, induced by the infection in the first season. Potter *et al.* (2004) established that cattle are able to develop immunity for some virulence factors of *Escherichia coli* O157:H7, i.e. secreted proteins that are assumed to play a role in colonization of host epithelial cells. Therefore, on the long term some resistance against O157 VTEC might have developed. Calves infected in the transmission experiment and put on a "clean" pasture a year later, did not start shedding again. So, it seems that these calves did not function as a reservoir of *E. coli* O157. Since samples from the soil were tested negative for *E. coli* O157, the calves were most likely not exposed to *E. coli* O157. Therefore, no conclusion can be made about long term resistance against the inoculated type of O157 VTEC the year before. Whether or not these calves would have started shedding again after experimental challenge with the same or a different strain remains unknown.

After grazing of susceptible calves on a pasture infected with O157 VTEC the year before, no infection occurred. For economic reasons, calves used for this experiment were bulls. Previous research reported no effect of sex on faecal prevalence and shedding of O157 VTEC (Looper *et al.*, 2003; Smith *et al.*, 2001; Donkersgoed *et al.*, 1999). Letting a pasture lie fallow for one winter therefore seemed to be sufficient to prevent spread to susceptible animals in the next spring.

To summarize, in this study previously infected calves and contaminated pastures did not initiate the infection in the next shedding season. Results of this study indicate that transmission of O157 VTEC occurs both in experimental and naturally infected cattle. Transmission rates differ considerably between weaned calves and dairy cows. Control strategies to reduce the infection rate probably have more impact in weaned calves than in older cattle. So, to reduce the number of infected animals on a farm, one should look for on-farm measures that reduce transmission within young calves or young stock. This was also concluded by Turner *et al.* (2003) on basis of a differential equation model that described transmission in a multigroup managed herd, although in their model the dynamics of the infectious organism itself was described rather than the dynamics of infectious and susceptible animals.

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Chapter 6

Prevalence of and risk factors for *Escherichia coli* O157 infection in Dutch dairy herds with known test history

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Abstract

The current retrospective cohort study was performed to find evidence for either persistence in or (re-)introduction of *E. coli* O157 at dairy farms. For this we established whether farms that were positive in the Dutch monitoring program were more likely to be found positive in a next shedding season compared to previously negative farms.

Of 49 previously positive farms, 31 were randomly selected. From 402 previously negative farms, 30 were randomly selected. Respectively, 30 and 23 farmers cooperated. Information about farm and management factors was collected. Exact multivariate logistic regression was performed to identify factors associated with *E.coli* in at least one shedding season after the first sampling. The variable "previously positive" was forced into the model because of the aim of the study.

Three variables remained in the final model. Previously positive herds were only slightly more often positive at second sampling (32%) than herds negative in the first sampling (29%) (OR=3.91; P=0.40). Significantly more herds with access to surface water on pastures were positive (64%) than herds in which cows had no access (14%) (OR=31.7; P=0.0003). Prevalence of positive farms with at least one cattle farm within 1 km distance was significantly increased (35%) compared to when no cattle farms were present in the neighbourhood (prevalence 0 %; OR=13.7; P=0.028).

Long-term persistence of *E. coli* O157 on dairy farms was not demonstrated in this study. Factors associated with a positive test at second sampling point in the direction of (re-)introduction rather than long-term persistence of infection.

6.1. Introduction

Escherichia coli O157 is an important human pathogen (Karmali, 1989; O'Brien and Kaper, 1998; Tesh and O'Brien, 1991). Major source of infection is consumption of contaminated cattle products or direct contact with animals or their faeces (Armstrong *et al.*, 1996; Chapman, 1999; Griffin and Tauxe, 1991; Keene *et al.*, 1997; Heuvelink *et al.,* 1999; Meng and Doyle, 1998). Monitoring indicated that *E. coli* O157 is endemic in Dutch cattle (Schouten *et al.* 2004; Schouten *et al.*, 2005a).

From transmission experiments and a longitudinal field study, it was concluded that transmission of *E. coli* O157 occurs in both experimentally and naturally infected cattle (Schouten *et al.*, submitted for publication). Positive animals were found in the same dairy herd in successive shedding seasons, but animals positive in the first season under study were not positive in the next (Schouten *et al.*, 2005b). It was concluded that farms can be positive for more than one shedding season.

The current retrospective cohort study was performed to find evidence for either persistence in or (re-) introduction of *E. coli* O157 at dairy farms. For this we established whether farms that had been tested positive before were more likely to be found positive in a next shedding season compared to previously negative farms.

6.2 Material and methods

Original data were derived from a monitoring program conducted by the National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands (Bouwknegt *et al.*, 2003). For this retrospective cohort study, 31 of the 49 Dutch farms that tested positive for *E. coli* O157 in this monitoring program in the months May until November (shedding season) in 1997-2000 (Schouten *et al.*, 2004), were randomly selected. From 402 farms that were found negative in the same period, 30 were randomly selected. Respectively, 30 and 23 farmers cooperated. These farms were sampled for the second time in July-September 2002 following the sampling procedure of the monitoring program (Schouten *et al.*, 2004). At second sampling, employees of the Inspectorate for Health Protection and Veterinary Public Health (KvW) collected about 2,520 faecal samples from the stable floor, which were pooled in 247 samples (3-5 pools per farm and 10-12 samples per pool).

Microbiological analysis of the samples was performed to determine whether *E.coli* O157 was present. Methods for isolation of *E. coli* O157 from faecal samples, using bacterial

culturing, Immuno Magnetic Separation (IMS), agglutination and serotyping, are described in detail by Schouten *et al.* (2004).

DNA fingerprints were made from the isolates using pulsed field gel electrophoresis (PFGE) based on Barrett *et al.* (1994), with *Xba* I as restriction enzyme. Cluster analysis of fingerprints was done with Bionumerics® (Dendrogram type=UPGMA, Similarity coefficient=Dice). Isolates were considered indistinguishable when 100% of the DNA-fragments were analogous and 'highly related' when at least 95% of the DNA-fragments matched.

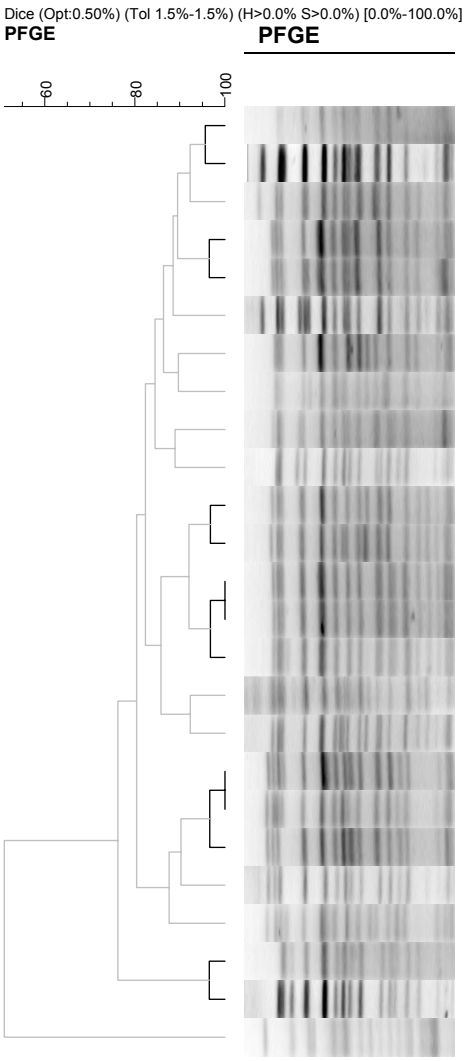
Information about farm and management factors was collected using the same questionnaire as in the monitoring program (Schouten *et al.*, 2004). Exact conditional logistic regression analysis (PROC LOGISTIC, SAS®, 2004) was performed to identify factors associated (Exact test $p<0.25$) with *E. coli* O157 status in at least one shedding season later. First, a univariable analysis was performed (Table 1), followed by a multivariate analysis using a backward procedure according to Hosmer and Lemeshow (1989). In the latter, the variable "previously tested positive" (yes/no) was forced into the model because of the aim of the study. Results of both univariable and multivariable analysis are presented, because a rather low power of the study is expected due to the small sample size.

6.3 Results

The average time between the first and second sampling was 176 weeks (± 55 weeks), varying from 86 to 273 weeks. Fifteen of 53 herds (28.3%) were found positive at the second sampling; 33.3% of farms that tested positive at earlier sampling were positive at resampling and 21.7% of earlier negative tested farms were positive (P=0.54, Table 6.1).

PFGE analysis indicated that six small clusters of PFGE types were found, comprising isolates from both first and second sampling (Figure 6.1). Isolates from clusters a, b, and e were all found in the North of the country, cluster d in the South and clusters c and f comprised isolates from both the North and the South. From farms that were positive in both samplings, no identical or highly related types of *E. coli* O157 were isolated.

Variables associated with *E. coli* O157 infection in univariable logistic regression analysis were 'other cattle farms within 1 km distance' (yes/no), 'access to surface water in the stables' (yes/no), 'access to surface water while on pastures' (yes/no), 'maize fed to the herd' (yes/no), 'farmyard pavements' (yes/no), 'hygienic measures regarding visitors' (yes/no), 'region' (North, Mid, West, South), and 'interval between samplings' (Table 6.1).



Farm*		Region#	First sampling	Second sampling date
K[a]	2	N	07-27-98, neg.	08-26-02
A[a]	1	N	07-06-98	
L	2	W	06-15-98, neg.	09-22-02
M[b]	2	N	06-15-99, neg.	07-29-02
B[b]	2	N		08-12-02
C	1	N	08-15-00	
N	2	W	05-18-98, neg.	07-23-02
D	2	E		09-30-02
E	2	S		09-23-02
F	1	M	07-13-99	
G[c]	2	N		09-09-02
H[c]	2	S		09-16-02
I[d]	2	S		09-10-02
J[d]	2	S		09-10-02
E[d]	1	S	07-10-00	
B	1	N	10-17-00	
I	1	S	06-02-98	
C[e]	2	N		07-22-02
O[e]	2	N	08-21-00, neg.	10-07-02
A[e]	2	N		07-08-02
G	1	N	09-26-00	
F	2	E		09-23-02
H[f]	1	N	05-29-00	
J[f]	1	S	11-30-99	
D	1	M	11-10-98	

Bionumerics® (Dendrogram type=UPGMA, Similarity coefficient=Dice)

*Farms A-J were found positive in both first (1) and second (2) sampling. Farms K, L, M, N, O were negative in the first sampling, so only PFGE results of second sampling (2) could be presented.

[#] North (N); Mid (M); West (W); South (S)

[a, b, c, d, e, f] Closely related isolates and indistinguishable isolates are defined as >95% and 100% fragments in common, respectively

Figure 6.1 PFGE patterns of all isolates of farms both positive at first (1) and second sampling (2) (n=10), and of farms only positive at second sampling (n=15)

Table 6.1 Factors associated (Exact test P<0.25) with *E. coli* O157 in univariable analysis, with frequencies (Freq), prevalences (Prev), odds ratios (OR), 90% confidence intervals, and P-values

Variable	Category	Freq (n)	Prev (%)	OR	90%-CI	P-value
Previously positive[a]	No	23	21.7	1.00	ref	0.538
	Yes	30	33.3	1.78	0.54-6.37	
Other cattle farms < 1km	No	6	0.0	1.00	ref	0.167
	Yes	47	31.9	3.62	0.64-∞	
Access to surface water in stables	No	50	26.0	1.00	ref	0.190
	Yes	3	66.7	5.47	0.40-169.2	
Access to surface water while on pastures	No	28	14.3	1.00	ref	0.003
	Yes	14	64.3	10.0	2.37-49.7	
Maize fed	No	8	62.5	1.00	ref	0.033
	Yes	45	22.2	0.18	0.32-0.86	
Easy to clean farmyard (asphalt, concrete, bricks)	No	5	80.0	1.00	ref	0.019
	Yes	48	22.9	0.08	0.03-0.68	
Hygienic measures for visitors	No	5	60.0	1.00	ref	0.131
	Yes	48	25.0	0.23	0.03-1.65	
Region	North	12	50.0	3.78	0.72-23.5	0.127
	Mid	15	20.0	1.00	ref	
	West	3	66.7	3.66	0.42-250.0	0.172
	South	23	17.4	0.85	0.16-5.00	1.000
Interval of sampling (weeks)	0-156	21	38.1	1.00	ref	0.227
	>156	32	21.9	0.46	0.14-1.50	

[a] P>0.25, but mentioned because of aim of the study

Three variables were highly correlated, 'access to surface water while on pastures', 'access to surface water in stables', and 'maize fed to the herd'. To avoid collinearity, the latter two were omitted from multivariable logistic regression. After multivariable analysis, three variables remained in the final regression model (Table 6.2).Herds that were previously positive were slightly more often positive at second sampling (32%) than herds that were negative in the first sampling (29%) (OR=3.91; 90%-CI: 0.50-98.4; P=0.40). Significantly more herds with access to surface water while on pastures were positive for *E. coli* O157 (64%) than herds in which cows had no access to surface water (14%) (OR=31.7; 90%-CI:

4.32-799.0; P=0.0003). Prevalence of positive farms with at least one cattle farm within 1 km distance was significantly increased (35%) compared to when no cattle farms were present in the neighbourhood (prevalence 0 %; OR=13.7; 90%-CI: 1.85-∞; P=0.028).

Table 6.2 Factors associated with *E. coli* O157 in multivariable logistic regression, frequencies (Freq), prevalence (Prev), Odds Ratio (OR), 90%-confidence interval, and Wald's *P*-values

Variable	Category	Freq (n)[b]	Prev (%)	OR	90%-CI	*P*-value (Wald's)
Previously positive[a]	No	17	29.4	1.00	ref	
	Yes	25	32.0	3.91	0.50-98.4	0.400
Surface water used	No	28	14.3	1.00	ref	
while on pastures	Yes	14	64.3	31.70	4.32-799.0	0.0003
Other cattle farms < 1km	No	5	0.0	1.00	ref	
	Yes	37	35.1	13.70	1.85-∞	0.028

[a] Forced into the model, because of aim of the study

[b] Due to missing values of the variable "Access to surface water" 11 observations could not be used (n=42)

6.4 Discussion

To investigate whether farms that previously tested positive in a monitoring program were more likely to be found positive again at least one shedding season later than previously negative farms, a random sample of both groups was tested for a second time.

There was no evidence found that previous positively farms were more likely to be positive again. This is in contrast with findings of Zhao *et al.* (1995) in which 22% of previously negative herds were positive, and 50% of previously positive herds. In the latter study, however, the time between samplings was approximately 1 year, while in our study it was on average 3 years. Because *E. coli* O157 is capable of surviving at a farm for years (Hancock *et al.*, 1997; Schouten *et al.*, 2005b; Shere *et al.*, 1998; Zhao *et al.*, 1995), it might be expected that once a farm was tested positive, it has an increased probability to be tested positive again in a next season. It is known that at farm level, the same clones can be detected on sampling occasions separated by as much as 17 months (Liebana *et al.*, 2005). Other DNA-types found at second sampling might either point to re-infection or to infection with multiple types from which one is dominant in the first season and another one in the second season. Phage typing and molecular characterisation showed that individual cattle can harbour multiple strains of *E. coli* O157 at the same time (LeJeune *et al.*, 2004). Isolated

types can vary in time (Zhao *et al*., 1995) and new subtypes can be found regularly within a farm (Hancock *et al.*, 1998). This might imply that animals can be infected with new strains from their environment or that in the gastrointestinal tract mutations of *E. coli* O157 occur (Wallace, 1999). In our study, isolates found in farms that were positive in both samplings, were neither identical nor highly related as shown by PFGE types. This might imply that there is no long-term persistence of the same type of *E. coli* O157 on a farm. However, only one isolate per pooled herd sample was typed by PFGE, so possible other types that were present could have been missed.

Farms that have other cattle farms in the vicinity or in which cows have access to surface water while on pastures were, however, more often positive for *E. coli* O157. This indicates that farms become positive because of a (re-)introduction of the agent. It should be noted that the variables 'maize fed' and 'access to surface water while in stable' were highly correlated with 'access to surface water while on pasture' and were omitted from the analysis. So, it would be expected that these variables also are risk factors for *E.coli* O157 infection. The results of the univariable analysis show a number of variables possibly associated with *E. coli* O157 infection of dairy farms. Due to the low power of the study (small sample size), most of the factors, although biologically very plausible, were only significant in the univariable, and not in multivariable, analysis. However, these variables might be of interest for persistence and/or (re-)introduction of *E.coli* O157 on dairy farms.

The small clusters that were found by PFGE comprised both clusters within a region and among regions. Indistinguishable subtypes can be found on farms that are as far as 100 km apart, without any demonstrable animal movements between them (Hancock *et al.*, 2001; Rice *et al*., 1999). Some isolates in the first sampling appeared highly related with isolates in the second sampling, although not found on the same farm and isolated respectively 2 and 4 years earlier.

To summarize, long-term persistence of *E. coli* O157 on dairy farms was not demonstrated in this study. Factors associated with a positive test at the second sampling point in the direction of (re-)introduction rather than persistence of infection.

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Chapter 7

General discussion

7.1 Introduction

O157 VTEC incidences and numbers of outbreaks in humans are increasing worldwide. Human infection can have severe consequences. The mean disease burden of O157 VTEC infections in The Netherlands, expressed as DALY's (Disability Adjusted Life Year; Chapter 1) is substantially higher per primary case per year than e.g. for campylobacteriosis, another foodborne public health problem in The Netherlands (55 vs. 4.4 DALY's per 1000 cases). Moreover, contamination of foods with O157 VTEC may lead to large outbreaks of disease. Because cattle appear to be an important reservoir, it is important to get insight into the epidemiological situation of VTEC in The Netherlands. Until now, diagnostic limitations and the complexity of often interrelated microbial, animal, herd, environmental and production factors have hindered the determination of the epidemiology of O157 VTEC. The objective of this thesis was to increase insight into prevalences, risk factors, and transmission of O157 VTEC in the primary sector, with emphasis on dairy and veal cattle. Results indicated that O157 VTEC appears to be endemic in cattle in The Netherlands (Chapters 2 and 3). To gain insight into the cause of this status, further studies described in this thesis aimed at the epidemiology and population dynamics of O157 VTEC.

7.2 O157 VTEC in cattle and humans

In The Netherlands, the annual incidence of recognised human VTEC infections (0.25 cases per 100,000 inhabitants) is relatively low compared to other countries like Germany, The United Kingdom, and Canada (Duynhoven *et al.*, 2002). Nevertheless, O157 VTEC is endemic in Dutch cattle herds. The herd-level prevalence appears to be similar to or only slightly lower than that of other countries (Chapter 1, 2 and 3).

Pulsed Field Gel Electrophoresis (PFGE) types of O157 VTEC isolates obtained from human cases, animals and herds (also from our studies) in The Netherlands from the time period 1997 up to 2005, are present in a database of the Institute for Public Health and the Environment (RIVM). Comparison of these PFGE types indicated that DNA-types found in human cases could also be identified in cattle (data not presented). This implies an association between human and cattle infection in The Netherlands. Avery *et al.* (2004) also typed O157 VTEC isolates from beef cattle, meat products and humans in The United Kingdom. O157 VTEC isolates that, based on epidemiological information, were expected to be from one origin, fell within a narrow PFGE profile. However, O157 VTEC isolates that, based on epidemiological information, were expected to be from more than one origin, sometimes also showed the same PFGE types. This shows that, although PFGE is considered

the gold standard for DNA fingerprinting for O157 VTEC (Swaminathan *et al.*, 2001), results should always be interpreted with careful regard to epidemiological data.

In summary, clusters of O157 VTEC types, including isolates from humans, cattle and food items, seem to exist. Outbreaks in humans might therefore be associated to infections in cattle.

7.3 O157 VTEC in cattle

7.3.1 Prevalences

Chapters 2 and 3 showed that prevalences of O157 VTEC in pigs and poultry herds were relatively low (<2%). In dairy and veal herds, though, a substantial part of the sampled farms appeared positive for O157 VTEC (7.2% and 9.3% respectively), especially in summer and fall (10.6% of dairy herds and 17.6% of veal herds). The longitudinal study in a dairy herd (Chapter 4) showed a within-herd prevalence of 0-29.5%, also with an obvious seasonal effect, i.e. no infections during winter.

Prevalences that are reported in this thesis, might underestimate true prevalences, as a result of sampling and laboratory methods. The measured within-herd prevalences might be affected by intermittent shedding of individual animals and the sensitivity and specificity of the microbiological test applied, which are not known. The calculated between-herd prevalences are probably lower than the true prevalences, because herds were only sampled once during the monitoring program. Since *E. coli* O157 are shed intermittently by cattle, strong fluctuations in point prevalences can occur (Hancock *et al.*, 1997; Mechie *et al.,* 1997). Also, the procedure of sampling a limited number of pooled faecal pats of the stable floors in the monitoring program implies that not all animals in a herd are sampled. Besides, the distribution of O157 VTEC in bovine faecal pats might impair sensitivity of the microbiological test (Pearce *et al.*, 2004). Furthermore, pooling a number of faecal pats to one pool sample before processing at the lab might dilute the bacteria, therewith also reducing the sensitivity of the test (Jordan, 2005). Jordan compares herd-level sensitivity of pooled sampling with sensitivity of individual sampling by simulation, using concentrations of bacteria in the faeces, and probability of infection as input of the model. In the model, specificity of the microbiological test was assumed to be 100%. The models show that, especially in case of low true prevalence in a herd (e.g. <5%), the sensitivity of pooled sampling can be increased by increasing numbers of samples per pool and/or number of pools per herd. Based on these results and our sampling procedure, possibly some gain in pooled sampling sensitivity might be reached in the monitoring program by increasing the number of

pools per herd (which was maximally 5, based on herd size), rather than the number of samples per pool. However, although sensitivity and specificity of our microbiological test are unknown, they are assumed to be sufficient as a result of the combination of immunomagnetic separation (IMS), selective media, agglutination and serotyping. Considering the aim of the monitoring, estimating between-herd rather than within-herd prevalences within the capacity regarding costs and workload for the laboratory, the pooled sampling used was supposed to suffice.

Despite the fact that prevalences might not be accurate, the monitoring program showed that O157 VTEC seems to be endemic in The Netherlands. This does not necessarily mean that O157 VTEC is endemic at farm level. Besides within-herd transmission, between-herd transmission is necessary for maintaining the endemic status in Dutch cattle populations.

7.3.2 Between-herd dynamics

The spread between herds might be affected by multiple factors. Possibly, some farms have a larger probability to get infected than others, as a result of risk factors for (re-) introduction. Concerning farm type, prevalences in the shedding season among veal herds were significantly higher than among dairy farms (17.6 % vs. 10.6%; Chapters 2 and 3). This might partly be explained by the age-difference; calves shed larger amounts of O157 VTEC and for longer periods compared to adult cattle (Cray and Moon, 1995). In veal herds O157 VTEC was also found during winter, while in dairy cattle no O157 VTEC was isolated during that time. Differences in prevalences were observed between white veal and pink veal systems (2.6% vs. 31.3%). White and pink veal systems differ considerably in management, feeding strategy, housing, and biosecurity measures; white veal farms are often connected to an integration, feed milk(-replacer), provide antibiotics (oxytetracycline), use mechanical ventilation and used to house the calves individually (prohibited since 2004). In contrast, pink veal farms often produce independent from any integration, supply concentrates and roughage, use less antibiotics, apply natural ventilation, and house calves in groups. Besides, calves for pink veal usually stay longer on the farm (35 wk vs. 25 wk, respectively), which increases the time that introduction and/or transmission can occur. Possibly, some of these variables might be underlying factors for the larger prevalence of O157 VTEC among pink veal herds than among white veal herds.

Within dairy herds, a positive association of cattle entry in a herd and (O157) VTEC contamination was affirmed (Wilson *et al.*, 1993; Nielsen *et al.*, 2002). Also in our risk factor analysis for dairy herds, herds that did purchase animals within 2 years before sampling were positively associated with the presence of O157 VTEC. From re-sampling previously tested dairy herds (Chapter 6), it seemed that previously positive herds tended to have a slightly

higher probability to be found positive than previously negative tested herds, although not significant. Besides possible persistence of bacteria, (re-)infection of O157 VTEC on previously infected herds might be more likely to occur as a result of the presence of factors related to an increased risk of introduction. The presence of other cattle farms within one km of the sampled farm and access of cows to surface water appeared to be risk factors for O157 VTEC, even when correcting for the previous status of that farm. This emphasizes the potential role of between-herd transmission in the dynamics of O157 VTEC and in maintaining the endemic status.

7.3.3 Within-herd dynamics

Based on our studies, within-herd dynamics seem to depend on several aspects. These are type of animal (dairy, veal), risk factors for spread or persistence within herds (management and animal factors), presence of other hosts, and environmental reservoirs. Transmission rates in weaned calves (experimental) appeared to be considerably larger than in dairy cows (field; Chapter 5). This might be the effect of both age of the animals and infection doses of O157 VTEC. For both dairy and veal herds, risk factors for O157 VTEC positive herds were analysed (Chapters 2 and 3). Risk factors that might affect spread or persistence within herds rather than introduction of O157 VTEC to a herd were e.g. type of feed supplied, presence of other animals (pigs), housing system, farm size, period that calves were on the farm before sampling, etc.

Regarding the seasonal effect, the question is whether repeated infection of a farm is a result of introduction of new bacteria on the farm or a matter of persistence. Persistence can be caused by presence of O157 VTEC in cattle, in other animals, or in the environment. Several PFGE types were observed during the period of sampling of the longitudinal field study (Chapter 4), but the predominating type in the second shedding season was already isolated in the first shedding season, although in low amounts, indicating possible persistence of the infection during winter. In Danish cattle herds one PFGE group was represented on each farm (Nielsen *et al.,* 2002). It was suggested that introduction and establishment of new O157 VTEC strains into these cattle farms was probably not common. The persistence and predominance of only a few PFGE types, observed by Lejeune *et al.* (2004) over the entire feeding period on a feedlot with high cattle population turnover, also highlight the importance of the farm environment, and not necessarily incoming cattle or other sources of introduction, as a potential source or reservoir of O157 VTEC on farms. These results indicate a certain level of persistence, but the duration is unknown. Although PFGE did not show any strains that persisted on resampled farms (Chapter 6), the slightly higher probability of previously positive farms to be found positive again might still be partly the

result of persistence of certain types of O157 VTEC types. For DNA typing only one colony per positive tested sample was typed by PFGE, while different strains might have been present. Also, in the gastrointestinal tract mutations of *E. coli* O157 might have occurred (Wallace, 1999).

The longitudinal field study on a dairy herd (Chapter 4) indicated that the population dynamics of O157 VTEC within a dairy herd might include cattle, other hosts, and the environment. This has been confirmed in literature (Nielsen *et al.*, 2004; Armstrong *et al.*, 1996; Beutin *et al.*, 1993; Chapman *et al.*, 1997; Hancock *et al.*, 1998b; Lahti *et al.*, 2003; Rasmussen and Casey, 2001; Wallace, 1999). Transmission within and between these groups might contribute to the persistence of O157 VTEC infection on a farm. However, it remains unclear in which direction transmission has taken place and whether O157 VTEC was able to multiply in the environment or animals other than cattle. It seems that in infected herds or in the environment, some niches occur that are able to function as reservoir for (low numbers of) O157 VTEC, also during winter. Young stock might keep carrying the bacteria (Chapter 3) and might be a reservoir in which O157 VTEC can survive during winter, when sufficient susceptibles (with a promoting contact structure) are present. Environmental sites with which cattle are in contact might play a role in transmission of O157 VTEC within a farm. Although in our study no isolates were found in water samples from troughs and pools on the pasture, other research indicated water troughs as reservoirs for O157 VTEC (Donkersgoed *et al*., 2001; Faith *et al.*, 1996; Hancock *et al,* 1998b; Lejeune *et al.,* 2001).

Considering the high prevalence found in faecal samples, not only from individual animals but also from the environment, the assumption of faeces playing a crucial role in transmission is supported. The isolation of O157 VTEC from samples of both the filling tube and the injectors of the manure injector indicates spread on pastures, possibly maintaining the infection on the farm or even introducing the infection to other farms. Although no transmission between shedding seasons could be demonstrated via previously contaminated pastures (Chapter 5), O157 VTEC is able to survive on pastures for at least a month (Fenlon *et al.*, 2000; Nicholson *et al.*, 2005).

Ultimately, infection in a herd might fade out because of limiting numbers of susceptible animals as a result of resistance built up for O157 VTEC. In our longitudinal field study, no dairy cows that were shedding in the first season were found positive in the second season (Chapter 4). Potter *et al.* (2004) established that cattle are able to develop immunity for some virulence factors of O157:H7 i.e. secreted proteins that are assumed to play a role in colonization of host epithelial cells. Higher titres might reflect earlier infection (Johnson *et al.*, 1996), which might have invoked resistance to O157 VTEC. Studies of Cray and Moon (1995) and Sanderson *et al.* (1999), however, showed renewed infection of calves after artificial re-inoculation with the same strain as from the first inoculation. The reported

periods of non-shedding before re-inoculation were 13 and 6 weeks, respectively, which is much shorter than the interval between shedding seasons in our field study. Still, a notably shorter period of shedding was observed following re-inoculation (2-4 weeks and <8 days respectively) than following initial inoculation (14-20 weeks and 20-43 days, respectively). This might also indicate increased resistance to O157 VTEC infection. In addition, artificially inoculated doses might be much higher than in the field, so the question remains whether the animals of these experiments would have been re-infected with natural exposure to O157 VTEC.

7.3.4 Dynamics O157 VTEC

In summary, results of our studies suggest that several types of O157 VTEC enter a farm and might persist for some time, even some years, with possibly different strains as the most prevailing types in subsequent shedding seasons. To maintain the infection, hosts other than cattle, and the environment of the herd might play a role. Ultimately, the within-herd infection might become extinct due to limiting numbers of susceptible cattle. After a while, the number of susceptibles can increase again (e.g. by replacement of culled cows by newly purchased ones or heifers), so that a new infection chain can occur (SIR-model with replacement at animal and farm level). Because negative tested herds can become positive (Chapter 6), between-herd transmission seems to occur. Therefore, the prevalence of O157 VTEC in The Netherlands maintains at a certain level (or even rises), but not always the same farms are positive. Possibly, when continuing monitoring indefinitely, all farms might be found positive at some time.

Within- and between-herd transmission of O157 VTEC during summer appears to be considerably different than during winter. The precise cause for this difference is yet unclear. Circumstances (temperature, feed, management) in winter might result in lower numbers of O157 VTEC in a herd. Lower shedding or impaired susceptibility of cattle might be the cause of different transmission rates in the different seasons. The parameter calculated from the transmission experiment (Chapter 5) should therefore be considered a transmission rate for summer/early fall (in which the experiment was carried out) and might differ substantially from the beta that would be found during winter/early spring. To gain more insight into this, a transmission experiment should also be performed in early spring, to observe whether the same extent of transmission would occur under the same experimental conditions.

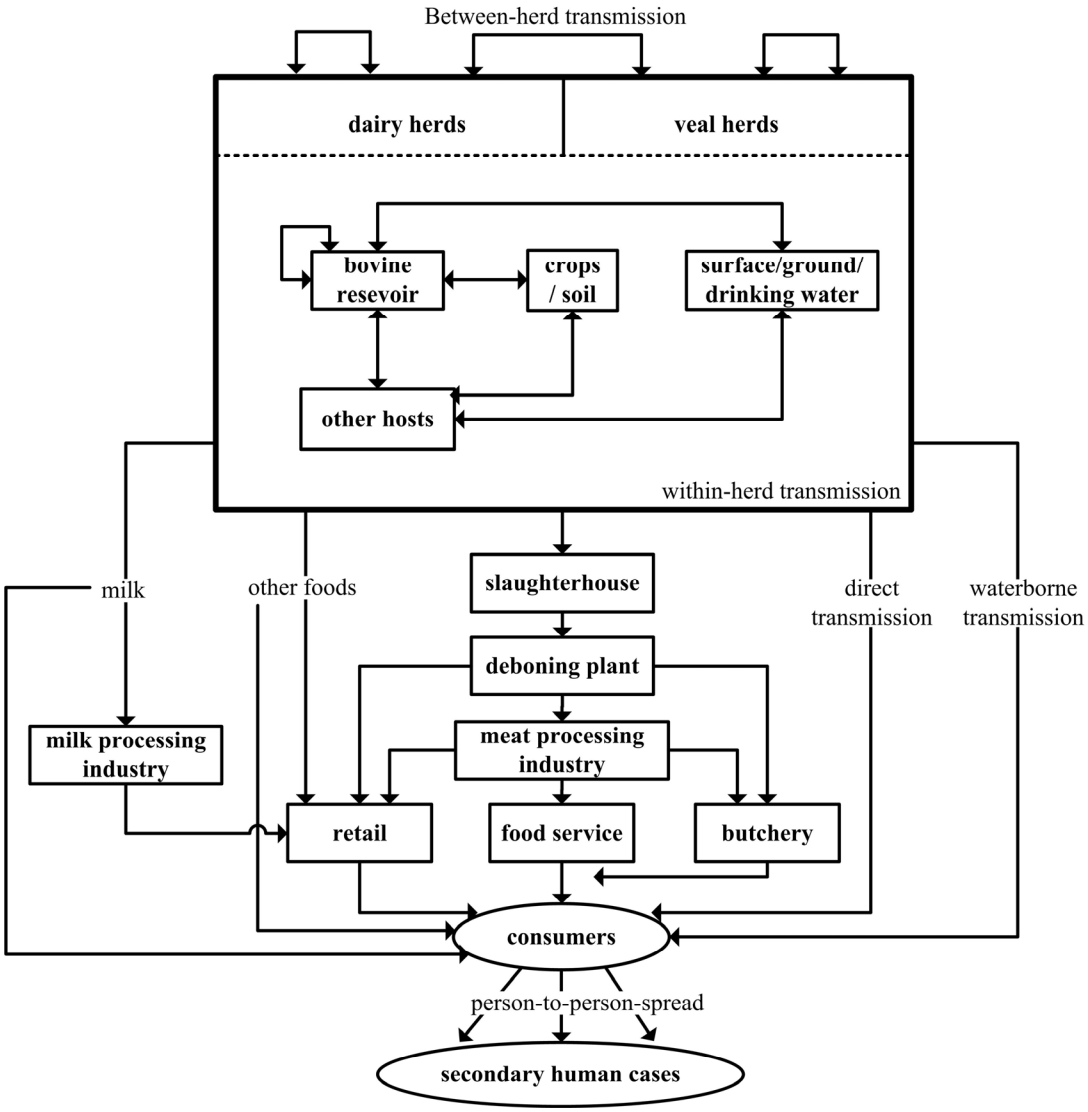

Figure 7.1 Hypothetical epidemiological model of O157 VTEC (adapted from Schouten and Kramer, 2002; Whittam *et al.*, 1998; Wallace, 1999)

7.4 Implications for humans in The Netherlands

Regarding the results of this thesis and literature, a hypothetical epidemiological model can be made (Figure 7.1). Because of the endemic status of O157 VTEC in cattle shown in this thesis, exposure of humans to O157 cannot be ruled out in The Netherlands. Although until now a relatively small part of the total food poisoning notifications in The Netherlands is related to O157 VTEC, the severe clinical human disease, mortality, and the continuous threat of human outbreaks ask for risk assessment and potential intervention strategies. As a result of intervention measures in the dairy industry (pasteurisation) and, in some lesser extent, the meat processing industry (zero-tolerance for visual faecal carcass contamination;

Heuvelink *et al.*, 2001), the risk of transmission by these routes has already been reduced in the Netherlands. However, meat as infection route has been reported and should not be ruled out (Heuvelink *et al.*, 2004). Furthermore, the risk of transmission directly from animals or products from the farm to humans can be considered substantial (Heuvelink *et al.*, 2000; Heuvelink *et al.*, 1998; Lier, 2003). Since O157 VTEC are spread via faecal excretion, it is important to think of ways to reduce faecal shedding, to prevent direct transmission to humans and to avoid faecal contamination of foods and water. Michel *et al.* (1999) already indicated an elevated risk of VTEC infection in a rural population, e.g. living in areas with high cattle density. This might suggest importance of direct contact with cattle, contaminated well water or locally produced food products.

7.4.1 Animal-to-person contact

Worldwide, O157 VTEC infection is associated with direct animal contacts, especially ruminants, also on petting zoos or open farms (Chapman„ 1999; Milne *et al.*, 1999; Renwick *et al.*, 1993; Trevena *et al.*, 1996). The number of petting zoos, care farms, farm camping sites and other farms open to the public (open farms), where intensive one-to-one contact with (farm) animals is common, is increasing. With this, the risk of human infection with O157 VTEC also increases and might be considered one of the most emerging risk factors for human infection with O157 VTEC. Insight into prevalence and behaviour of O157 VTEC on these farms is therefore required. Research of the The Food and Consumer Product Safety Authority (VWA) aims at making an inventory of *Escherichia coli* O157 and other zoonotic pathogens at petting zoos and open farms, with emphasis on the availability of hygiene facilities and consumption of raw dairy products. Little is known about ways and levels of introduction and transmission of O157 VTEC, though. Furthermore, registration of animals on such farms is inaccurate, resulting in lack of control on introduction and transmission of pathogens, which also might affect the animal production sector in case of animal disease outbreaks in The Netherlands.

To protect the general public of O157 VTEC infection via this route, possibly extra measures should be taken on petting zoos, care farms, farm camping sites and open farms. Although since 2001 a hygiene code for petting zoos is in force and the development of a certification system is already intended for (Anonymous, 2001), this system does not warrant measures for O157 VTEC yet. From the most important pathogens, risks of infection and transmission routes should be more quantified and surveillance might be implemented. Next, optimal prevention strategies for introduction and transmission of zoonotic pathogens can be developed and tested. An example of a measure preventing transmission of O157 VTEC is certification of farms that provide animals (cattle, goats, sheep) to petting zoos, care farms,

farm camping sites and other farms open to the public. Until now, little or no control, registration or canalisation is performed, so there is a probability of receiving animals from possibly O157 VTEC infected farms.

7.4.2 Faecally contaminated products and waterborne infections

For consumers, the largest risk for infection with VTEC by dairy products is when buying these products from the farm directly. Especially products that are not sterilised or pasteurised, like raw milk or unpasteurised cheeses. In contrast to results of Mechie and Chapman (1997) and Rahn *et al.* (1997), fore stream milk samples, bulk milk tank samples and milk filters which were tested in the longitudinal field study (Chapter 4), were not found positive for O157 VTEC while animals were positive. This might be due to a good hygienic milking management on this specific farm, which might prevent faecal contamination of milk, or due to a too low density of bacteria for detection with the method applied. Nevertheless, the possibility exists that raw milk products are faecally contaminated with O157 VTEC. When stored incorrectly, possible bacterial contamination might even increase. Farms that are registered to sell their products to consumers directly are inspected regularly in The Netherlands and are obliged to point out the risks of consuming raw products to their consumers. However, not all farms that sell their products are registered.

Meat can also be faecally contaminated, both directly and as result of cross-contamination during slaughter (Avery *et al.*, 2004; Jordan *et al.*, 1999) or when products are stored together. Especially consumption of rendered or raw meat products is a risk for O157 VTEC infection. The ability of O157 VTEC to survive in animal faeces, on pastures and in associated (aquatic) environments can also induce spread of O157 VTEC to crops by direct application of infected manure or by irrigation with contaminated water. Therefore, faecally contaminated crops or products (e.g. unpasteurised apple juice, filet americain) form a risk for consumers when not handled properly.

Worldwide, waterborne infections with O157 VTEC are emerging (Bopp *et al.*, 2003; Bruce *et al.*, 2003; Harrison and Kinra, 2004; Hrudey *et al.*, 2003; Licence *et al.*, 2001; Samadpour *et al.*, 2002), indicating faecal O157 VTEC contamination of both consumption (especially well/surface) and recreational waters.

In summary, attention should be paid to the risks of consuming raw cheeses and unpasteurised drinks (raw milk, fruit juices), raw meat products, fertilized vegetables and fruits and contaminated waters. Also the consumers of products from (organic) farms, which are fertilized directly with cattle slurry, should be aware of the risks.

7.4.3 Possible intervention strategies at farm-level

When aiming at reducing risks for humans by interventions at farm-level, it is of most importance to reduce the number of positive animals and farms. Simulation models predicted that reduction of O157 VTEC prevalence in cattle would result in substantial reduction in contamination of beef products (Jordan *et al.*, 1999). To determine what prevention and control strategies would be effective, more specific research for such measures should be done. Many types of VTEC are widely disseminated in intermittently shedding cattle, showing no clinical disease, but also in other animals and the environment. Therefore, the question is whether traditional means of controlling infectious agents, such as test and cull of carrier animals, would be cost-effective.

In general, measurements aiming at reduction of faecal/oral transmission (e.g. for *Mycobacterium Avium* subspecies *paratuberculosis*, *Leptospira interrogans* serovar *hardjo*, or *Salmonella* Dublin) might possibly be associated with an overall reduction of zoonotic agents, including O157 VTEC. An effective measure to reduce spread of O157 VTEC within farms might be separating age groups in cattle herds; compared to adult cattle, calves shed higher numbers of O157 VTEC for longer periods of time (Cray and Moon, 1995) and show a higher transmission rate (Chapter 5). That might be the reason that veal calf herds have larger proportions of positive herds (Chapter 3), and possibly higher within-herd prevalence.

Besides general applicable intervention measures, specific measures for O157 VTEC might be possible. In literature, different intervention strategies are described. Examples are use of probiotics (Brashears *et al.*, 2003a,b; Tkalcic *et al.*, 2003; Zhao *et al.*, 1998; Zhao *et al.*, 2003), phage therapy (Callaway *et al.*, 2003c), vaccination (Finlay, 2003, Moxley *et al.*, 2003; Potter *et al.*, 2004), chlorate addition (Anderson *et al.*, 2000; Callaway *et al.*, 2002; Edrington *et al.*, 2003), feeding management (Callaway *et al.*, 2003b), and other management strategies, like manure management (Nicholson *et al.*, 2005) and water management (Hancock *et al.*, 1998a). A combination of some of these strategies might achieve a reduction in O157 VTEC in cattle (Callaway *et al.*, 2003a). Based on our results and literature, it seems worthwhile to (experimentally) investigate the potential role of risk factors at farms, like water (troughs), effect of feed and presence of other animal species, and their effect on transmission of O157 VTEC. For the latter, transmission experiments could be performed to test whether O157 spreads in other animals or between other animals and cattle. Treatment with a potential vaccine to bring the reproduction ratio below 1 could be efficient and therefore worthwhile testing. However, to be able to decide which control measures are most cost-efficient, a sensitivity and cost-benefit analysis should be performed.

Considering intervention strategies, one should keep in mind that 50% of the veal calves in The Netherlands is imported. Although within Europe O157 VTEC belongs to the category

A zoonoses of the Zoonoses Directive (2003/99/EC), a test for O157 VTEC is not part of the control procedure preceding import. Imported veal calves might therefore be a cause of introduction of O157 VTEC on Dutch farms. The majority of veal calves imported on veal farms, however, end up directly in the slaughterhouse, when reaching slaughter weight. This seems to minimize the risk of transmission to cattle farms. Nevertheless, infected veal calves still enter the food chain and therefore remain a threat to public health.

7.3.4 Other VTEC types

Worldwide, most reported human illness outbreaks due to VTEC have been attributed to serotype O157. However, extensive reports of these outbreaks and use of O157 VTEC selective media at laboratories around the world might have created the general perception that this serotype is the only VTEC of significant importance to public health. While some other serotypes are increasingly reported as human pathogenic (e.g. O111, O26, O113), approximately 60 VTEC serotypes have been associated with human illness (Bettelheim, 2003). Some of these types might even be more commensal in domestic animals than O157 VTEC. To gain information on the occurrence of other human pathogenic VTEC types in domestic animals, detection might be based on tests for virulence factors (e.g. serological or DNA tests).

7.5 Conclusions

In conclusion, this thesis showed that O157 VTEC is endemic in the Dutch cattle population. Both within-herd and between-herd transmission seem to contribute to this status. Although the dynamics of VTEC are not completely clear yet, some specific properties are described in literature; lack of host-specificity (although cattle are the main host), seasonal shedding, nearly omnipresent occurrence in cattle farms, (transient) residence in the gastro-intestinal tract of animals not associated with disease, high prevalence in young cattle, and persistence of the organism on a farm for years (Hancock *et al.*, 2001). Some of these properties were confirmed in this thesis, and extra data were provided on risk factors for infection of dairy and veal herds and within-herd transmission. However, more research on transmission, persistence, and potential control measures is recommended.

Important for control is the current state of knowledge on introduction routes and survival of the bacteria on the farm. As long as this is not clear, standard monitoring of cattle and typing of O157 VTEC isolates, both in animals and human patients, should be continued. Herewith, more insight can be gained in the different types of O157 VTEC prevailing in The Netherlands and in ways of transmission among animals and herds, and the routes of human

infection. In past outbreaks, typing supported the implementation of measures to control the spread of infection, e.g. product withdrawal or temporary closure of open farms (Willshaw *et al.*, 2001). Therefore, co-operation of the human and animal production sector is very important, especially in case of a suspected outbreak.

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Summary

Introduction

An important human pathogenic *Escherichia coli* is O157 VTEC. VTEC are defined as all *E. coli* bacteria that produce verocytotoxins. When additional virulence factors are present, infection in humans can lead to clinical signs. The O157 VTEC strains identified in the studies of this thesis, are diagnosed as O157 serogroups that *(I)* have genes for the production of one or both of the most frequently occurring vero(cyto)toxins, verotoxin 1 (VT1) and verotoxin 2 (VT2), and *(II)* possess the gene that may cause attaching-effacing (AE) laesions in the colon of humans. Clinically, more (potential) virulence factors of VTEC are described, e.g. several VT2 subtypes and the production of haemolysin (Hly). In humans, infection with VTEC can lead to asymptomatic infection, mild diarrhoea, bloody diarrhoea or haemorrhagic colitis (HC), with as possible complications the haemolytic uraemic syndrome (HUS) or trombocytic thrombocytopenic purpura (TTP). Since 1982, in several countries VTEC outbreaks are reported. Besides sporadic cases, varying incidences were reported in countries in all continents from 0.1 to 9.85/100,000 population (VTEC) or 2.0 to 7.8/100,000 children younger than 5 years (HUS). Large outbreaks have occurred in North-America (USA, Canada), Asia (Japan), and Europe (Scotland, England, and Wales). Potential sources of (often foodborne) infection varied from animal products to vegetables and other, not always consumption related, sources of infection.

In The Netherlands, each year about 30 cases of HUS are reported, with two-third of the cases in children younger than five. The incidence of HUS is 2.0 per 100,000 children younger than 5 years. Although not all cases of HUS are associated with VTEC, O157 VTEC is the bacteria most frequently isolated from HUS patients. The mean disease burden of O157 VTEC infections in The Netherlands, expressed as DALY's (Disability Adjusted Life Year; Chapter 1) is substantially higher per primary case per year than for e.g. campylobacteriosis, another foodborne public health problem in The Netherlands (55 vs. 4.4 DALY's per 1000 cases). Because of the severe consequence of human infection, the large range of products that can be contaminated O157 VTEC is a realistic threat for public health.

From literature, cattle appear to be important reservoirs of O157 VTEC. Prevalences of O157 VTEC in cattle populations vary considerably between countries. Also other animals, e.g. sheep, pigs, horses and chickens, were found to be carriers of O157 VTEC. Literature indicates that the bacteria can be found at several locations on and in vicinity of the farm, including other animals, water, soil, feed etc. From the moment O157 VTEC colonizes in the gastro-intestinal tract of cattle, intermittent periods of faecal excretion occur. Strong evidence exists for seasonal excretion and transmission, with periods of maximum numbers of

shedding coinciding with peaks in human infection. In general, because VTEC is excreted in faeces, faeces might play an important role in transmission of VTEC.

To understand the dynamics of O157 VTEC in cattle populations, it is important to have insight in prevalences, risk factors, transmission and persistence of O157 VTEC in cattle. Until the start of this research, little was known on the occurrence and behaviour of O157 VTEC in the Dutch primary animal production sector. This thesis principally aims at increasing this insight in O157 VTEC in the primary sector, with emphasis on dairy and veal, by literature research, observational epidemiology (field studies), and transmission experiments.

O157 VTEC dynamics

To estimate the prevalence of O157 VTEC on Dutch dairy herds, laying hen and broiler flocks, finishing-pig herds and veal herds, faecal samples were collected as part of a national monitoring program to test for O157 VTEC (Chapters 2 and 3). This monitoring program for zoonotic agents in farm animals started in The Netherlands in 1996 at the National Institute for Public Health and the Environment (RIVM). A questionnaire about farm characteristics resulted in variables that could be analysed to identify and quantify factors associated with presence of O157 VTEC.

In total, 7.2% of dairy farms and 9.3% of veal farms were positive, with higher prevalences found during summer and early fall. Of broiler flocks, laying flocks and finishing-pig herds, respectively, 1.7%, 0.5% and 0.4% were O157 VTEC positive. For dairy and veal herds, logistic regression was performed on variables from the questionnaire, comparing positive herds to negative herds. To account for season, a sine function was included in logistic regression as offset variable. In the final model for dairy herds, the presence of at least one pig at the farm (OR=3.4), purchase of animals within the last 2 years before sampling (OR=1.9), supply of maize (OR=0.29) to the cows, and sampling a herd in the year 1999 or 2000 (compared to sampling in 1998; OR=2.1 and 2.9, respectively) had associations with the presence of O157 VTEC. In the final model for veal herds, pink-veal production (OR=6.1, compared to white-veal production), group housing of the sampled herd (OR=8.9, compared to individual housing), more than one stable present (OR=5.0), hygienic measures regarding visitors (OR=4.3), interval arrival-sampling of a herd of >20 weeks (OR=8.5, compared to < 10 wks), and presence of other farms within 1 km distance (OR=0.1) showed associations ($P<0.05$) with the presence of O157 VTEC.

To gain insight in the dynamics of O157 VTEC on a farm, several studies were carried out. Within-herd prevalence, potential environmental reservoirs, intermediate hosts and DNA

types of O157 VTEC isolates were investigated in a longitudinal field study of a dairy farm that was found positive in the monitoring program (Chapter 4). From July 1999 till November 2000, this study was conducted on a dairy farm in The Netherlands. Faeces, blood, milk and environmental samples were collected 14 times with intervals varying from 4-10 weeks during the study period. Isolates were tested for most common virulence genes and typed by pulsed field gel electrophoresis. In total, 71 isolates were obtained from 2164 samples, of which 49 were from dairy cows, eight from young stock, five from other animals and nine from the environment. Positive samples were all detected in summer and early fall. VT- and eae-genes were found in all tested isolates, except in one. DNA typing showed that three clusters of O157 isolates could be identified. One of these clusters contained samples of two shedding seasons, indicating possible persistence on the farm during winter and spring.

Repeated measures analysis of variance showed that cows with O157 VTEC infection had higher daily milk production in the period preceding sampling ($p=0.0055$). There was no association between the results of the LPS-ELISA on serum samples from dairy cows and their O157 status.

Data from the longitudinal field study of fourteen months in a dairy herd and data from an experiment with calves were used to quantify transmission of O157 VTEC in cattle (Chapter 5). For the latter, two groups of ten calves were placed on two pastures. Of each group, five were inoculated with 10^9 CFU O157 VTEC and, when considered infectious, placed back into their group. All contact calves became positive within six days after reunification. The estimate of the basic reproduction ratio $R_{0,\ experiment}$ was 7.3 (95% CI 3.9-11.5), indicating that each infectious calf will infect on average seven other calves during an assumed infectious period of 28 days in a fully susceptible population. $R_{0,\ dairy\ herd}$ appeared to be about 10 times lower (0.71; 95% CI 0.53-0.99), possibly indicating an age-effect.

Also the role of previously infected calves and pastures in the transmission of O157 VTEC was tested in experiments (Chapter 5). After the transmission experiment, six contact-infected animals that were shedding continuously during the experiment were housed in a tying stall during winter. After forty days, all six were negative for O157 VTEC. In June, after a period of 34 weeks in which the heifers stayed negative, they were placed on a clean and isolated pasture to observe whether they started shedding again. On each pasture that was infected with high doses of O157 VTEC during the transmission experiment the previous summer, newly purchased susceptible calves were placed. None of the heifers or calves started shedding and none of the pasture samples were positive during the 14 weeks from the start of these experiments, indicating that both the heifers and the previously contaminated pasture did not function as reservoir of O157 VTEC.

A retrospective cohort study was performed to find evidence for either persistence in or (re-)introduction of *E. coli* O157 at dairy farms. For this we established whether farms that

were positive in the Dutch monitoring program were more likely to be found positive in a next shedding season compared to previously negative farms (Chapter 6). Of 49 previously positive farms, 31 were randomly selected. From 402 previously negative farms, 30 were randomly selected. Respectively, 30 and 23 farmers co-operated. Information about farm and management factors was collected. O157 VTEC isolates were DNA-typed. Exact multivariate logistic regression was performed to identify factors associated with *E. coli* O157 in at least one shedding season after the first sampling. Three variables remained in the final model. Previously positive herds were only slightly more often positive at second sampling (32%) than herds negative in the first sampling (29%) (OR=3.91; P=0.40). Significantly more herds with access to surface water on pastures were positive (64%) than herds in which cows had no access (14%) (OR=31.7; P=0.0003). Prevalence of positive farms with at least one cattle farm within 1 km distance was significantly increased (35%) compared to when no cattle farms were present in the neighbourhood (prevalence 0 %; OR=13.7; P=0.028). Long-term persistence of *E. coli* O157 on dairy farms was not demonstrated in this study. Factors associated with a positive test at second sampling point in the direction of (re-)introduction rather than long-term persistence of infection.

Discussion and main conclusions

In cattle, O157 VTEC seems to be endemic in The Netherlands. Besides within-herd transmission, between-herd transmission seems necessary for maintaining the endemic status in Dutch cattle populations. The spread between herds might be affected by multiple factors. Differences in prevalences are e.g. observed between farm types (e.g. dairy vs. veal; white veal vs. pink veal). Sampling of previously tested dairy herds (Chapter 6) indicated that (re-)infection of O157 VTEC on previously infected herds might be likely to occur as a result of the presence of factors related to an increased risk of introduction. This emphasises the potential role of between-herd transmission in the dynamics of O157 VTEC and in maintaining the endemic status.

Within-herd dynamics seems to depend on several aspects; type of animal (dairy, calves), risk factors for spread or persistence within herds (management and animal factors), presence of other hosts, and environmental reservoirs. Transmission was shown to occur, although transmission rates in weaned calves (experimental) appeared to be considerably larger than in dairy cows (field; Chapter 5).

The question is whether a farm is found to be infected in subsequent samplings because of introduction of new bacteria on the farm or because the bacteria persist on the farm. The longitudinal field study on a dairy herd indicated that the population dynamics of O157

VTEC within a dairy herd might include cattle, other hosts, and the environment. The assumption of faeces playing a crucial role in transmission is supported. Several PFGE types were observed during the period of sampling of the longitudinal field study (Chapter 4), but the predominating type in the second shedding season was already isolated in the first shedding season, although in low amounts, indicating possible persistence of the infection during winter. These results indicate a certain level of persistence, which is confirmed in literature, but the duration is unknown.

Ultimately, infection in a herd might fade out because of limiting numbers of susceptible animals being a result of resistance built up for O157 VTEC. In our longitudinal field study, no dairy cows that were shedding in the first season were found positive in the second season (Chapter 4). It was established in literature that cattle are able to develop immunity for some virulence factors of O157:H7, which might invoke resistance to O157 VTEC.

In summary, results of our studies suggest that several types of O157 VTEC enter a farm and might persist for some time, even some years, with possibly different strains as the most prevailing types in subsequent shedding seasons. Within- and between-herd transmission of O157 VTEC during summer might be considerably different than during winter. Ultimately, the within-herd infection might become extinct due to limiting numbers of susceptible cattle (SIR-model with too low replacement). Because herds, where O157 VTEC was not found before, can become infected (Chapter 6), between-herd transmission seems to occur. Between-herd transmission of O157 VTEC can thus lead to persistence in a larger region e.g. The Netherlands.

Because of this endemic status in cattle, exposure of humans to O157 VTEC cannot be ruled out in The Netherlands. Although until now a relatively small part of the total food poisoning notifications in The Netherlands is related to O157 VTEC, the severe clinical human disease, mortality, and the continuous threat of human outbreaks ask for risk assessment and potential intervention strategies. With increasing numbers of petting zoos, care farms, farm camping sites and open farms, especially the risk of transmission directly from animals or products from the farm to humans can be considered emerging. Little is known about ways and levels of introduction and transmission of O157 VTEC, though. To protect the general public of infection via this route, possibly extra measures should be taken on petting zoos, care farms, farm camping sites and open farms.

Dairy products and meat can be faecally contaminated. For consumers, the largest risk for infection with VTEC by dairy products is when buying these products from the farm directly. The ability of O157 VTEC to survive in animal faeces, on pastures and in associated (aquatic) environments can also induce spread of O157 VTEC to crops by direct application of infected manure or by irrigation with contaminated waters.

When aiming at reducing risks for humans by interventions at farm-level, it is of most importance to reduce the number of positive animals and farms. To determine what prevention and control strategies would be effective, more specific research for such measures should be carried out. In general, measurements aiming at reduction of faecal/oral transmission might possibly be associated with an overall reduction of zoonotic agents, including O157 VTEC. An effective measure to reduce spread of O157 VTEC within farms might be separating age groups in cattle herds. In literature, alternative intervention strategies are described. Examples are use of probiotics, phage therapy, manure storage, vaccination, and feeding management. To be able to decide which control measures would be most cost-efficient, more (experimental) research is necessary and a sensitivity and cost-benefit analysis should be performed.

In summary, the main conclusions of this thesis are:

- O157 VTEC is endemic in the Dutch cattle population. Both within-herd and between-herd transmission seem to contribute to this status.
- Cattle are the main host for O157 VTEC. However, the bacteria can also be found in other animals and in the environment.
- In The Netherlands, O157 VTEC is shed with a seasonal pattern.
- The proportion of positive veal cattle herds is higher than that of dairy cattle herds.
- Risk factors concerning farm characteristics could be identified for O157 VTEC infection of both dairy (e.g. animal purchase and maize supplementation) and veal farms (e.g. pink-veal production and group housing).
- Transmission in calves is higher than in dairy cows.
- O157 VTEC can persist on a farm for more than one shedding season. However, there was no clear evidence for long term-persistence. (Re-)introduction of O157 VTEC on farms, i.e. between-farm transmission, appears more likely.
- Important for control of O157 VTEC is more knowledge on introduction routes and survival of the bacteria on the farm, and insight in the effect of specific intervention measures on these aspects.

Samenvatting

Deze samenvatting is een vereenvoudigde bewerking van de Engelse, wetenschappelijke samenvatting (Summary)

Inleiding

"Als je kinderen hiervan wilt redden, richt je inspanningen dan in de eerste plaats op het voorkomen dat ze geïnfecteerd worden. Doe iets om de hoeveelheid E. coli O157 bacteriën daar buiten te verminderen…!"
Uit: E. coli O157; The true story of a mother's battle with a killer microbe (Mary Heersink, 1996).

De bacterie in het bovenstaande citaat, *E. coli* O157 (ook wel O157 VTEC genoemd), is een steeds vaker voorkomende ziekteveroorzaker bij de mens. VTEC's zijn *Escherichia coli* bacteriën die giftige verocytotoxinen (VT) produceren. Besmetting bij mensen kan leiden tot zeer ernstige symptomen. Mary Heersink beschrijft in bovengenoemd boek het zeer ernstige verloop van de ziekte van haar zoontje na besmetting met deze bacterie door het eten van een besmette, niet goed doorbakken hamburger. Besmetting met O157 VTEC kan bij mensen leiden tot een infectie zonder zichtbare verschijnselen, milde diarree of bloederige diarree (haemorrhagische colitis; HC), met als één van de mogelijke complicaties het haemolytisch uremisch syndroom (HUS). Dit syndroom komt vooral voor bij jonge kinderen en kan leiden tot blijvende nierschade of zelfs overlijden. Vanaf 1982 zijn besmettingen met VTEC in diverse landen beschreven. De frequentie van infecties wereldwijd varieert over landen van 0,1 tot 9,85 per 100,000 inwoners (O157 VTEC infecties algemeen) of van 2,0 tot 7,8 per 100.000 kinderen onder de vijf jaar (HUS). Grote uitbraken hebben plaatsgevonden in Noord-Amerika (VS, Canada), Azië (Japan) en Europa (Schotland, Engeland en Wales). Mogelijke bronnen van infectie (veelal via voedsel) zijn dierlijke producten, groenten, drinkwater en andere infectieroutes, zoals direct contact met besmette dieren en het zwemmen in besmet water. Veelal wordt hierbij uitgegaan van besmetting via (runder)mest.

In Nederland worden jaarlijks zo'n 30 gevallen van HUS gerapporteerd, waarvan tweederde bij kinderen jonger dan vijf jaar. De frequentie van het voorkomen van HUS is hiermee 2,0 per 100.000 kinderen jonger dan vijf jaar. Hoewel niet alle gevallen van HUS geassocieerd worden met VTEC, wordt O157 VTEC wel het meest gevonden bij HUS patiënten. Vanwege de ernstige gevolgen van humane besmetting en het brede scala aan

producten dat besmet kan zijn met O157 VTEC, vormt deze kiem een realistische bedreiging voor de volksgezondheid.

In de literatuur wordt rundvee als belangrijk reservoir van O157 VTEC beschreven. De mate van voorkomen van O157 VTEC onder rundvee verschilt aanzienlijk tussen landen. In de literatuur wordt ook aangegeven dat de bacteriën op verscheidene plaatsen op of rondom een veebedrijf aanwezig kunnen zijn, zoals in andere dieren dan rundvee (bijv. schapen, varkens, paarden en kippen), in het water, in de grond, in voer etc. Zodra O157 VTEC zich nestelt in de darmen van rundvee, kunnen runderen de bacterie periodiek uitscheiden. Er is sterk bewijs voor seizoensgebonden uitscheiding en verspreiding, waarbij de periode met maximale uitscheiding samen lijkt te vallen met de piek van humane besmettingen. Omdat VTEC via mest wordt uitgescheiden, kan in het algemeen worden gesteld dat mest een belangrijke rol speelt in de verspreiding van VTEC op bedrijven.

Om het gedrag (dynamiek) van O157 VTEC in de rundveepopulatie te begrijpen, is het belangrijk om inzicht te hebben in de mate van voorkomen van de bacterie (prevalentie), risicofactoren voor besmetting, verspreiding (transmissie), en handhaving (persistentie) van O157 VTEC besmettingen in rundvee in Nederland. Tot de start van dit onderzoek was hierover weinig bekend. Het doel van het onderzoek beschreven in dit proefschrift is voornamelijk dit inzicht te vergroten, in het bijzonder binnen melkvee- en vleeskalverbedrijven, door middel van literatuuronderzoek, epidemiologisch (veld)onderzoek en transmissie-experimenten.

O157 VTEC dynamiek

Om de prevalentie van O157 VTEC te schatten op Nederlandse melkvee-, leghen-, vleeskuiken-, vleesvarkens- en vleeskalverbedrijven, is een landelijk monitoringsprogramma opgezet waarbij mestmonsters afkomstig van deze bedrijven worden getest op O157 VTEC (Hoofdstuk 2 en 3). Dit monitoringsprogramma voor humane ziekteverwekkers bij boerderijdieren is in Nederland gestart in 1996, door het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Een vragenlijst over bedrijfskenmerken werd ingevuld met de veehouder. Dit resulteerde in een uitgebreid gegevensbestand waaruit door middel van statistische analyse risicofactoren voor de aanwezigheid van O157 VTEC vastgesteld konden worden d.m.v. het vergelijken van positieve en negatieve bedrijven. Het effect van de risicofactoren werd uitgedrukt in Odds Ratios (OR). Een OR van 1 betekent dat er geen verband kan worden aangetoond tussen een factor en besmetting met O157 VTEC. Een OR groter dan 1 betekent dat er een positief verband is aangetoond, deze factor vormt dus een risico voor

besmetting. Een OR kleiner dan 1 ("negatief verband") houdt in dat een factor wellicht preventief kan werken tegen besmetting.

In totaal is over de jaren heen bij 7,2% van de bemonsterde melkveebedrijven en 9,3% van de vleeskalverbedrijven ten minste één besmet mestmonster gevonden. In de zomer en het vroege voorjaar lag de prevalentie zelfs hoger. Van de vleeskuiken-, leghen- en vleesvarkensbedrijven werd respectievelijk 1,7%, 0,5% en 0,4% positief gevonden voor O157 VTEC. Binnen de risicofactoren analyse, die uitgevoerd werd voor melkvee en vleeskalverbedrijven, werd rekening gehouden met het seizoenseffect. Uit de analyse bleek dat voor melkveebedrijven de aanwezigheid van minimaal 1 varken op het bedrijf (OR=3,4), aankoop van vee binnen de laatste 2 jaar voor monstername (OR=1,9), het voeren van maïs aan de koeien (OR=0,29) en monstername in 1999 en 2000 (vergeleken met monstername in 1998; OR=2,1 en 2,9 respectievelijk) in verband werden gebracht met de aanwezigheid van O157 VTEC. Voor vleeskalverbedrijven bleken rosévlees productie (OR=6,1; vergeleken met witvlees productie), groepshuisvesting (OR=8,9; vergeleken met individuele huisvesting), aanwezigheid van meer dan 1 stal (OR=5,0), het toepassen van hygiënemaatregelen voor bezoekers (OR=4,3), een periode van meer dan 20 weken tussen aankomst van de kalveren op het bedrijf en de monstername (OR=8,5; vergeleken met minder dan 10 weken), en de aanwezigheid van andere bedrijven binnen een straal van 1 km (OR= 0,1) een verband te tonen met de aanwezigheid van O157 VTEC.

Voor dit proefschrift zijn verschillende studies uitgevoerd om meer inzicht te krijgen in de dynamiek van O157 VTEC op een veebedrijf. In hoofdstuk 4 is een onderzoek beschreven waarin op een Nederlands melkveebedrijf, dat positief was voor O157 VTEC, alle runderen, hun omgeving en andere dieren herhaaldelijk onderzocht werden op O157 VTEC. Dit bedrijf werd gevolgd van juli 1999 tot november 2000, waarbij mest-, bloed-, melk- en omgevingsmonsters 14 keer werden verzameld met tussenpozen van 4 tot 10 weken. Mest-, melk- en omgevingsmonsters werden getest op de aanwezigheid van O157 VTEC bacteriën, bloedmonsters werden getest op afweerstoffen tegen O157 VTEC. Ook werden DNA-types van de gevonden bacteriën (isolaten) onderzocht. In totaal werd O157 VTEC in 71 monsters gevonden, waarvan 49 mestmonsters van melkvee, 8 mestmonsters van jongvee, 5 mestmonsters van andere dieren en 9 monsters vanuit de omgeving. Alle positieve monsters werden gevonden in de zomer en het vroege voorjaar. Door DNA typering werden 3 groepen, bestaande uit dezelfde O157 DNA-typen, op dit bedrijf geïdentificeerd. Eén van deze clusters bevatte isolaten uit 2 opeenvolgende seizoenen, wat kan duiden op persistentie op het bedrijf van dit type gedurende winter en voorjaar.

Gegevens van bovengenoemd onderzoek op een melkveebedrijf, samen met gegevens van een experiment met kalveren, zijn gebruikt om de transmissie van O157 VTEC tussen dieren te berekenen, uitgedrukt in een getal dat de mate van transmissie aangeeft (R_0-waarde;

Hoofdstuk 5). Voor het experiment zijn twee groepen van elk tien kalveren op aparte weilanden geplaatst. Van elke groep werden vijf kalveren kunstmatig besmet met O157 VTEC en teruggeplaatst in de groep. Alle (niet kunstmatig besmette) contactdieren in beide groepen werden binnen zes dagen ook positief. Hieruit werd een R_0 van 7,3 berekend, wat betekent dat één infectieus kalf gemiddeld 7 andere kalveren zal besmetten in een volledig gevoelige (niet-besmette) populatie tijdens een aangenomen infectieuze periode van 28 dagen. De R_0 voor melkvee bleek ongeveer tien keer zo laag (0,71), wat kan duiden op een leeftijdseffect.

In het onderzoek van hoofdstuk 5 is door middel van experimenten ook gekeken naar de rol van voormalig besmette kalveren en weilanden op de transmissie van O157 VTEC. Na het transmissie-experiment werden zes contact-besmette dieren, die tijdens het experiment continu de bacterie uitscheidden, aangebonden in een stal gehuisvest in de winterperiode. Na 40 dagen waren alle zes dieren negatief voor O157 VTEC. In juni, na een periode van 34 weken waarin de dieren negatief bleven, werden de pinken op een 'schoon' en geïsoleerd weiland gezet om te zien of ze opnieuw zouden gaan uitscheiden. Daarnaast werden op elk weiland dat tijdens het experiment van de vorige zomer besmet was met een hoge dosis O157 VTEC, nieuw aangekochte gevoelige (niet eerder besmette) kalveren geplaatst. In beide proeven ging geen enkel dier de O157 VTEC bacterie uitscheiden tijdens de 14 weken durende proef, wat erop duidt dat zowel de voorheen besmette pinken als het voorheen besmette weiland niet als O157 VTEC reservoir dienden.

Een laatste studie (Hoofdstuk 6) werd uitgevoerd om bewijs te vinden voor persistentie dan wel (her)introductie van *E. coli* O157 op melkveebedrijven. Hiervoor werd vastgesteld of bedrijven die voormalig positief waren in het Nederlandse monitoringsprogramma, meer kans hadden om in een volgend seizoen (tenminste één uitscheidingsseizoen na de eerste monstername) positief te worden gevonden dan voormalig negatieve bedrijven. Uit 49 voormalig positieve bedrijven werden willekeurig 31 bedrijven geselecteerd en uit 402 voormalig negatieve bedrijven werden willekeurig 30 bedrijven geselecteerd. Hieruit wilden respectievelijk 30 en 23 bedrijven meewerken aan een nieuwe bemonstering en het invullen van een nieuwe vragenlijst om informatie over het bedrijf te verzamelen. Het DNA van nieuw gevonden O157 VTEC isolaten werd getypeerd en statistische analyse werd opnieuw uitgevoerd om factoren te vinden die verband houden met *E. coli*. Drie variabelen waren aanwezig in het uiteindelijke statistische model. Voorheen positief bevonden bedrijven werden slechts iets vaker positief bevonden bij de tweede monstername (32%) dan voorheen negatieve bedrijven (29%). Bedrijven met toegang tot oppervlaktewater vanuit het weiland werden significant vaker positief bevonden (64%) dan bedrijven waarbij de dieren geen toegang tot oppervlaktewater hadden (14%) (OR=31,7). Tenslotte waren bedrijven met tenminste 1 ander bedrijf binnen een straal van 1 km significant vaker positief (35%) dan

bedrijven zonder buurtbedrijven (0%; OR=13,7). Lange termijn persistentie van *E. coli* O157 op melkveebedrijven kon met deze studie niet worden aangetoond. De factoren die geassocieerd werden met een positieve tweede monstername duidden eerder op (her)introductie van de infectie vanuit de omgeving.

Discussie en belangrijkste conclusies

O157 VTEC lijkt endemisch te zijn bij rundvee in Nederland, wat betekent dat de infectie blijft voorkomen met een relatief constant aantal besmette dieren/bedrijven; het aantal nieuwe besmettingen wordt gecompenseerd door het aantal dieren/bedrijven waar de ziekte verdwijnt. Naast transmissie binnen een bedrijf lijkt transmissie tussen bedrijven noodzakelijk te zijn voor handhaving van de endemische status in de Nederlandse rundveepopulatie. De verspreiding tussen bedrijven wordt mogelijk door meerdere factoren beïnvloed. Verschillen in prevalentie werden bijvoorbeeld gevonden tussen bedrijfstypen (bijv. melkvee- versus vleeskalverbedrijven; witvlees versus rosévlees kalveren). Bemonstering van eerder geteste melkveebedrijven (Hoofdstuk 6) gaf aan dat een infectie van O157 VTEC op bedrijven die eerder al positief waren op langere termijn meest waarschijnlijk wordt veroorzaakt door de aanwezigheid van factoren die gerelateerd zijn aan een verhoogde risico op (her)introductie. Dit benadrukt de potentiële rol van verspreiding tussen bedrijven in de dynamiek van O157 VTEC en voor het handhaven van de endemische status.

De dynamiek binnen een bedrijf blijkt van verscheidene zaken af te hangen, waaronder diersoort (melkkoe, kalf), risicofactoren voor verspreiding en persistentie binnen een koppel (management en dierfactoren), aanwezigheid van andere gastheren (dieren) en reservoirs in de omgeving. Transmissie in runderen is aangetoond, hoewel de transmissie in jonge kalveren (experimenteel) aanzienlijk hoger bleek dan in melkvee (veldstudie; Hoofdstuk 5).

De vraag was of een bedrijf in achtereenvolgende bemonsteringen besmet wordt bevonden vanwege introductie van nieuwe bacteriën op het bedrijf of vanwege persistentie van de bacterie op het bedrijf. Het veldonderzoek op een positief melkveebedrijf gaf aan dat de aanwezigheid van O157 VTEC mede wordt bepaald door het vee, andere gastheren en de omgeving. De aanname dat mest een cruciale rol speelt in de transmissie wordt ondersteund. Verscheidene DNA typen zijn waargenomen tijdens de monsterperiode in dit veldonderzoek (Hoofdstuk 4), maar het overheersende type in het tweede uitscheidingsseizoen was reeds in kleine hoeveelheden geïsoleerd in het eerste uitscheidingsseizoen, wat mogelijk duidt op persistentie van de infectie tijdens de winter. Deze resultaten tonen dus een zekere mate van persistentie aan, wat wordt bevestigd in de literatuur, maar de duur ervan is onbekend.

Uiteindelijk zou een infectie in een koppel dieren kunnen uitdoven vanwege een afname van gevoelige (mogelijk te besmetten) dieren door een opgebouwde weerstand tegen O157 VTEC. In het veldonderzoek is geen van de melkkoeien die in het eerste seizoen positief was, ook positief bevonden in het tweede seizoen (Hoofdstuk 4). In de literatuur is reeds beschreven dat rundvee in staat is immuniteit op te bouwen voor enkele virulentiefactoren van O157 VTEC, wat weerstand tegen infectie zou kunnen bewerkstelligen.

Samenvattend suggereren de resultaten van dit proefschrift dat verscheidene typen O157 VTEC op een bedrijf kunnen binnenkomen en voor enige tijd aanwezig kunnen blijven, zelfs meer dan 1 jaar, met mogelijk verschillende stammen als de meest dominant aanwezige in achtereenvolgende uitscheidingsseizoenen. Transmissie van O157 VTEC binnen en tussen bedrijven zou in de zomer heel anders kunnen zijn dan in de winter. Uiteindelijk zal de transmissie binnen een bedrijf kunnen uitdoven vanwege afname in gevoelige dieren. Omdat bedrijven waar voorheen geen O157 VTEC was gevonden wel besmet kunnen worden (Hoofdstuk 6), lijkt transmissie tussen bedrijven voor te komen. Transmissie van O157 VTEC tussen bedrijven kan zo dus wel leiden tot persistentie in een groter gebied, bijv. Nederland.

Vanwege de endemische status in rundvee, kan blootstelling van mensen aan O157 VTEC niet worden uitgesloten in Nederland. Hoewel tot op heden een relatief klein deel van de gemelde voedselvergiftigingen in Nederland in verband staat met O157 VTEC, vragen de mogelijk ernstige ziekteverschijnselen of sterfte bij mensen en de constante dreiging van uitbraken om een risico-inschatting en preventieve maatregelen. Door toenemende aantallen kinder-, zorg- en kampeerboederijen en boerenbedrijven die zich openstellen voor publiek, kan vooral directe overdracht van dieren naar mensen als een toenemend risico worden beschouwd. Om het publiek te beschermen tegen besmetting via deze route, zouden mogelijk extra maatregelen moeten worden genomen op deze boerderijen. Wat betreft besmetting via voedsel: vlees en melkproducten kunnen via mestdeeltjes met O157 VTEC besmet zijn. Voor melkproducten is het grootste risico voor consumenten wanneer deze producten direct van het bedrijf worden gekocht. Het vermogen van O157 VTEC om te overleven in dierlijke mest, op weilanden en in aangrenzende (water)omgeving kan echter ook overdracht naar gewassen (bijv. groenten) veroorzaken door directe toepassing van besmette mest of door beregening met besmet water.

Wanneer als doel wordt gesteld het risico voor mensen te reduceren door het nemen van maatregelen op bedrijfsniveau, is het met name belangrijk om het aantal positieve dieren en bedrijven terug te brengen. Om vast te stellen welke maatregelen effectief zijn, zal meer specifiek onderzoek voor dergelijke maatregelen noodzakelijk zijn. In het algemeen kunnen maatregelen die zich richten op afname van transmissie door mest of via met mest besmette producten mogelijk een algehele afname van ziekteverwekkers, inclusief O157 VTEC,

bewerkstelligen. Een voorbeeld van een effectieve maatregel om verspreiding van O157 VTEC binnen bedrijven te reduceren kan het scheiden van leeftijdsgroepen zijn. In de literatuur worden alternatieve strategieën beschreven, zoals aangepaste mestopslag, vaccinatie van runderen en aanpassing van voermanagement, wat effect zou hebben op de mate van voorkomen van O157 VTEC. Om te kunnen beslissen welke maatregelen meest kosten-effectief zijn, is meer (experimenteel) onderzoek nodig waarbij kosten-baten analyse uitgevoerd moet worden.

Samenvattend zijn de belangrijkste conclusies van dit proefschrift:

- O157 VTEC is endemisch in de Nederlandse rundveepopulatie. Deze status lijkt een gevolg van zowel transmissie binnen als tussen bedrijven.
- Rundvee is de belangrijkste gastheer voor O157 VTEC, hoewel de bacterie ook bij andere dieren en in de omgeving kan worden gevonden.
- In Nederland is de uitscheiding van O157 VTEC seizoensgebonden: vooral in de zomer en in het najaar kan de bacterie worden aangetoond.
- Het aandeel besmette vleeskalverbedrijven is groter dan dat van melkveebedrijven.
- Risicofactoren zijn geïdentificeerd voor infectie van zowel melkvee- (bijv. dieraankoop of maïs voeren) als vleeskalverbedrijven (bijv. rosévlees productie of groepshuisvesting).
- Transmissie in kalveren ligt hoger dan in melkvee.
- O157 VTEC kan langer dan één uitscheidingsseizoen op een bedrijf aanwezig zijn. Er is echter geen bewijs voor langdurige persistentie. Herintroductie van O157 VTEC op een bedrijf, als gevolg van transmissie tussen bedrijven, lijkt het meest waarschijnlijk.
- Belangrijk voor de bestrijding van O157 VTEC is kennis van de introductieroutes en overleving van de bacterie op een bedrijf, en inzicht in de effectiviteit van specifieke interventiemaatregelen.

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About the author

Jannigje Maria (Marije) Schouten werd op 11 mei 1974 geboren in Nijmegen. Tot 1992 groeide zij achtereenvolgens op in Wijchen (1974-1978), Nijmegen (1978-1989) en Beuningen (1989-1992). In 1992 behaalde zij het VWO diploma aan het Dukenburg College te Nijmegen. Datzelfde jaar begon zij met de opleiding Zoötechniek aan de toenmalige Landbouwuniversiteit Wageningen. Binnen haar studietijd was ze actief binnen de studievereniging van Wageningse Zoötechniek studenten 'De Veetelers', onder meer binnen het bestuur en in het bijzonder op het gebied van onderwijs. Met de specialisatie Veehouderij, met haar hoofdonderwerp op het gebied van veterinaire epidemiologie en na een halfjaarlijkse stage bij North Carolina State University in Raleigh (USA), studeerde zij in september 1997 met lof af. Vanaf januari 1998 was zij werkzaam als assistent in opleiding bij de huidige leerstoelgroep Kwantitatieve Veterinaire Epidemiologie van Wageningen Universiteit en Research Centre (WUR). Dit leidde tot het voor u liggende proefschrift. Als lid van de WIAS Associated PhD Council vertegenwoordigde ze de AIO's/PhD's binnen het (dagelijks) bestuur van WIAS. Tevens was ze in 2003-2004 coördinator van het consortium voor Veterinaire Epidemiologie en Economie (cVEE), een samenwerkingsverband van de leerstoelgroepen Kwantitatieve Veterinaire Epidemiologie (QVE-WUR) en Bedrijfseconomie (BE-WUR) en de Faculteit Diergeneeskunde (FD-Universiteit Utrecht). Sinds februari 2004 is zij als postdoc werkzaam bij de leerstoelgroep Humane Voeding en Epidemiologie van Wageningen Universiteit. Hier vervult zij de functie van projectmanager op een internationaal onderzoeksproject van het World Cancer Research Fund op het gebied van voeding en kanker.

Training and Supervision Plan

Name PhD student	Marije Schouten		**Graduate School WIAS**
Project title	Development, implementation and benefits of a monitoring system for verocytotoxin producing *E. coli* (VTEC) O157 in humans, foods of animals origin and livestock		
Group	QVE		
Daily supervisor(s)	E.A.M. Graat, A. v.d. Giessen		
Supervisor(s)	M.C.M. de Jong		
Project term	from 01-98	until 02-04	
Submitted	23-8-2005	first plan / midterm / **certificate**	

EDUCATION AND TRAINING (minimum 30 credits)

The Basic Package (minimum 3 credits)	**year**	**credits ***
WIAS Common Course (mandatory)	1999	
Course on philosophy of science and/or ethics (mandatory)	2001	
Subtotal Basic Package		**5**

Scientific Exposure (conferences, seminars and presentations, minimum 8 credits)	**year**
International conferences** (minimum 3 credits) **& presentations	
Foodborne pathogens; detection and typing. Internat. Symp. Den Haag	1998
Society Veterinary Epidemiology and Preventive Medicine (SVEPM) 2001, Noordwijkerhout	2001
Oral; "Risk factor analysis of *Escherichia coli* O157 on Dutch dairy farms; preliminary results"	
SVEPM 2002, Cambridge, Poster: "Transmission of O157 VTEC within a calf population"	2002
Seminars and workshops & presentations	
WIAS Science day 2000, 2001, 2002, 2003	2000-2003
Oral: "Results of a monitoring system for VTEC in livestock"	2001
Klankbordgroep Integr. Werkgroep zoonosen, Den Haag;	2001
Oral: "Experimentele transmissie van *E. coli* O157 in rundvee"	
Workshop "Contamination Shigatoxin producing *E. coli* (STEC) O157"; RIVM	2001
WIAS seminar "VTEC"; November 2002	2002
Oral: "Development, implementation and benefits of a monitoring system for VTEC in humans, foods of animal origin and livestock"	
Diergezondheid, kwestie van Europees beleid? Veetelers symposium, Ede	2002
Annual meeting Dutch Society for Vet. Epidemiology and Economics (VEEC)	2000-2002/ 2004
Symposium “STEC”; RIVM	2003
Oral: "Transmissie van STEC O157 binnen runderpopulaties"	
Veal sector information day	2003

Oral: "Monitoring of VTEC in livestock"		
Workshop "Weging risicofactoren voor in- en versleep van VTEC op melkveebedrijven"	2004	
Subtotal International Exposure		**15**
In-Depth Studies (minimum 6 credits, of which minimum 4 at PhD level)	**year**	
Disciplinary and interdisciplinary courses		
"Broaden your horizon"; debating course	2001	
Population Dynamics of Infectious Diseases, Utrecht, November	2001	
PhD students' discussion groups *(optional)*		
Discussion and Working Group "Transmission of infectious diseases", QVE	2000	
Subtotal In-Depth Studies		**6**
Statutory Courses	**year**	
Use of Laboratory Animals (mandatory when working with animals)	1999	
Subtotal Statutory Courses		**4**
Professional Skills Support Courses (minimum 3 credits)	**year**	
Course Techniques for Scientific Writing (advised)	1999	
WCFS/VLAG course "your next job"; (project)management, writing skills etc.	2004	
Subtotal Professional Skills Support Courses		**3**
Research Skills Training (optional)	**year**	
Preparing PhD research proposal for WIAS-call	2004	
Subtotal Research Skills Training		**1**
Didactic Skills Training (optional)	**year**	
Lecturing		
Lecture "Integratievak Gezondheidsleer en Reproductie"	1999-2002	
Supervising practicals and excursions		
Supervising groups of the "Farm project"	2002/2003	
Supervising MSc theses *(maximum 2 credits per major, 1.5 credits per minor)*		
6 x major (2000-2003)		
Subtotal Didactic Skills Training		**19**
Management Skills Training (optional)	**year**	
Organisation of seminars and courses		
WIAS seminar "VTEC"	2002	
Membership of boards and committees		
WAPS/WIAS Board	1999/2000	
Coordinator consortium Veterinary Epidemiology and Economics	2003/2004	
Subtotal Management Skills Training		**19**
Education and Training Total (minimum 30 credits)		**72**

* one ECTS credit equals a study load of approximately 28 hours

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