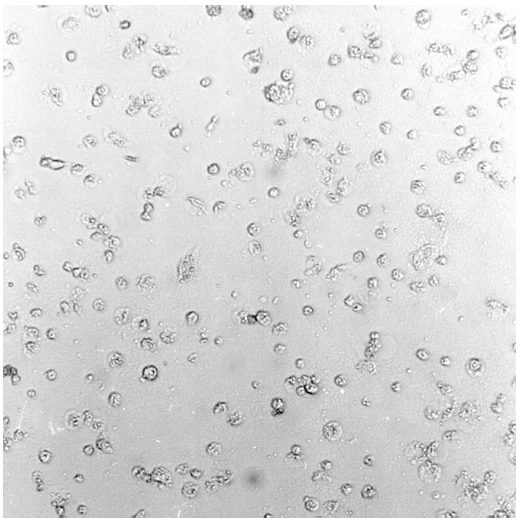
Vero cytotoxin producing

Escherichia coli

(VTEC)

isolated from Danish patients

Vero cell monolayer
Negative control



Vero cell monolayer
Typical cytotoxic effect of Vero cytotoxin 1 produced by strain H19, serotype O26:H11

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Ph.d. thesis, 10. October 1997

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Preface

This ph.d. thesis was conducted at The WHO Collaborative Centre for Reference and Research on *Escherichia* and *Klebsiella*, Department of Gastrointestinal Infections, Division of Microbiology, Statens Serum Institut, Copenhagen, Denmark, where I am employed as a Research Assistant.
Matriculation took place at the Faculty of Medicine, University of Copenhagen on the 1st January 1994.
Niels Høiby, Professor, MD, D.Sc. (Med.), Head of Department of Clinical Microbiology, Rigshospitalet, Copenhagen, and Peter Gerner-Smidt, MD, D.Sc. (Med.), Head of Department of Gastrointestinal Infections, Statens Serum Institut, Copenhagen have been my supervisors during the project. I thank both of them for their enthusiasm, inspiration and support throughout the whole study period.
I especially want to express my sincere and very warm gratitude to the entire staff at the International *Escherichia* and *Klebsiella* Centre (WHO), also known as the coli-laboratory, Statens Serum Institut. In particular the technical assistance, skilfulness and encouragement of Susanne Jespersen, Bente Klestrup Thomsen and Betty Wulff has been highly appreciated.

My very warm thanks are also extended to Lothar Beutin, Department of Microbiology, Robert Koch Institute, Berlin and Drs. Bernard Rowe, Henry R. Smith, John E. Trelfall and Thomas Cheasty, Laboratory of Enteric Pathogens, Central Public Health Laboratory, Colindale, London who invited me to study in their laboratories and with whom I have had many inspiring discussions.

Many others have helped me during this project and I thank them all.

Most of all, I thank my wife Sofie and my three daughters Andrea, Johanne and Gertrud for their support and extreme patience.

Finally, I would like to thank Ida Ørskov, MD, D.Sc. (Med.), and Frits Ørskov, MD, D.Sc. (Med.), for their excellent training and inspiration.

Flemming Scheutz, Copenhagen, 10. October 1997.

Purpose

The purpose of this ph.d. thesis has been:

- to determine the prevalence of Verotoxin producing *E. coli* (VTEC) in Denmark.
- to present a detailed serological, genotypical and phenotypical characterisation of VTEC isolated from Danish patients.
- to obtain information on the clinical symptoms of Danish patients infected with VTEC, compared with EPEC.

Three different approaches were used:

- Prospective screening of diarrhoeal stools using DNA probes specific for Verotoxin and the detection of free faecal cytotoxin in stool specimens.
- Prospective and retrospective sero-, geno- and phenotypical characterisation of selected isolates with special emphasis on EPEC O groups.
- Direct contact by questionnaire with patients who had been infected with VTEC or selected attaching and effacing *E. coli* strains.

Results and conclusion are summarised in **9. General summary**, p. 98.

The behaviour of these by far numerically predominant rods seems to follow no fixed pattern; some exist as single individuals, some unite into clearly identifiable double rods, or are simply tangled together in chaotic heaps.

Theodor Escherich (1857-1911).

1. Introduction

Escherichia coli is a natural and essential part of the bacterial flora in the gut of humans and animals. Most *E. coli* strains are non-pathogenic and will normally reside in the colon, yet *E. coli* is one of the most common causes of a variety of both intestinal and extraintestinal diseases throughout the world. The possession of a large and varied repertoire of specific virulence factors, which we have only recently begun to understand, accompanied by the extraordinary ability to survive in the environment and colonise it's many hosts make this species an all together very heterogeneous group and an excellent opportunistic pathogen. Anyone who has had the opportunity to study this organism will at some point in time experience the fascination that arises when beginning to comprehend the baffling number of biochemical, genetic, physiological and pathogenic mechanisms. *E. coli* is one of the most studied micro-organisms of modern times yet our knowledge and understanding ranges from the well studied area of the intricate host-parasite relationship between the development and turnover of receptors in the gut of the new-born piglet and the well adapted adhesins and toxins of *E. coli* strains causing piglet diarrhoea to areas characterised by our complete ignorance.

It is beyond the scope of this presentation to give an overview of the role and virulence factors of *E. coli* in extraintestinal diseases but following a short historical introduction there is a brief presentation of the enteropathogenic types of *E. coli*.

1.1. Historical background

Bacterium coli commune was first described by Theodor Escherich in a lecture held in the Society of Morphology and Physiology in Munich on the 14. VII. 1885 [1]. The lecture entitled “Die Darmbacterien des Neugeborenen und Säuglings” described a ... *single species of slender, sometimes slightly curved, short rods* ... which ... *vary quite considerably in length (from 1 to 5 µ)*. The bacteria was decolourized by Gram’s method and ... *on glucose solution they exhibit marked properties of fermentation* ... and ... *violent gas formation*...[2]. ... *in addition to facultative anaerobiosis* ... they exhibit ... *the ability to satisfy their very low nitrogen requirements from the simplest ammonium compounds*.
In his concluding remarks Escherich writes that *These bacteria can be regarded as harmless parasites as long as the functions of the digestive tract proceed normally* [2]. It was soon realised however, that the bacteria might be pathogenic to both humans and animals.
In 1923 Adam noted a relation between certain sucrose fermenting dyspepsiecoli and diarrhoea in children without being able to demonstrate serological similarities [3]. Ten years later Goldschmidt found a large number of Adam’s type and was able to demonstrate that they were serologically related (cited in [4]). In a case control study in 1945 Bray demonstrated the relation between *Bacterium coli var. neapolitanum* and diarrhoea in humans [5]. A few years later Bray and Beaven used slide agglutination to type 95% of the same bacteria isolated from stool cultures from children with diarrhoea but only 4/100 from healthy control children. The children with diarrhoea had an average age of 6 months. The mortality rate was more than 40% and most cases were seen during the summer months and thus referred to as *summer diarrhoea* [6].
At the same time in 1947 a large hospital outbreak occurred among babies in Aberdeen [7]. The mortality rate was 51%. The isolated bacteria was diagnosed as *Bacterium coli var. neapolitanum* [8] and the association between certain serologically distinct types of *Bact.coli* and infantile gastro-enteritis was confirmed [9]. In London the same type of outbreaks were described among small children [10]. The breakthrough in typing these outbreak strains which by then had been isolated in England, Scotland, USA and Germany was achieved in 1950 when the *E. coli* serotyping scheme developed by Kauffmann [11] was used to show that most of the strains belonged to either serogroup O55 or O111 [12]. Neter *et al.* were the first to use the term enteropathogenic *E. coli* (EPEC) to refer specifically to strains that caused infantile diarrhoea [13].
During the 1950s several new serogroups were added to the list of those epidemiologically incriminated as causing diarrhoea [4]. During this period volunteer studies furthermore confirmed the hypothesis that EPEC strains were pathogenic [14], [15], [16], [17]. Yet almost three decades should pass before the pathogenic mechanisms of EPEC were slowly unravelled.
Meanwhile the enterotoxins of enterotoxigenic *E. coli* (ETEC) and the invasive properties of enteroinvasive *E. coli* (EIEC) had been described (see below).

In 1977, Konowalchuk *et al.* [18] discovered a marked irreversible cytopathic effect in Vero cells (African green monkey kidney cell line) produced by ten of 136 culture filtrates from *E. coli* that was distinct from other enterotoxins. Seven of the 10 strains were from children with diarrhoea and belonged to EPEC O groups. The effect could only be seen in Vero cells and not in Y1 mouse adrenal cells and Chinese hamster ovary (CHO) cells, and it was distinctly different from that of heat-labile enterotoxin. The cytotoxic effect could be neutralised by rabbit immune antisera and subsequently shown to be caused by one or more cytotoxins referred to as Vero toxins (VT) or Vero cytotoxins [19]. It was suggested that VT could contribute to diarrhoeal disease in human infants and possibly in young pigs [18].

The clinical and public health significance of this discovery remained uncertain until 1983, when verotoxin producing *E. coli* (VTEC) became associated with two conditions of hitherto unknown aetiology: haemorrhagic colitis [20],[21] and the haemolytic uraemic syndrome [22]. Two outbreaks - one in Oregon and Michigan, USA [20] and one in a Canadian nursing home [21] - were linked with what at that time was considered a “rare” *E. coli* serotype, O157:H7. The Canadian *E. coli* O157:H7 strains produced VT and O’Brien *et al.* subsequently confirmed the American isolates of *E. coli* O157:H7 to be positive for a Shiga-like cytotoxin [23]. O’Brien’s group had already made the important observation that the cytotoxin (VT) from Konowalchuk’s reference strain H30, serotype O26:H11 was very similar to Shiga toxin, produced by *Shigella dysenteria* serotype 1, in terms of biological properties, physical characteristics and also antigenically [24],[25]. Hence, the term Shiga-like toxin (SLT) was used to describe this toxin and both terms, VT and SLT have been used widely until recently when yet a very logical third term, Shiga toxin (ST), has been proposed [26]. In spite of this, the term VT is still in use and will also be used throughout this presentation in order to avoid confusion. Furthermore it could be argued that VT specifically refers to *E. coli* strains.

Since the initial description of VT as a potential virulence factor in childhood diarrhoea, VTEC has been studied intensively and the role of these organisms in disease is now well established.

VTEC, and serotype O157:H7 in particular, is one of the leading causes of bloody diarrhoea in many countries and of haemolytic uraemic syndrome (HUS), a life threatening condition, which again is the major cause of acute renal failure in childhood.

It has been the purpose of this ph.d. to investigate the role of VTEC in Denmark.

1.2. Taxonomy and biochemical reactions

The genus *Escherichieae* belongs to the family of *Enterobacteriaceae* and includes five species [27]:
- *Escherichia coli* found in both animals and humans.
- *E. fergusonii*, *E. hermanii*, *E. vulneris* which have only been isolated from humans.
- *Escherichia blattae* which is only found in cockroaches.

Enterobacteriaceae consists of non-spore forming Gram negative rods, 0,3-1,0 X 1,0-6,0 µm. They are facultative anaerobic and oxidase negative.

Most *E. coli* have 0-15 (typically 8) flagella in a peritrichous arrangement. *E. coli* from clinical specimens are characterised by the reactions shown in table 1.1.

The biochemical reactions of certain *E. coli* strains may be difficult to differentiate from those of strains of the genus *Shigella.* Based on the DNA content, *E. coli* (DNA mol% of G+T is 50-51%) and the four *Shigella* species represent only one species. However , for reasons of historical precedent and convenience the distinction prevails.

The term coliform bacteria has no taxonomical significance but is usually used to describe lactose positive, Gram negative bacteria with the apparent colony morphology of *E. coli i.e.* circular, convex smooth colonies with a sharp, well defined edge on solid medium. In the field of hydrobiology the definition of coliform bacteria also includes the formation of gas within 48 hours at 35°C. Coliforms will include species within *Escherichia, Klebsiella, Citrobacter, Enterobacter* and *Hafnia*.

Table 1.1. The biochemical reactions of *E. coli* *

	Sign	*E. coli* %+	*E. coli* inactive § %+
Fermentation of:			
Adonitol	-	1-5	3
Dulcitol	d	49-60	40
Sorbitol	d+	80-95	75
Raffinose	d	49-50	15
Xylose	d+	83-95	70
Rhamnose	d+	80-84	65
Maltose	+	91-95	80
Salicin	d	36-40	10
Inositol	-	1	
Lactose	d+	89-95	25
Sucrose	d	50-54	15
Sorbose	+		
Mannitol	+	98	93
D-glucose, acid	+	100	100
D-glucose, gas	+	92-95	5
Trehalose	+	98	90
Cellobiose	-	1-4	2
Melibiose	d	75	40
Arabinose	+	99	85
α-Methyl-D-Glucoside	-	0	0
Erythritol	-	0	0
Glycerol	d+	75-89	65
Mucate	+	92-95	30
Indole production	+	96-98	80
KCN, growth	-	0-3	1
H_2S production [a]	-	0-1	0-1
Gelatin liquified (22°C)	-	0	0
NH_4 glucose	+	95	
NH_4 citrate (Simmons')	-	<1	<1
KNO_3	+	100	98
Voges-Proskauer	-	0	0
Methyl red	+	99-100	95
Urease production [b]	-	0-1	0-1
Motility (36°C)	+ or -	62-69/95 [c]	5
Malonate utilisation	-	0	0
PGUA [d]	+	96	
ONPG [e]	+	94-95	45
ONPX [f]	-	1	

Table 1.1. - continued

	Sign	*E. coli* %+	*E. coli* inactive § %+
Lysine decarboxylase	d+	81-90	40
Arginine dihydrolase	d-	16-21	3
Ornthine decarboxylase	d	57-65	20
Phenylalanine deaminase	-	0	0
DNase (22°C/25°C)	-	0	0
Jordan's D-tartrate	+	95-98	85
Acetate utilisation	d+	84-90	40

Key:
- = <= 10% positive
d = 26-74% positive
d+ = 75-89% positive
+ = >= 90% positive

* The numbers indicate range in percentage of positive reactions from ref.s [28],[29] and [30] after 2-3 days incubation at 36°C.
§ The distinction between *E. coli* and *E. coli*, inactive - also referred to as *Shigella*-like *E. coli* - is made only in ref. [30] and is based on significant differences known to occur in clinical specimens.
[a] A few occational strains will exhibit plasmid-mediated hydrogen sulfide production.
[b] A few occational strains will exhibit plasmid-mediated hydrolysis of urea.
[c] 62-69% from ref.s [28] and [29]; 95% from ref. [30].
[d] β-glucuronidase production.
[e] *o*-Nitrophenyl-β-D-galactopyranoside production.
[f] β-xylosidase production.

Since, here too, there was a similar lack of uniformity, and in all other respects the cultures were similar I have termed them collectively Bacterium coli commune.

Theodor Escherich (1857-1911).

1.3. Types of *Escherichia coli* enteropathogens

E. coli isolated from intestinal diseases have been grouped into many different types, by the use of acronyms, some more devious than others. The grouping has been based on epidemiological evidence, clinical features, phenotypical traits and more recently virulence factors which have primarily been identified using molecular biology, animal models and *in vitro* assays. In the following the currently recognised types of *E. coli* enteropathogens are reviewed with emphasis of virulence factors and mechanisms pertaining to verotoxin producing *E. coli* (VTEC).

1.3.1. Enteropathogenic *E. coli* (EPEC)

The term **e**ntero**p**athogenic ***E. c****oli* (EPEC) was originally used to refer specifically to strains associated with infantile diarrhoea [13]. This rather weak definition was used for decades and was the cause of much controversy. It became increasingly problematic as other mechanisms by which *E. coli* may cause diarrhoea were discovered. A definition of exclusion adopted at a workshop held at the National Institute of Health, USA in September 1982 allowed for the inclusion of a very heterogeneous group of pathogens: EPEC was defined ... *as diarrheagenic E. coli belonging to serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanisms have not yet been proven to be related to either heat-labile enterotoxins or heat-stable enterotoxins or Shigella-like invasiveness* [31].
For many years O:H serotyping of the somatic lipopolysaccharide O antigen and the flagella H antigen was the only way that EPEC could be identified. A summary of the most important O:H serotypes thought to represent EPEC is shown in table 1.2.
Even though a table like table 1.2. appears quite complicated at first glance it should be emphasized that only 13 out of 173 possible O groups are represented and that only a few H antigens *i.e.* H2, H6, H7, H12 and H21 occur within several of these 13 O groups. However, recent advances has allowed a much more precise description of EPEC based on specific virulence characteristics.

1.3.1.1. Localised adherence (LA)

The first breakthrough in understanding the pathogenesis of EPEC infection was the observation that EPEC adhere to epethelial cells in tissue culture [32] and did so in a distinctive pattern termed **l**ocalised **a**dherence (LA) [33]. It was soon realised that LA was plasmid mediated [34] and high molecular weight plasmids were found in many EPEC serotypes [35],[36]. Furthermore, through volunteer studies it was established that plasmid-cured EPEC strains were less diarhoeagenic than the parental plasmid containing strains

[37]. A 1 kb DNA fragment originally thought to encode an **E**PEC **a**dherence **f**actor (EAF) necessary for LA was cloned [38] and used as a DNA probe to study the prevalence of EPEC [38],[39],[40],[41],[42],[43],[44],[45], [46],[47],[48]. EAF plasmids were found in many EPEC serotypes and ranged in size from 26 to 76 MDa [49].

Table 1.2. O:H serotypes traditionally regarded as classical EPEC O:H serotypes.

O group	H antigen[a)]	Comments
O18ab	H^-; H14	O18 strains are probably not EPEC [50],[51]
O18ac	H^-; H7	
O26	H^-; H11; H34 *	O26:H^- and O26:H11 may also be VTEC [52],[53],[54]
O44	H^-; H18; H34*	O44:H18 is probably EAggEC [55]
O55	H^-; H6; H7	O55:H7, H10 and H^- may also be VTEC [56]
O86	H^-; H34	
O111	H^-; H2; H7; H12; H21	O111:H^- may also be VTEC [52],[57],[58],[59],[60],[61] O111:H12 is probably EAggEC [62]
O114	H^-; H2	
O119	H^-; H2; H6	
O125ac	H^-; H6; H21	
O126	H^-; H2; H21; H27	O126:H27 often EAggEC [55],[63]
O127	H^-; H6; H21	
O128ab	H^-; H2; H7; H12	O128:H2 may also be VTEC
O142	H^-; H6	
O158	H^-; H23	

Data from [32],[37],[40],[49],[50],[51],[63],[64],[65],[66],[67].
[a)] Non motile strains of *E. coli* are regarded as descendants of motile strains that have lost their motility by mutation(s). Their original H antigen was often deduced from comparison of biochemical reactions [12],[68].
* O18, O26:H34 and O44:H34 only in [63].
EPEC: enteropathogenic *E. coli*; VTEC: Vero cytotoxin producing *E. coli*; EAggEC: enteroaggregative *E. coli*.

However, not all EPEC strains that showed localised adherence were positive with the EAF gene probe [55],[63],[69],[70]. Sequencing of the EAF probe finally revealed no significant homology to any known genes. However, a small sequence of 86 base pairs showed an 82,8% homology to the insertion sequence IS*630* of *Shigella sonnei* [71].
The next major breakthrough in the understanding of localised adherence was the discovery of a plasmid mediated inducible bundle-forming pilus [72], a flexible rope-like fimbriae that intertwines, linking individual bacteria. As a result microcolonies are formed and the bundle-forming pilus is thus responsible for the LA phenotype. Sequencing soon revealed that the gene (*bfp*A) encoding the major structural subunit, termed bundlin, of this fimbriae encodes a protein related to the type IV fimbriae family [73],[74], which includes the pili of

Vibrio cholerae, Pseudomonas aeroginosa, Neisseria gonorrhoea, as well as other pathogens [72]. There is evidence that at least 14 genes on the EAF plasmids are required for the complete biogenesis of the bundle-forming pilus [75],[76]. Additionally the chromosomal *dsb*A gene encoding an oxidoreductase enzyme necessary for efficient disulphide bond formation in the periplasmic space is required for localised adherence [77].

1.3.1.2. Attaching and effacing (A/E) lesions

The hallmark of EPEC infection is the ability of the organism to cause attaching and effacing (A/E) lesions of enterocytes in the intestinal tract. These lesions were first seen in pigs [68] and named by Moon *et al.* after studies of EPEC strains in pigs and rabbits [78]. On electron micrographs of jejunal biopsies from children infected with EPEC, bacteria are seen intimately attached to epithelial cells on cup-like pedestals composed of depolymerised cytoskeletal proteins [79]. Microvilli are disrupted as a result of the cytoskeletal rearrangements and effaced by vesiculation (Figure 1.1).

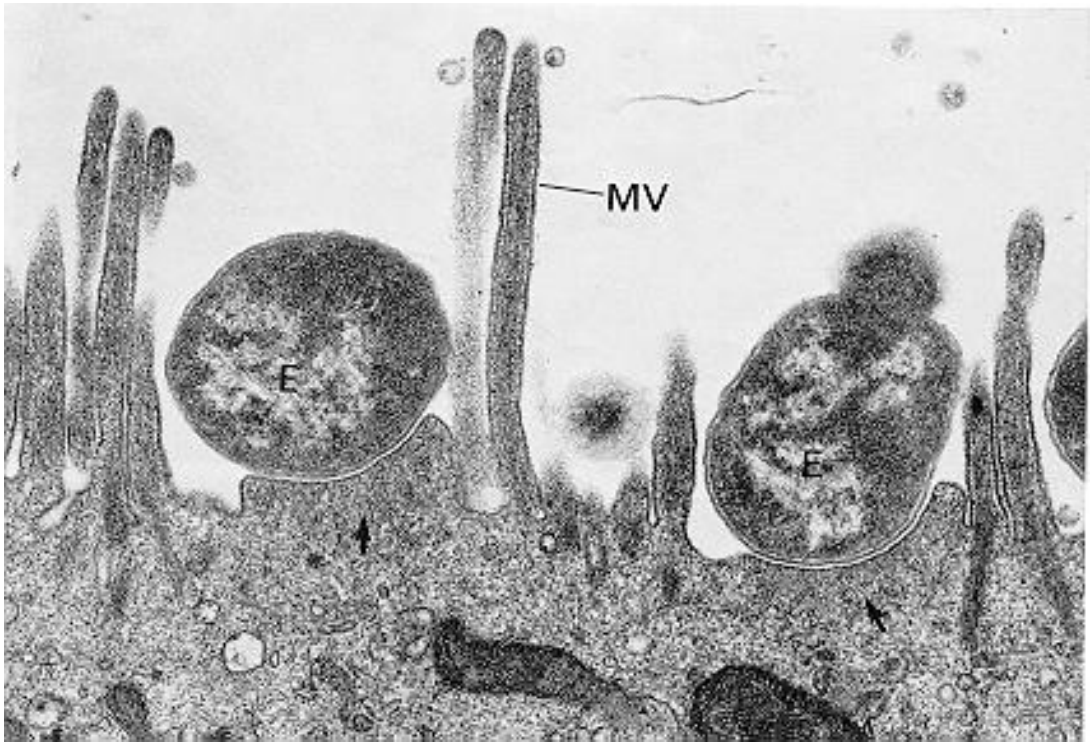

Figure 1.1. EM of cultured human intestinal mucosa infected with EPEC strain E2348 (E) for 8 hours. Bacteria are seen intimately attached to the epithelial cells on cup-like pedestals composed of depolymerised short filament cytoskeletal proteins (arrows). Brush border microvilli (MV) are disrupted as a result of the cytoskeletal rearrangements and effaced by vesiculation (used with permission from ref. [79]).

The intimate attachment of EPEC is mediated by a protein known as intimin which is a 94 kDa outer membrane protein encoded by the *eae*A gene (***E****. coli* **a**ttaching and **e**ffacing) [80]. A 1 kb fragment of the eaeA gene referred to as CVD434 has been cloned [81] and

used to screen for A/E *E. coli* [82], and to characterise enteropathogenic *E. coli* [83]. However the *eae*A gene is only one of many genes located on a **pa**thogenicity **i**sland (pai) known as the **l**ocus of **e**nterocyte **e**ffacement (LEE) [84].
EPEC secretes at least five proteins encoded by LEE. Two of these **E**PEC **s**ecreted **p**roteins, EspA and EspB (formerly known as eaeB), are involved in the activation of signal transduction pathways of the host epithelial cells [85],[86],[87] while the role of the other three proteins is undetermined [88]. Four of these proteins are secreted by a type III secretion system mediated by the *sep*A and *sep*B (**s**ecretion of **E**PEC **p**roteins) genes [89]. The fifth protein is coregulated with the *sep*-mediated protein secretion but by an alternative mechanism [90].
Protein secretion, the transcription of the intimin gene (*eae*A) and the synthesis of the bundle-forming pilus (*bfp*A) are all affected by growth conditions [89],[90],[91] and a plasmid-encoded regulatory region. The plasmid locus is either referred to as the *per* (**p**lasmid-**e**ncoded **r**egulator) genes [92] (*per*ABCD) or as an integrated part of the bundle-forming pilus (bfp) operon, genes *bfp*TVW [93]. I spite of the dispute over nomenclature and to some degree over the functions of these genes [94],[95], there is general agreement that *per*A/*bfp*T encodes a protein belonging to the AraC family of transcriptional activators [92],[93].

The A/E lesion of EPEC in human epithelial enterocytes is also seen *in vitro* when attaching bacteria adhere to a variety of human tissue culture cell lines [51]. In both cases depolymerised actin accumulate in the apical cytoplasm beneath attaching bacteria. The site-specific concentrations of cytoskeletal actin are characteristic of the A/E membrane lesion and the accumulated actin is easily made visible by staining with fluorescein isothionate-phalloidin. This has formed the basis of a simple, highly sensitive test, the fluorescein actin stain (FAS) test, for A/E *E. coli* [51] which has been used to screen for EPEC [63],[96].

1.3.1.3. Signal transduction

EPEC has been shown to interfere with normal pathways of signal transduction in epithelial cells. EPEC will induce tyrosine phosphorylation, phosphorylation of myosin light chain, vinculin and α-actinin, *in vitro* release of intracellular calcium, phospholipase C activity resulting in elevated levels of inositol phosphates and diacylglycerol in turn activating protein kinase C, reactions that induce host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake [64],[97],[98].

1.3.1.4. Cellular invasion

EPEC is not invasive in the way that *Shigella*, *Yersinia* and *Salmonella* are [99]- yet some EPEC strains may exhibit *in vitro* invasion of epithelial cells at levels comparable to those seen in EIEC [100]. A 4.5 kb fragment from a large EPEC plasmid of an O111:H^- strain negative for both EAF and *eae*A will confer epithelial cell invasivity and the attaching and effacing ability on a non-invasive laboratory strain [101],[102]. Only 11/49 EPEC strains would hybridise with the cloned fragment.

1.3.1.5. Clinical illness

Clinically, EPEC produce diarrhoea with malaise, vomiting, and often low-grade fever. The infection is clearly both type- and host dependant ranging from acute watery to chronic, persistent diarrhoea, usually with mucus but no gross blood [30],[64],[103]. Particularly in children, the diarrhoea may be protracted and severe leading to dehydration, an important factor in mortality due to diarrhoeal illness.
Experimental infection of adults results in watery diarrhoea approximately 7-16 hours after inoculation and lasts on average less than two days [14],[37],[104]. Abdominal cramps, nausea, vomiting, malaise, and fever are common. Faecal leukocytes are sometimes seen [104].

1.3.1.6. Epidemiology

EPEC is an important cause of world-wide childhood diarrhoea, particularly in the developing world [64]. In the developed world EPEC has traditionally been associated with institutional outbreaks in the 1950s and 1960s which seemed to cease as hygienic standards were improved. However, despite the fact that EPEC are not often sought, outbreaks and sporadic cases of EPEC were recently reported [63],[105],[106],[107],[108],[109]. Most recently it has been demonstrated that *E. coli* with enteropathogenic characteristics are relatively common (app. 5%) in stools
from children in a North American hospital [82].

1.3.2. Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) are an important cause of diarrhoea in both humans and domestic animals. ETEC does not invade or damage epithelial cells but produce one or more enterotoxins that are either heat-labile (LT) or heat-stable (ST) [110]. These enterotoxins will cause intestinal secretion either by activation of guanylate- (ST) or adenylate (LT) cyclase and are subdivided on the basis of their biological activities, receptors and chemical and antigenic properties. ST_h and ST_p is used to indicate strains of human or porcine origin. ETEC produce at least 19 different surface structures (colonisation factors), usually plasmid encoded, that allows them to adhere to intestinal mucosal receptors [111].

Clinically ETEC produce an afebrile, watery diarrhoea, sometimes not unlike a mild case of cholera, that usually resolve within days [30]. ETEC are the most common cause of traveller's diarrhoea and an important cause of infant diarrhoea in the developing world [103].

1.3.3. Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) are very similar to *Shigella*, and like *Shigella* they are capable of invading and multiplying in the distal large bowel host cells in the human gut. The

molecular basis is very complex and multiple chromosomal and plasmid genes are associated with virulence [112]. EIEC is restricted to a very limited number of serotypes, most of which are non-motile.

As with *Shigella*, EIEC produce a dysenteric illness characterised by fever and blood and pus in the stool. Epidemiological studies have shown EIEC to be a relatively rare cause of diarrhoea, although outbreaks occur, including food-borne outbreaks [103]

1.3.4. Enteroaggregative *E. coli* (EAggEC)

Enteroaggregative *E. coli* (EAggEC) are characterised by a distinct aggregative adherence (AA) pattern to HEp-2 cells *in vitro* first described by Nataro *et al.* [113]. This pattern is distinguished by the prominent autoagglutination of bacterial cells to each other, to the surface of the HEp-2 cells as well as to the glass cover slip in a characteristic layering best described as a “stacked brick” configuration. The AA pattern is plasmid mediated [114] and was suspected to be a putative agent of diarrhoeal disease as early as 1988 [115]. Volunteer studies with EAggEC have indicated that certain types will cause diarrhoea and other enteric symptoms including borborygmia and cramps (summarised in [116]), and epidemiological studies have implicated EAggEC as a cause of travellers’ diarrhoea [117],[118]. Most recently EAggEC have been associated with acute and chronic diarrhoea and abdominal colic in young children in Germany [119], and with 4 outbreaks of gastro-enteritis in the UK [120]. A DNA probe referred to as CVD432 has been cloned from the plasmid of strain O42, serotype O44:H18 (formerly an EPEC O:H serotype) and used to identify EAggEC [121]. A plasmid encoded flexible, bundle-forming fimbrial structure, designated aggregative adherence fimbria I (AAF/I), is encoded by two regions [122],[123]. Region 2 encodes a transcriptional activator of the AraC family of DNA-binding proteins [123]. At least one toxin similar to the heat-stable toxin of ETEC, ST designated EAST1 has been identified in an EAggEC strain [124],[125]. The gene encoding EAST1 has been shown to be broadly distributed among diarrhoeagenic *E. coli*: 100% of 75 O157:H7 VTEC, 41% of 227 EAggEC, 41% of 149 ETEC, 22% of 65 EPEC, and 38% of 47 *E. coli* from asymptomatic children hybridised with the EAST1 DNA probe (SS126) [126]. The significance of these findings remains to be established.

EAggEC have been associated with chronic diarrhoea in many parts of the world. In children, EAggEC intestinal illness is characterised by watery diarrhoea, vomiting, dehydration, and, occasionally, abdominal pains, fever, and bloody stools [30].

1.3.5. Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) are defined by the presence of the diffuse adherence (DA) pattern of *E. coli* strains to HEp-2 cells [33],[114]. A surface fimbriae designated F1845 confers the DA phenotype and a DNA probe has been cloned [127]. Another adhesin (designated AIDA-I) has also been associated with DA of *E. coli* of serotype O126:H27 [128]. The role of DAEC in diarrhoea is unclear.

1.3.6. Vero cytotoxin producing *E. coli* (VTEC)

Vero cytotoxin producing *E. coli* (VTEC) are characterised by ability to produce either one or both of at least two antigenically distinct , bacteriophage-mediated cytotoxins referred to as VT1 (SLTI or Stx1) and VT2 (SLTII or Stx2). Whereas VTEC refers to all *E. coli* strains that produce VT in culture supernatants [18],[19], the term **e**ntero**h**aemorrhagic ***E. c****oli* (EHEC) has been used to refer to strains that have the same clinical and pathogenic features associated with the prototype organism *E. coli* O157:H7 [129]. In practice EHEC is used to describe a subgroup of VTEC that causes haemorrhagic colitis.

1.3.6.1. Toxin structure, function and mode of action

Verotoxin belongs to the Shiga toxin family with the following members: Shiga toxin which is produced by *Shigella dysenteria* type 1, VT1, VT2 and VT2 variants (VT2v). Like many other bacterial toxins, Shiga toxin and VT is composed of an A (active) subunit and 5-6 B (binding) subunits. The molecular weights of the A and B subunits are approximately 33,000 and 7,500 respectively, but differ in each of the VTs as summarised in table 1.3. One of the variants, VT2v is only produced by VTEC causing oedema disease, a usually fatal disease, in weanling pigs and referred to as VT2e. Unlike all other VTs this is not cytotoxic to HeLa cells and binds to a different receptor, Gb_4. The functional receptor for human VTs is the glycolipid globotriosyl ceramide Gb_3 (galactose-α-(1-4)-galactose-β-(1-4)-glucose ceramide) [130],[131] found in human renal endothelial cells [132]. Slightly different species of Gb_3 are also present on human monocytes, and VT1 induces the synthesis of cytokines IL-1β, TNF-α, IL-6 and IL-8 in non-stimulated monocytes by an LPS-independent cell activation [133].
VTs are 28S rRNA N-glycosidases which inhibit the elongation factor 1 (EF-1)-dependent aminoacyl-tRNA binding to 60S ribosomal subunits [134] thus inhibiting protein synthesis. In Vero cells the result is cell death by apoptosis [135]; One molecule of VT is sufficient to induce cell death (G. Keusch, personal communication).

Table 1.3. Designations, molecular weight, biological activity, A subunit active site and B subunit receptor of some of the reported VT types summarised from ref.s [134] and [136].

		Molecular weight		Cytotoxicity to		A subunit active site	B subunit receptor
		A subunit	B subunit	HeLa	Vero		
VT1	(SLT-I)	32,226	7,690	+	+	Glu167	Gb_3
VT2	(SLT-II)	33,193	7,817	+	+	Glu166	Gb_3
VT2vha	(VT2v-a)	33,147	7,772				Gb_3
VT2vhb	(VT2v-b)	33,165	7,772				
VT2e	(VTe,VT2vp)	33,068	7,583	-	+		Gb_4
VT2vp2	(SLT-IIva)	33,090	7,555		++		
VT2v	(SLTIIc)						
VT2ev	(SLTIIva)						

Gb_3: Globotriocyl ceramide.
Gb_4: Globotetraocyl ceramide.

1.3.6.2. Molecular biology and genetics of VT

There are two main types of VT: VT1 and VT2 [137]. With the exception of one amino acid VT1 is identical to Shiga toxin from *Shigella dysenteria* type 1 and is neutralised by Shiga toxin antiserum [138],[139].
The genes controlling production of VT are phage-encoded in most *E. coli* strains. There are at least two morphologically distinct phages, one encoding VT1, phage H19J from strain H19, serotype O26:H11, and two morphologically identical phages 933J and 933W from strain 933, serotype O157:H7 encoding VT1 and VT2 respectively [140]. VT phages have also been detected in several other serotypes [141],[142],[143],[144],[145]. The structural genes for VT1 and VT2 share 58% overall nucleotide and 56% amino acid sequence homologies [138] and the homology between the different VT2 genes range from 71% (VT2e/VT2ev) to 98.9% [146].
The genes for VT1 and VT2 have been cloned by many different groups [147],[148],[149],[150],[151], and used as polynucleotide DNA probes to identify VTEC. Subsequent sequencing of VT genes has been used to produce numerous oligonucleotide probes for diagnosis of VTEC and especially subdivision of VT2 [152],[153]. PCR primers are even more numerous and comprises consensus primers amplifying all VTs (VT_{321}) [154], VT1 (MK1) and VT2 (MK2) genes, specific amplification of VT1 and VT2 and human variants VT2vha and VT2vhb [155],[156], and porcine variants [157].
For a brief presentation and practical use of the subtyping of VT genes with 4 VT1 and 9 VT2 oligonucleotide DNA probes, and 3 VT1 and 8 VT2 primer pairs see ref.s [146], [158] and [159].

1.3.6.3. Attaching and effacing lesions of VTEC

Like EPEC, VTEC have been shown to cause attaching and effacing lesions both *in vivo* [160], in animal models [161], [162],[163] and *in vitro* [51]. Two separate groups have cloned, sequenced and characterised the *eae* homologue from EHEC O157:H7 [164],[165]. Sequence homology between EPEC and EHEC sequences was 86% and 83% at the nucleotide and amino acid levels respectively [164] and the EHEC *eae* sequence was 97% homologous to the EPEC *eae* gene for the first 2200 bp and 59% homologous over the last 800 bp [165]. Both *eae* sequences show 50 % homology to the central region of the *Yersinia pseudotuberculosis inv* gene [81],[165], and the predicted amino acid sequence of the EHEC *eae* gene share 31% identity and 51% similarity with the invasin molecule of *Yersinia pseudotuberculosis* [164].
The 1 kb *eae*A probe of EPEC, CVD434 [81] has been compared with a 410 bp region at the 3' end of the *eae*O157 gene [166]. Both probes hybridised with all 246 O157:H7 or H^- VTEC strains tested. A further 10 strains hybridised with both probes but not with VT gene probes. The *eae*A of EPEC but not the *eae*O157 sequence hybridised with 18 O157:H8 or H39 strains, predominantly from human diarrhoea [166]. Further serotype specific heterogeneity of the *eae* gene in VTEC strains O55:H7 or H^-, O111:H8 and O157:H7 or H^- has been demonstrated [167]. Restriction enzyme digestion profiles of PCR products generated by amplification of the 5' end have likewise demonstrated serotype specific differences in VTEC O groups O26, O103 and O157 [168].

1.3.6.4. EHEC plasmids

Almost all O157:H7 strains harbour a large 60-65 MDa plasmid [21], designated pO157, that plays a role in virulence [169]. O26:H11 strains also possess at least one plasmid in the range of 55-70 MDa. Restriction enzyme patterns of plasmids from other O:H serotypes (including O5, O91, O103, O111, O121 and O127) show a notable similarity with the large plasmids in O157 and O26 strains [53]. A 3.4 kb fragment from a large plasmid of O157:H7 (prototype EDL 933) has been cloned and used as a DNA probe (referred to as CVD419) to identify EHEC plasmids [53] *i.e.* large plasmids found in Verotoxin producing *E. coli* strains.
DNA probing with gene probes defining the incompatibility group of plasmids indicate that the EHEC plasmids share an approximately 23 kb fragment with EPEC plasmids and that the large plasmids of both EPEC and EHEC constitute a family of transfer-deficient Inc F-IIA plasmids [170] while sequencing of pO157 reveals high homology to the orf1 of the RepFIB replicon [171].
One study described the EHEC plasmid as required for the expression of a fimbrial antigen and necessary for the adherence to epithelial cells [169] but other studies have not been able to confirm this [172],[173]. Apparently the EHEC plasmid does not play an essential role in a number of animal models using gnotobiotic piglets [174], mice [175] or rabbits [176]. However, one recent study indicated that patients infected with O111:H^- VTEC strains carrying enterohaemolysin encoding plasmids are at a higher risk for developing HUS than patients infected with haemolysin- negative O111:H^- VTEC strains [177]. The large plasmid of O157 encode EHEC-haemolysin which is homologous to the *E. coli* α haemolysin [178],[179] and a novel catalase-peroxidase, KatP which is closely associated to the EHEC-haemolysin [180].

Small plasmids of 6.6 kb found in O157:H7 VTEC strains isolated from patients with bloody diarrhoea and HUS appear to synthesise colicin D [181].

1.3.6.5. Haemolysins

Certain strains of *E. coli* have the ability to lyse erythrocytes of different mammalian species. The best characterised is the α haemolysin which is secreted and can be demonstrated in culture fluid filtrates [182]. α haemolysin is often associated with urinary tract infections [183]. Different types of haemolysins, called enterohaemolysins, are often found in VTEC strains. In contrast to α haemolysin, enterohaemolysins can be detected only on blood agar plates containing washed sheep erythrocytes. The lysed zones on sheep blood plates are smaller and more turbid than α haemolysin [182].
The blood lysis of α haemolysin is usually detected after 3-4 hour of incubation of bacteria, whereas the enterohaemolysin requires overnight incubation before it becomes detectable [184],[185].
Three types of enterohaemolysin have been described. The large plasmid pO157 encodes an EHEC-haemolysin (EHEC-hly) similar to the α haemolysin but exhibits the enterohaemolysin phenotype. The genes encoding the EHEC-hly constitutes a typical RTX (**R**epeats in **T**o**x**in) determinant, the EHEC-hly operon, with the gene order CABD [171]. The EHEC-*hly*A gene encodes the active protein [179] and EHEC-*hly*B and *hly*D share high sequence homology with other RTX transport proteins [171]. Like α haemolysin, the EHEC-hly is a highly active cytolysin of the RTX family with a similar but not identical pore-forming capacity [186]. The EHEC plasmid DNA probe (CVD419) covers the EHEC-*hly*A and part of the *hly*B gene [179].
Two other enterohaemolysins Ehly1 and Ehly2 have been described [187],[188]. Ehly1 is a 33 kDa cell associated protein encoded by a bacteriophage, ϕC3888, found in O26:H11 VTEC [189]. Ehly1 has no known sequence homology to any other DNA or protein sequence [187].
The Ehly2 enterohaemolysin is also encoded by a bacteriophage, ϕC3208, found in O26:H11. It is in part homologous to DNA of bacteriophage λ but completely unrelated to Ehly1 [188].
The enterohaemolytic phenotype is associated with VTEC [190] and has been proposed as a good screening test for the identification of VTEC[191].

1.3.6.6. Epidemiology

Most information on the transmission has been learned from outbreak investigations. During the period from 1982-1993 at least 20 outbreaks of O157:H7 have been reported in the USA (summarised in ref.s [192],[193]). These outbreaks have affected 1,509 patients resulting in the hospitalisation of 346 patients, 86 cases of HUS and 19 deaths. This exploded in the following years with 13 outbreaks in 1993 and 30 outbreaks in 1994 [194]. The largest multistate outbreak in the USA occurred in early 1993 with more than 700 illnesses and four deaths [195],[196]. It is estimated that 20,000 illnesses and 250 deaths occur each year in the USA [197]. In Canada 15 outbreaks were reported in 1982-1987 with 242 cases, 24 cases of HUS and 15 deaths [198]. The first recognised community outbreak of O157:H7 in Europe occurred in the UK in the summer of 1985 affecting at least 24 persons. Eleven patients were hospitalised and one died [199]. In England and Wales O157:H7 was isolated from 39% of sporadic cases of haemorrhagic colitis [200], 33% of sporadic HUS cases [201]. In an outbreak of HUS in the West Midlands, O157:H7 was isolated from 33% of cases [202]. Subsequent outbreaks and sporadic cases in the UK have been reported [203]. Scotland has one of the highest rates of infection with O157 increasing from 1.37/100,000 of the population in 1989 [204] to 32.3/100,000 in 1996 [205].

The worst food poisoning outbreak with O157 VTEC in Scotland occurred in 1996 affecting 415 individuals and hospitalised 151 of whom 18 elderly died [206].

O157 VTEC has been isolated from outbreaks and from sporadic cases of diarrhoea and HUS in many parts of the world: Canada, UK, Argentina, Germany, Central Europe, Chile, Italy (summarised in [192]). Japan experienced the largest outbreak of O157 VTEC ever recorded in 1996 with approximately 10,000 cases and 8 deaths [207].

Other VTEC O:H serotypes have caused outbreaks of diarrhoea and HUS: O111:H^-, O145:H^- and O?:H19 in Japan [61], O26:H11 in the Czech Republic [208], O111:H^- in Australia [59], Italy [57] and France [209]. A new clone of sorbitol fermenting O157:H7 VTEC has been isolated from patients with diarrhoea and HUS [210],[211], and Verotoxin producing *Citrobacter freundii* has caused an outbreak in Germany [212].

Many other serotypes have been isolated from sporadic cases of HUS, some of which are summarised in table 1.4.

Table 1.4. O:H serotypes and VT-type (if available) of non-O157 verotoxin producing *E. coli* (VTEC) strains isolated from patients with haemolytic uraemic syndrome (HUS).

O group	H antigen	VT1	VT2	VT1+VT2	HUS	references
O1	H$^-$;		3		3	[213]
O2	H5			1	2	[56],[198]
O5	H$^-$;	2			2	[213]
O8	H14				1	[214]
O9ab	H$^-$				1	[215]*
O15	H$^-$;		1		1	[214]
O18	H?	1			1	[213]
O22	H8			1	1	[216]
O26	H$^-$; H11	5	5		12	[198],[201],[213] [214],[216],[217],[218]
O55	H$^-$; H6, H7, H10, H?		4		7	[56],[213],[216],[219]
O91	H21				2	[151],[198]
O101	H$^-$				1	[215]*
O103	H$^-$; H2				6	[220], this study
O104	H2		1		1	[201]
O105ac	H18				1	[219]
O111	H$^-$; H8	6	5	17	32	[213],[217],[218] [221],[198],[216],[222]
O113	H21		1		5	[198],[217],[218]
O115	H10				1	[219]
O117	H4				1	[198]
O121	H19				2	[198],[218]
O128ab	H2, H25				2	[219]
O145	H$^-$; H25, H28	1			3	[198],[218],[219], this study
O153	H25				1	[201]
O163	H19		1		1	[201]
O165	H19, H25				2	[219]
OX3	H2, H21		1	1	2	[223], this study
OR	H$^-$; H5		1		2	[201],[218]
ONT	H$^-$; H2, H25	1	1	1	6	[198],[201],[216],[224]
Sum		16	24	21	102	

* Two serotypes from the same patient.

Outbreak investigation has shown that O157 VTEC can be transmitted by food, water, drinks and person-to-person spread. Most outbreaks have been caused by foods of bovine origin such as minced beef, undercooked hamburgers, roast beef and raw milk [196],[198],[192],[225],[226],[227],[228],[229],[230], or pasteurised milk [231] or yougurt [232] contaminated after pasteurisation. Many other sources suspected of having been contaminated from an original bovine source include fresh-pressed apple cider [233], commercial apple juice [234], new potatoes [199], vegetables [235], sandwiches [203],[236], supermarket meat products [237], and sausages [59],[238]. The spread by water was demonstrated in a large outbreak caused by unchlorinated drinking water [239], contaminated well water [240], swimming in lakes [241],[242], [243],[244], and children's paddling pool [245]. Person-to-person spread was observed in a nursing home outbreak, [236], in a day care centre outbreak [246] and during food-borne outbreaks [196] indicating a very low infectious dose. Similar associations have been found in sporadic cases of VTEC infections [247]. Contact with farm animals and cattle in particular represents a risk factor for infection with VTEC [248], but is not necessarily associated with clinical illness [249].
The primary reservoir of O157 and other VTEC is cattle [219],[250] but VTEC is found in most ruminants [251],[252].

1.3.6.7. Clinical illness

E. coli O157:H7 VTEC causes non-bloody, watery diarrhoea, bloody diarrhoea (haemorrhagic colitis), haemolytic uraemic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), and death [253]. One or two days after a prodromal illness consisting of crampy abdominal pain and a short-lived fever non-bloody diarrhoea ensues. Vomiting and nausea may occur at any time during the illness. Within 1 or 2 days the illness progresses into a grossly bloody diarrhoea which is often accompanied by increased abdominal pain. Abdominal tenderness is the most prominent feature at this stage of the infection and other diagnoses are often considered. Bloody diarrhoea lasts between 4 and 10 days [254],[255]. In 2-10% of the cases with bloody diarrhoea the infection develops into haemolytic uraemic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) [247]. Both HUS and TTP are characterised by a triad of acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia and it is often difficult to distinguish between the two syndromes [256].
The clinical features of O157 VTEC infection may lead clinicians to suspect non infectious causes, such as inflammatory bowel disease, ischaemic colitis, or - in children - intussusception, appendicitis, ulcerative colitis, Crohn's disease, diverticulosis and pseudomembranous colitis. Infectious colitis caused by *Campylobacter, Clostridium difficile, Salmonella, Shigella* and *Yersinia* may also be suspected [254],[255]. A few cases of extraintestinal VTEC infections have been described. These include haemorrhagic cystitis and balanitis [257], bacteremia associated with urosepsis and HUS [258], a non-complicated human urinary tract infection (UTI) [259], and non-bacteremic UTI followed by HUS [260]. Two human UTIs complicated by HUS caused by unusual VTEC strains are briefly described in this study and due for publication [261].

1.3.6.8. Diagnosis of VTEC

The diagnosis of VTEC should be considered in any patient with bloody stools or HUS but also when a food-borne infection or other exposure to the organisms is suspected.
The sorbitol- and β-glucuronidase negative phenotypic property of O157:H7 VTEC has facilitated detection in mixed flora on different selective media such as sorbitol-MacConkey (SMAC) agar [262] or 4-methylumbelliferyl-β-D-glucuronide (MUG) containing medium [263]. Modifying SMAC medium by the addition of cefexime, rhamnose, and tellurite has also been reported to improve detection [264],[265]. However, the overall diagnostic strategy should be directed towards detecting VTEC in general. The best way to diagnose VTEC is by isolating colonies that are positive in the Vero cell assay (VCA) but this usually requires screening of up to 20 individual colonies because the proportion of VTEC colonies is often as low as 5 to 20% [22],[218], sometimes down to 1% [200],[266]. The detection of free faecal Verotoxin (FVT) may improve the detection rate [267] and an even more sensitive method for screening stool cultures for VTEC employing polymyxin B to release VT from colony sweeps (VT/PECS) has been described [268]. A method using mitomycin C as an inducing agent to enhance the production of VT has also been used [269],[270]. Enzyme-linked immunosorbent assay (ELISA) methods using antibodies or receptors specific for VT have been developed [271],[272],[273],[10],[274],[275]. Polynucleotide and oligonucleotide DNA probes for VT1 and VT2 have been used [83],[153],[210], [276],[277],[278],[279], as well as PCR to detect VT sequences [154],[155],[276],[280], [281],[282]. Immunomagnetic separation has been used and compared with other methods [283],[284]. Many other techniques and methods have been described but are not reviewed here.
No matter what method is used it is important that the presumptive finding of a VT producing organism is confirmed by positivity in the Vero cell assay and relevant biochemical characterisation.
When VTEC can not be isolated from the stool, serodiagnosis, measuring serum antibodies to the O antigen (LPS) type, has turned out to be very useful. Both ELISA, immunoblotting and indirect haemagglutination have been described [57],[213],[285],[286],[287],[288],[289],[290],[291],[292],[293],[294],[295].

2. Screening of diarrhoeal stools

2.1. Purpose

The purpose of this study was to test and develop methods that would improve the diagnosis of Vero toxin producing *E. coli* (VTEC) in Denmark and to characterise VTEC strains that were isolated during the study.

2.2. Materials and methods

Population: Patients from Denmark whose diarrhoeal stools were submitted for bacterial culture to the Department of Gastrointestinal Infections at SSI.

Faecal specimens: Faecal specimens were randomly collected in series of either 40 or 80 specimens from the same day on 16 independent sample days during an eight months period from March 4 to November 8 in 1994. Different procedures were tested in the first pilot project in which 55 faecal specimens were collected on January 11. Based on results from the pilot project and different procedures performed on specimens collected on the first two sample days, February 23 and March 22, the following protocol was applied to all specimens (a precise description of each of these procedures is given in Methodological studies: **8.1. Screening**).

Microbiology: Approximately 1 g of faeces from stool specimens was suspended in 2 ml saline followed by inoculation of approximately 10 µl on SSI enteric medium ("Red plate", SSI; Cat. No. 785). The specimens were routinely examined for the presence of *Salmonella, Yersinia, Vibrios* and *Shigella.* The faecal suspension was also inoculated on modified Drigalski agar ("blue plate", SSI; Cat. No. 783) and examined for enteropathogenic *E. coli* (EPEC) if requested specifically by the submitting physician.

From all the red plates that were negative for *Salmonella*, *Yersinia*, *Vibrios* and *Shigella* - and from all the blue plates - 9-19 randomly selected lactose- and non-lactose fermenting coliform colonies and a sweep from an area with confluent growth was examined by colony or sweep dot blot hybridisation with a pool of DNA probes for VT1, VT2 and *eae*A genes.

Strain characterisation: Single colonies reacting positively in the DNA pool were sero-, pheno- and genotyped as described below. In specimens where only the sweep dot blot was positive bacterial culture from a zone of dense growth on the primary plate was reinoculated on either red or blue plates. Single colonies from this secondary plate were examined by colony dot blot hybridisation until colonies reacting with the probes could be identified.

Biochemistry: The strains were confirmed as being *E. coli* using the Minibact E kit (SSI; Cat. No. 905 and 906) [296] and testing for ß-glucuronidase production on PGUA plates (SSI; Cat. No. 722) [297].

Serotyping: O:H serotyping was done by microtiter plate- and tube agglutination using methods described by Ørskov & Ørskov [298].

Vero cytotoxin production: Production of verotoxin was examined using the Vero cell assay (VCA) [18]. The precise procedure and associated experiments are described in Methodological studies: **8.2. Vero cell assay**.

Haemolysin production: Haemolysin production was examined on washed blood agar plates using 5% defibrinated sheep blood [185].

The Fluorescein Actin Stain test: The FAS test was done according to methods described by Knutton *et al.* [51].

Genotyping: The strains were examined for the presence of virulence genes using DNA probes for VT1 [147], VT2 [152], *eae*A [81], EHEC (CVD419) [53], Agg [121], DA [127] and EAF [38]. The probes were labelled by random priming with digoxigenin-d-UTP (Boehringer) and colony blots were hybridised under stringent conditions according to the manufacturer's prescriptions. The experimental procedures for optimal stringency are described in Methodological studies: **8.5. DNA probes**.

The original DNA probes and their host strains are listed in table 2.1.

Free faecal cytotoxin (ffct): 1 ml of the faecal suspension was transferred to microfuge tubes and mixed thoroughly followed by centrifugation at 15.000 g for 30 minutes. 30 µl of the supernatant at 1:1, 1:2 and 1:4 dilutions was added in duplexes to wells in microtiter-plates containing Vero cells. The cytotoxic effect of supernatants was read each day for three days.
During the first three sample days enrichment of faecal specimens in brain heart infusion (BHI) broth followed by Mitomycin C induction of Verotoxin producing phages was tested. This procedure was abandoned for the rest of the study period because it was found to be very laborious without increasing the sensitivity of the ffct test.

Clostridium difficile cytotoxin: Selected stool specimens that were positive for *Clostridium difficile* were also examined for the presence of free faecal cytotoxin in the Vero cell assay.

Pilot project: A pilot project tested different procedures which are described in Methodological studies: **8.1. Screening**.

Table 2.1. **Table of original DNA probes.**Fejl! Bogmærke er ikke defineret.

Probe	Vector Plasmid	Selection Criteria †	Name of Construct	Probe size in kb	Restriction Enzyme	Reference	Host No.	Geno- and Phenotype	D number
*eae*A	pUC19	Amp^r	pCVD434	~1	*Sal*I-*Kpn*I [a]	[81]	DH52		D2202
EHEC	pBR325	Amp^r,Cm^r	pCVD419	3.4	*Hin*dIII	[53]	HB101		D2195
EAgg	pUC19	Amp^r	pCVD432	~1	*Eco*RI-*Pst*I [b]	[121]	DH52a		D2196
DA [c]	pUC8	Amp^r	pSLM862	390 bp	*Pst*I	[127]	JM83		D2197
EAF	pCVD315[d]	Amp^r	pJPN16	~1	*Bam*HI-*Sal*I	[38]	HB101		D2203
VT1	pACYC177	Km^r	NTP705	0.75	*Hin*cII	[147]	60R746	*E. coli* K12 F$^-$ [e]	D2208
VT2	pGEM1	Amp^r	pDEP28	0.85	*Sma*I-*Pst*I	[152]	62R310	*E. coli* K12 F$^-$ [e]	D2207

† Recommended concentrations of antibiotics are specified in the "**ANTIBIOTICS**" table 11.1., see **Appendix**.

[a] This fragment will include 2 bases of pUC19 (not 3 bases as stated in [81]). The probe originates as a *Sal*I-*Stu*I fragment cloned into the *Sal*I-*Sma*I site of pUC19.

[b] This fragment will include 21 bases (left arm) and 22 bases (right arm) of pUC19. The probe originates as a ~700 bp fragment from 17-2 cloned into the *Bam*HI site of pUC19.

[c] This is the *daaC* gene which Steve Moseley cites as a 390 bp *Pst*I fragment (It was first described as a ~280 bp *Pst*I fragment). Peter Echeverria calls his DA pW22, cuts it with *Pst*I which results in a 450 bp fragment.

[d] This is a derivative of pBR322.

[e] Prototrophic

2.3. Results

2.3.1. Specimens and episodes

A total of 1.270 stool specimens were included. Four specimens were not examined by neither DNA pool or for the presence of free faecal cytotoxin (ffct) and were excluded from further analysis.
310 specimens were not examined by DNA hybridisation for one of the following reasons:

- Primary plates yielded enteropathogenic bacteria other than *E. coli*.
- No - or very sparse - growth on primary plates.
- Colonies from primary plates showed no growth on hybridisation membranes on agar plates.
- Primary plates were forgotten, discarded of or otherwise mislaid. This was the main reason for not being able to do the DNA hybridisation.

34 specimens were not examined for the presence of ffct primarily because there was not enough stool sample left *i. e.* less than 1 g to perform a satisfactory ffct test.

The 1.266 specimens represented 930 episodes of diarrhoea and were examined for the presence of virulence genes with the DNA pool, for the presence of ffct or both. The distribution and number of specimens examined are shown in table 2.2.
Data identifying each patient by CPR number was obtained from the department's patient register (dBase IV) for patients whose stools were culture positive and from filed patient slips for culture negative stools. CPR numbers were checked for validity using a dBase application (see Appendix: **11.2. dBase applications**). CPR numbers were valid for 1.125 specimens. The last four digits were missing or temporary for 16 and 6 specimens respectively. Names were available however and used for analysis of age and sex based on the assumption that the date of birth was correct. 17 specimens had invalid CPR numbers and were not used for age and sex analysis. They were registered as episodes based on names. It should be noted that due to the study design, all stools from patients submitting more than one stool were received on the same day except for five patients. These five patients submitted two (2 patients), three (2 patients) and five (1 patient) stools one week to 3 months apart. 14/15 of these stools were negative for bacterial pathogens and were considered as 5 episodes even though they did not meet the standard definition of an episode. No CPR number was available for 106 specimens and will be referred to as non-episodal specimens. The number of specimens, the type of validity of CPR numbers and the resulting number of episodes are shown in table 2.3. Two, three and five stool specimens were examined from 170 (18%), 62 (7%) and 1 (0.1%) patients respectively. As a result 913 episodes could be described by both age and sex and 915 episodes by age only. The seasonal distribution of the 930 analysed episodes are shown in figure 2.1. The overall distribution of 913 episodes by age and sex is shown in figure 2.2. Unfortunately negative episodes were not registered in 1994 rendering a direct comparison between the screening distribution and the general distribution of 1994 impossible.

Instead age and sex distribution from the screening is compared to similar data from the two following years 1995- July 1996 when episodal registration of all submitted stools had been implemented. A similar distribution of age and sex during the screening period and the period 1995- July 1996 is shown in table 2.4.

Table 2.2. Distribution of 1270 stool specimens examined with the DNA pool (VT1, VT2 and *eae*A), for the presence of free faecal cytotoxin (ffct) or both.

		Stool specimens examined with:					
Week	Date	DNA pool and ffct	DNA pool only	ffct only	ND	Total number of specimens	Total number of episodes
9	04. mar. 94	25	7	15	3	50	42
12	22. mar. 94	18		7		25	23
21	25. maj. 94	85	8	8	1	102	80
24	15. jun. 94	29	14	11		54	41
25	22. jun. 94	57		23		80	64
27	05. jul. 94	61		19		80	64
34	24. aug. 94	62		18		80	56
35	30. aug. 94	71		9		80	59
36	07. sep. 94	59		21		80	60
37	14. sep. 94	60		20		80	40
38	21. sep. 94	66	1	13		80	56
39	28. sep. 94	63		49		112	78
40	05. okt. 94	60		32		92	59
43	25. okt. 94	71		25		96	65
44	01. nov. 94	78		21		99	63
45	08. nov. 94	65		15		80	63
	Sum	930	30	306	4	1270	913

ND: Not done.
ffct: free faecal cytotoxin.

Table 2.3. Number of specimens, validity of CPR numbers and the resulting number of diarrhoeal episodes.

CPR number	Number of specimens	control code	Number of episodes with:				Total number of episodes
			One stool	Two stools	Three stools	Five stools	
None	106	9					
Valid	1125	1	730	105	60	1	896
Last 4 digits missing *	16	9	13		1		14
Last 4 digits temporary	6	0	4	1			5
Invalid **	17	0	13	2			15
Sum	1270		760	108	61	1	930

control code: number given by MODULUS.PRG (see appendix: **11.2. dBase applications**).

* : Two episodes could not be sex determined because names were ambigously associated with sex.

**: All 17 specimens were registered as episodes based on names but were not used for age and sex analyses.

Table 2.4. Comparison of age and sex distribution from 913 episodes of diarrhoea during the screening in 1994 compared with the overall distribution of all submitted stools, representing 141,491 episodes in the two following years 1995- July 1996.

	1994 screening			1995- July 1996		
Age groups	Episodes No.	Episodes %	% males	Episodes No.	Episodes %	% males
< 1	57	6%	61%	6292	4%	54%
1-5	155	17%	52%	19175	14%	56%
5-10	32	4%	66%	5074	4%	54%
10-20	45	5%	51%	8169	6%	49%
20-30	131	14%	41%	22335	16%	41%
30-40	116	13%	46%	18493	13%	47%
40-50	93	10%	49%	16516	12%	45%
50-60	92	10%	43%	14060	10%	43%
60-70	64	7%	42%	11752	8%	41%
>70	128	14%	41%	19625	14%	34%
	913	100%	47%	141491	100%	45%

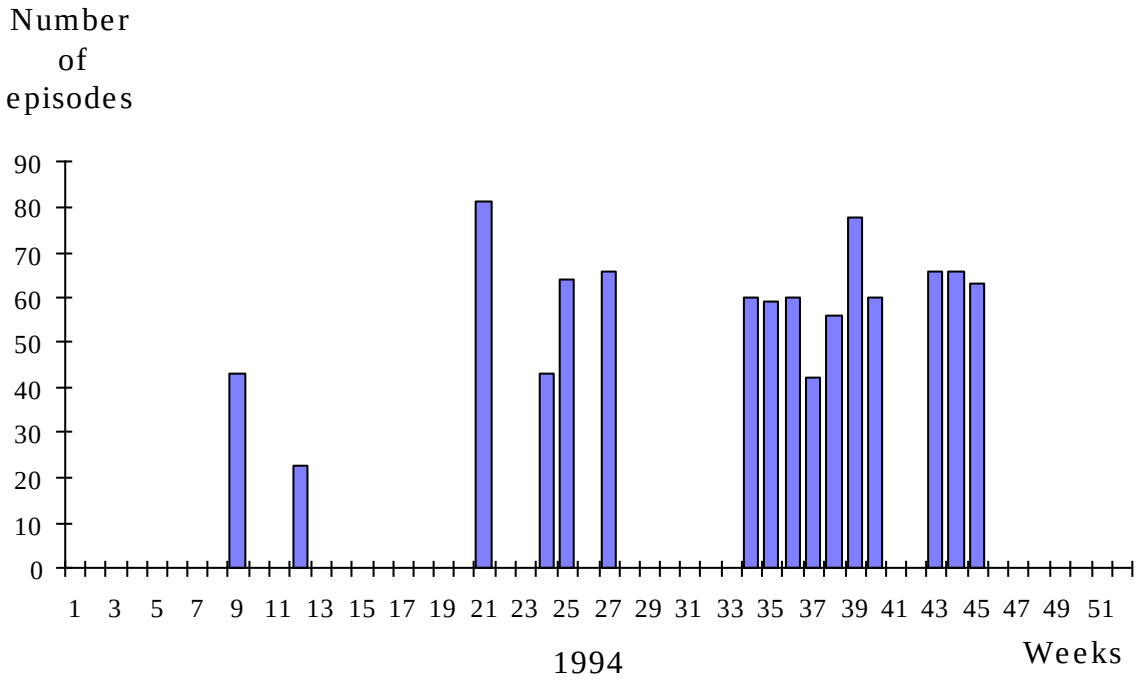

Figure 2.1. Calendar time distribution of 930 episodes analysed during screening of diarrhoeal stools during 1994.

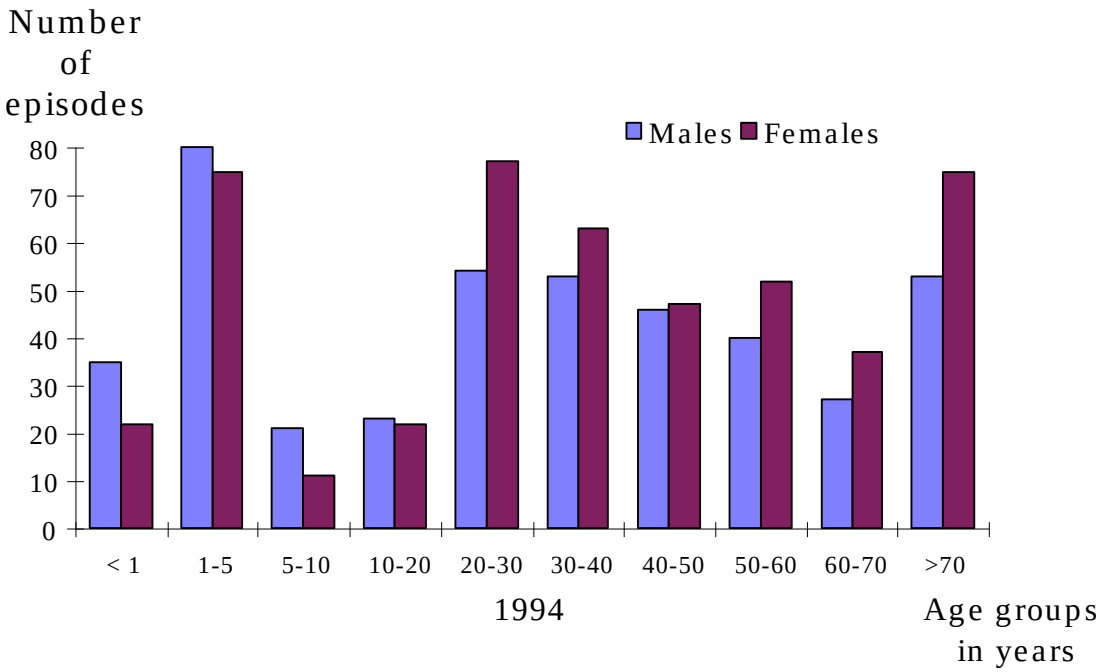

Figure 2.2. Distribution of 913 diarrhoeal episodes by age and sex.

2.3.2. Screening with DNA probes

9.928 dot blots from 960 specimens representing 737 episodes and 46 non-episodal specimens were probed with the DNA pool. An average of 10.3 (range 1-20) dot blots per specimen were probed.
387 dot blots (average 5,4 per specimen) from 72/960 (7.5%) specimens representing 66 episodes were positive with the DNA pool. The number of positive dot blots per specimen ranged from 1 in 20 specimens to 19 in one specimen. The prevalence of the number of positive dot blots in positive specimens in relation to the number of dot blots tested with the DNA pool is shown in table 2.5.A.
The number of positive and negative specimens in relation to the number of tested dot blots are shown in table 2.5.B.
Table 2.5. shows that 2/15 (13.3%) specimens were positive by one dot blot each when 1 to 5 dot blots were tested (blue hatching in table 2.5.). Three out of 35 (8.6%) specimens were positive by one dot blot each when 6-9 dot blots were tested (green hatching in table 2.5.). Fifty eight out of 859 (6.8%) specimens were positive by 1-10 dot blots when 10 dot blots were tested (red hatching in table 2.5.), and 9/51 (17.6%) specimens were positive by 1-19 dot blots when 17-20 dot blots were tested (yellow hatching in table 2.5.). The probe positive rate in specimens ranged from 5% to 100% with a median value of 40% (mean 48.1%) suggesting that the number of positive dot blots per number of dot blots tested were not normal distributed.
This is quite evident in figure 2.3. where the distribution of number of positive specimens in relation to the percent positive dot blots is shown. Instead a bimodal distribution seems more likely as illustrated in figure 2.4. which shows that two thirds of the positive specimens were either represented by 1-2 positive dot blots per specimen (38%) or 9-10 positive dot blots per specimen (24%) when 10 dot blots were tested.

Table 2.5. A: The number of VT1, VT2 or *eae*A (DNA pool) positive dot blots per specimen from 960 specimens in relation to the number of dot blots tested per specimen.
B: The number of specimens that were positive or negative by dot blot in relation to the number of dot blots tested per specimen.

A

Number of positive dot blots per specimen	Number of dot blots tested per specimen													Number of positive specimens
	1	2	3	4	5	6	8	9	10	17	18	19	20	Sum
1			1	1				1	16				1	20
2						1		1	6					8
3									3					3
4									5					5
5									5					5
6									1		1			2
7									4		1			5
8									4			1		5
9									7					7
10									7					7
11											1			1
13													1	1
16											1			1
18											1			1
19													1	1

B

Number of specimens:														
Positive by dot blot			1	1		1		2	58		5	1	3	72
Negative by dot blot	4	3	2	1	3	1	7	24	801	1	3	6	32	888
Sum	4	3	3	2	3	2	7	26	859	1	8	7	35	960
% positives	13.3					8.6			6.8	17.6				

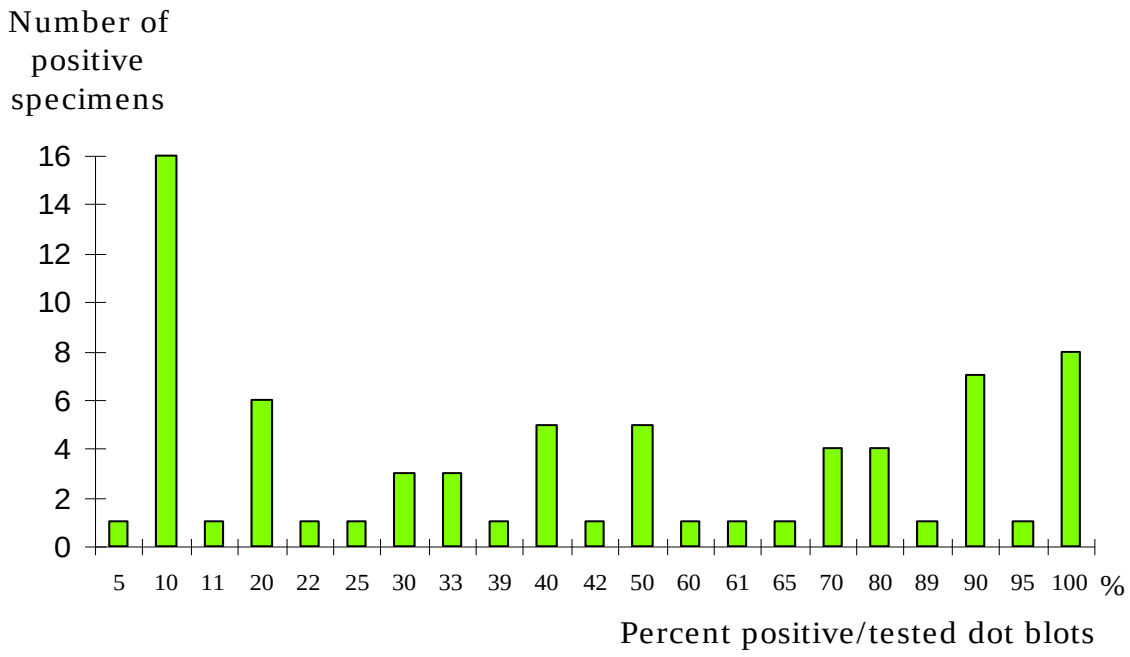

Figure 2.3. Distribution of percent positive dot blots per specimen in 72 specimens.

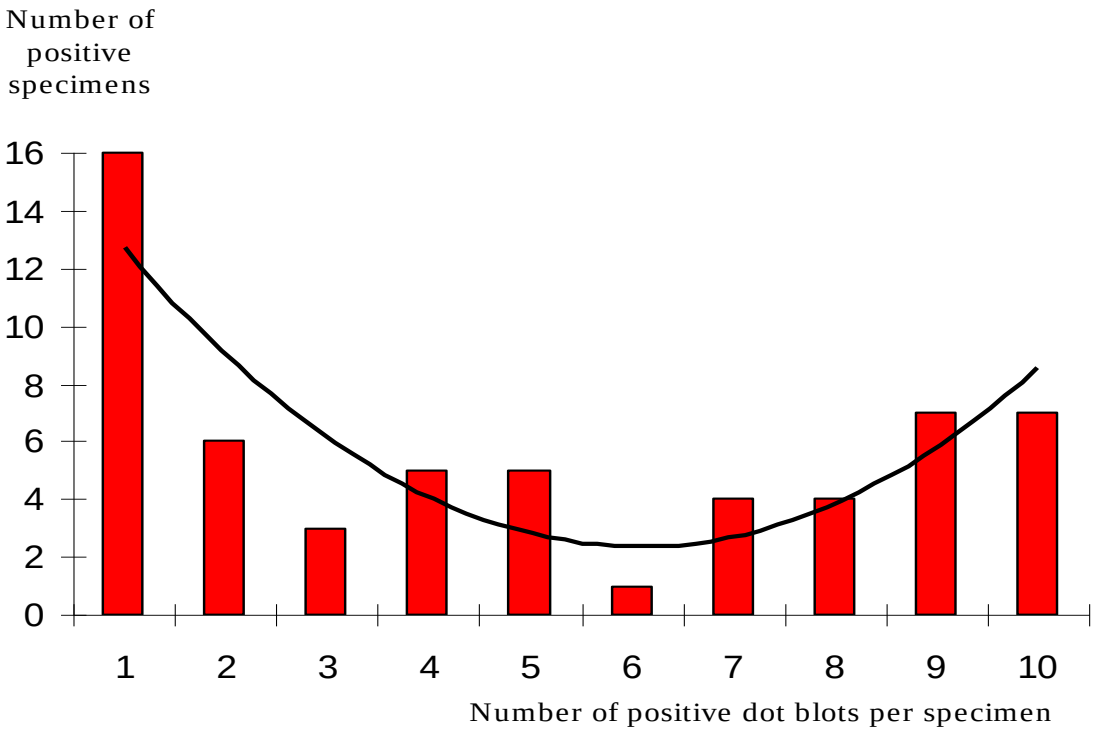

Figure 2.4. The figure shows the number of positive specimens in relation to the number of positive dot blots per specimen in the 58 specimens where 10 dot blots were tested. The bimodal distribution is suggested with the black line.

2.3.3. Free faecal cytotoxin (ffct)

1.236 stool specimens representing 905 diarrhoeal episodes and 106 non-episodal specimens were tested for the presence of free faecal cytotoxin. The undiluted supernatants were too difficult to read whereas dilutions 1:2 and 1:4 could be read in most cases. The readings could be grouped into the following 10 main categories:

VT:
Vero cytotoxic effect. Cell death by apoptosis is observed as cells shrink to a minimal small, round “raisin-like” shape. Early positives were seen on the first day and late positives on the third day.
A clear VT effect (VT) and a possible VT effect (VT?) was observed in 14 (1.1%) and 39 (3.2%) specimens respectively.

***Clostridium* cytotoxin:**
Clostridium difficile cytotoxic effect was usually seen within 4 hours. The Vero cells swelled and assumed a large round form.
A clear *Clostridium* cytotoxin effect (Clost.) and a possible *Clostridium* effect (Clost.?) was observed in 64 (5.2%) and 15 (1.2%) specimens respectively.

RC:
Rounded cells. Similar to the *Clostridium difficile* cytotoxic effect but less pronounced and delayed. Usually not observed until the first day or later.
Rounded Vero cells was observed in 118 (9.5%) specimens.

Aggregation:
The Vero cells aggregated in the form of large lumps.
Aggregation was observed in 2 (0.2%) specimens.

Dead:
Cell death was observed - almost as a fixation. The effect is similar to that of Na-azide.
Vero cells in 8 (0.6%) specimens were dead.

Flocculation:
Vero cells were not clearly affected but flocculation occurred and increased with time. The flocculation may represent unspecified Gram-positive bacterial growth and was observed in 7 (0.6%) specimens.

Contamination:
Bacterial contamination caused by the presence of gentamycin resistant bacteria.
20 (1.6%) specimens were contaminated.

Mould:
Growth - usually dense - of mould fungi was observed in 27 (2.2%) specimens.

Negative:
No effect on Vero cells.
916 (74.1%) were negative.
?:
An ambiguous effect was observed in 6 (0.5%) specimens.

Each category was graded with scores ranging from + (minimal visible effect) to +++ (maximum and typical effect) where all Vero cells were affected. Combinations of the different categories could be seen in the same specimen. The graded categories from the 1:2 and 1:4 dilutions of supernatants resulted in 102 different combinations which were carefully evaluated and reduced into 5 main free faecal cytotoxin (ffct) result groups of interpretation summarised in table 2.6.

Table 2.6. Summary of the results of the free faecal cytotoxin (ffct) tests on 1.236 diarrhoeal specimens showing the number of specimens within each of the 5 main ffct result groups (see text), and the type of enteropathogenic bacteria isolated from each group.

ffct results	Specimens		*E. coli*			*C. difficile* with free toxin	*C. difficile* without free toxin	Other entero-pathogen	No entero-pathogen
	No.	%	**VTEC**	*eae* A positive					
				Single	Double	No.	No.	No.	No.
VT	14	1,1%	**2**						12
VT?	39	3,2%	**1**	5		1	3	4	25
Clost.	64	5,2%		1	a, b, c	30	5		25
Clost.?	15	1,2%		1	b	7	4		2
Other §	188	15,2%		6	b, b	4	29	11	136
Negative	916	74,1%	**1**	39	c, d, d, d	1	29	91	751
Sum	1236	100%	**4**	52	10	43	70	106	951
Not done	34			5	c			6	22

Single: *eae* A positive *E. coli* only.
Double: *eae* A positive *E. coli* co-isolated with other enteropathogen.
OR: Orough.
VT: Vero toxigenic effet on Vero cells.
Clost.: *Clostridium* cytotoxic effect.

Other enteropathogens include c through d:
a: C. difficile with free toxin
b: *C. difficile* without free toxin
c: *Campylobacter jejuni/coli*
d: *Salmonella*

§: Other: Unspecific categories grouped here are: Rounded cells, aggregation, dead, flocculation, contamination, mould, and ? as specified in the text.

VTEC was isolated from two VT positive specimens (O26:H11 and OR:H21) and from one VT? specimen (O166:H28). VTEC O128:H2 was isolated from one of the negative specimens. See table 2.6.

2.3.4. *Clostridium difficile* cytotoxin

188 stool specimens that were positive for *Clostridium difficile* representing 125 diarrhoeal episodes and 13 non-episodal specimens were included and tested for the presence of free faecal cytotoxin. The readings were grouped into the same 10 main categories and graded as previously described. This resulted in 4 main ffct result groups which are summarised in table 2.7.

Table 2.7. Results of ffct tests on 188 diarrhoeal specimens that were positive for *Clostridium difficile* showing the number of specimens within each of the 4 main free faecal cytotoxin (ffct) result groups (see text), and the type of enteropathogenic bacteria isolated from each group.

ffct results	Specimens		*E. coli* VTEC	*C. difficile* with free toxin	*C. difficile* without free toxin	Other entero-pathogen	Notes
	No.	%	**No.**	No.	No.	No.	
VT	3	1,7%	**1** ¤	1	1		
Clost.	63	35,6%		54	8	1	d
Clost.?	6	3,4%		4	2		
Other §	64	36,2%		16	44	4	c, d, d
Negative	41	23,2%		3	32	6	c, d
Sum	177	100%	**1**	78	87	11	
ND	11			5	5	1	c

¤: *C. difficile* without free toxin co-isolated with VTEC O22:H8
VT: Vero toxigenic effet on Vero cells.
Clost.: *Clostridium* cytotoxic effect.
§: Other: Unspecific categories grouped here are: Rounded cells, aggregation, dead, flocculation, contaminationj, mould and ?.
Notes: c: *Campylobacter jejuni/coli*
d: *Salmonella*

2.3.5. Follow up on results from the free faecal cytotoxin (ffct) tests

The 53 specimens from the screening which had any indication of a VT effect in the ffct test but had not yielded VTEC during the DNA pool screening and the 3 VT positive specimens from the *Clostridium difficile* cytotoxin tests with a VT effect were re-examined with the purpose of isolating VTEC. The frozen, preserved stool specimens were thawed and plated from saline suspensions on red and blue plates. Original supernatants from the ffct tests with pellets of saline stool suspension were thawed, resuspended thoroughly and plated on blue plates. The latter did not yield any growth and were discarded. 17/56 (30%) of the thawed stool specimens showed growth and 5 single colonies and a sweep was tested in the Vero cell assay (VCA) and probed with VT1 and VT2 DNA probes on separate nylon membranes. 5 specimens were also examined by replica on nylon membranes that were hybridised with the VT1 probe with negative results.
One sweep from a stool that was positive for *Clostridium difficile* without free toxin was positive in the VCA and with VT1 and VT2 DNA probes. Single positive colonies were isolated from the sweep, identified as *E. coli* and serotyped as O22:H8.

2.3.6. Bacterial pathogens

Enteropathogenic bacteria were isolated from 207/930 (22.3%) patients and from 5 non-episodal specimens. VTEC and *eae*A positive *E. coli* were isolated from 4 (0.4%) and 50 (5.4%) patients respectively. *eae*A positive *E. coli* were co-isolated from 11 (1.2%) patients infected with *Campylobacter coli/jejuni* (3 patients), *Clostridium difficile* with free toxin (1 patient), *Clostridium difficile* without free toxin (4 patients), and *Salmonella* (3 patients). There was no record of a relation to an outbreak. Culture positive stools are listed in table 2.8. 13 individual colonies from one specimen were positive with the DNA pool but all 13 colonies gave negative results when tested with the single gene probes for VT1, VT2 and *eae*A. No explanation of this phenomenon was found. *Shigella* was isolated from another patient where 19/20 dot blots were positive with the DNA pool.

Table 2.8. Enteropathogens isolated from patients during screening of 1.270 diarrhoeal stool specimens. Some of the patients from whom enteropathogens had been identified on primary red and blue plates on the first day *i.e*. *Salmonella, Shigella, Yersinia* are not included in the table.

Enteropathogens isolated	Patients	Non-episodal specimens
Campylobacter coli/jejuni	19	
Clostridium perfringens	1	
Clostridium difficile with free toxin	36	3
Clostridium difficile without free toxin	63	2
Verotoxin producing *E. coli* (VTEC)	4	
eae A positive *E. coli*	50	
eae A positive *E. coli* and other pathogen*	11	
Salmonella	63	
Shigella ssp.	5	
Yersinia enterocolitica (O:3)	9	
Sum	261	5

* : Other enteropathogens were *Campylobacter coli/jejuni* (3 patients), *Clostridium difficile* with free toxin (1 patient), *Clostridium difficile* without free toxin (4 patients), and *Salmonella* (3 patients).

The patient characteristics, the pathogenic *E. coli* belonging to either VTEC or to classical EPEC O:H serotypes and the detection results are summarised in table 2.9.

Table 2.9. Characteristics of 12 patients from whom pathogenic *E. coli* belonging to either VTEC or classical EPEC O:H serotypes were isolated during screening of diarrhoeal stools in 1994, and their bacteria.

Patients	Age Years or Months (= m)	Sex	Diarrhoeal duration in days	Travel abroad	*E. coli* Serotypes	DNA	Positive/ tested dot blots		ffct	Notes
							No.	%		
					VTEC:					
F39744	1	M	30		O22:H8				VT	b
F41507	23	F	4		O26:H11	++	6/20	30%	VT	
F70095	30	M			O128ab:H2	+	7/10	70%	-	
F22333	32	M	30	Portugal	O166:H28	+	6/18	33%	VT?	
F18123	65	F			OR:H21	+	11/18	61%	VT	
					EPEC:					
F57624	11 m	M	21		O26:H-	+	5/10	50%	-	
F66738	1	F	20		O26:H11	+	5/10	50%	-	
F68570	2	F			O55:H7	+	10/10	100%	-	
F68551	37	M			O111:H-	+	1/10	10%	-	
F62333	1	M			O127:H40	+	1/10	10%	-	b
F66671	1	M	42		O128ab:H2	- +	5/10	50%	RC	b
F58035	28	F			O128ab:H2	+	4/10	40%	-	

OR: Orough.
H-: Non motile strain.
DNA: Number of stool specimens examined from each patient.
+: first stool, ++: first and second stool.
- +: second stool only
ffct: Free faecal cytotoxin.
VT: Vero cytotoxic effect on Vero cells.
RC: Rounded cells
Notes: b: co-isolated with *C. difficile* without free toxin

The characteristics of the isolated VTEC and classical EPEC strains are shown in table 2.10.

50 different O:H serotypes of *eae*A positive *E. coli* not belonging to any well defined pathogenic group of *E. coli* were isolated from 51 patients. The most common serotypes were O2:H40, ONT:H6 (ONT: O not typeable) (4 patients each), O76:H7, O129:H11 and O145:H- (3 patients each), and 26 other O groups (1-5 isolates from each O group), 5 Orough (OR) and five ONT isolates. 39 patients had only one serotype but up to 4 different *eae*A positive *E. coli* serotypes were found in the remaining 12 patients.

Table 2.10. Characteristics of VTEC and classical EPEC O:H serotypes isolated during screening of diarrhoeal specimens in 1994.

C number	Serotype	Hly	VCA	VT1	VT2	*eae*A	EAF	EHEC	EAgg
VTEC									
C679-96	O22:H8	Ent.	+	+	+	-	-	+	-
C456-94	O26:H11	Ent.	+	+	-	+	-	+	-
C744-94	O128ab:H2	Ent.	+	+	+	-	-	+	-
C369-94	O166:H28	Ent.	+	+	+	+	-	+	-
C340-94	OR:H21	Ent.	+	+	+	-	-	+	-
EPEC									
C596-94	O26:H-	α	-	-	-	+	-	-	-
C688-94	O26:H11	-	-	-	-	+	-	+	-
C707-94	O55:H7	-	-	-	-	+	-	-	-
C692-94	O111:H-	-	-	-	-	+	+	-	-
C651-94	O127:H40	-	-	-	-	+	-	-	-
C614-94	O128ab:H2	-	-	-	-	+	-	-	-
C728-94	O128ab:H2	-	-	-	-	+	-	-	-

Hly: Haemolysin production. Ent.: Enterohaemolysin; α: α haemolysin.
VCA: Verocell assay. VT1, VT2, *eae*A, EAF, EHEC and EAgg: DNA probes as listed in table 2.1.

2.3.7. Clinical data

The duration of diarrhoeal episodes from patients with VTEC, EPEC and *eae*A positive *E. coli* ranged from 4-30 (median: 30), 20-42 (median: 21) and 2-90 (median: 26) days respectively. One out of 4 (25%) patients infected with VTEC (O166:H28), none of the patients infected with EPEC and 11/55 (20%) patients with *eae*A positive *E. coli* stated travel abroad as being related to their diarrhoea. Three (27%) of these travellers had other enteric pathogens isolated from their stools.
Twenty four (45%) of the patients infected with 30 different serotypes of *eae*A positive *E. coli* were less than 4 years of age with a median diarrhoeal duration of 26 days. The median diarrhoeal duration was 60 and 52 days in adult patients in their twenties and thirties respectively. The clinical data from patients infected with *eae*A positive *E. coli* are summarised in table 2.11.

Table 2.11. Summary of clinical data from 53 patients from whom *eae*A positive *E. coli* was isolated during screening of diarrhoeal stools in 1994.

Age groups in years	Patients		males/ females	Duration in days	Travel abroad	Number of serotypes
	No.	%	%	median	No.	No.
0-4	24	45%	50/50	26	2	30
4-16	5	9%	60/40	3		4
20-27	10	19%	30/70	60	6	10
31-38	4	8%	25/75	52	1	5
47-59	5	9%	40/60	7	2	4
> 70	5	9%	80/20	41		6
Sum	53	100%			11	59

According to the Danish patient register (Department of health, see **7. Questionnaire investigation**) one of the patients included in the screening, and from whom no entero-pathogen was identified, developed HUS. Information on the patient slip also indicated that the patient had HUS and that an examination for enterohaemorrhagic *E.* coli was requested - yet VTEC was not identified by standard procedures. The ffct test from this patient was VT positive, yet none of 10 tested dot blots were positive in the DNA pool.

2.4. Discussion

This study examines 1.266 stools using two different methods to describe the prevalence of VTEC in Danish patients during 1994. Although there are no specimens from the winter season and spring is slightly underrepresented the stools examined are generally representative both in terms of age, sex and the year 1994.

Using DNA probes the study identified 4/737 (0.5%; 95% confidence interval: 0.1-1.4%) patients with VTEC infection. Inclusion of the 46 non-episodal specimens did not change this prevalence significantly. If this prevalence is generally applicable to the 60,000-80,000 diarrhoeal episodes per year in Denmark it means that 300-400 patients are infected with VTEC each year. At present the number of VTEC infections that are diagnosed per year in Denmark is less than 10.

The prevalence in this study is somewhat lower but comparable to similar investigations in other studies, but different methods and selection criteria renders direct comparisons difficult. Using SMAC agar (see p. 25) and examination of 3 sorbitol-positive colonies for VT production one prospective study isolated 166/5,415 (3%) VTEC strains. 29 (18%) were non-O157 VTEC of serotypes O26:H11 (11 isolates), O103:H2 (five isolates), O?:H21 (three isolates), O91:H^- (two isolates), and O145:H^-, O111:H^-, O38:H21, O6:H^- and O5:H^- (one isolate each) [299].
A prospective study in Seattle children using DNA probes to detect VT genes examined 5 colonies from each stool specimen and detected 18/445 (4%) VTEC strains. Five strains were of serotypes O26:H^- (two isolates), and O68:H^-, O153:H2 and O-:H11 (one isolate each). The remaining 13 children were infected with O157 [300].
In the USA many surveys and screenings have been focused on the detection of O157 VTEC and found a prevalence of 0,08% in unselected stools during one year [301] which was similar in the same area 9 years later [302]. Other studies have identified O157 VTEC in 19,6% of patients with bloody diarrhoea but none from patients with non-bloody diarrhoea [303], 0,4% [304] and 0,46% [305] of all stools. 54% of laboratories performing stool cultures in the USA screen either all stools or all bloody stools submitted for culture for O157 VTEC [306]. Studies of bloody stools in the UK have reported isolation rates of VTEC ranging from 5% [307] to 78% [308]. The two dominating serotypes found are O157:H^-, and O157:H7, but serotypes O26:H^-, O26:H11, and O111:H^- also seem to have a high prevalence [190],[201],[309].

Using the free faecal cytotoxin (ffct) test indicated that 14/905 (1.5%; 95% confidence interval: 0.8-2.6%) patients, (1.4% including the 106 non-episodal specimens), might be infected with VTEC because their stool supernatants had a clear VT effect on Vero cells. A further 39 specimens, corresponding to 3.8-4.3% depending on exclusion/inclusion of non-episodal specimens, had a possible VT effect on Vero cells. This rather high number of presumptive VT positive specimens clearly stresses the necessity of the use of neutralising antisera (or monoclonal antibodies) or other sensitive confirmative methods such as PCR when performing the ffct test. Neither of these supplementary methods were used in this study rendering the interpretation rather speculative. Neutralising antisera were not available during the screening period of 1994. However, as described in the Methodological studies: **8.4. VT1 and VT2 neutralising rabbit antisera**, neutralising rabbit antisera against both VT1 and VT2 have been produced and preliminary testing warrants their use in the future diagnosis of VTEC.
The isolation rate of VTEC from $ffct^+$ stools might possibly have been improved if the stool specimens had been store at 4°C (instead of the long term freezing at -20°C), and if a more immediate effort had been devoted to the isolation of VTEC either by examination of more than twenty colonies in the VCA or by dot blot/replica hybridisation. Requesting another stool specimen from the patient was also a possibility that was not examined.
The fact that VTEC was isolated from 2 of the VT and one VT? positive ffct tests clearly indicates that the ffct test may be used as a good screening method for VTEC. Furthermore the ffct test was VT positive from a patient with HUS and from whom no other enteropathogen, including VTEC, was isolated. Presumptive positive specimens should be examined with the neutralising antisera and examination for VTEC by DNA probing

intensified. However, VTEC was also isolated from a ffct test negative specimen indicating that the ffct test may fail in identifying all VTEC positive stools.

The results of the ffct screening in this study can not be directly compared with other studies because most studies have examined for free faecal Verotoxin (FVT) using neutralising antisera or antibodies supplemented with other methods. Nevertheless it is evident from both this study and others that the detection of ffct or FVT is a more sensitive method than any other method used [267]. In a Canadian study where detection of FVT was employed, evidence of VTEC infection was found in 80/9,449 (0.85%) unselected stools [310]. Stools from 6/67 (9%) FVT positive specimens yielded non-O157 VTEC isolates of O groups O26, O103 and O128. VTEC was not isolated from 13 FVT positive stools, eleven of whom had diarrhoea, including bloody diarrhoea and HUS (4 patients).
Another prospective Canadian study during 10 weeks in the summer detected free faecal Verotoxin (FVT) in 74/3,577 (2.1%) consecutive stool specimens [311]. Sixty nine FVT^+ and O157-positive stool specimens were probed with VT genes by colony blot hybridisation and yielded 38 VT gene probe positive isolates of which 22 were non-O157. Fourteen of 22 gene probe positive isolates were negative in the Vero cell assay! The 8 VCA positive isolates were of serotypes O26:H11 and O103:H25 (2 strains each) and O2:H29, O103:H2, O153:H11 and OR:H? (one strain each). In this study it was concluded that the detection of FVT in stools would enhance the diagnosis of VTEC infection threefold over cultures for *E. coli* O157 VTEC.
Another sensitive method is PCR which has been used in Belgium for over five years on 17,296 unduplicated faecal samples [158]. VT genes were found in 1.02% of the samples with non-O157 VTEC being more frequently isolated at 0.66% than O157 VTEC with an isolation rate of 0.17%. O157 was the most common serotype with 29 isolates followed by O26 and O103 (12 isolates each), O91 (11 isolates), O111 (6 isolates), O145 (5 isolates), O128 (4 isolates), and other serotypes (64 isolates).
A recent 25-week survey in a Virginia community hospital used a new EIA method for the detection of VT on selected stools (abnormal appearance) and found 11/270 (4.1%) positives. Six isolates were O157 and five were O88:H^-, O103:H^-, O111:H^- and two O-:H^- [312] .
In the paediatric population FVTs were detected in 6/227 (2,6%) children with diarrhoea but not in controls [313]. FVT^+ specimens were only examined for non-sorbitol fermenting *E. coli* and identified 3 O157. The other 3 VTEC isolates were not serotyped.

In Austria, using enzyme immunoassay (EIA) for VT1 and VT2 and SMAC on stool samples from 280 paediatric patients, three (1.1%) O157 VTEC and 4 (1.4%) VTEC of serotypes O111:H^- (two isolates), O113:H53 and O146:H^- were isolated [58].

In Germany using PCR for VT1, VT2, EHEC-hly and *eae*A, 13/468 (2.8%) of children with diarrhoea were infected with VTEC, two of which were O157 [314]. The 11 (2.4%) remaining strains were serotypes O1:H7, O9:H^-, O26:H^- (two isolates), O26:H11, O111:H^-, O119:H5, O133:H53, and ONTs with H antigens H28, H1 and H^-.

From an epidemiological point of view the DNA method is clearly to be preferred because it allows for the isolation and subsequent typing of the causative organism. The one serious drawback using this method is that the sensitivity is unknown and low because VTEC is often present in very low numbers. The proper procedure would require screening of up to 20 individual colonies or replicas.
However the ffct test with the use of neutralising antisera is a good supplement when clinical suspicion indicates a more elaborate diagnostic examination. This could be in relation to outbreaks of bloody diarrhoea, particularly in institutions, to travel abroad in areas where VTEC infections or outbreaks are reported, or when examination of stool specimens from HUS/TTP patients is performed.

Even though this study succeeds in documenting that VTEC infections are not unusual in Denmark it clearly underestimates the prevalence of VTEC. First of all the sampling procedures do not follow good collection practice which recommends that three consecutive stools should be examined from each patient. In the present study only 62/930 (6.7%) and 170/930 (18.3%) patients had three and two stools examined respectively. Except for five stools these were all received on the same day which questions whether they were taken separately. This study also excluded examination of specimens where other enteropathogens had been identified, thus underestimating concomitant infections. That these do occur was illustrated by the isolation of VTEC from a patient who was positive for *Clostridium difficile*. Furthermore the distribution of DNA probe percent positives per specimen, figure 2.3. (p. 35), and the suggested bimodal distribution of dot blot positive specimens, figure 2.4. (p. 35), gives reason to suspect that the lower detection limit was not properly identified in this study. Other studies have found that the proportion of VTEC colonies is often as low as 5 to 20% [22],[218], sometimes down to 1% [266] or less than 1% [200]. The significance of this warrants further studies.

*eae*A positive *E. coli* was isolated from approximately 5% of patients with diarrhoea making this organism the presumably second most common enteropathogen. 55% of the patients infected with *eae*A positive *E. coli* were children less than 16 years, table 2.11. (p. 43), and the mean diarrhoeal duration in adults was 1-2 months. These findings could be taken as an indication that the enteropathogenic potential of *eae*A positive *E. coli* is age-related and that they are associated with persistent diarrhoea but clearly warrants further investigation in the form of case-control studies. The inclusion of the *eae*A probe served to illustrate problems related to sensitivity. This was clearly desirable as the number of VTEC isolated would not have allowed any conclusions in this respect.

2.5. Conclusion

VTEC is not an unusual enteropathogen in Danish patients but the prevalence is low. It may be isolated from at least 0.5% of patients with diarrhoea, probably more. VTEC O157 was not identified during the prospective screening period indicating that sporadic cases of O157 are rare in Denmark.
DNA probes for VT1, VT2 and *eae*A may be pooled and used in the diagnosis of VTEC, classical EPEC and other *eae*A *E. coli*. The dot blot technique allows for the isolation of the causative organism within a time frame acceptable to a routine diagnostic laboratory and for further characterisation and typing of the strains. The sensitivity of the dot blot hybridisation method needs to be more accurately determined. As of 10th February 1997 the dot blot hybridisation method using 2 DNA probe pools and the

*eae*A DNA probe alone has been adopted as a routine procedure in the diagnosis of VTEC, EIEC, EPEC, ETEC and *eae*A positive *E. coli* in the Department of Gastrointestinal Infections, SSI. Some preliminary results from the diagnosis of VTEC in the first half of 1997 are presented in **5. Other Vero cytotoxin producing *E. coli* (VTEC)**.
The ffct test may be used as a supplement to other diagnostic tests when clinical suspicion indicates infection with VTEC. It should be replaced with the free faecal Vero toxin (FVT) test.

3. Study of selected EPEC O groups

3.1. Purpose

1. To examine a collection of *E. coli* strains originally diagnosed as EPEC and belonging to EPEC O groups O26, O55, O111 and O128 for Vero cytotoxin production.
2. Genotypical characterisation of all VTEC and a representative number of EPEC strains from the aforementioned collection, as well as strains collected during the prospective period 1994-96.

3.2. Strains

All isolates of EPEC O groups O26, O55, O111 and O128 that had been entered into the International *Escherichia* and *Klebsiella* Centre's database by the end of August 1997 were included. The isolates were isolated from faeces - primarily from children with diarrhoea - during the years 1959-1996 in Denmark. From 1994-96 only one isolate per patient was included whereas two isolates from a few patients during the period from 1959-93 were occasionally encountered and not left out of the general distribution pattern. The general distribution of a total of 1.714 *E. coli* isolates belonging to EPEC O groups O26, O55, O111 and O128 is shown in figure 3.1.

3.3. Methods

The following DNA probes were used to genotype the strains by colony dot blots: VT1 (NTP705), VT2 (DEP28), *eae*A (CVD434), EAF (JPN16), EHEC (CVD419), and EAgg (CVD432).
The FAS test was done according to methods described by Knutton *et al.* [51].

Biochemistry and phenotyping was done as described previously in Screening of diarrhoeal stools: **2.2. Materials and methods**.

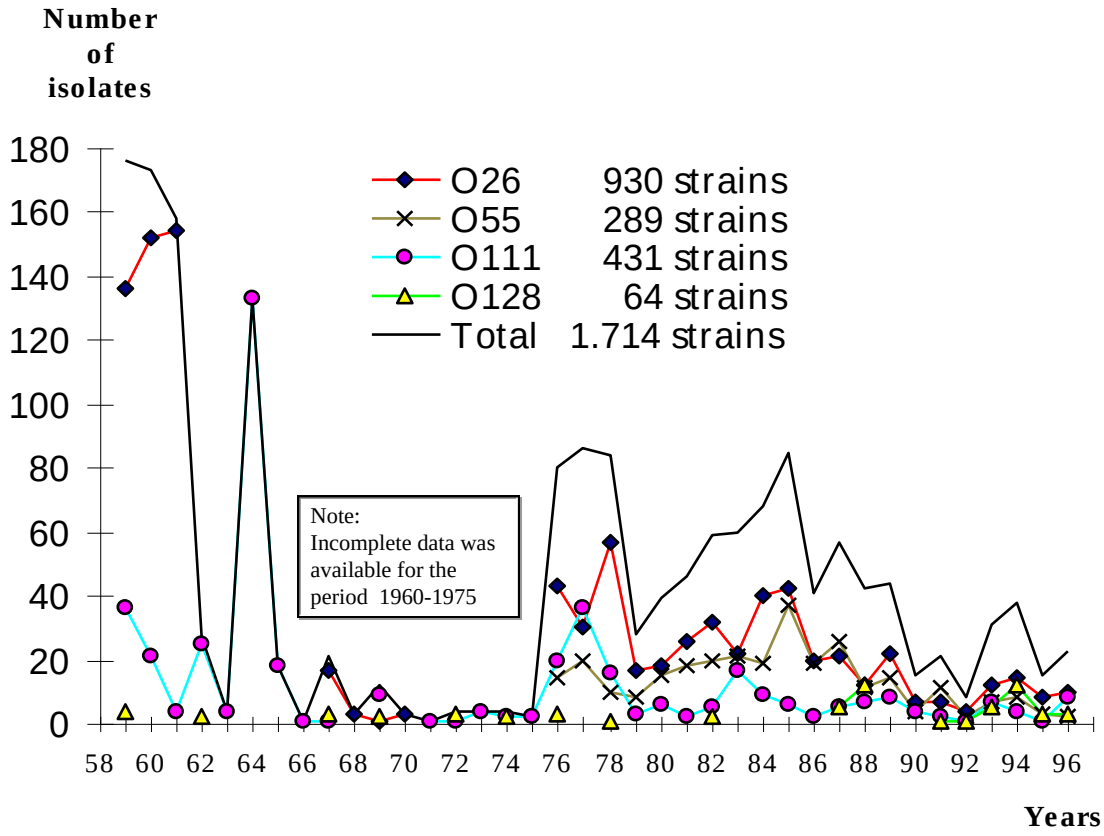

Figure 3.1. Distribution of 1.714 EPEC strains of O groups O26, O55, O111 and O128 isolated in Denmark from 1959-1996.

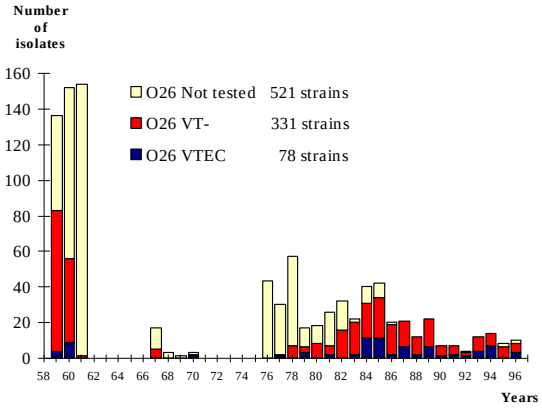

Figure 3.2. Distribution of 930 isolates of EPEC O group O26.

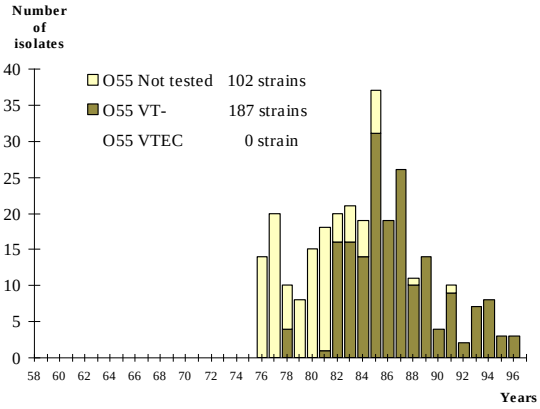

Figure 3.3. Distribution of 289 isolates of EPEC O group O55.

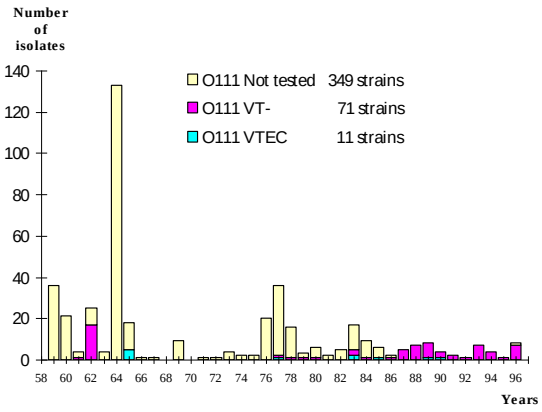

Figure 3.4. Distribution of 431 isolates of EPEC O group O111.

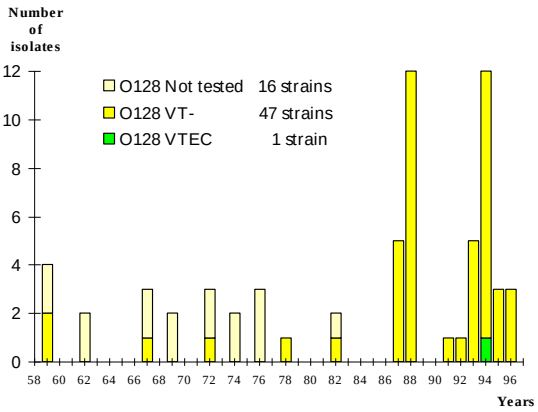

Figure 3.5. Distribution of 64 isolates of EPEC O group O128.

3.4. Results

A total of 729/1.714 (43%) *E. coli* strains from this collection were tested for Vero cytotoxin production in the Vero cell assay. Four hundred and nine out of 930 (44%) O26, 190/289 (66%) O55, 82/431 (19%) O111, and 48/64 (75%) O128 isolates were tested. Priority was given to strains that were isolated within the most recent decade but some early strains were also examined.
The distribution of available and tested isolates from each of the four O groups is shown in figures 3.2, 3.3, 3.4, and 3.5.

Ninety out of 729 (12,3%) of the tested strains were positive in the Vero cell assay. The 90 isolates originated from 83 patients and one strain from each of these patients were further analysed.
None of the 190 O55 strains were VTEC. 171 of the tested O55 strains belonged to serotypes O55:H6, O55:H7 and O55H-. The remaining 19 isolates were of various other H types. Therefore the O55 strains were not further analysed.

One hundred and seventy nine (all 83 VTEC, all available VT^- strains from the last decade (81) and 15 earlier strains from EPEC O groups O26, O111 and O128 were genotyped with DNA probes. The majority of these strains belonged to the classical EPEC O:H serotypes. Only one isolate per patient was characterised. The distribution of the 179 genotyped VTEC and VT^- strains is shown in figure 3.6.

Of 179 strains, many of which were originally characterised as EPEC, 58% (69 VTEC and 14 VT^- strains) were $EHEC^+$ whereas only 3.5% (0 VTEC and 5 VT^-) were EAF^+.

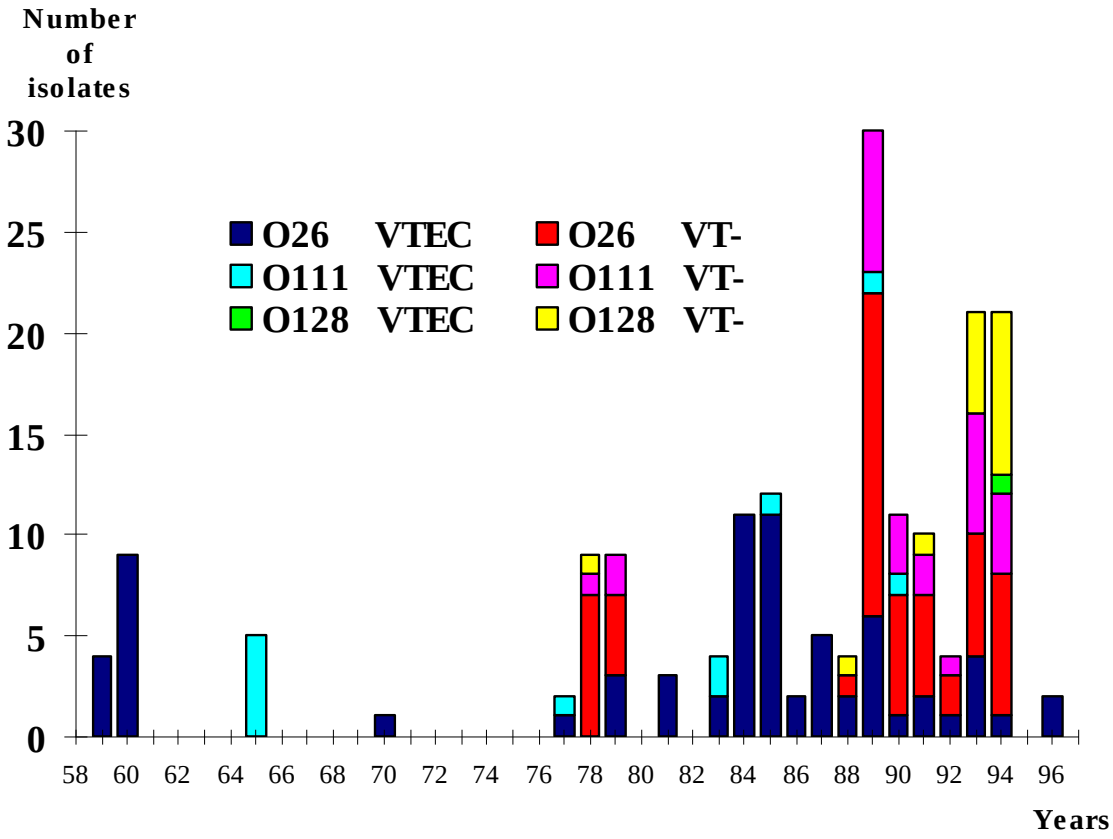

Figure 3.6. Distribution of 179 strains of EPEC O groups O26, O111 and O128 and their ability to produce Vero cytotoxin.

3.4.1. O group O26

The earliest VTEC strain serotype O26:H11 was isolated from a one-and-half year old child in 1959.

Figure 3.2. shows the distribution of O group O26.

Two hundred and fifty seven (27%) and 577 (62%) strains were H11 and H^- respectively. The remaining 96 strains belonged to 14 different H-types and this group was considered to be too heterogeneous to allow for any general conclusions. Consequently these strains were not included in the testing for VT production.
Seventy eight out of 409 (19%) of O26 strains tested in the Vero cell assay were VTEC.

One hundred and twenty five (71 VTEC and 54 VT$^-$) strains of O:H serotypes O26:H11, H21, H46 and O26:H$^-$ were further characterised.
Figure 3.7. shows the characteristics of O26 VTEC and VT$^-$.

Sixty four out of 71 (90%) of the O26 VTEC strains were *eae*A$^+$, VT1$^+$ and EHEC$^+$.
Only 2 (3%) VTEC O26 strains were *eae*A$^+$, VT2$^+$, EHEC$^+$ and one strain *eae*A$^+$, VT1$^+$, VT2$^+$, EHEC$^+$.
Sixty seven (94%) VTEC O26 strains were EHEC$^+$.
All motile strains were H11 except one H21.

Fifty three out of 54 (98%) VT$^-$ O26 strains were *eae*A$^+$, and a subgroup third, 18/54 (33%), were also EHEC$^+$. One strain was negative with all the DNA probes.

Eighty five out of 125 (68%) O26 strains hybridised with the EHEC (CVD419) probe whereas none hybridised with the EAF or EAgg probes.

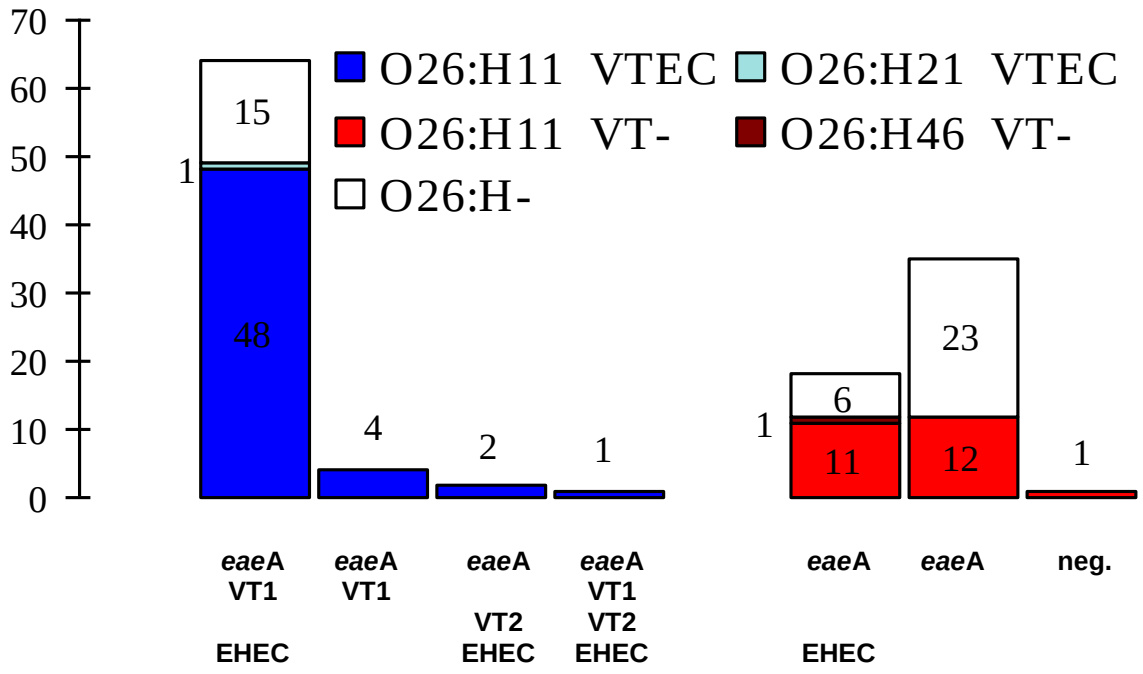

Figure 3.7. H types and genotypical characteristics of EPEC O group O26: 71 VTEC and 54 VT$^-$ strains.

3.4.2. O group O111

The earliest VTEC strain serotype O111:H$^-$ was isolated from an infant in 1965.

Figure 3.4. shows the distribution of O group O111.
Eleven out of 82 (13%) of O111 strains tested in the Vero cell assay were VTEC and were of serotypes O111:H8 and H$^-$.
Thirty seven (11 VTEC and 26 VT$^-$) strains of O group O111 with H antigens H2, H8, H9, H11, H12, H21, H25 and H$^-$ were further characterised.

Figure 3.8. shows the characteristics of O111 VTEC and VT$^-$.

All 11 (100%) O111:H8 or H$^-$ were VT1$^+$.
Three out of 11 (27%) VTEC strains were *eae*A$^+$, EHEC$^+$.
Seven (64%) VTEC strains were *eae*A$^+$.
One (9%) strain did not hybridise with any other probe than VT1.

Five out of 26 (19%) VT$^-$ O111:H2 or H$^-$ were *eae*A$^+$, EAF$^+$.
Four (16%) VT$^-$ O111:H25 or H$^-$ strains were *eae*A$^+$.
Six (23%) VT$^-$ O111:H21 or H$^-$ strains were Agg$^+$.
Eleven (42%) VT$^-$ O111:H9, H11, H12, H21 or H$^-$ strains were DNA probe negative.

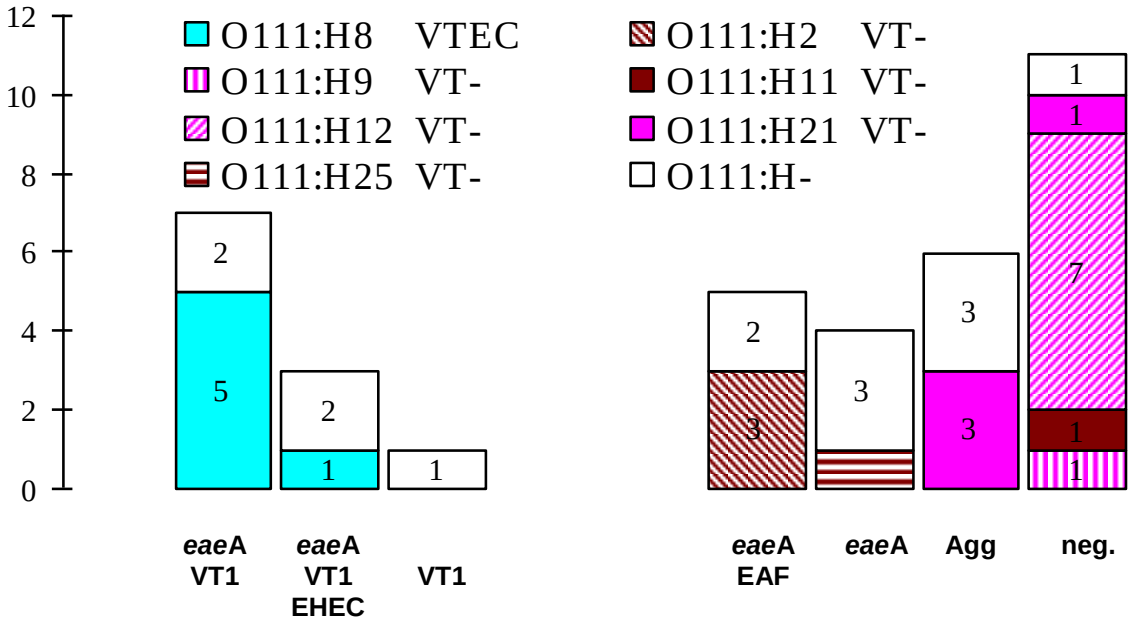

Figure 3.8. H types and genotypical characteristics of EPEC O group O111: 11 VTEC and 26 VT$^-$ strains.

Nine out of 26 (35%) VT$^-$ O111 strains with H antigens H2 or H25 possessed virulence genes that are associated with EPEC and 6 (23%) with H21 to EAggEC.
Eleven out of 26 (42%) VT$^-$ O111 strains with H antigens H9, H11, H12, H21 or H$^-$ did not possess any of the virulence genes tested for.

3.4.3. O group O128

The only VTEC strain serotype O128:H2 was isolated from a 20-year-old man in 1994.

Figure 3.5. shows the distribution of O group O128.
One out of 48 (2%) O128 strains serotype O128:H2 tested in the Vero cell assay was VTEC.

Eighteen (1 VTEC and 17 VT$^-$) strains of O group O128 with H antigens H2, H8, H10, H12, H35 and H45 were further characterised.

Figure 3.9. shows the characteristics of O128 VTEC and VT$^-$.

One out of 18 (6%) VTEC O128 strains was serotype O128:H2 and VT1$^+$, VT2$^+$, EHEC$^+$.

Nine out of 17 (53%) VT$^-$ O128:H2 or H8 strains were *eae*A$^+$.
One (6%) VT$^-$ O128:H2 strain was *eae*A$^+$, EAF$^+$.
One (6%) VT$^-$ O128:H35 strain was Agg$^+$
Six (35%) VT$^-$ O128:H2, H10, H12, H45 or H$^-$ strains were negative with all DNA probes.

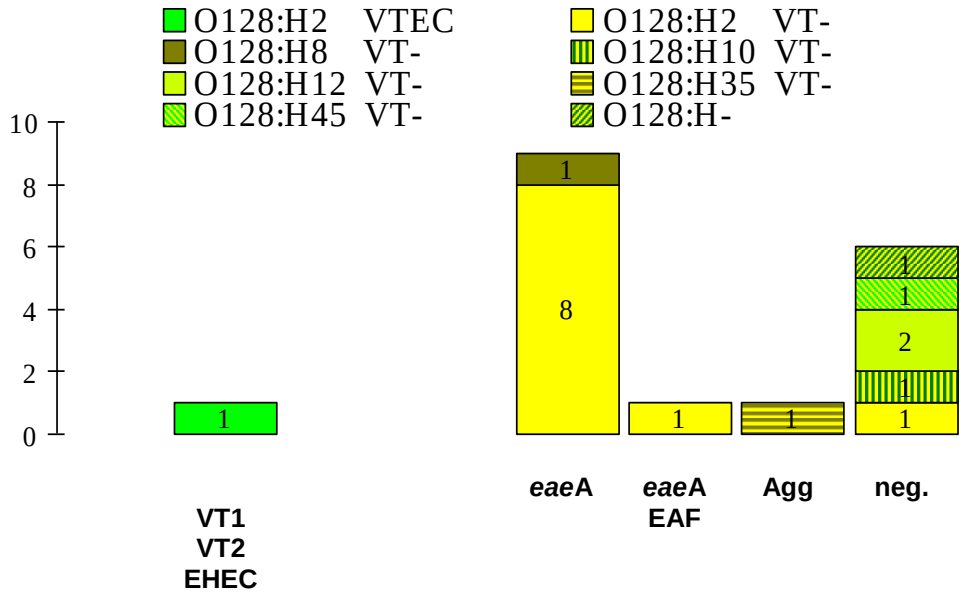

Figure 3.9. H types and genotypical characteristics of EPEC O group O128: 1 VTEC and 17 VT$^-$ strains.

3.5. Discussion

The data presented here are in no way representative for the period studied, however no outbreaks with any of the examined types were evident. The study demonstrates that VTEC, and VTEC O26 in particular, has been present in Denmark for at least the past four decades, probably longer. There is therefore no obvious evidence that VTEC as such is an emerging pathogen. The prevalence of the four examined O groups, O26, O55, O111 and O128, seem to be declining with time, figure 3.1. This could either represent a true decrease of the prevalence of EPEC/VTEC or it could reflect a declining awareness of EPEC/VTEC as pathogens. This trend has certainly been amplified by a change in routine diagnosis of EPEC in 1993. Before 1993 all stools from children less than 4 years of age were slide agglutinated in standard EPEC antisera but in 1993 this was changed so that EPEC was only examined for when requested by the submitting physician. Because EPEC were traditionally only considered relevant in relation to institutional outbreaks, which have not occurred since the 60s and 70s, the number of requests may have dropped even further. The decentralisation of the diagnosis of enteropathogens in the beginning of the 1990s has also resulted in a decrease in the number of isolates that are received for typing at the International *Escherichia* and *Klebsiella* Centre. It is quite conceivable that EPEC, and attaching and effacing (A/E) *E. coli* in general, may be increasingly overlooked and much more common as diarrhoeal pathogens than currently recognised. This has been illustrated and discussed in the previous chapter and is supported by new evidence from a North American paediatric hospital, Seattle, Washington where *eae*A$^+$ *E. coli* was isolated from 25/445 (5.6%) children in a prospective study [82]. Five of these were VTEC. The etiologic role in both childhood and adult diarrhoea of *eae*A$^+$ *E. coli* warrants further elucidation.

For certain O:H serotypes the study illustrates very nicely that currently recognised virulence factors of *E. coli* enteropathogens belong to a limited number of O:H serotypes: O111:H8 and H$^-$ is VTEC, O111:H2 is EPEC and O111:H21 is EAggEC. At the same time the study challenges this notion because O26:H11 and H$^-$ are shown to consist of strains that are quite heterogeneous with respect to virulence genes and that O26 strains may either be classified as EPEC or VTEC. A third of the O26 VT$^-$ strains were EHEC$^+$ which raises the question of whether these strains represent original EPEC strains that have acquired an EHEC virulence factor or if they represent original VTEC strains that have lost their VT genes. The loss of the VT property was recorded in a few strains (O26 and O128) during this study but was not examined in detail.

The findings of this study are supported by other similar retrospective examinations of classical EPEC strains. The reclassification of EPEC strains as VTEC began in 1977 when Konowalchuk and colleges first described the production of Vero cytotoxin by O26 and O111 strains [18]. In a study of O26:H11 from infant diarrhoea (25 patients), infant cases of HUS (4) and calves with scours (8) in the UK 27/37 (73%) were VTEC. 26 strains were VT1 and one was VT2. 24/27 (89%) VTEC and 5/10 (50%) VT$^-$ were positive with the EHEC (CVD419) probe [54]. EHEC positivity corresponded 100% with the production of enterohaemolysin. All strains were negative with the EAF probe but 36/37 (97%) strains showed LA and were FAS positive.
More recently, examination of *E. coli* from 925 cases of diarrhoea using gene probes and adhesion tests identified 41/128 (32%) O26 as *eae*A$^+$ and VTEC. Thirty five, 1, 1, and 4 strains were of H types H11, H21, H32 and H$^-$ respectively [83]. Two out 116 (1.7%) O55:H7 strains were *eae*A$^+$ VTEC, 0/92 O111 strains were VTEC (but O111:H8 were not included in the study), and 13/140 (9%) O128 strains were VTEC (1 *eae*A$^+$ H25, 12 H2 and 1 H$^-$). Less than 10% of all the strains hybridised with the EAF probe.

Clonal examination of serogroup O111 in Brazil also found 4 VT1, *eae*A$^+$, three of which also were positive with EHEC (CVD419) and enterohaemolysin positive [315]. Recently in Italy 8/55 (15%) EPEC strains from children were actually VTEC of O groups O26, O111 and O128 [107]. A retrospective examination of 3.065 *E. coli* isolated between 1958 and 1992 from different countries in Asia identified 64 VTEC comprising 11 O groups. O26 (6 VTEC strains) and O111 (8 strains) were among the four most common VTEC O groups [316].
No VTEC strains within EPEC O group O55 were found in this study. O55 VTEC has been reported from England [309],[83], Germany [317] and the Czech Republic [213]. These have been individual cases of H types H7, H10, H? and H$^-$. In view of the fact that only the more recent isolates of the Danish O55 isolates were tested for VT production it is not surprising that VTEC was not found.

3.6. Conclusion

The study demonstrated that approximately 20% of Danish strains within O group O26 originally diagnosed as EPEC was actually VTEC and that VTEC was also found within O groups O111 and O128. The earliest VTEC strains were isolated in 1959 and the study does not provide evidence that VTEC as such is an emerging pathogen. VTEC was not found in a limited number of O55 strains.
The characterised VTEC strains belong to a few O:H serotypes which do not possess virulence factors that are usually associated with EPEC but more so with EHEC, a subgroup of VTEC causing bloody diarrhoea.
These results raise questions about the aetiology of diarrhoea in Denmark.
They confirm that EHEC$^+$ strains are quite common among diarrhoeagenic *E.* coli.
It is confirmed that EAF$^+$ *E. coli* strains are rare in Europe.
Laboratories which routinely diagnose EPEC should also have presumptive positive strains tested for the production of Vero cytotoxin.

4. O group O157

4.1. Methods and strains

Humans: Diagnosis of O157 VTEC began in 1986 at SSI with the implementation of the Vero cell assay and routine screening by slide agglutination. During an unknown period from the end of the 80s to the beginning of the 90s blue plates (modified Drigalski), where lactose was replaced by sorbitol, were used when O157 was suspected. The only other hospital which has screened for O157 is the Herlev county hospital which covers the Copenhagen county area. However due to the low number of O157 infections this practice was abandoned. Therefore, diagnosis of O157 in humans has primarily been based on clinical criteria.

Animal and retail food: Very limited data about the prevalence of O157 in the animal reservoir and retail food has been presented in Denmark. Most of the presumptive positive findings of O157 have been submitted to The International *Escherichia* and *Klebsiella* Centre (WHO) for typing. Permission to present preliminary data on these strains has been granted by each of the individuals concerned (see appendix: **11.3. Permissions.**

Characterisation: The strains were characterised as previously described in the study of selected EPEC O groups. In addition all strains were hybridised with ETEC toxin gene probes LT, ST_h and ST_p, and some strains with the enteroaggregative heat-stable toxin probe (EAST1), see tables 8.2 and 8.3 (p. 85-86) of DNA probes in Methodological studies: **8.5. DNA probes**.

Phage typing was kindly performed by Thomas Cheasty, Laboratory of Enteric Pathogens (LEP), Central Public Health Laboratory at Colindale Avenue in London.

4.2. Results

A total of 66 (46 strains of human origin, 7 strains of bovine and porcine origin and 13 strains from retail minced beef), representing of 45 VTEC O157:H7 or H^- strains and 21 VT^- O157 of various H types were included.

4.2.1. O157 VTEC

None of the O157 strains hybridised with any of the ETEC toxin gene probes.
Five of 5 tested VTEC O157 strains and 9/15 (60%) VT^- O157 of human origin were positive with the EAST1 probe. All 7 animal strains were $EAST1^+$, and 10/13 (77%) strains from retail meat were $EAST1^+$.
The characteristics of VTEC O157 strains of human, bovine and retail minced beef origin are shown in figure 4.1. All O157 VTEC were sorbitol negative within 24 hours and all but one did not produce β-glucuronidase on PGUA plates. Two strains produced urease.

All 45 VTEC O157 strains hybridised with the *eae*A and EHEC (CVD419) probes.
18/45 (40%) were positive with both VT1 and VT2, 25 (56%) with VT2 alone and 2 (4%) with VT1 alone.

Clinical data was available for 14 patients. 3 HUS cases and one presumed HUS case were reported. All three confirmed cases were caused by O157:H7 *eae*A^{+}, VT2^{+}, EHEC^{+} strains. Nine patients had bloody diarrhoea and one was a healthy carrier.

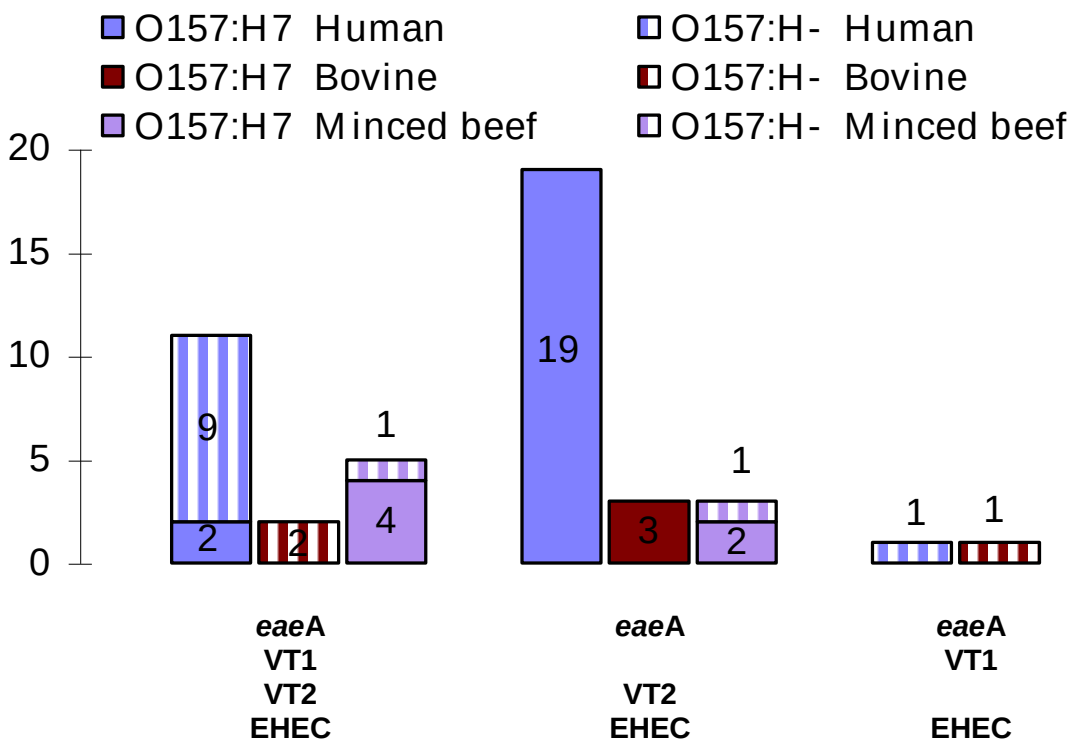

Figure 4.1. H types and genotypical characteristics of 45 VTEC O157:H7 and H^{-} strains: 31 human, 6 bovine and 8 retail meat; 6 minced beef and 2 minced pork with traces of bovine serum albumin (BSA).

4.2.2. O157 VT^{-}

A total of 21 O157 VT^{-} strains were also submitted for typing. 15 were isolated from patients with diarrhoea, 1 from pig diarrhoea and 5 were isolated retail minced beef or pork meat.
The characteristics of VT^{-} O157 strains of human, bovine and retail meat origin are shown in figure 4.2. Seventeen (81%) of the VT^{-} strains were sorbitol positive and produced β-glucuronidase on PGUA plates. One strain did not produce β-glucuronidase and 3 were sorbitol negative.

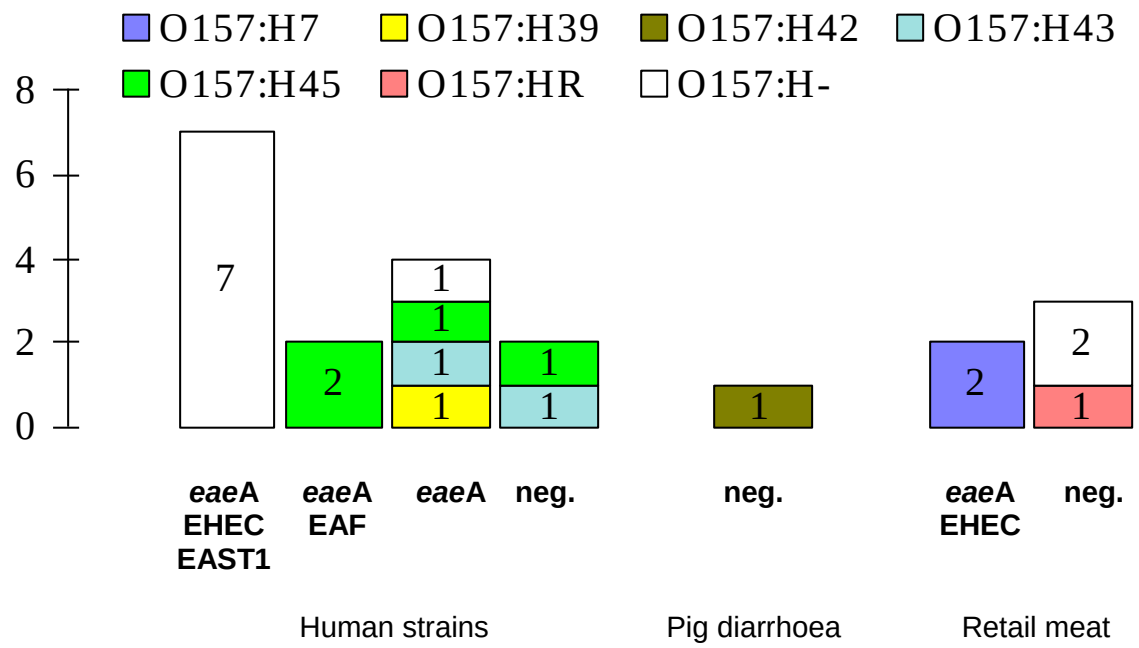

Figure 4.2. H types and genotypical characteristics of 21 VT$^-$ O157: 15 human, 1 pig diarrhoea and 5 retail bovine and pork meat.

4.2.3. Phage typing of O157

51 (49 VTEC and 2 VT$^-$) strains were phage typed at LEP, PHLS in London. This number was reduced to 43 after weeding out duplicate- and control strains.
The most common phage types (PT) were PT4 with 12 strains (28%), PT14 with 6 strains (14%), and PT 2 and PT23 with 4 strains (9%) each.
All the phage types were evenly distributed over the study period from 1987-1997 except for PT23 which was found only in retail meat during a screening for VTEC O157 conducted by The Danish Food Agency in 1996, see table 4.2.
Eleven of 34 (32%) VTEC strains were VT2 of PT4. All other combinations were represented by only 1-4 strains each. The correlation of VT production with phage type is shown in table 4.3.

Table 4.2. Distribution of phage types of O157 by year.

Phage type	1987	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	Total
NT		1										1
2							1	1	1		1	4
4	2			1	2	1	2		1 *	1	2	12
8			1			1						2
14				1		2		1	2			6
21									1			1
23										4		4
34					1							1
41						1						1
49					1					1		2
50								1				1
54										1		1
82										1		1
RDNC					1					1	1 *	3
Rough								1		2		3
Total	2	1	1	2	5	5	3	4	5	11	4	43

*: VT^- strains.
RDNC: Reactions do not conform to standard patterns; NT: Not typed due to poor growth.

Table 4.3. Correlation of VT production with phage type of O157 from humans, cattle and retail meat.

VT production	Phage type												
	2	4	8	14	21	23	34	41	49	50	54	82	Total
VT1 & VT2	1		2	4	1	4			1	1			14
VT1				1									1
VT2	3	11		1			1	1	1		1	1	20
VT^-		1											1
No. strains	4	12	2	6	1	4	1	1	2	1	1	1	36

4.2.4. Discussion

The isolation of a few O157 VTEC strains each year since 1987 indicates that O157 is not a big public health problem in Denmark. No Danish outbreak of O157 has been recorded in the last decade. One HUS case was related to a recent outbreak of O157 on the island of Fuerteventura . The patient, a four-and-a-half year old girl developed symptoms more than a month after returning from a holiday on the Canarian island and was supposedly infected by her younger brother who was a healthy carrier for more than one month.
All the human VT2$^+$ strains were O157:H7 whereas 9/11 VT1, VT2 strains were non-motile. The phage types were different from O157 phage types found in the UK where the majority of VT1, VT2 strains were phage type 1, or the related types 4, 8 and 14 and VT2 strains were phage type 2 or 49 [318]. The heterogeneous distribution of phage types (table 4.2.) and genotypes (table 4.3.) indicated that there is no common source for O157 infection in Denmark.
Almost half of the human strains were VT$^-$ and 13/15 strains were *eae*A$^+$. These findings stresses the importance of having presumptive O157 VTEC strains examined for VT production. However, the sorbitol-negative-β-glucuronidase negative phenotype is highly indicative of O157 VTEC (Fisher's exact test: $p < 0.00001$) in human isolates. The isolation of *eae*A$^+$ O157 VT$^-$ highlights the relevance of further examination of the etiologic role of *eae*A$^+$ *E. coli*.
The reasons for the low prevalence of O157 in Denmark are unknown. One possible explanation could be a low prevalence in the bovine reservoir. However, VTEC has been found in 16.8% of Danish cattle. Unfortunately the isolates were not serotyped [319]. Approximately 1% of Danish cattle presented at slaughter are positive for O157 VTEC (Jeppe Boel, personal communication) which is comparable to that of other countries. Another reason for the low prevalence could be good slaughtering practises. One study was not able to isolate O157 in retail beef meat [320] but as described in this chapter O157 has been isolated from 2/1,584 (0.13%) minced beef and 2/528 (0.4%) pork meat samples [321]. It has also been speculated that because the Danes eat meat from older animals compared to other countries the infection pressure would be lower because the carriage rate has been described as higher in young animals than in older animals.
A third reason could be a high standard hygiene in private as well as commercial kitchens. Danish O157 VTEC could be less virulent than O157 from other countries but this does not seem plausible as the HUS attack rate of Danish O157 VTEC, 2/46 (4.3%), is comparable to what is recorded world-wide [247],[255],[322],[323].

5. Other Vero cytotoxin producing *E. coli* (VTEC)

5.1. Strains and methods

Strains: In this context other Vero toxin producing *E. coli* (VTEC) were defined as non-O157 strains which were **not** described in **3. Study of selected EPEC O groups** O26, O55, O111 and O128. Five of these strains were received for typing at the International *Escherichia* and *Klebsiella* Centre based on clinical criteria in which a VTEC infection had been suspected prior to the study period. Ten strains were isolated during the most recent prospective period in the Department of Gastrointestinal Infections. In the prospective period which began 10th February 1997 all bloody stools from children less than 4 years were routinely examined for the presence of VTEC by the colony dot blot hybridisation procedure described in **8.5. DNA probes** with a DNA probe pool consisting of VT1, VT2 and *ipa*H (which detects both *Shigella* spp. and EIEC) instead of VT1, VT2 and *eae*A as described in Screening of diarrhoeal stools: **2.2. Materials and methods**.

5.2. Results

In 1993 two cases of urinary tract infection (UTI) caused by Verotoxin producing *E. coli* (VTEC) were diagnosed at Rigshospitalet in Copenhagen. None of the two patients had any previous history of diarrhoea.
VTEC OX3:H2 VT1, VT2 and EHEC$^+$ was isolated from the urine of a 33-year-old man with a urinary tract infection developing into haemolytic uraemic syndrome (HUS) requiring dialysis, plasma exchange and assisted ventilation.
VTEC O145:H28 VT1, EHEC$^+$ and *eae*A$^+$ was isolated from the urine of a 3-week-old boy with a UTI developing into HUS.
Both strains produced enterohaemolysin.
The third UTI strain was of serotype O103:H2 VT1, EHEC$^+$ and *eae*A$^+$ and was isolated from an elderly woman in 1968. Very limited clinical data was available but anaemia was indicated.

O103:H2 VT1, EHEC$^+$ and *eae*A$^+$ in two other patients resulted in incomplete HUS (preliminary clinical data) in a three-year-old girl with bloody diarrhoea, and HUS in a one-year-and-four-months-old boy.

For the remainder of strains clinical data was not yet available at the end of this study.

Seven patients were less than 5 years and infected with 4 VT1, EHEC$^+$ and *eae*A$^+$ strains of serotypes O26:H$^-$, O123:H49, O145:H4, O145:H28 (two patients) and OR:HR, and one VT2, EHEC$^+$ and *eae*A$^+$ of serotype O121:H19.
No common types were isolated from the remaining four adults from whom O11:H49 VT2, EHEC$^+$, O91:H$^-$ VT1, VT2, EHEC$^+$, O113:H4 VT2, EAF$^+$ (!), and O117:K1:H7 VT1 were isolated.

Table 5.1. The table shows other VTEC as defined in strains and methods isolated in Denmark and submitted for typing before 1997 and strains isolated in the routine diagnosis of VTEC in the Department of Gastrointestinal Infections, SSI in the first six months of 1997.

Strain designation					DNA probes					
C number	Serotype	Patient age	Sex	Source	VT1	VT2	*eae* A	EHEC	EAF	EAST1
C 246-97	O11:H49	22 yrs	M	F	-	+	-	+	-	-
C 492-97	O26:H-	1 yr 10 m	F	F	+	-	+	+	-	+
C 260-97	O91:H-	43 yrs	M	F	+	+	-	+	-	-
C 524-68	O103:H2	69 yrs	F	U	+	-	+	+	-	-
C 198-94	O103:H2	3 yrs	F	BD	+	-	+	+	-	-
C 232-97	O103:H2	1 yr 4 m	M	HUS, F	+	-	+	+	-	-
C 86-97	O113:H4	37 yrs	F	F	-	+	-	-	+	-
C 363-97	O117:K1:H7	24 yrs	F	F	+	-	-	-	-	-
C 390-97	O121:H19	1 yr 4 m	F	F	-	+	+	+	-	+
C 488-97C	O123:H49	1 yr	M	F	+	-	+	+	-	+
C 357-97	O145:H4	1 yr 9 m	M	F	-	+	-	+	-	+
C 603-93	O145:H28	1 m	M	HUS, U	+	-	+	+	-	
C 123-97	O145:H28	1 yr 5 m	F	F	+	-	+	+	-	+
C 258-93	OX3:H2	33 yrs	M	HUS, U	+	+	-	+	-	
C 479-94	OR:HR	1 yr 5 m	F	F	+	-	+	+	-	ND

Sex. M: male; F: female. Source: F: faecal specimen; U: extract stab culture isolated from the urine of a patient with a urinary tract infection; BD: extract stab culture isolated from stool of a patient with bloody diarrhoea and preliminary data suggesting incomplete HUS; HUS, F: extract stab culture isolated from stool of a patient with haemolytic uraemic syndrome (HUS); HUS, U: extract stab culture isolated from the urine of a patient with haemolytic uraemic syndrome (HUS). ND: Not done.

5.3. Discussion

The O:H serotypes isolated from two of the three patients with UTI in this study do not belong to any of the well-established groups of pathogenic *E. coli* that may cause intestinal or extraintestinal disease. They are unique in the sense that they have not previously been isolated from humans.
In Australia OX3:H21 VTEC was isolated from a case of sudden infant death syndrome (SIDS) [324], and recently in Finland from a woman with acute bloody diarrhoea followed by HUS [223]. OX3:H2 has also been isolated from healthy sheep [252].
O145:H^- was described as being associated with HUS already in 1985 [218] and has been isolated from patients with diarrhoea [190], hemorrhagic colitis [325] and HUS [57]. O145:H^- has also caused an outbreak of HC (HUS) in Japan [61]. O145:H28 has been isolated from diarrhoea calves [326] but never before from humans. However, both OX3:H2 and O145:H28 VTEC have been isolated from the faeces of Danish cattle at slaughter (Jeppe Boel, Danish National Food Agency, personal communication).

O103:H2 was isolated from three patients which either had HUS or indications of incomplete HUS. O103:H2 has been identified as a potential agent of HUS in France [220] and isolated from a six-year-old girl with HUS after a UTI [260]. Patients with HUS in Italy develop antibodies to the O103 LPS O antigen [295].

As reviewed in the discussion in Screening of diarrhoeal stools, **2.4. Discussion** the isolation of a variety of VT positive sero- and genotypes is to be expected when detection of VT genes is being applied in the diagnosis of VTEC. In this respect VTEC infections in Danish patients do not differ from VTEC infections found in other parts of the world. Nine of the isolated O groups in this study have been associated with the development of HUS as shown in table 1.4. (p.23). This emphasises the importance of the continued diagnosis of VTEC from Danish patients.

6. Haemolysin production

6.1. Strains and methods

A total of 224 strains characterised in the EPEC study (158 strains) and the O157 study (66 strains) were included. The strains were described by their serotype and genotype as recorded previously, and by their phenotype on washed blood agar using 5% defibrinated sheep blood [185].

6.2. Haemolysin production of O groups O26, O111 and O128

Sixty out of 83 (72%) VTEC and 17/75 (23%) of VT^- *E. coli* produced enterohaemolysin on sheep blood plates, see table 6.1. 22 VT^- strains from the EPEC study were not tested.

Table 6.1. Phenotypical and genotypical characterisation of haemolysin production of VTEC and $eaeA^+$ *E. coli* belonging to EPEC O groups O26, O111 and O128.

Haemolysin production on washed sheep blood agar	EHEC (CVD419) DNA probe	VTEC			VT^-			
		O26	O111	O128	O26	O111	O128	Total
α	-				11			11
α	+	2			1			3
Ent.	+	55	2	1	11			69
Ent.	-	1	1		4	2		8
+	+	2	1		3			6
+	-	2						2
-	+	8			1			9
-	-	1	7		8	20	14	50
Sum		71	11	1	39	22	14	158

α: α haemolysin.
Ent.: Enterohaemolysin.
+: Haemolysin positive but no clear determination of α- or enterohaemolysin.

Of 87 strains hybridising with the EHEC probe 69 strains (79%) were phenotypically positive for haemolysin production. 58 of these (84%) were VTEC. A further 6 strains (7%) showed haemolytic activity - usually weak - on sheep blood agar plates which could not be characterised as α- or enterohaemolysin.

The production of α haemolysin indicated by a clear, haemolytic zone on the sheep blood agar plates after 3-4 hours of growth, was found only in O group O26. Whereas only 2/71 (2,8%) of VTEC strains produced α haemolysin, 12/39 (31%) tested VT^- strains produced α haemolysin. Thus α haemolysin producing O26 strains were VT^- ($\chi^2 = 17.71$; $p < 0.0001$).

6.3. Haemolysin production of O157

Forty two out of 45 (93%) O157 VTEC and 4/21 (19%) VT^- O157 *E. coli* produced entero-haemolysin on sheep blood plates, see table 6.2. Three VTEC strains were α haemolysin positive. O157 VTEC was associated with the enterohaemolytic phenotype ($\chi^2 = 37,41$; $p < 0.0001$).

Table 6.2. Phenotypical and genotypical characterisation of haemolysin production of VTEC and VT^- *E. coli* belonging to O group O157 isolated from patients, cattle and retail meat.

Haemolysin production on washed sheep blood agar	EHEC (CVD419) DNA probe	VTEC O157			VT^- O157			
		human	bovine	retail	human	pig diarrhoea	retail	Total
α	+	3						3
Ent.	+	28	6	8	2		2	46
-	+				5			5
-	-				8	1	3	12
Sum		31	6	8	15	1	5	66

ND: Not done; retail: Minced beef and pork meat.
α: α haemolysin production; Ent.: enterohaemolysin production.

Forty six out of 54 (85%) of $EHEC^+$ O157 strains were enterohaemolysin positive, 3 (6%) α haemolysin producing and 5 (9%) were haemolysin negative.

6.4. Discussion

The use of sheep blood agar plates has been suggested as a diagnostic tool for the detection of VTEC because of the close association between enterohaemolysin- and verotoxin production [185],[252], and because of the increased probability of diagnosing both O157 and non-O157 VTEC strains [190],[191],[327]. This would undoubtedly increase the overall isolation rate of VTEC in general but would also miss a certain percentage of VTEC and identify false positives. In a study of German O111 VTEC the primary screening on blood agar plates was found unsuitable because only 66% of the strains showed the enterohaemolytic activity [177]. In this study 72% of VTEC were enterohaemolysin producing which is somewhat lower but comparable to other studies where 88% [190] and 91% [327] of VTEC produced enterohaemolysin. However, the 18% of VT^- *E. coli* that were enterohaemolysin positive in this study is somewhat higher than that found in one other study which found 3,4% of VT^- *E. coli* strains enterohaemolytic [191]. This difference could certainly reflect a strong selection bias in the present study. The phenotypic sheep blood agar assay failed to detect 13/71 (18%) of VTEC and 5/16 (31%) of VT^- *E. coli* that hybridised with the EHEC probe thought to be specific for the enterohaemolysin gene. There may be several explanations for this. First of all it has been observed that most enterohaemolysin positive O26:H11 strains may occasionally produce variant colonies that do not give zones of haemolysis and that these strains are EHEC probe negative due to the loss of the EHEC plasmid [54]. The loss of plasmids could also occur in O groups O111 and O128. In this study the same colonies were not always used for geno- and phenotyping which could explain some of the differences. Some O111 VTEC strains apparently carry silent EHEC Hly genes [177]. This could also occur in other O groups. Batch to batch differences in the sheep blood plates and the significance of plate age were not studied and might account for some discrepancies. The activity of the O157 enterohaemolysin is calcium dependent [328] and both secretion [328] as well no secretion [179] of the toxin has been described. The EHEC enterohaemolysin locus in non-O157 strains has only been characterised in one O111 strain [177] and showed nearly 100% identity with the O157 EHEC Hly gene. O26 EHEC enterohaemolysin genes have not been characterised but genetic variation and differences in the secretion is a possibility.

Eight strains (2 VTEC and 6 VT^-) gave enterohaemolytic phenotypes but did not hybridise with the EHEC probe indicating that the enterohaemolytic phenotype may be determined by other enterohaemolysin genes such as the Ehly1- and Ehly2 genes [187],[188].

Eleven out of 14 (79%) α haemolysin positive O26 strains did not hybridise with the EHEC probe. It may be concluded that the production of α haemolysin in O26 strains is encoded by genes different from the plasmid encoded O157 enterohaemolysin. It is important to note that the 61,4% homology between the EHEC enterohaemolysin structural gene (EHEC-*hly*A) and the chromosomally encoded α haemolysin gene (α-*hly*A) as well as the 64% homology of the corresponding *hly*B genes [179] do not result in cross reactivity between the EHEC gene probe (CVD419) which covers the EHEC-*hly*A and part of the EHEC-*hly*B genes, and the α-*hly*AB genes. The enterohaemolysin phenotype is not detected in α haemolysin producing strains on sheep blood agar. Therefore the α phenotype of VTEC will always require further analysis either by using plates containing unwashed sheep erythrocytes, which are only sensitive to α haemolysin, or by hybridisation with gene probes specific for α haemolysin and the other enterohaemolysins.

7. Questionnaire investigation

7.1. Applications and approvals

This part of the project has been approved by the Scientific Ethical Committee (Det videnskabsetiske komité system, journal nr. (KF) 01-036/97). The approval includes permission to contact patients directly by questionnaire and a permission from the Register superintendence (Registertilsynet) to register patient data by their CPR (Civil Registry System) number.
Patients that were eligible to be included in the project were patients with *E. coli* infections and patients with any of the following syndromes:

- Haemolytic uraemic syndrome (HUS).
- Thrombotic thrombocytopenic purpura (TTP).
- Idiopathic thrombocytopenic purpura (ITP).

Data on patients with HUS, TTP and ITP were obtained from the Department of Health's Danish Patient Register (Sundhedsstyrelsens landspatientregister). Names and addresses were obtained from the Ministry of interior's CPR office.

7.2. Patients with *E. coli* infections

7.2.1. Purpose

1. To identify patients from whom VTEC, *E. coli* of O groups O26, O111, O128 and *eae*A positive isolates of *E. coli* had been isolated.
2. To obtain information on clinical symptoms of patients infected with *E. coli* from the above mentioned groups by direct contact with patients or their parents trough questionnaires.
3. To characterise the strains and to correlate and compare virulence factors to clinical symptoms.

7.2.2. Materials and methods

Patients: All patients from whom VTEC, *E. coli* of EPEC O groups O26, O111, O128 and *eae*A positive isolates of *E. coli* had been isolated were eligible for inclusion in the questionnaire study. Identification of patients required the entry of either a valid CPR number or birthday and name of the patient into the database of The International *Escherichia* and *Klebsiella* Centre (WHO).

Questionnaires: A cover letter of information and a two page questionnaire was sent by ordinary mail to all eligible patients, see appendix: **11.4. Questionnaires**.

Strains: All VTEC, *E. coli* of EPEC O groups O26, O111, O128 and *eae*A positive isolates of *E. coli* from the collection of The International *Escherichia* and *Klebsiella* Centre (WHO) which had been entered into the above mentioned database.

Characterisation: Biochemical, genotypical and phenotypical characterisation of *E. coli* isolates was done using the same methods as described in Screening of diarrhoeal stools: **2.2. Materials and methods** and in Study of selected EPEC O groups: **3.3. Methods**.

Statistical analysis: The crude association between major clinical signs and symptoms, and main types of *E. coli* (O157 VTEC, non-O157 VTEC, and *eae*A^+ EPEC) was assessed by Chi-square analysis. However, because of age-imbalances, age-adjusted logistic regression was carried out for associations with a p-value $\leq$ 25% in the initial crude analyses.

7.2.3. Results

For the period from 1978-1997 CPR numbers were available from 141 patients and for the period from 1959-1996 the birthday and name was available from 304 patients. 24 CPR numbers were invalid and the unequivocal identity by birthday and name of 64 patients could not be established. When names, addresses and status had been obtained a further 31 patients were excluded because they had either emigrated (17) or they had died (14), thus reducing the number of questionnaires to 185. Dunning letters were only sent once.

111 (60%) patients returned useable questionnaires with enough data to be included in the study. 17 (9%) indicated that they did not want to participate and 56 (30%) did not reply at all. One returned questionnaire (1%) referred to medical records which were not yet available at the end of the study period.

Among the 111 patients, the isolates included 13 VTEC O157, 20 VTEC O26, 11 other VTEC, 29 classical EPEC O:H eaeA^+ serotypes, and 6 classical EPEC O:H *eae*A^- serotypes. In addition there were 31 *eae*A^+ and 1 *eae*A^- *E. coli* strains not belonging to the classical EPEC O:H serotypes. The isolates were grouped into 6 groups A-F. The distribution of the six groups is listed in table 7.1.

The clinical data for all main types are summarised in table 7.2.

Table 7.1. Distribution of main types A-F of *E. coli* enteropathogens by year.

Group	1979-89	1990	1991	1992	1993	1994	1995	1996	1997
A: O157 VTEC	2		3	2		1	1	2	2
B: O26 VTEC	15		1		2	1		1	
C: Other VTEC [a]	2	1			2	4			2
D: EPEC, *eae*A^{+} [b]	11	2	3	2	2	9			
E: EPEC, *eae*A^{-} [c]				1	2	2		1	
F: *eae*A^{+} [d]						32			
Total	30	3	7	5	8	49	1	4	4

Serotypes (number of strains):

[a] O103:H2 (1), O111:H8 (1), O111:H- (2), O113:H4 (1), O128ab:H2 (1), O145:H28, (2), O166:H28 (1), OX3:H2 (1) and OR:HR (1).

[b] O26:H11 (8), O26:H- (8), O55:H7 (3), O55:H34 (1), O111:H2 (2), O111:H- (2), O127:H40 (1), O128ab:H2 (5).

[c] O111:H12 (2), O111:H21 (1), O126:H33 (1), O127:H40 (1) and O128ab:H10 (1)

[d] 27 different serotypes isolated during the screening of diarrhoeal stools in 1994 were included. One *eae*A^{-} strain was erroneously included because it was originally submitted as a presumptive EPEC O group O126 isolate.

Table 7.2. Clinical data for all main types of *E. coli* enteropathogens.

		Patients with					
		A	B	C	D	E	F
	All patients	O157 VTEC	O26 VTEC	Other VTEC[a]	EPEC, *eae*A$^+$	EPEC, *eae*A-	*eae*A$^+$
Number of patients	111	13	20	11	29	6	32
Sex ratio (M/F)	62/49	7/6	12/8	6/5	20/9	1/5	16/16
Age: median	1yr 4m	4 yrs 6m	11m	1 yr 5m	10m	1 yr 1m	14 yrs 11m
Age: range	36 days-78 yrs	1-64 yrs	6m-23 yrs	36 days-37 yrs	4m-14yrs	4m-56 yrs	11m-78 yrs
Age: number ≤ 5 yrs	80	7	19	7	16	4	13
Clinical data [a]							
HUS	6	3		2		1	
Diarrhoea:							
Duration: median	14 days	12 days	20 days	14 days	20 days	12 days	15 days
Duration: range	2 days-7 yrs	3-28 days	2-45 days	5-90 days	7-165 days	10-14 days	3 days-7 yrs
Bloody	23/65 (35%)	10/11 (91%)	3/11 (27%)	3/10 (30%)	3/15 (20%)	0	4/16 (25%)
Slimy	55/74 (74%)	6/8 (75%)	11/14 (79%)	6/9 (67%)	15/19 (79%)	3 (100%)	14/21 (67%)
Watery	67/91 (74%)	4/6 (67%)	16 (100%)	7/9 (78%)	7/9 (78%)	4/5 (80%)	21/24 (88%)
Loose	52/68 (76%)	1/2 (50%)	11 (100%)	7 (100%)	16/19 (89%)	1/2 (50%)	15/18 (83%)
Other symptoms:							
Nausea	25/81 (31%)	1/9 (11%)	3/15 (20%)	3/8 (38%)	7/18 (39%)	1/5 (20%)	10/26 (38%)
Vomiting	26/102 (25%)	2/13 (15%)	2/18 (11%)	5/11 (45%)	6/24 (25%)	1/5 (20%)	10/31 (32%)
Joint symptoms	8/78 (10%)	1/9 (11%)	0	1/8 (13%)	0	1/5 (20%)	5/26 (19%)
Headache	11/65 (17%)	2/7 (29%)	1/10 (10%)	2/7 (29%)	0	1/4 (25%)	5/26 (19%)
Fever	38/86 (44%)	4/10 (40%)	6/11 (55%)	2/7 (29%)	11/24 (46%)	3/6 (50%)	12/28 (43%)
Hospitalisation	47/109 (43%)	10/13 (77%)	7/20 (35%)	5/11 (45%)	12/29 (41%)	1/6 (17%)	12/30 (40%)

[a] Clinical data: Number of positives/total number (percent positive).

For statistical analysis three main types were compared: O157 VTEC (group A), non-O157 VTEC (groups B and C) and *eae*A$^+$ EPEC (group D). Age groups were less than one year (group 1), 1-5 years (group 2) and more than 5 years (group 3).

The relative risk of developing HUS was higher for patients infected with O157 VTEC (OR: >1,000) and non-O157 VTEC (OR: >1,000) than for patients infected with *eae*A$^+$ EPEC. Age-adjustment did not change this correlation significantly and was related to main type (p = 0.0468) rather than to age (p = 0.6559).

This correlation was reflected by the relatively higher risk of patients being hospitalised when infected with O157 VTEC (OR: 5.2) but not so for patients infected with non-O157 VTEC (OR: 0.98) compared with patients infected with *eae*A$^+$ EPEC. Hospitalisation was related to both main type (p = 0.0018) but also to age (p = 0.0195) , see table 7.3. Children less than 1 year were more likely to be hospitalised (OR: 5.37).

Table 7.3. Relative risk of being hospitalised by main type of *E. coli* enteropathogens. The table shows crude and age-adjusted odds ratios for hospitalised patients infected with O157 VTEC, non-O157 VTEC and *eae*A$^+$ EPEC.

	Hospitalisation	N	OR *	95% CI	Age-adjusted OR **	95% CI
O157 VTEC	10	13	5.2	(1.15-23.01)	16.6	(2.7-101.8)
Non-O157 VTEC	12	31	0.98	(0.34-2.78)	1.1	(0.35-3.33)
*eae*A$^+$ EPEC	11	28	1	(referent)	1	(referent)

* Logistic regression odds ratio; p = 0.0415

** Age-adjusted logistic regression odds ratio: P value for *E. coli* main type 0.0018. P value for age 0.0195, log likelihood ratio tests.

N: Total number of patients from each main type

OR: Odds ratio

CI: Confidence interval

The relative risk of having bloody diarrhoea was much higher for patients infected with O157 VTEC (OR: 36.7) and slightly higher for patients infected with non-O157 VTEC (OR: 1.5) compared with patients infected with *eae*A$^+$ EPEC, see table 7.4.
In the age-adjusted analysis these differences were still significant and related to main type (p = 0.0036) rather than to age (p = 0.7238), see table 7.4.

Table 7.4. Relative risk of bloody diarrhoea by main type of *E. coli* enteropathogens. The table shows crude and age-adjusted odds ratios for bloody diarrhoea in patients infected with O157 VTEC, non-O157 VTEC and *eae*A$^+$ EPEC.

	Number with bloody stool	N	OR *	95% CI	Age-adjusted OR **	95% CI
O157 VTEC	10	11	36.7	(83.3-412.3)	30.0	(2.3-392.5)
Non-O157 VTEC	6	21	1.5	(0.3-7.2)	1.4	(0.3-7.0)
*eae*A$^+$ EPEC	3	14	1	(referent)	1	(referent)

* Logistic regression odds ratio; p = 0.0003

** Age-adjusted logistic regression odds ratio: P value for *E. coli* main type 0.0036. P value for age 0.7238, log likelihood ratio tests.

N: Total number of patients from each main type

OR: Odds ratio

CI: Confidence interval

The relative risk of persistent diarrhoea (more than two weeks) was weakly associated with infection of *eae*A$^+$ EPEC and more likely to occur in children aged one to five years. This association became stronger when adjusted for age, see table 7.5.

Table 7.5. Relative risk of persistent diarrhoea (more than two weeks) by main type of *E. coli* enteropathogens. The table shows crude and age-adjusted odds ratios for persistent diarrhoea in patients infected with O157 VTEC, non-O157 VTEC and *eae*A$^+$ EPEC.

	Persistent diarrhoea	N	OR *	95% CI	Age-adjusted OR **	95% CI
O157 VTEC	5	10	0.28	(0.05-1.5)	0.06	(0.004-0.9)
Non-O157 VTEC	16	22	0.76	(0.18-3.26)	0.7	(0.15-3.52)
*eae*A$^+$ EPEC	14	18	1	(referent)	1	(referent)

* Logistic regression odds ratio; p = 0.3054

** Age-adjusted logistic regression odds ratio: P value for *E. coli* main type 0.0554. P value for age 0.0567, log likelihood ratio tests.

N: Total number of patients from each main type

OR: Odds ratio

CI: Confidence interval.

7.3. Patients with Haemolytic uraemic syndrome (HUS), Thrombotic thrombocytopenic purpura (TTP) or Idiopathic thrombocytopenic purpura (ITP)

7.3.1. Preliminary results

Time has not allowed for a detailed analysis of patients with HUS, TTP or ITP. However, the preliminary age- and sex distribution is presented below because the distributions allow for some preliminary speculations. HUS in Danish patients does not appear to be a childhood disease as generally accepted, see figure 7.1. A total of 33 HUS cases were recorded during the period of 1994 to 1996 and only six of those were examined for the presence of VTEC. One HUS case was diagnosed at SSI and was not recorded by the register.
TTP and ITP (data not shown) appear to be associated with old age, see figure 7.2.

7.3.2. Preliminary conclusion

Very few of the Danish HUS and TTP cases are examined for VTEC. It is recommended that HUS is made a notifiable disease so that all cases may be subjected to microbiological examination.

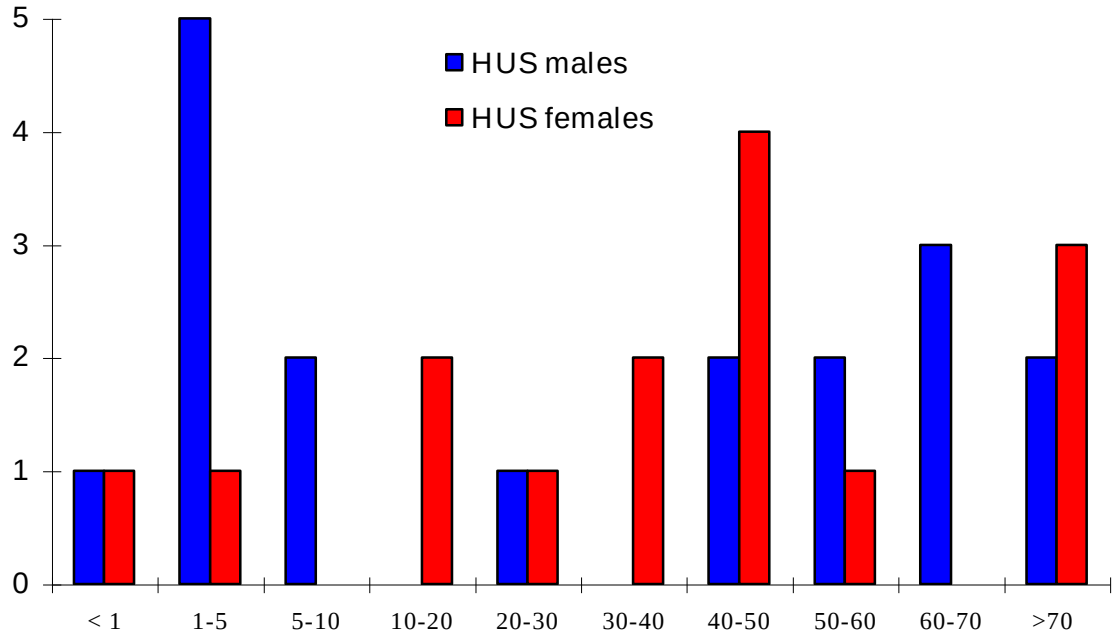

Figure 7.1. Age and sex distribution of patients with HUS as recorded in the Danish National patient register during the period of 1994 to 1996 (preliminary data).

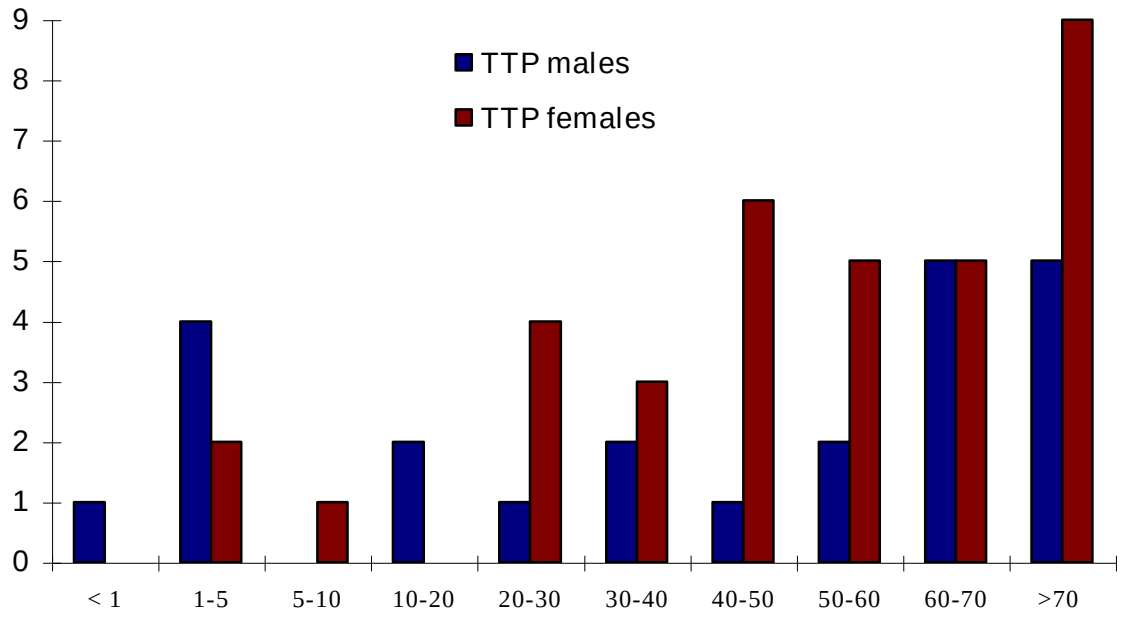

Figure 7.2. Age and sex distribution of patients with TTP as recorded in the Danish National patient register during the period of 1994 to 1996 (preliminary data).

8. Methodological studies

8.1. Screening

8.1.1. Pilot project **January 11, 1994**

The pilot project had the purpose of testing different procedures in relation to the ffct test and hybridisation with the DNA probes VT_{321} and *eae*A.
It was performed as described in **8.1.2. Screening procedures** with some exceptions:

- Hybridisation was done on two sets of nitro-cellulose (NC) paper. One set was probed with VT_{321} and the other with *eae*A.
- The saline suspensions to be used in experimental series A, B and C were stored frozen at -20°C for three weeks until they could be processed.
- 1 ml of the saline suspension was used to inoculate 5 ml of the Brain heart infusion (BHI) broth starter cultures which were incubated overnight in a waterbath at 37°C without shaking.
- 143 µl starter culture was inoculated into 1 ml of BHI in a microfuge tube and incubated for 1 hour in a rotating device.
- The rest of the starter culture was transferred into 250 ml flasks with 35 ml of BHI and incubated in a waterbath at 37°C with shaking.
- Mitomycin C was added to flasks and microfuge tubes at a final concentration of 1 µg/ml and flasks and microfuge tubes were incubated in waterbath and with rotation as previously described.
- All BHI Mitomycin C induced cultures were frozen at -20°C until the following day when they were centrifuged at 15.000 rpm for 30 minutes after thawing. Supernatants were tested in two-fold dilutions from 1:1 to 1:1.024 in the Vero cell assay.

8.1.1.1. Results

55 stool specimen were included. All 55 BHI / Mitomycin C induced cultures from the microfuge tube supernatants were negative and it was decided to abandon this procedure. One BHI / Mitomycin C induced culture supernatant from the flask cultures was VT positive in a dilution of 1:4. Supernatants from the saline suspensions showed a cytotoxic effect at dilutions 1:1, 1:2, 1:4 and 1:8 in 3, 12, 7 and 1 specimens respectively. One of these had a VT-like effect. 7 specimens could not be described in a definitive manner and 25 were negative. It was decided to categorise the ffct readings more precisely .
All the DNA probing results with the *eae*A probe were negative or impossible to interpret from the NC papers. Almost all dot blots with the VT_{321} were positive - including the negative control. At the same time Jeppe Boel (the Royal Veterinary and Agriculture University, Copenhagen) who had provided me with the VT_{321} PCR fragment had come to the conclusion that the VT_{321} was very difficult to work with.

8.1.1.2. Conclusion

Based on these results it was decided to categorise the ffct readings more precisely and to proceed with the polynucleotide probes for VT1 and VT2 instead of the VT_{321} probe. Furthermore it was decided to pool the three probes into a DNA pool, to optimise stringency and to use nylon membranes during the screening period.
Examination of the 55 specimens from the first pilot project was not pursued any further.

8.1.2. Screening procedures **February 23, 1994**Fejl! Bogmærke er ikke defineret.

Three experimental series were performed:

A. BHI / Mitomycin C induced amplification of Verotoxin production.
B. Direct detection of free faecal cytotoxin.
C. Hybridisation of dot blots with a pool of DNA probes VT1, VT2 and *eae*A.

A. Faeces (approximately 1 g) was suspended in 3 ml of saline in a disposable centrifuge tube - Whirlimix vigorously.
1 ml of the saline suspension was inoculated into 5 ml of BHI broth in test tubes and incubated at 37°C overnight without shaking. This was the starter culture.
The first 18 starter cultures (use all of the starter culture ~ 6 ml) are reinoculated into 35 ml of BHI broth in 250 ml flasks and incubated at 37°C in a waterbath with shaking for 1 hour.
Additional starter cultures are stored frozen at -80°C after the addition of 1 ml of glycerine (~ 12 - 14% glycerine) until further examination.
After 1 hour of shaking 82 ml of Mitomycin C (STOCK solution: 0,5 mg/ml) was added to the cultures which are incubated at 37°C without shaking for 6 hours.

> On **March 22, 1994** (third sample day) this was changed to **Fejl! Bogmærke er ikke defineret.*with shaking over night and a small red plate was seeded with the over night broth for control of growth.***

Remove 1,5 ml to microfuge tube; Spin 15.000 x g, 30 min.
The supernatants are transferred to clean microfuge tubes and tested in two-fold dilutions on Vero cells.
The rest of the supernatants are stored frozen at -20°C.

B. The rest of the saline suspension (approximately 2 ml) was centrifuged at 15.000 rpm in the Sorval centrifuge, 30 min. The supernatant was transferred to microfuge tubes and tested in two-fold dilutions on Vero cells.
The remainder was stored frozen at -20°C.

The Vero cells from A and B were read each day for three days.

C. A saline suspension was routinely seeded on a big red plate.
After reading of the big red plates the worksheets are photocopied. From all the plates that are negative for Enteropathogenic bacteria - and from all the blue plates - 10-20 single colonies of different appearance are circled and numbered on the plates and then seeded unto a nylon membrane (Amersham, Hybond N-) for DNA probing with a pool of VT1, VT2 and *eae*A gene probes. The membranes are placed on beef extract agar plates and set at 37ºC as late as possible in the afternoon, incubated overnight and taken out by the first one at work the following morning. The plates with membranes are stored in the refrigerator until lysis and fixing of DNA. After secondary reading of the small red plates (selenite), *Clostridium difficile* and *Campylobacter* all positive findings of Enteropathogenic bacteria are noted on the worksheets.
Hybridisation with VT1, VT2 and *eae*A. All positive colonies are streaked out for purification and a single colony was picked for storing on extract agar stabs. All further characterisation was done from the purified plates or from the stabs.

A, B and C.
After analysis of the notes on the worksheets, the results from the Vero cell assay and the hybridisation experiment all specimens that are negative in all three of the experimental series (A, B and C) are marked and discarded of including frozen starter cultures, supernatants and plates. Specimens that are positive in the Vero cell assay but negative with the DNA probes are examined again for the presence of possible positive colonies.

8.2. Vero cell assay

Vero cells are grown in plastic culture flasks at 37ºC in growth medium (see below). During the project period it was decided to add amfotericin (0,25 µg/ml) in order to reduce growth of fungi which disturbed the readings when performing the ffct test on stool specimens.
Vero cells are washed once with 20% Trypsine versene and trypsinised with 4 ml 20% Trypsin versene for 5-6 minutes. After very careful decantation of Trypsine versene the cells are loosened from the bottom by hammering the flask into one's hand several times. After the addition of 15-20 ml of growth medium it was checked that a proper cell suspension has been obtained and the suspension was transferred to a reservoir. From the reservoir 150 µl are transferred to each of 96 wells in a microtiter plate and incubated over night at 37°C in a moist chamber. The following day wells are emptied by inverting the plates onto a thick layer of soft tissue and 200 µl of test medium (see below) was added to each well. 30 µl of supernatant from either a pure culture grown in 1 ml of Evan's medium in a microfuge tube (37°C on a rotator overnight) or from a saline stool suspension was added in duplicate at the desired dilutions. Supernatants from verotoxin producing strains and from VT negative control strains grown in Evan's medium are always added in duplicate to at least one plate in an assay.
The cytotoxic effect was read each day for three days - sometimes four days when an assay spanned a weekend.

Growth medium:	Eagles minimal medium (MEM) with 10 % heat inactivated foetal calf serum (FCS), 4 mM L-glutamine, gentamycin (60 µg/ml) and amfotericin B (0.25 µg/ml).
Test medium:	Eagles minimal essential medium (MEM) with 3 % heat inactivated foetal calf serum (FCS), gentamycin (60 µg/ml) and amfotericin B (0.25 µg/ml).

8.3. Labelling of Vero cells with europium

8.3.1. Background and hypothesis

Background: Vero toxin belongs to a family of bacterial toxins that can inhibit translation in eukaryotic cells. This inhibition of protein synthesis causes profound physiological changes in the cells. *In vitro* evidences suggest that this induces cell death by apoptosis [329]. Apoptosis is characterised by morphologic changes, including cell shrinkage, condensation of nuclear materials and deformation of the nucleus, and the internucleosomal fragmentation of DNA into units of 180-200 base pairs.
^{51}Cr has been and is still widely used by immunologists as a target cell marker in cytotoxicity assays. As the radioactive ^{51}Cr isotope has certain drawbacks an alternative approach was adopted.
The use of lanthanide chelate labels with unique fluorescence properties have been used as the first real alternative to the ^{51}Cr release assay. Europium, Eu^{3+}, is the one which is mainly used.

Hypothesis: Apoptosis in Vero cells induced by Vero toxin can be measured by the specific release of a chelate labelled component such as europium.

Principle of VT mediated cytotoxicity assay, see figure 8.1.
The procedure may be divided into three main steps:

1. Labelling of Vero cells.
2. Release of the marker from Vero cells by apoptotic processes caused by Vero cytotoxin.
3. Detection of released marker.

Labelling of Vero cells was performed in a calcium free buffer at room temperature in the presence of dextran sulphate. The cells were then washed with a buffer containing calcium and glucose.

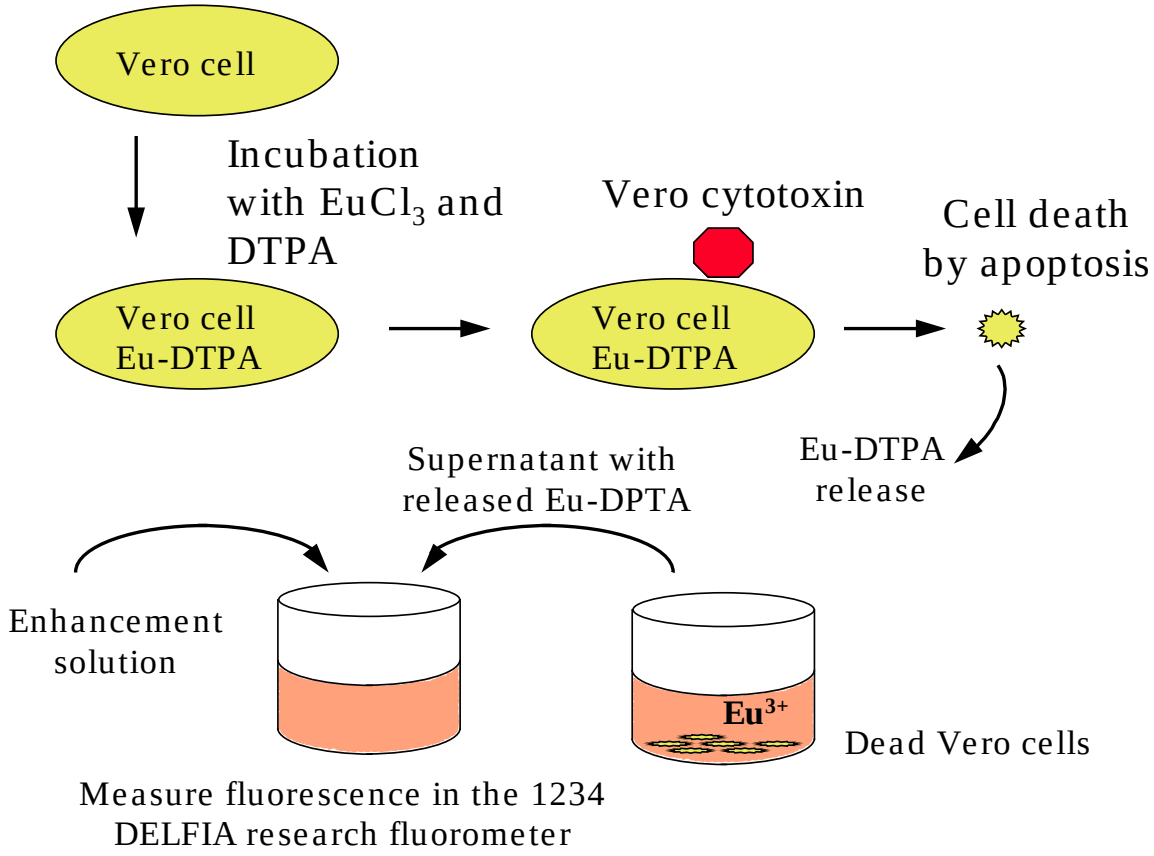

Figure 8.1. Principle of the Eu-release assay.

8.3.2. Preliminary results

Preliminary testing according to the above principles indicated that Vero cells may be labelled with europium and release this marker when exposed to Vero toxin in supernatants from reference strains in a dose dependent manner. The Verotoxin-specific release ranged from 30 to 45%. However the small number of assays did not allow for a more general conclusion as regards the applicability of europium labelled Vero cells for detection of VT or free faecal cytotoxin.
Further experiments and prospective testing on both control strains and on clinical stool samples is needed.

8.4. VT1 and VT2 neutralising rabbit antisera

Immunisation protocol and vaccine strains were kindly provided by Dr. Alfredo Caprioli, Istituto Superiore di Sanitá, Roma, Italy.
In brief the VT-neutralising antisera were prepared in rabbits by multiple sub-cutaneous injections of culture supernatants of reference VTEC vaccine strains, which were concentrated by ammonium sulphate precipitation and emulsified with equal volumes of Freundt's complete and incomplete adjuvant [293].

8.4.1. Protocol

A new immunisation protocol was developed in collaboration with Lis Bruun, The Animal Department, SSI, in order to minimise the risk associated with the injection of crude extract of VT.

Vaccine strains were:
H30 (=D2242). Serotype O26:H11, VT1 producer
E32511 (=D2244). Serotype O157:H^-, VT2 and VT2c producer

Preparation of crude extract VT antigen:

1. 0.5 l TSB over-night culture with shaking
2. Centrifuge at 12.000 x g 20 min.
3. Supernatant is precipitated by 60% saturated NH_4SO_4 and centrifuged.
4. The precipitate is resuspended in 10 ml PBS and dialysed.
5. Centrifuge at maximum (18.000 rpm) for 30 min.
6. The supernatant is the crude extract antigen, stock VT, which is stored at -20°C. The stock VT extract is titrated in the Vero cell assay.

Immunisation:

40 ml of pre-immune serum is drawn to be used as control serum.

1. First immunisation : 0,5 ml stock VT + 0,5 ml of Freundt's complete adjuvant is emulsified and injected sub-cutaneously applying 0,1 ml at 6 separate spots on NZ rabbits.
2. This injection is repeated at two weeks intervals using Freundt's **in**complete adjuvant.
3. After the third immunisation the rabbit is bled 10 ml from the ear in order to measure the VT-neutralising effect. Titration of the VT-neutralising antiserum is done by two-fold dilutions of antiserum against the supernatant of reference strain(s) grown in Evan's medium. The VT supernatant is diluted to one titer step higher (stronger) than the end titration point. For example a VT supernatant with end-titration point at 1:512 must be tested at a dilution of 1:128. All reference strain VT supernatants must be carefully titrated and kept as stock solutions at -20°C. The VT titers are stable for a long time.

4. Immunisation is repeated every two weeks until VT-neutralising antiserum can be diluted 1:256 - 1:512 and still show a satisfactory neutralisation of VT supernatants from VT reference strains.

For diagnostic purposes it is sufficient to produce anti-$VT2_c$ antiserum because this antiserum will neutralise both VT2 and $VT2_c$.

Definitions: One unit of verotoxin activity was defined as the amount present at the highest dilution of a VT preparation which caused any cytotoxic effect on the confluent monolayer of Vero cells after three days [201].
One unit of neutralising antiserum was defined as the amount present at the highest dilution of antiserum which neutralised 1-5 units of VT after three days [201].

Neutralisation: Equal volumes of VT preparation and VT-neutralising antiserum is mixed and incubated at 37°C for one hour. 60 μl of this mixture is added to the Vero cells. Equal volumes of VT preparation and pre-immune serum is used as control.

8.4.2. Results: Testing of VT1 and VT2 neutralising rabbit antisera

Two pairs of rabbits were given 11 injections over a period of 5 months. One rabbit died for reasons not associated with VT immunisation.
VT crude extract used for immunisation contained 1,024 units in the VT1 and 128 units in the VT2 stock solutions. This is lower than supernatants from reference strain cultures (D2163, D2164 and D2165) grown in Evan's which contained 10,000-40,000 units of VT1 and 5,120-10,240 units of VT2.
The VT1-neutralising antiserum contained 400-500 units when tested on 2-4 units of VT1 stock solution.
The VT2-neutralising antiserum contained approximately 4,800 units when tested on 1-5 units of VT2 stock solution.

Time did not allow for any clinical testing.

8.4.3. Conclusion

VT1- and VT2 neutralising rabbit antisera were produced and shown to neutralise the corresponding VT stock solutions used for immunisation. Further testing is required in order to determine the applicability of VT-neutralising antisera in the diagnosis of VTEC.

8.5. DNA probes

DNA probes were kindly provided by the following individuals:
The VT_{321} unlabelled PCR fragment from Jeppe Boel, the Royal Veterinary and Agriculture University, Copenhagen, Denmark.
VT1 and VT2 from Henry R. Smith, Laboratory of Enteric Pathogens, Central Public Health Laboratory, Colindale Avenue, UK.
*eae*A, EHEC, EAF, Agg, DA, ST_p, ST_h, LT, EIEC, CDT from James B. Kaper, Center for Vaccine Development (CVD), University of Maryland, Baltimore, USA.
*bfp*A from Phil Tarr, Children's Hospital and Medical Center, Seattle, Washington, USA.
ST_p, ST_h, LT from Halvor Sommerfelt and Hans Steinsland, Senter for international helse, Bergen Universitet, Norway.
*ipa*H from Malabi M. Venkatesan, Department of Bacterial Immunology, Walter Reed Army Institute of Research, Washington DC, USA.
EAST1 from Steve Savarino and Daniel A. Scott, Enteric Diseases Program, Naval Medical Research Institute-Annex, Rockville, Maryland, USA.

All the original probes including the VT_{321} PCR fragment were initially labelled by random priming and tested on a panel of positive and negative control strains.
Some control strains were already present in the culture collection others were kindly provided together with the DNA probe strains. All the control strains are listed in table 8.1.

During the project the following DNA probes were sequenced: VT1, VT2, *eae*A, Agg, EAST1, EIEC, ST_p and ST_h. Upon confirmation of sequence homology with existing sequences in Genebank the probe fragments were recloned into either the pBluescript ® SK + phagemid (Stratagene) or the very similar pBS + phagemid (Stratagene) vector. Sequencing, Genebank searches and cloning was done by Martin Borre or Kit Boye, Department of Clinical Biochemistry, SSI except for cloning of DNA probe fragments LT, ST_p and ST_h into the pBS vector which was done by Hans Steinsland, University of Bergen, Norway. Fragments residing in the pBluescript or pBS- vectors were labelled by PCR.

Recloned DNA probes are shown in tables 8.2. (Random priming labelling) and 8.3. (PCR labelling).

Table 8.1. **Control strains**

D number	Original No.	FAS	VCA	VT1	VT2	*eae*A	EHEC	*bfp*A	EAF	Agg	EAST1	DA	EIEC	*ipa*H	LT	ST_h	ST_p	Serotype	Comments
D1826	E20513/0	+	-	-	-	+	-	+	+	-		-	-	-	-	-	-	O111:H2	EPEC control; LA+
D1827	E20518/0	+P	-	-	-	+	-	+	-	-			-	-				O128:H2	EPEC †
D1923	HB101	-	-	-	-	-	-	-	-	-		-	-	-	-			OR:H-	HB101 for cloning
D2103	MC1061	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OR:H48	K-12
D2163	E32511	-	+	-	+	+	+		-	-	+	-	-	-	-	-	-	O157:H-	VTEC
D2164	E30480	-	+	+	+	+	+		-	-	+	-	-	-	-	-	-	O157:H7	VTEC
D2165	H19	+	+	+	-	+	+		-	-	+	-	-	-	-	-	-	O26:H11	VTEC. VCA control
D2168	PB-176 CFA/II									-	+	-			+	+	+	O6:K15:H16	ETEC
D2188	933	-	+	+	+	+	+		-	-	+	-	-	-	-	-	-	O157:K-:H7	VTEC. PGUA-
D2189	17-2	-	-	-	-	-	-		-	+	+	-	-	-	-	-	-	O3:K-:H2	EAggEC control
D2190	R551-1	-	-	-	-	-	-		-	-	-	+	-	-	-	-	-	O21:K-:H4	DA control. α Hly
D2191	H10407	-	-	-	-	-	-		-	-	+	-	-	-	+	+	+	O78:K-:H11	ETEC control
D2192	#7	-	-	-	-	-	-		-	-	-	-	+	+	-	-	-	O124:H30	EIEC control. PAL+
D2194	H5(neg)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	O9:K+:H4	Negative control
D2224	H38a	-	-	-	-	-	-		-	-		+	-	-	-	-	-	O36:H4	DA control
D2226	"042"	-	-	-	-	-	-		-	+	+	+	-	-	-	-	-	O44:H18	Agg+, DA+
D2240	17-2		-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	O3:K-:H2	EAST1 control
D2241	FN414		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OR:K?:H25	Negative control
D2258	290C		-	-	-	-				-	+	-	-	-	-	-	+	O27:K?:H7	ETEC. cs6+
D2259	144B		-	-	-	-			-	-	-	-	-	-	-	+	-	O115:K?:H5	ETEC. cs5+, cs6+
D2260	F14C		-	-	-	-			-	-	-	-	-	-	+	-	-	O8:K+:H9	ETEC. CFA & cs unknown
D2261	405A		-	-	-	-				-	+	-	-	-	-	+	-	O148:H28	ETEC. cs6+
D2262	H10407+			-	-	-			-	-	+	-	-	-	+	+	+	O78:K-:H11	ETEC. STp, ST_h and LT
D2263	F360A		-	-	-	-			-	-	+	-	-	-	-	-	+	O27:K-:H20	ETEC. cs6+
D2264	439A		-	-	-	-				-	-	-	-	-	+	-	-	O25:K+:H-	ETEC. CFA & cs unknown
fr1292	No2 VIR	-	-	-	-	-	-		-	-		-	+	+	-	-	-	O143:H-	EIEC control. Pal-test+
fr1294	No2135 AVIR	-	-	-	-	-	-		-	-		-	+	+	-	-	-	O143:H-	Pal-test-
fr1368	BH 2232-5	-	-	-	-	-	-		-	-		-	+	+	-	-	-	O172:H-	EIEC. Pal-test +
fr1237	4288-84		+	+	+	+	+	-	-	-	+		-	-	-	-	-	O157:H7	"HUS" control. α-hly

VCA: Verocell assay
FAS: +P = FAS poor *i.e.* FAS positive but only a few bacteria per HeLa cell.

† Originally described as non-adherent but I have found it weakly adherent and FAS poor.

Table 8.2. **DNA probes labelled by random priming**Fejl! Bogmærke er ikke defineret.

Probe	Vector Plasmid	Selection†	Name	Size	Restriction Enzyme	Total in kb	D Number	Reference
*eae*A	pBluescript	Amp^r	pCVD434	~1 kb	*Sal*I-*Kpn*I	~4,0	D2229	[81]
EHEC	pBR325	Amp^r,Cm^r	pCVD419	3.4 kb	*Hin*dIII	9,4	D2195	[53]
Agg	pBluescript	Amp^r	pCVD432	~1 kb	*Hin*CII-*Kpn*I	~4,0	D2228	[121]
DA	pUC8	Amp^r	pSLM862	390 bp	*Pst*I	3,45	D2197	[127]
EAF	pCVD315	Amp^r	pJPN16	~1 kb	*Bam*HI-*Sal*I	5,0	D2203	[38]
*bfp*A	pBluescript	Amp^r	pMSD207	~0,9kb	*Eco*RI	3,8	D2234	[330]
VT1	pBluescript	Amp^r	NTP705	~0.75 kb	*Eco*RI-*Sal*I	3,71	D2209	[147]
VT2	pBluescript	Amp^r	pDEP28	0.85 kb	*Sma*I-*Pst*I [a]	3,81	D2227	[152]
ST_p (STIa)	pBluescript	Amp^r	pCVD426	157 bp	*Bam*HI-*Eco*RI	3,07	D2230	[331]
ST_h (STIb)	pBluescript	Amp^r	pCVD427	216 bp	*Bam*HI-*Hin*dIII	3,13	D2231	[331]
LT	pEWD299	Amp^r	G119	850 bp	*Hin*dIII	3,2	D2256	[331]
EAST1	pUC18	Amp^r	pSS126	125 bp	*Eco*RI-*Pst*I	7,71	D2251	[126]
EIEC	pBluescript	Amp^r	pPS2.5	2.5 kb	*Hin*dIII	5,41	D2215	[332]
*ipa*H	pBluescript	Amp^r	pWR390	~0,77 kb	*Hin*CII-*Pst*I	3,73	D2233	[333]

[a] cut at 25°C.

Table 8.3. **DNA probes labelled by PCR**

Vector plasmids : pBluescript or pBS-. Selection : Amp^r

Probe	Name	Size		Total in kb	Cloned from	D Number
*eae*A	pCVD434	~1 kb		~4,0	D2202	D2229
Agg	pCVD432	~1 kb		~4,0	D2196	D2228
DA	pSLM862	390 bp		3,0	D2197	D2235
*bfp*A	pMSD207	~0,9kb		3,8	C441-95	D2234
VT1	NTP705	~0.75 kb		3,71	D2208	D2209
VT2	pDEP28	0.85 kb		3,81	D2207	D2227
ST_p (STIa)	pDAS101	157 bp		3,2	pDAS101	D2230
ST_h (STIb)	pDAS100	216 bp		3,2	pDAS100	D2231
LT	pWD299	0,32 kb	[a]	3,3	pWD299	D2232
EAST1	pSS126	125 bp	[b]	3,1	D2251	D2255
*ipa*H	pWR390	~0,77 kb	[c]	3,73	pWR390	D2233

[a] Cloned as a fragment of 320 bp but migration on gels indicate a smaller fragment size of 250 bp.

Note: PCR labelled fragments will migrate slower than the orignal DNA probe fragments because the labelling agent (Digoxigenin (DIG)-dUTP), and the multiple cloning site and primers increase the molecular weigth. Differences depend on fragment size and labelling procedure. Examples:

[b] Total size of labelled fragment is 215 bp but fragment migrates on agarose gel as a larger fragment of 350 bp.
[c] Total size of labelled fragment is 860 bp but fragment migrates on agarose gel as a larger fragment of 1 kb.

8.5.1. DNA probe purification

Plasmid purification: Plasmid vectors containing the DNA probe fragment were purified using Qiagen Maxi tips (500 mg) according to the manufacturer's description(s). The size was confirmed by control agarose gel (1%) electrophoresis of sample DNA vector and DNA control marker of known size.

Determination of DNA concentration: Concentration of the purified plasmid DNA was determined spectrophotometrically by diluting the DNA plasmid stock solutions 50-100 fold and measuring the absorbance at 260 nm.
45 - 50 mg DNA/ml results in A_{260} = 1,00.
Purity of DNA was estimated by the A_{260}/A_{280} ratio. An A_{260}/A_{280} ratio of 1,8 - 1,9 was considered to reflect purity good enough to be used for restriction enzyme digestion.
The purified plasmids were cut with appropriate restriction enzyme(s) according to the manufacturer's description(s).
Assessment of the DNA concentration was also routinely done by visual estimation of the DNA concentration from agarose gel intensity of plasmid band by comparison of control DNA marker - usually *Hin*dIII cut λ phage and 100 bp ladders of DNA - of known concentration.

Restriction enzyme digest: Cutting with one restriction enzyme followed the below procedure:

X ml DNA (0,1 - 10 mg DNA in H_2O (milli-Q) or TE buffer)
2 ml 10 X digestion buffer according to restriction enzyme
18-***X*** ml H_2O (milli-Q)
1 ml restriction enzyme (RE).

Volumes were adjusted according to the following rules of thumb:
Normally 1 ml contains 8-12 units/ml which is sufficient to cut 10 mg of DNA.
Definition : 1 unit (U) RE cuts 1 mg purified DNA completely in one hour.

Examples of restriction enzyme digest with two enzymes (Gibco BRL):

*eae*A	EAF
2,5 ml (7,5 - 8,5 mg total DNA)	2 ml (7 - 8 mg total DNA)
2 ml buffer REact 4	2 ml buffer REact 3
15,5 ml H_2O (milli-Q)	16 ml H_2O (milli-Q)
1 ml *Kpn*I (RE)	1 ml *Bam*HI (RE)
	1 ml *Sal*I (RE)
21 ml; 1 hour, 37°C.	22 ml; 1 hour, 37°C.
Followed by the addition of:	
2 ml buffer REact 10	1 ml buffer REact 10
1 ml *Sal*I (RE)	
24 ml; approx. 1 hour, 37°C.	23 ml; approx. 1 hour, 37°C.

Control of restriction enzyme digest: After cutting of vector plasmid with appropriate restriction enzyme(s) (RE) for 1-2 hours at 37°C the fragments were separated by electrophoresis in 1% agarose gel (SeaKem GTG) with 0,5 mg/ml Ethidium bromide (EtBr) at 80-100 V. Complete cutting and separation was initially controlled after approx. half an hour with an UV hand lamp. Electrophoresis was stopped when the probe fragment was clearly visible from the larger vector plasmid band. The correct size was checked by comparison to marker DNA on an UV illuminator - preferably 365 nm (or 312 nm) and the correct fragment was cut out of the gel.

Purification and concentration of probe fragment: The pDNA probe fragment was eluted and purified with a Prep-A-Gene kit (Bio Rad) according to the description by the manufacturer, but an extra step of ethanol precipitation to ensure optimal purity was added.
Recovery and concentration of DNA probe fragment was visually estimated by comparison of sample DNA probe fragment band with marker DNA bands of known size after control agarose gel electrophoresis.

8.5.2. Labelling and detection

Labelling, quantification of labelling efficiency and detection: Both small- and large scale random priming labelling of purified DNA probes with digoxigenin (DIG)-dUTP, estimation of the yield of DIG-labelled DNA and detection of hybrids by enzyme immunoassay was done according to the manufacturer's description(s) (Boehringer Mannheim).

PCR labelling: PCR labelling used primers T3 and T7 (see below) for amplification of probes residing in the pBS and pBluescript vector.
The following application protocol was used to generate 100 µl mixtures.
Prepare amplification mixture by adding reagents to a sterile microfuge tube in the following order:

For the small DNA probes:
ST_p, ST_h, LT, EAST1 and DA.

20 µl 5X conc. PCR buffer
63 µl Milli-Q H_2O
2 µl 20 µM T3 17 bp primer
2 µl 20 µM T7 19 bp primer
10 µl 10X conc. PCR DIG probe synthesis mix
Vortex gently
2 µl 100 pg/µl plasmid DNA
4 drops (approximately 70 µl) of mineral oil.

For the larger DNA probes: VT1, VT2, *eae*A, EAgg, EIEC, *bfp*A and *ipa*H.

20 µl 5X conc. PCR buffer
63 µl Milli-Q H_2O
2 µl 20 µM T3 17 bp primer
2 µl 20 µM T7 19 bp primer
5 µl 2 mM dNTP
5 µl 10X conc. PCR DIG probe synthesis mix
Vortex gently
2 µl 1 ng/µl plasmid DNA
4 drops (approximately 70 µl) of mineral oil.

The amplification mixture was heated to 94°C and 1 µl Taq DNA polymerase at 5 Units/µl was added to the mixture. At the end of the project period this was changed to a hot start in which the amplification mixture was heated to 94°C for 5 minutes before the addition of Taq polymerase.

30 cycles were run at 94°C 1 minute (denaturation), 50°C 1 minute (annealing) and 65°C 2 minutes (polymerisation) and ended with one step at 72°C 10 minutes before cooling to 4°C.

The lower water phase is transferred to a clean microfuge tube and 1 µl is run on an agarose gel (1%) for estimation of correct size and concentration. The PCR labelled probe DNA is precipitated with ethanol (5 µl Na-Acetate + 137 µl 96% ETOH at -20°C over night, alternatively -80°C for 30 minutes) and resuspended in 50 µl TE buffer.

For recent routine PCR labelling a master mixture is made by mixing PCR buffer, Milli-Q H_2O, 20 µM T3 17 bp primer, 20 µM T7 19 bp primer, PCR DIG probe synthesis mix (and 2 mM dNTP mixture for larger probes) at appropriate volumes which are stored in aliquots at -20°C.

Primers:

T3: 5' - ATT AAC CCT CAC TAA AG - 3' 17 bp
T7: 3' - GGA TAT CAC TCA GCA TAA T- 5' 19 bp

8.5.3. Colony dot blots

Nylon membranes (Hybond N+, Amersham) were placed on beef broth agar plates (SSI) and inoculated with bacterial culture from single colonies on primary plates and incubated 6-18 hours. Lysis, denaturation of bacterial DNA is achieved by placing the membranes on filter papers wetted with the following solutions:

1. 10% SDS, 10 minutes
2. 0,5 N NaOH, 10 minutes.
3. 1,5 M NaCl, 0,5 M NaOH, 10 minutes.
4. 1 M Tris-HCl, pH 7,4, 5 minutes. Repeat twice.
5. 1,5 M NaCl, 0,5 M Tris-HCl, pH 7,4, 10 minutes.

The membrane is dried and the DNA fixed by baking at 80°C for 2 hours. Colony debris is removed using a cotton bud soaked in the Tris-HCl buffer or a 2 X SSC with 1% SDS.

8.5.4. Prehybridisation and hybridisation

The membrane was prehybridised in approximately 20 ml per 100 cm^2 membrane with a solution of 5 X SSC, 0,5% Blocking reagent (Boehringer's kit), 0,1% N-Laroylsarcosine, 0,02% SDS in big and small hybridisation tubes according to membrane size and placed in a HYBAID hybridisation oven with a rôtisserie at 65°C for a minimum of 1 hour. Hereafter the prehybridisation solution - except 6-7 ml - was removed from the tube and freshly denatured (10 minutes in boiling water followed by cooling to approximately 65°C) Digoxigenin-labelled DNA probe was added. Hybridisation was run from 1 hour to over night at 65°C.
The hybridisation solution was removed and kept stored at -20°C for reuse. The solution was used 8-10 times depending on membrane size and number of positive colonies.

8.5.5. Washes

The membrane(s) were washed 2 X 5 minutes in 2 X SSC, 0.1% SDS at room temperature (first wash) followed by 2 X 30 minutes in 0,1 X SSC, 0,1% SDS at 65°C (second wash) with a minimum of 50 ml washing solution in the tube per wash. The membranes were used directly for detection or air-dried until later.

8.5.6. Optimising stringency

Temperature: All DNA probes were tested at hybridisation temperatures at 60°C, 65°C and 68°C. The upper safety limit for the hybridisation tubes is 70°C.
Repeated assays indicated that all probes could be hybridised at 65°C (data not shown) and that washing could be used to increase the specificity of DNA probes.

Washes: Three washing solutions were tested at the second washing step and compared for both random priming and PCR labelled DNA probes.

1. Increased stringency using 0.01 X SSC, 0.1% SDS.
2. The stringency was kept at the original stringency using a 0.1 X SSC, 0.1% SDS.
3. The stringency was lowered using a 0.6 X SSC, 0.1% SDS washing solution.

Washing was done at the same temperature (65°C) for 30 minutes as previously described, see figure 8.2. A, B and C.

Further experiments showed that all probes could be hybridised and washed under the same conditions of stringency (2.: 0.1 X SSC, 0.1% SDS) whether they were PCR labelled or labelled with random priming (data not shown).

1	2	3	4	5	6	7	8	9	10
VT2 pBS	Agg pBS	*eae*A pBS	*ipa*H pBS	*bfp*A pBS	Agg EAST1	EAST1	EAST1	EAST pBS	**VT1** VT2 EHEC
VT1 VT2 EHEC	Negative control	**VT1** VT2 *eae*A EHEC EAST1	Agg EAST1	DA	LT ST_h ST_p	VT2 *eae*A EHEC	**VT1** VT2 *eae*A EHEC	**VT1** *eae*A EHEC	
11	12	13	14	15	16	17	18	19	20

	C or D-number	Gene probe or strain designation	Vector or wild type serotype	**Expected DNA probe result**
1.	D2227	pDEP28	pBluescript	VT2
2.	D2228	pCVD432	pBluescript	Agg
3.	D2229	pCVD434	pBluescript	*eae*A
4.	D2233	pWR390	pBluescript	*ipa*H
5.	D2234	pMSD207	pBluescript	*bfp*A
6.	D2240	17-2	O3:K-:H2	Agg, EAST1
7.	D2250	pSS106	pUC19	EAST1
8.	D2251	pSS126	pUC18	EAST1
9.	D2255	pSS126	pBluescript	EAST1
10.	C1023-91	DG 194-2	O128ab:H2	**VT1**, VT2, EHEC
11.	C 258-93	F2306	OX3:H2	**VT1**, VT2, EHEC
12.	D2103	MC1061	OR:H48	Negative
13.	D2188	933	O157:K-:H7	**VT1**, VT2, *eae*A, EHEC, EAST1
14.	D2189	17-2	O3:K-:H2	Agg, EAST1
15.	D2190	R551-1	O21:K-:H4	DA
16.	D2191	H10407	O78:K-:H11	ETEC: LT, ST_h, ST_p
17.	D2163	E32511	O157:H^-	VT2, *eae*A, EHEC, EAST1
18.	D2164	E30480	O157:H7	**VT1**, VT2, *eae*A, EHEC, EAST1
19.	D2165	H19	O26:H11	**VT1**, *eae*A, EHEC, EAST1
20.	Membrane identification field.			

Figure 8.2. A. Legend. The top table lists the expected DNA probe hybridisation results according to the control strains listed below. The table should be compared with Figure 8.2. A on the following page.

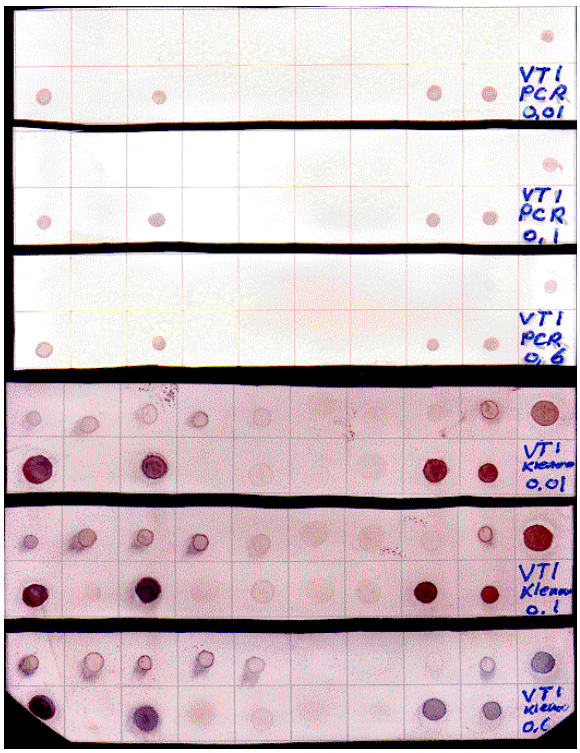

Figure 8.2. A Nylon membranes inoculated with control strains as listed in figure 8.2. A Legend. The three top membranes were hybridised with PCR labelled VT1 probe and the three lower membranes with random priming labelled VT1 DNA probe.

The membranes were washed at the second washing step as follows:

1. Top membrane and fourth membrane:
   Increased stringency using 0.01 X SSC, 0.1% SDS.
2. Second and fifth row:
   The stringency was kept at the original stringency using a 0.1 X SSC, 0.1% SDS.
3. Third and sixth row:
   The stringency was lowered using a 0.6 X SSC, 0.1% SDS washing solution.

1	2	3	4	5	6	7	8	9	10
VT2 pBS	Agg pBS	*eae*A pBS	*ipa*H pBS	*bfp*A pBS	Agg EAST1	EAST1	EAST1	EAST pBS	VT1 **VT2** EHEC
VT1 **VT2** EHEC	Negative control	VT1 **VT2** *eae*A EHEC EAST1	Agg EAST1	DA	LT ST_h ST_p	**VT2** *eae*A EHEC	VT1 **VT2** *eae*A EHEC	VT1 *eae*A EHEC	
11	12	13	14	15	16	17	18	19	20

	C or D-number	Gene probe or strain designation	Vector or wild type serotype	**Expected DNA probe result**
1.	D2227	pDEP28	pBluescript	**VT2**
2.	D2228	pCVD432	pBluescript	Agg
3.	D2229	pCVD434	pBluescript	*eae*A
4.	D2233	pWR390	pBluescript	*ipa*H
5.	D2234	pMSD207	pBluescript	*bfp*A
6.	D2240	17-2	O3:K-:H2	Agg, EAST1
7.	D2250	pSS106	pUC19	EAST1
8.	D2251	pSS126	pUC18	EAST1
9.	D2255	pSS126	pBluescript	EAST1
10.	C1023-91	DG 194-2	O128ab:H2	VT1, **VT2**, EHEC
11.	C 258-93	F2306	OX3:H2	VT1, **VT2**, EHEC
12.	D2103	MC1061	OR:H48	Negative
13.	D2188	933	O157:K-:H7	VT1, **VT2**, *eae*A, EHEC, EAST1
14.	D2189	17-2	O3:K-:H2	Agg, EAST1
15.	D2190	R551-1	O21:K-:H4	DA
16.	D2191	H10407	O78:K-:H11	ETEC: LT, ST_h, ST_p
17.	D2163	E32511	O157:H^-	**VT2**, *eae*A, EHEC, EAST1
18.	D2164	E30480	O157:H7	VT1, **VT2**, *eae*A, EHEC, EAST1
19.	D2165	H19	O26:H11	VT1, *eae*A, EHEC, EAST1
20.	Membrane identification field.			

Figure 8.2. B. Legend. The top table lists the expected DNA probe hybridisation results according to the control strains listed below. The table should be compared with Figure 8.2. B on the following page.

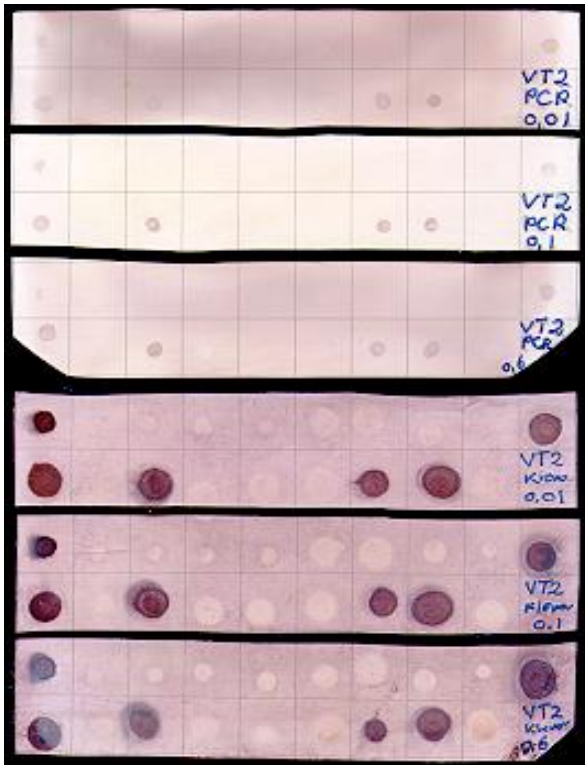

Figure 8.2. B Nylon membranes inoculated with control strains as listed in figure 8.2. B Legend. The three top membranes were hybridised with PCR labelled VT2 probe and the three lower membranes with random priming labelled VT2 DNA probe.

The membranes were washed at the second washing step as follows:

1. Top membrane and fourth membrane:
   Increased stringency using 0.01 X SSC, 0.1% SDS.
2. Second and fifth row:
   The stringency was kept at the original stringency using a 0.1 X SSC, 0.1% SDS.
3. Third and sixth row:
   The stringency was lowered using a 0.6 X SSC, 0.1% SDS washing solution.

1	2	3	4	5	6	7	8	9	10
VT2 pBS	Agg pBS	***eae*A** pBS	*ipa*H pBS	*bfp*A pBS	Agg EAST1	EAST1	EAST1	EAST pBS	VT1 VT2 EHEC
VT1 VT2 EHEC	Negative control	VT1 VT2 ***eae*A** EHEC EAST1	Agg EAST1	DA	LT ST_h ST_p	EIEC *ipa*H	? *	Negative control	
11	12	13	14	15	16	17	18	19	20

	C or D-number	Gene probe or strain designation	Vector or wild type serotype	**Expected DNA probe result**
1.	D2227	pDEP28	pBluescript	VT2
2.	D2228	pCVD432	pBluescript	Agg
3.	D2229	pCVD434	pBluescript	***eae*A**
4.	D2233	pWR390	pBluescript	*ipa*H
5.	D2234	pMSD207	pBluescript	*bfp*A
6.	D2240	17-2	O3:K-:H2	Agg, EAST1
7.	D2250	pSS106	pUC19	EAST1
8.	D2251	pSS126	pUC18	EAST1
9.	D2255	pSS126	pBluescript	EAST1
10.	C1023-91	DG 194-2	O128ab:H2	VT1, VT2, EHEC
11.	C 258-93	F2306	OX3:H2	VT1, VT2, EHEC
12.	D2103	MC1061	OR:H48	Negative
13.	D2188	933	O157:K-:H7	VT1, VT2, ***eae*A**, EHEC, EAST1
14.	D2189	17-2	O3:K-:H2	Agg, EAST1
15.	D2190	R551-1	O21:K-:H4	DA
16.	D2191	H10407	O78:K-:H11	ETEC: LT, ST_h, ST_p
17.	D2192	#7	O124:H30	EIEC, *ipa*H
18.	D2193*	2348/69	O127:H6	?
19.	D2194	H5 (neg)	O9:K^+:H4	Negative control
20.	Membrane identification field.			

Figure 8.2. C. Legend. The top table lists the expected DNA probe hybridisation results according to the control strains listed below. The table should be compared with Figure 8.2. C on the following page.

***** This strain was recieved as being the EPEC control strain 2348/69 serotype O127:H6, *eae*A, EAF, *bfp*A, but did not conform with the given genotype.

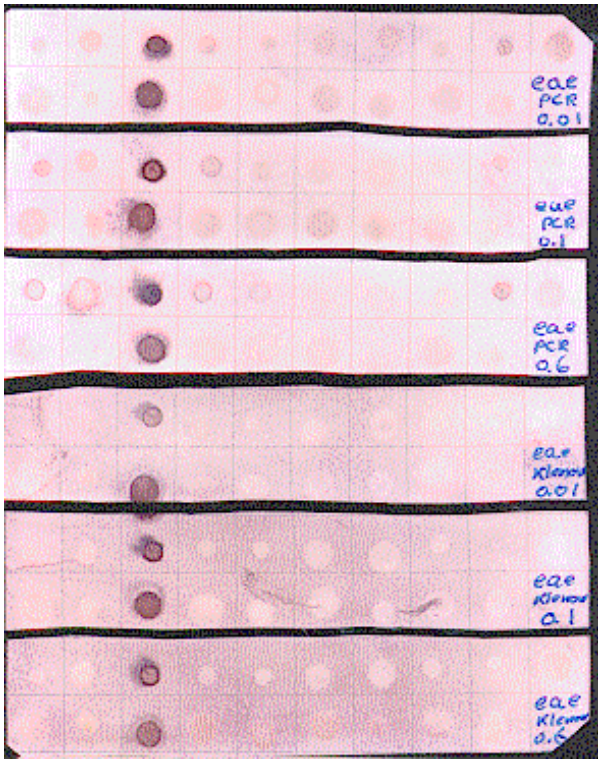

Figure 8.2. C Nylon membranes inoculated with control strains as listed in figure 8.2. C Legend. The three top membranes were hybridised with PCR labelled *eae*A probe and the three lower membranes with random priming labelled *eae*A DNA probe.

The membranes were washed at the second washing step as follows:

1. Top membrane and fourth membrane:
   Increased stringency using 0.01 X SSC, 0.1% SDS.
2. Second and fifth row:
   The stringency was kept at the original stringency using a 0.1 X SSC, 0.1% SDS.
3. Third and sixth row:
   The stringency was lowered using a 0.6 X SSC, 0.1% SDS washing solution.

9. General summary

9.1. Purpose

The purpose of this ph.d. thesis has been:

- to determine the prevalence of Verotoxin producing *E. coli* (VTEC) in Denmark.
- to present a detailed serological, genotypical and phenotypical characterisation of VTEC isolated from Danish patients.
- to obtain information on the clinical symptoms of Danish patients infected with VTEC, compared with EPEC.

Three different approaches were used:

- Prospective screening of diarrhoeal stools using DNA probes specific for Verotoxin and the detection of free faecal cytotoxin in stool specimens.
- Prospective and retrospective sero-, geno- and phenotypical characterisation of selected isolates with special emphasis on EPEC O groups.
- Direct contact by questionnaire with patients who had been infected with VTEC or selected attaching and effacing *E. coli* strains.

9.2. Results

Using DNA probes for VT in a prospective screening of 960 stool specimens representing 737 episodes and 46 non-episodal specimens identified 4 (0.5%) patients infected with VTEC.
Using the free faecal cytotoxin test indicated that 14 out of 905 (1.5%) patients might have been infected with VTEC but this was not confirmed by the isolation of VTEC.
The retrospective study demonstrated that 19% of Danish strains within O group O26 originally diagnosed as EPEC were VTEC strains and that VTEC was also found within O groups O111 and O128. The study provided evidence that VTEC is not an emerging pathogen.
Only a few O157 VTEC strains were isolated per year in Denmark during the last decade. The clinical data indicated that patients infected with O157 VTEC were at a higher risk of developing HUS than patients infected with *eae*A$^+$ EPEC and that this risk was reflected by the relatively higher risk of patients being hospitalised.
The relative risk of having bloody diarrhoea was much higher for patients infected with O157 VTEC and slightly higher for patients infected with non-O157 VTEC compared with patients infected with *eae*A$^+$ EPEC.
The relative risk of persistent diarrhoea (more than two weeks) was weakly associated with infection of *eae*A$^+$ EPEC and more likely to occur in children between the age of one to five years.
The VTEC strains isolated in Denmark during the last decade are shown in table 9.1. (p.100).

9.3. Conclusions

VTEC is not an unusual enteropathogen in Danish patients but the prevalence is low. It may be isolated from at least 0.5% of patients with diarrhoea. VTEC O157 was not identified during the prospective screening period indicating that sporadic cases of O157 are rare in Denmark. The retrospective part of the study confirms that O157 is not a big public health problem in Denmark. No Danish outbreak of O157 has been recorded in the last decade.

The sorbitol-negative-β-glucuronidase negative phenotype is highly indicative of O157 VTEC in human isolates.

The aetiologic role in both childhood and adult diarrhoea of *eae*A^+ *E. coli* warrants further elucidation. The isolation of *eae*A^+ O157 VT^- highlights the relevance of further examination.

The characterised VTEC strains belong to a few O:H serotypes which possess virulence factors that are usually associated with EHEC, a subgroup of VTEC causing bloody diarrhoea which is occationally complicated by HUS.

The results raise questions about the aetiology of bloody diarrhoea in Denmark. Laboratories which routinely diagnose EPEC should also have presumptive positive strains tested for the production of Vero cytotoxin.

Nine of the isolated O groups in this study have been associated with the development of HUS. The number of HUS cases from patients infected with O157 VTEC was similar to the number of patients with other VTEC serotypes. This emphasises the importance of the continued diagnosis of all VTEC strains from Danish patients.

As very few of the Danish HUS and TTP cases are examined for VTEC it is recommended that HUS is made a notifiable disease so that all cases may be subjected to microbiological elucidation.

Table 9.1. VTEC O:H serotypes diagnosed in Denmark from 1986 to July of 1997.

O:H serotype	<= 1 year	1-4 years	> 5 years	Total
O11:H49			1	1
O22:H8†		1		1
O26:H11†	13	3	1	17
O26:H21	1			1
O26:H-	5	3		8
O91:H-			1	1
O103:H2		2		2
O111:H-	2			2
O113:H4			1	1
O117:K1:H7			1	1
O121:H19		1		1
O123:H49		1		1
O128:H2†			1	1
O145:H28 *	1	1		2
O157:H7	6	7	7	20
O157:H-	1	2	8	11
O166:H28†			1	1
OX3:H2 *			1	1
OR:H21†			1	1
OR:HR	1			1
	31	21	23	75

* Two unusual cases of urinary tract infection complicated by HUS.
† Serotypes isolated during prospective screening of dierrhoeal stoools in 1994.

OR: O rough
HR: H rough

10. References

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11. Appendix

11.1. Antibiotic selection for plasmid vectors containing DNA probes

Antibiotics were used for selection of plasmid vectors containing DNA probes by spreading of relevant dilutions from stock solutions as listed in table 11.1.

Storage of stock solutions:
All stock solutions were divided into aliquots (usually 0,5 ml per migrofuge tube) and stored at -20°C.

Production of plates with antibiotics :
Autoclaved medium was cooled to 55°C and antibiotic in the desired concentration was added immediately before pouring the plates. The plates with tetracycline were stored **in the dark**. Approx. 30 ml was poured into each plate (9 cm plates).

[334] Pp. 71-72 and 444.
[335] Pp. 129 and 147.

Table 10.1. **ANTIBIOTICS**

Name	Abbreviation	**STOCK** solution in mg/ml	Final concentration in (μg/ml**Fejl! Bogmærke er ikke defineret.**): broth	Plates	Comments γ = μg/ml
Ampicillin (Na-salt)	**Ap**	25	(35)-50	50	H_2O; Sterile filtration. Plates with 25-50γ are kept at 4°C and must be used within 1-2 weeks [334] p. 71-72 and 444.
Chloramphenicol	**Cm**	30-(35)	10-30	10-20	100% ethanol; Sterile filtration. For amplification of plasmids: 170-300γ. Stock-solution can be stored for at least one year. Plates with 10γ are kept at 4°C and must be used within 1-5 days [334] p. 71-72 and 444.
Kanamycin	**Km**	25-50	50	30	H_2O; Sterile filtration. (Jeppe Boel: 80γ for VT1). High copy vectors with **Km^R** results in an increased **Km^R** : up to 300γ versus the normal 50γ. The frequency of spontaneous **Km^R** (= background) can be reduced by using a rich medium, e. g. LB. A higher conc. of Km does not work [335] p. 129 and 147.
Streptomycin (Sm-sulphate)	**Sm**	20	40	25	H_2O; Sterile filtration.
Tetracycline	**Tc**	12,5	12,5-15	10	Ethanol/H_2O (50% v/v); Sterile filtration. Stored in the dark (use tinfoil). Mg ions have an antagonistic effect. Use LB or c1776 [334] with NaCl (6 g/l) in stead of MgCl. High copy vectors with Tc^R reduces the Tc^R compared to the low copy vectors (opposite of Km).
Nalidixic acid	**Nal**			50	High conc. (up to 100γ) is *nal*A (42,5 minutes). Low conc. (up to 4γ) is *nal*B (51 minutes).
Sulphathiazol	**Su**			200	Use 10 % blood agar plates without peptone.

11.2. dBase applications

Validity of CPR numbers was checked with a self made dBase application called MODULUS.PRG. The last digit in a valid CPR number is called the control number. The control number, which also indicates sex (even control numbers indicate female), is calculated using the 9 preceding numbers which are birth-date without the century plus 3 random number as described below.
A randomly selected male born on the 7. July 1961 serves as an example.
The birth-date and the last randomly chosen numbers are multiplied with the following:

0	7	0	7	6	1	4	2	8
*	*	*	*	*	*	*	*	*
4	3	2	7	6	5	4	3	2

0+ 21+ 0+ 49+ 36+ 5+ 16+ 6+ 16+

sum = 149

The sum is divided by 11: 149 / 11 = 13
143

Remainder 6

The control number is found by subtracting the remainder from 11:
11 - 6 = 5

The CPR number for this male is: 070761-4285 [336].

The dBase application results in the following values in the control field:

Valid CPR numbers:	control = 1
Invalid CPR numbers:	control = 0
No CPR number:	control = 9
Last four digits of CPR number missing or temporary:	control = 9

Age and sex were calculated using valid CPR numbers with a self made dBase application. Age was calculated using the first 6 digits *i.e.* date of birth from CPR numbers where the last four digits were missing or temporary.

11.3. Permissions

On the following pages the signing individuals have kindly given their permission to use typing results from strains that they have submitted to the International *Escherichia* and *Klebsiella* Centre (WHO).

11.4. Questionnaires

The Danish original questionnaires are enclosed on the following pages.