Virulence factors of Verotoxin-producing *Escherichia coli* O157:H7

by

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This thesis is presented for the Degree of Doctor of Philosophy, in the Department of Biological Sciences, University of Warwick.

September 1993
IN COLOUR

NUMEROS ORIGINALES
DEDICATION

To my family, and in memory of my parents.
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DECLARATION

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr C S Dow. All work was carried out at the University of Warwick apart from studies on the human colonic tissue were carried out at the George Eliot Hospital, Nuneaton under the supervision of Dr N Bajallan. All sources of information have been specifically acknowledged by means of reference.
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I am grateful to all those who gave me helpful advice and answered many questions. I must thank Mrs. Vicky Cooper for her help with raising antisera.

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<th>Definition</th>
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<tbody>
<tr>
<td>AE</td>
<td>Attaching-effacing</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Diaminoethane tetraacetic acid</td>
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<td>EHEC</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FVT</td>
<td>Free Verotoxin</td>
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<tr>
<td>g</td>
<td>Gramme</td>
</tr>
<tr>
<td>Gb₃</td>
<td>Globotriaosylceramide</td>
</tr>
<tr>
<td>Gb₄</td>
<td>Globotetraosylceramide</td>
</tr>
<tr>
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<td>Haemorrhagic colitis</td>
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<td>HUS</td>
<td>Haemolytic uremic syndrome</td>
</tr>
<tr>
<td>KDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile toxin</td>
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<tr>
<td>M</td>
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<tr>
<td>µg</td>
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<td>mM</td>
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<td>OD</td>
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<td>PAGE</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
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<tr>
<td>ST</td>
<td>Heat-stable toxin</td>
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<td>Transmission electron microscope</td>
</tr>
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<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-hydroxymethylaminomethane</td>
</tr>
<tr>
<td>TIP</td>
<td>Thrombotic thrombocytopenic purpura</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylenesorbitan monolaurate</td>
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<tr>
<td>VTEC</td>
<td>Verotoxin-producing <em>Escherichia coli</em></td>
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<td>VT</td>
<td>Verotoxin(s)</td>
</tr>
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Escherichia coli O157:H7 is one of several E.coli serotypes that produce Verocytotoxins (VTs); they are collectively called "Verocytotoxin-producing E.coli"(VTEC). VTEC are medically important bacteria which have been implicated in cases of haemorrhagic colitis and haemolytic uremic syndrome. Two distinct VTs are known, VT1 and VT2, and variants of VT2 have been described. They are potent exotoxins which kill mammalian cells by inhibiting protein synthesis.

The virulence properties manifested by these organisms include the elaboration of VT1 or VT2 (or both), and the adherence to intestinal epithelial cells via an attaching-effacing mechanism. Many strains carry a 60 MDa plasmid which is thought to be involved in adhesion.

Initial data demonstrated that the VTEC O157:H7 isolates under study possess two virulence factors, production of VTs and adherence to epithelial cells. However, effort has focused on investigating bacterial adherence, largely because attachment of VTEC is thought to be an important pathogenic mechanism since it allows colonisation, which facilitates toxin delivery, and adherence may be sufficient to cause diarrhoea in experimental animals in the absence of VTs. Moreover, a better understanding of the adhesion mechanism should help in finding ways by which adherence can be prevented.

Since the bacterial-mucosal interactions are complicated in vivo by events and conditions that are not reproduced in current in vitro tests, a series of experiments were designed to investigate bacterial adherence to epithelial cells under conditions which are as close as possible to the in vivo situation. Significantly different data were obtained when quantitative adherence assays were performed under different physiological conditions, (different growth media, growth phase,
pH values, low iron and oxygen limitation). Both iron-restricted, and oxygen-limited media induced a reduction in the final cell density, however, anaerobiosis significantly increased the adherence capacity of VTEC O157:H7 to HeLa cells while low iron caused a reduction in the number of adherent bacteria. Actively growing cells in the exponential phase were more adherent to HeLa cells than cells in the stationary phase.

Since adhesion results from mutual recognition of surface structures from both the bacterial cell (adhesin) and the host cell (receptor), the bacterial cell envelope, and the HeLa cell outer membranes were investigated.

Results of the preliminary characterisation of VTEC O157:H7 surface components which have been implicated as adherence factors indicated that these strains are not fimbriated, however, they have been shown to be capable of binding to epithelial cells. Further studies were therefore, focused upon the identification of nonfimbrial adhesin(s).

The use of competitive inhibitors, such as bacterial outer membrane extracts (OMPs), isolated lipopolysaccharides (LPS) and rabbit antisera to the H-7 flagella, OMPs, and LPS suggested that the role of H-7 flagella is insignificant, the LPS may in part be involved, but the OMPs seemed to have the major role in mediating attachment of O157:H7 to HeLa cells.

The expression of OMPs under variable cultural conditions was examined, and significant differences were detected by the SDS-PAGE analysis of these extracts. The expression and repression of certain proteins was apparent under anaerobiosis, iron-restriction, different pH values and different bacterial growth phases.

HeLa cell outer membranes were studied to identify the receptors on the host cell. Purified outer membranes were analysed by SDS-PAGE and used as inhibitors of bacterial adherence. Two proteins were identified by immunoblotting as a potential receptors.
CHAPTER 1

INTRODUCTION
CHAPTER 1

INTRODUCTION

1.1 General historical background

*Escherichia coli* was first described by Doctor Theodor Escherich in 1885 as "Bacterium coli commune". His achievement however, extended far beyond this important description (Escherich, 1885).

The primary habitat of *E.coli* is the gastrointestinal tract, principally the bowel of mammals and birds. Colonisation of human intestine takes place soon after birth, once established, *E.coli* remain as a member of the normal flora of man. Most of these organisms are harmless, however, there are fully pathogenic strains that cause distinct syndromes of diarrheal diseases.

*E.coli* is the causative agent of most human cases of urinary tract infections and bacterial gastro-enteritis. Uropathogenic strains usually originate in the colon, colonise the perineum, cross the urethra to the bladder and occasionally ascend to the kidney (Fowler & Stamey, 1977).

From the early days of the 1940s when *E.coli* were first convincingly associated with human diarrhoea, much has been discovered about the several categories of diarrhoeagenic *E.coli*, including information on their clinical features, epidemiology, O:H serotypes, and most particularly, their pathogenesis.

From a previous state of some confusion about their role as enteric pathogens, diarrhoeagenic *E.coli* are now recognised as being the best understood bacterial enteropathogens.
1.2 Discovery of Verotoxin-producing *E. coli* (VTEC)

In 1977 Konowalchuk and colleagues showed that culture filtrates of some strains of *E. coli* produced cytotoxic effects on Vero cells (line of African green monkey kidney cells). Two other cell lines, Y1 (mouse adrenal cells) and CHO (Chinese hamster ovary cells) were not affected by these filtrates. Therefore, the activity of the new toxins could be distinguished easily from that of the *E. coli* heat-labile enterotoxin (LT). The cytopathic effect on Vero cells was shown to be due to the action of one or more toxins subsequently termed Vero cytotoxins (VTs). VT produced by *E. coli* strain H30 of serotype O26:H11 was shown to be very similar to Shiga toxin (ShT) produced by *Shigella dysenteriae* of serotype 1, in terms of biological properties and antigenicity (O'Brien *et al.*, 1982, O'Brien & LaVeck, 1983). As a consequence of these similarities, the term shiga-like toxin (SLT) was also used. However, in this study, the term VT will be used throughout to avoid confusion.

The VT neutralised by anti-Shiga toxin was designated VT1, and a second VT which was not neutralised by anti-Shiga toxin, designated VT2 (Scotland *et al.*, 1985). Variants of VT2 have been identified recently (VT2vh) from humans (Oku *et al.*, 1989) and porcine VT (VT2vp) (Marques *et al.*, 1987).

Vero cytotoxin producing strains of *E. coli* (VTEC) have been isolated primarily from human and animal infections. Subsequently, VTEC predominantly of serogroup O157, have been associated with cases of haemorrhagic colitis (HC) characterised by severe bloody diarrhoea (Riley *et al.*, 1983) and haemolytic uremic syndrome (HUS) which is characterised by acute renal failure, haemolytic anaemia, and thrombocytopenia (Karmali *et al.*, 1983).
VTEC have also been implicated as a cause of disease in animals, particularly calves and pigs, however, O157 strains do not appear to cause significant disease in animals.

It is important to note that other VTEC serotypes are being isolated more frequently and also that a great variety of *E.coli* serotypes can acquire the gene coding for VT, although it is assumed to be the level of expression of these genes which is important in the pathogenesis of diseases associated with VTEC infection.

The discovery that VTEC are associated with two life threatening diseases (HC & HUS) of previously unknown aetiology, generated great interest in these organisms and their toxins.

During the last ten years the study of VTEC has increased dramatically and the role of these organisms in disease is now well established.

The first international symposium on VTEC infections was held in 1987 in Canada. That meeting reflected the great interest in the subject in many countries of the world. It hoped that an increased knowledge of these organisms can be exploited to prevent and treat VTEC infections in man and animals.

### 1.3 Distinction of VTEC from other diarrhoeagenic *E.coli*

Diarrhoea may be a matter of inconvenience to healthy adults, but can be life threatening in the very young and the elderly, as well as in the undernourished and immuno compromised individuals.

All enteric pathogens share an oral route of infection, after ingestion of contaminated food or drink, pathogenic *E.coli* must overcome a number of non specific local defences, such as the gastric acid barrier in the stomach, the peristaltic mechanism of the small intestine, and must
interact with the mucus layer protecting the epithelial cells, before they are able to colonise the intestinal mucosa and cause illness. 

*E.coli* strains that cause diarrhoea in humans have been grouped into five major categories (table 1.1). Organisms in these categories differ in their pathogenesis, epidemiology, and O:H serotype (Levine, 1987).

### Table 1.1 Patterns of illness and mechanisms of virulence associated with *Escherichia coli* groups that cause human enteric illness

<table>
<thead>
<tr>
<th><em>E.coli</em> group</th>
<th>Pattern of illness</th>
<th>Virulence mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteropathogenic (EPEC)</td>
<td>Watery diarrhoea, low-moderate fever.</td>
<td></td>
</tr>
<tr>
<td>Enterohaemorrhagic (EHEC)</td>
<td>Watery-bloody diarrhoea, no-low fever.</td>
<td>Verotoxins, &amp; A/E</td>
</tr>
<tr>
<td>Enteroaggregative (EAggEC)</td>
<td>Persistent infant diarrhoea.</td>
<td>Unknown.</td>
</tr>
</tbody>
</table>

1.3.1 Enterotoxigenic *E.coli* (ETEC)

ETEC are a major cause of travellers' diarrhoea and infant diarrhoea in the Third World Countries. The clinical features are watery diarrhoea, nausea, abdominal cramps, and low grade fever. ETEC colonise the small intestine after adhering to the mucosal epithelial cells by means of specific fimbriae or colonisation factors. They are non-invasive, and
produce one or both of two cholera-like heat labile toxins (LT), or heat stable (ST) enterotoxins, which act specifically on the enterocytes, causing fluid secretion resulting in watery diarrhoea.

1.3.2 Enteroinvasive *E. coli* (EIEC)

EIEC are an important cause of dysenteric forms of diarrhoeal illness, they do not produce LT or ST but like *Shigella*, with which they share antigens, they have the ability to invade and multiply within the colonic epithelial cell and cause eventual death of the cell.

Clinical symptoms include fever, severe abdominal cramps, toxaemia and watery diarrhoea followed by gross dysentery consisting of scanty stools of blood and mucus. The ability of these bacteria to invade epithelial cells is due to the presence of a large, approximately 140 megadalton (MDa) enteroinvasive plasmid (plnv) coding for the production of several outer membrane proteins involved in the invasion of mammalian cells (Harris *et al.*, 1982, Hale *et al.*, 1983).

Recently it has been found that all major EIEC O serogroups produce low levels of VTs that are immunologically distinct from VT1 and VT2 (Fasano *et al.*, 1990). However, the significance of low level cytotoxin production is uncertain, while epithelial cell invasiveness is clearly the predominant virulence property responsible for the dysenteric syndrome. The presence of these cytotoxins may indicate the severity of the symptoms.

1.3.3 Enteropathogenic *E. coli* (EPEC)

EPEC strains are considered to be a homogeneous group distinct from other diarrhoeagenic *E. coli* strains chiefly because they lack specific
virulence attributes which are characteristic of the others, namely invasiveness and toxin production.

The characteristic way in which all EPEC strains associate with the intestinal mucosa is known as the attachment-effacement (A/E) mechanism (Moon et al., 1983). In the region of bacterial attachment, the microvillous border is lost, the cytoskeletal elements are disrupted, and bacteria viewed by electron microscopy, partially surrounded by cup-like projections (pedestals) from the enterocyte surface (Fig. 1.1).

Figure 1.1 Representation of the attaching-effacing mechanism of enteropathogenic E. coli (EPEC) adherence to epithelial cells.

Bacteria destroy the brush border of the epithelial cells and closely adhere to the apical surface, causing cupping and pedestal formation by the cell membrane.
These cellular changes, the mucosal response to them and their effect on the physiology of digestion and absorption, are presumably the key to the pathogenesis of the disease. However, it was found (Cleary et al., 1985) that some EPEC strains, particularly those of serogroup O26, commonly produce VT, but the biological significance of this remains unclear.

Clinically EPEC infection occurs most frequently in infants and young children and is characterised by fever, vomiting and diarrhoea with a prominent amount of mucus but without gross blood.

1.3.4 Enterohaemorrhagic E.coli (EHEC)

EHEC are defined by their ability to produce Verotoxin in culture supernatants. These organisms are also known as Verotoxin producing E.coli (VTEC), and they are the subject of this study, in which the nomenclature (VTEC) will be adhered to.

VTEC have been shown to produce a spectrum of illness ranging from asymptomatic infection and uncomplicated diarrhoea to haemorrhagic colitis (HC) which is characterised by the sudden onset of grossly bloody diarrhoea with low grade or no fever, sometimes preceded by abdominal pain and cramps. These symptoms also describe the prodromal phase of haemolytic uremic syndrome (HUS), where acute renal failure, thrombocytopenia, haemolytic anaemia and even death may result (Fig. 1.2)

In addition to VT production, VTEC adhere to epithelial cells by an attaching-effacing mechanism characteristic of EPEC, and many strains carry a 60 MDa plasmid thought to be required for the expression of fimbrial antigen and for adhesion to epithelial cells (Karch et al., 1987).
Figure 1.2 Pathogenic properties of *E.coli* O157:H7

Virulence factors of VTEC O157:H7

- Mucosal colonisation
- Verotoxins production

**Haemorrhagic colitis**
- Bloody diarrhoea
- Low temperature
- Abdominal pain

**Haemolytic uraemic syndrome**
- Renal failure
- Thrombocytopenia
- Haemolytic anaemia

Although several serotypes have been classified as VTEC, most human strains belong to the O157 serogroup, and of these a high percentage possess the H7 flagellar antigen (O157:H7). The virulence factors of this serotype have been studied in detail in this project.

1.3.5 *Enteroaggregative* *E.coli* (EAggEC)

EAggEC strains are a recently described class of bacteria (Nataro *et al.*, 1985) epidemiologically associated with infant gastroenteritis, especially in the developing world being most prominent among cases that persist more than 14 days.
EAggEC strains are characterised by an aggregative pattern of adherence (AA) to HEp-2 cells, wherein bacteria are seen in "stacked brick" aggregates attaching to HEp-2 cells and usually, to the glass surface between cells in tissue culture.

Little is known about their pathogenicity. They do not induce the attaching-effacing lesions, nor do they elaborate Cholera-like or Shiga-like toxins. Some strains of EAggEC have been shown to secrete a heat-labile protein that is antigenically related to haemolysin (Baldwin et al., 1992).

1.4 Clinical manifestations of VTEC infection

Although VTEC were first described in 1977, their association with human disease remained unknown until 1982. Infection with VTEC may cause a severe and even fatal illness especially in the elderly and in children. Milder forms of illness are also likely, but may not come to medical attention.

The clinical conditions associated with VTEC infection range from mild diarrhoea to severe bloody diarrhoea known as haemorrhagic colitis (HC). Some patients develop the haemolytic uremic syndrome (HUS), mainly infants and young children. In adults this may take the form of thrombotic thrombocytopenic purpura (TTP), which reflects probable overlap between the two syndromes. Some VTEC strains have also been implicated in post-weaning diarrhoea in piglets.

The association of VTEC with ulcerative colitis has also been reported. Wulffen et al. (1989) detected 4 VTEC strains of antigenic type O2:H5 from 17 patients with ulcerative colitis. VTEC O2:H7 has also been associated with a baby who died of Sudden Infant Death (Cot Death) in New Zealand (Wilson & Bettelheim 1980).
1.4.1 Haemorrhagic colitis (HC)

HC is characterised by grossly bloody diarrhoea, usually in the absence of the usual signs of inflammation such as fever, or pus cells in the stool. It is frequently preceded by abdominal cramps and a watery diarrhoea. The cause of the syndrome was unknown until 1982 when two separate outbreaks of HC in Michigan and Oregon were investigated and led to the discovery of a particular serotype of *E.coli*, O157:H7, as the aetiologic agent (Riley *et al.*, 1983). It soon began to emerge that *E.coli* O157:H7 was by no means a rare serotype as initially thought, but rather a common isolate from patients with HC and non-specific bloody diarrhoea.

Although the precise role of VTs in HC is not fully understood there is substantial evidence suggesting that the toxins are important virulence factors in the development of bloody oedematous vascular lesions in the colon (Fontaine *et al.*, 1988). The toxin may also participate in direct killing of colonic epithelial cells and may provoke fluid secretion and diarrhoea in the host (O'Brien & Holmes, 1987).

The use of antibiotics to treat VTEC associated HC is not beneficial and the early use of antimotility agents to control the diarrhoea may increase the mortality and morbidity (Ryan *et al.*, 1986). This is thought to be because a decreased intestinal motility may allow the multiplication of organisms and the increased production of VT.

1.4.2. Haemolytic Uremic Syndrome (HUS)

HUS is characterised by three features: kidney damage and possible failure, haemolytic anaemia and low platelet counts "thrombocytopenia".
HUS occurs in all age groups but more commonly in infants and young children, and it is one of the major causes of renal failure in childhood. A close association between VTEC and HUS was first reported by Karmali et al. (1983). Studies since then have established that VTEC are the major cause of the classical form of HUS (Karmali et al., 1985a; Scotland et al., 1988) which is preceded by a prodromal phase of bloody diarrhoea resembling HC.

The mechanism by which VT may be involved in the pathogenesis of HUS is at present unknown, although accumulating evidence suggests that the toxin may have a direct effect on endothelial cells (Obrig et al., 1988; Milford and Taylor, 1990).

Histopathological studies of the kidneys from HUS patients have shown profound alteration in the glomeruli and it has been hypothesised that VTs produced by VTEC strains are disseminated from the gut by the blood stream and target the kidneys were they generate damage to the glomerular endothelial cells (Wadolkowski et al., 1990).

The value of antimicrobial treatment during illness remains conflicting. Studies by Ryan et al. (1986) suggested that antimicrobial therapy was ineffective, whereas some authors have found that anti-microbial treatment during prodromal illness was associated with a mild clinical course and a good outcome (Martin et al., 1990).

1.4.3 Thrombotic thrombocytopenic purpura (TTP)

TTP is another serious but uncommon condition associated with VTEC infection in young adults. The distinction between HUS and TTP is sometimes blurred. In TTP the clinical features of HUS (renal failure, haemolytic anaemia and thrombocytopenia) are accompanied by neurological involvement and fever. The peak incidence of TTP is
between 30 and 40 years of age, while HUS is most common in children younger than four years of age. Most cases of TTP present without an antecedent illness, whereas a prodromal diarrhoeal illness is an essential feature of HUS. In patients with HUS or TTP, *E.coli* O157:H7 strains producing VT2 only have been the most common isolate (Ostroff *et al*., 1989).

1.4.4 **Diseases of animals due to VTEC.**

VTEC have been isolated from both diseased and healthy cattle, however, VT-producing *E. coli* were isolated from 27% and 29% of diarrhoeic cattle and buffalo calves, respectively, in comparison to less than 6% of healthy control animals (Mohammad *et al*., 1985).

VT-producing strains have also been detected in pigs with diarrhoea or Oedema disease, a severe and often fatal disorder in weaned piglets (Dobrescu, 1983; Smith *et al*., 1983). This is a neurological disease which begins with a staggering gait, limb paralysis and progresses to convulsion, coma and finally death. The disorder is characterised by oedema which is restricted mainly to the stomach wall and colonic mesentery with profound vascular damage. The "Porcine VT" can be clearly differentiated from the two "Human VTs" on the basis of antigenic cross reactivity and heat stability (Linggood & Thompson, 1987). A vaccine is currently under development, based on a toxoid preparation, which should protect against oedema disease (Macleod *et al*., 1991).

1.5 **Epidemiology of VTEC infection**

*E.coli* O157:H7 infection is a major public health problem in the United States and Canada. In addition there are signs it may be on the increase
in the UK (Fig 1.3). Food and unpasteurised milk are thought to be the most important source of infection. As a consequence of the sharp summer peak in incidence of the disease, and the association of the organisms with hamburger meat, a common assumption is to link the disease with common summer food preparation practices. Thus the lay press has referred to it as "Hamburger syndrome" or "Barbecue syndrome".

**Figure 1.3 Number of VTEC O157:H7 isolates in the UK 1982-1992**

![Bar graph showing number of VTEC O157:H7 isolates in the UK from 1982 to 1992](image)


**1.5.1 Incidence of HC and HUS due to *E. coli* O157:H7**

**1.5.1.1 America**

VTEC association with HC and HUS has occurred over a wide geographical area and has affected males and females of all age groups.
The majority of infections are with strains belonging to serogroup O157, and have been reported primarily in the summer months. Outbreaks with VTEC have occurred in the community, in nursing homes for the elderly and in day care centres for young children, with most severe clinical manifestation usually seen in paediatric and geriatric populations (Ryan et al., 1986; Krishnan et al., 1987).

In Canada there has been an exponential increase in the number of isolates of E.coli O157:H7 since its identification in 1978. Only five isolates had been obtained between 1978 and 1981, while the number of isolates from cases of diarrhoea or HUS reached 1,342 in 1987. In addition, the number of reported outbreaks due to O157:H7 has also increased each year since 1982 (Hockin & Lior 1988).

In 1985 there was an outbreak of E.coli O157:H7 infection in a nursing home for the elderly in Ontario, 55 of 169 resident, and 18 of 137 staff members were affected. HUS developed in 12 residents, 11 of whom died (Carter et al., 1987). Two institutional outbreaks were reported in July 1988. In one, in Regina, there were 81 suspected cases, of which 40 were confirmed due to O157:H7. The other outbreak occurred in Keswick, Ontario; 11 patients had positive culture results, 6 including 1 who died, had bloody diarrhoea (Hockin & Lior 1988).

In the USA, VTEC O157 is associated with 15% - 30% of all cases of bloody diarrhoea. However, 75% - 90% of patients with HUS in North America have associated VTEC infection of the bowel (Edelman et al., 1988). One of the recent outbreaks, traced to consumption of undercooked fast-food hamburgers in early 1993, has moved the US Congress to address the issue of microbial contaminants in food supply (Knight, 1993), 500 culture-confirmed cases were identified, 41 persons developed HUS and 4 children died.
HUS is relatively uncommon in most parts of the world, however, Argentina has the highest reported frequency of HUS, and, it is the most common cause of acute renal failure in young children. Argentinean children were prospectively studied during the HUS season for evidence of Verotoxin-related diseases. On the basis of serology, faecal cytotoxin neutralisation, stool cultures, and DNA hybridisation of colony lysates, most children with HUS had evidence of infection with Verotoxin-producing organisms (Lopez et al., 1989).

1.5.1.2 In Britain:

All cases of HC and HUS associated with VTEC have been investigated during the last ten years. The first noted outbreak of HUS associated with the VTEC serogroup O157 in Britain, occurred in 1982 in the West Midlands (Taylor et al., 1986). The first recognised outbreak of HC due to O157:H7 in the UK occurred in 1985 in East Anglia (Morgan et al., 1988), at least 24 people were affected over a 2-week period. Eleven patients were admitted to hospital and there was one death. Unlike previously reported outbreaks hamburgers were not the vehicle of infection, and a case-control study suggested that handling vegetables, and particularly potatoes, was the important risk factor.

A prospective study of HC was conducted in England and Wales from October 1985 to October 1986. Cases were distributed widely throughout the country, and were not noticeably related to urban or rural areas. VTEC were detected by DNA probe analysis in 32 (39%) of 83 specimens. Thirty of the 32 VTEC belonged to serogroup O157 confirming the importance of this serogroup as a cause of HC in areas other than North America (Smith et al., 1987).
In July 1987 an outbreak of HC occurred in Birmingham after a Christening party, and the public health laboratory service (PHLS) investigation showed that VT producing *E.coli* was the causative organism (Gross, 1992). Soon afterwards in the same area a number of people became ill after a barbecue and again VTEC including *E.coli* O157 were isolated.

In 1991, there were 7 outbreaks (Thomas *et al.*, 1993) the largest of which occurred in the Preston area. There were 23 confirmed cases of infection nationally, with an unusual urease-producing strain of *E.coli* O157:H7 of phage type 31 being isolated. A descriptive epidemiological study of these cases revealed that 11 had eaten a burger at an outlet of the same burger chain within the previous week, 9 of these had eaten at the same outlet in Preston on the same day. Another major outbreak occurred in the Scottish borders, affecting 2 residential homes and 3 people in the community. Preston was the location of another outbreak later in that year affecting 17 people. In addition to the general outbreaks, there were 45 household outbreaks involving 97 cases. National surveillance provided by the PHLS-VTEC working group has shown that the number of *E.coli* O157 isolates, referred to the enteric pathogen-division, is on the increase in the UK.

1.5.3 **Reservoir, source and transmission of VTEC**

VTEC are widely distributed in the intestine of animals, predominantly cattle, and food products of animal origin are the primary source of human infection.

Most outbreaks of *E.coli* O157:H7 have been linked epidemiologically to the consumption of undercooked ground beef and to a lesser extent to the drinking of unpasteurised milk.
Studies by Wells et al. in 1991 suggest that dairy cattle, a source of both raw milk and beef products, are the principal reservoir of *E. coli O157:H7*. Examination of retail fresh meat and poultry for strains of O157:H7 showed that these organisms were present in 3.7% of ground beef, 1.5% of poultry and 2% of lamb samples (Doyle & Schoeni 1987), so it is possible that animals other than cattle may serve as the reservoir of this organism.

Additionally, in a report on the first community outbreak of HC in the UK due to *E. coli O157:H7* in which 24 persons were affected, 11 were admitted to hospital and one person died. Vegetables were thought to be the source of infection (Morgan et al., 1988). Raw food of animal origin may be contaminated with the organisms via faecal contact during slaughter or the milking procedure.

The first documented food-born outbreak of HC due to *E. coli O157:H7* associated with the consumption of heat-processed meat patties, occurred among students attending a Minnesota school in October 1988 (Belongia et al., 1991). This outbreak was unusual since heat-processed products should be pathogen-free; and in response to this outbreak specific requirements have been proposed for the manufacture of heat-processed meat patties.

A recent outbreak of diarrhoeal disease due to *E. coli O157:H7* resulting in 4 deaths, 32 hospitalisations and a total of 243 documented cases was linked epidemiologically to the public water supply in Cabool (Geldreich et al., 1992). Another outbreak associated with water borne transmission of this pathogen occurred in Scotland during the summer of 1990 (Dev et al., 1991).

While VTEC infection in most cases is probably food-borne, there is increasing evidence that infection can also be acquired through person to
person transmission, indicating that a relatively small dose of the infectious agent may be sufficient to initiate disease, but this is a less common mode of transmission.

1.5.4 Host susceptibility to VTEC infection

Little is known about factors that determine susceptibility to VTEC infection and the risk factors for the development of systemic complications. The peak age incidence (very young and elderly) of classical HUS is a reflection of the age incidence of VTEC infection as a whole, so it could be due to the lack of specific immunity, possibly to VT. Patients with VTEC-associated HUS develop a rising level of VT-neutralising antibodies. However, there is no definite information on the significance of these antibodies in long term immunity (Karmali et al., 1987). Specific host factors such as the presence of specific receptors like the glycolipid globotriaosyl ceramide (Gb3) in target tissue could be a factor in the development of HUS in susceptible individuals. On the other hand, the hypothesis that the development of complications is related to the inoculum size of micro-organisms is supported by the occurrence of a high incidence of HUS among elderly residents in a large nursing home outbreak, and the correlation of previous gastrectomy with the more severe form of HUS (Carter et al., 1987). In the same outbreak the use of antibiotics prior to the onset of symptoms was considered to be a risk factor for acquiring the infection and the development of HUS. The antibiotics might be involved in the enhancement of toxin production, by alteration of the normal competing bowel flora, leading to overgrowth by VTEC.
The influence of blood group status on the development of HUS in children was studied by Rose et al., (Warwick Hospital, personal communication). They have speculated that the expression of the P1 blood group antigen is in some way protective against the development of HUS.

The hypothesis is that the receptors for VT have been shown to require a terminal galactose α (1-4) galactose disaccharide, which in human is expressed in the P1 blood group antigen (Lindberg et al., 1987). If a P1 +ve person is exposed to VT, his/her erythrocytes will adsorb the free toxin and so reduce the burden on other target cells. Once internalised within the erythrocytes the toxin may be relatively innocuous, as there is no ribosomal activity for the toxin active A subunit to interrupt (section 1.8.1.1).

Additionally expression of various blood group antigens is age related. P1 +ve individuals have reduced expression both in their pre-school year and in senescence. 18 of the 22 HUS children studied showed remarkable reduction in the P1 antigen. Furthermore, patients with severe renal damage showed either the absence of the P1 antigen or a marked reduction in expression, compared to normal children. This may explain why HUS is so common in Argentina while in neighbouring Brazil it is rare, a consequence of P1 antigen expression being very low in the Argentinean population (11%), whilst in parts of Brazil antigen expression is found in 75% of individuals.

1.6 Pathogenesis of VTEC infections

The various components involved in the expression of bacterial pathogenicity have been summarised in figure (1.4).
Figure 1.4 Factors in pathogenicity and infection with VTEC O157:H7.

Beef, milk, or infected person | Consumption of contaminated food or drink, or person to person transmission | Susceptible host develop illness

The source of infectious agent is the natural habitat of the pathogen, then there is the stage of transmission of the infectious challenge dose to the new host, followed by a process of infectivity of the agent (incubation period). Thereafter, the organisms damage the host and express virulence by essentially invasive or toxic mechanisms or a combination of these leading to signs and symptoms of the disease. The outcome of such an attack on the host depends upon the many variables which influence the host-parasite association.

The possible mechanism by which VTEC causes disease is illustrated in figure (1.5).
Figure 1.5 Routes by which *E.coli* O157:H7 causes disease

Ingestion of contaminated food or drink

\[ \downarrow \]

Adherence of VTEC to colonic epithelium

\[ \downarrow \]

Release of VT into the bowel

\[ \downarrow \]

Absorption of VT into the blood

\[ \downarrow \]

Endothelial damage of small blood vessels

\[ \downarrow \]

Local intra-vascular coagulation

\[ \Leftrightarrow \]

Colon (HC) Kidney (HUS)

1.6.1 Initiation of infection

Little is known about the inoculum size required to initiate infection. The infective dose for this pathogenic strain of *E.coli* is estimated to range from 10-100 organisms (Krishnan *et al.*, 1987). Presumably, after ingestion of contaminated food, the organisms have to overcome the gastric acid barrier. The low gastric pH has a major role in reducing the inoculum size of infecting organisms. The importance of
this was highlighted in a nursing home outbreak of *E.coli* O157:H7 infection in which previous gastrectomy was correlated with an increased risk of acquiring the infection (Carter *et al.*, 1987)

### 1.6.2 Colonisation of intestinal mucosa

No information is available from human cases to assess directly the nature, site or mechanisms of intestinal colonisation by VTEC. Nor are there data on the manner in which VTEC interact with, and penetrate, the intestinal mucus barrier. On the other hand a well-defined pattern of colonisation has emerged from studies of natural and experimental VTEC infections in animals that is consistent with the attaching-effacing adherence (A/E) characteristic of human EPEC infection (Moon *et al.*, 1983; Robins-Browne, 1987). While the EPEC associated A/E adherence in animals has been demonstrated in the small bowel, the VTEC associated adherence has been observed mainly in the large bowel.

### 1.6.3 Development of Haemorrhagic Colitis

There is more than one possible mechanism, which might account for the development of diarrhoea that is the main clinical manifestation of HC. One hypothesis is that diarrhoea results from local action of VT on the intestinal mucosa, which is supported by evidence provided by experiments on animals.

Feeding of VT alone to infant rabbits results in clinical symptoms and histological changes almost identical to those seen after challenge with live bacteria. Furthermore, bloody diarrhoea and extreme mucosal and submucosal congestion, characteristic of HC in humans infected with *E.coli* O157:H7, were seen after administration of VT (Pai *et al.*, 1986).
The toxin purified by Padhye et al. (1986) damaged the epithelial cells in the mouse colon, but not other areas of the gastrointestinal tract, sloughing-off of the surface and crypt cells was accompanied by haemorrhage as seen in human infection. Similar effects on the mouse colon and kidney have been reported in the study by Beery et al. (1984), with culture supernatant of *E. coli* O157:H7.

More recent studies have demonstrated the effect of intravenous injection of purified VT in rabbits. The clinical features included watery diarrhoea. Pathologically, there was oedema with haemorrhage in the mucosa and submucosa of the caecum (Richardson et al., 1992).

The other possible mechanism is related to the attaching-effacing adherence of VTEC to the intestinal epithelial cells. The development of diarrhoea in animal models, in association with characteristic attaching-effacing lesions in the large bowel mucosa, is strong suggestive evidence that this may be a mechanism by which VTEC cause diarrhoea in humans.

Tzipori and colleagues (1987) investigated the role of two putative virulence factors, a fimbrial antigen specified by a 60 MDa plasmid and phage mediated VT production. They found that neither VT production nor the presence of the 60 MDa plasmid was required to cause diarrhoea. The latter was correlated with the attaching-effacing lesions which occurred independently of the plasmid or toxin associated factors.

### 1.6.4 Development of HUS

Classical HUS consists of a triad of clinical features, acute renal failure, thrombocytopenia and haemolytic anaemia. Pathologically HUS is characterised by microvascular angiopathy that affects not only the
kidney but a variety of other tissues and organs including the gastrointestinal tract, central nervous system and pancreas. Current opinion indicates that verotoxin is the factor that causes primary vascular endothelial cell damage, assuming that the toxin passes intact into the circulation from the bowel after enteric infection by VTEC, and then interacts specifically with endothelial cells in the target tissues (Richardson et al., 1992).

Both VT1 and VT2 have the ability to bind to a specific glycolipid globotriosyl ceramide (Gb3) receptor which is a major component of the glycolipid in human renal tissue (Boyd & Lingwood 1989). The pathophysiological abnormalities in HUS include not only endothelial cell damage but also, reduction in the platelets count, an increase in plasma platelet aggregation activity (Monnens et al., 1985) and the occurrence of an abnormal factor VIII (Rose et al., 1985). It has been suggested that the abnormal factor VIII related antigen leads to platelet aggregation and thrombocytopenia. The fact that factor VIII related antigens are synthesised by endothelial cells, suggests that the action of VT on these cells may be responsible for factor VIII aberration in patients with HUS.

Another manifestation of HUS is microangiopathic haemolytic anaemia, characterised by the presence of fragmented erythrocytes. The mechanism of erythrocyte damage has yet to be resolved. Erythrocytes in most individuals express the P antigen, which has structural similarities to Gb3 (Lindberg et al., 1987), the possibility therefore that VT binds to erythrocytes and therefore has a role to play in the development of haemolytic anaemia has yet to be investigated.
1.7 Virulence Factors of *E. coli*

For *E. coli* to cause illness in a susceptible host, it must possess more than one of the following virulence determinants:

### 1.7.1 Colonisation factors

Colonisation of mucous membranes is a prerequisite for most diseases caused by *E. coli*, and a number of fimbrial colonisation factors have been described (Evans *et al.*, 1975; Honda *et al.*, 1984; Tacket *et al.*, 1987). However, non-fimbriated *E. coli* may be capable of attaching to mucosal surfaces by mechanisms that have yet to be elucidated (Sussman *et al.*, 1982). On one hand, mucosal colonisation may be asymptomatic if the host is immune to the pathogenic mechanisms exhibited by the infecting strain. On the other hand bacterial adherence to intestinal mucosa may itself be sufficient to cause disease (Riley, 1987).

### 1.7.2 Endotoxins

Endotoxins are the cell envelope lipopolysaccharide (LPS) of Gram-negative organisms. The endotoxicity of LPS is expressed when it is released in the body by the break down of the infecting bacterial cells. Endotoxins are directly involved in the production of symptoms of disease. Several lines of evidence suggest that bacterial endotoxins enhance both the diarrhoeagenic and lethal effect of VT2 when given to rabbits after VT2 exposure (Barrett *et al.*, 1989). LPS was also found to protect rabbits from VT2 toxicity when given before a VT2 challenge.
1.7.3 Exotoxins

Some *E. coli* strains produce a number of toxins, the best recognised of these are the plasmid coded heat-labile (LT), and heat-stable (ST) enterotoxins produced by strains that cause diarrhoeal disease in man. Their effect on the intact intestinal mucosa is to give rise to a net secretion of water and electrolytes. Other strains of *E. coli* produce heat-labile toxins called Vero cytotoxins (VTs) due to their toxic effect on Vero cells in tissue culture. These toxins will be discussed further, later in this chapter.

1.7.4 Invasiveness

Certain strains of *E. coli* are able to invade and multiply within the epithelial mucosal cells of the colon in a similar manner to *Shigella* and produce a dysentery like disease in man (Harris *et al.*, 1982).

1.8 Virulence factors of VTEC O157:H7

Several virulence properties have been described in the literature that may contribute to the intestinal and systemic diseases caused by *E. coli* O157:H7. Two such factors are:-

a) Verotoxin production:
All clinical isolates produce one or two verotoxins which are cytotoxic to Vero cells in tissue culture.

b) Adhesive properties:
In addition to toxin production *E. coli* O157:H7 has the ability to adhere to intestinal mucosa in a characteristic attachment-effacement manner.
The importance of each factor and its relevance to bacterial pathogenicity will be discussed in detail in the following sections.

1.8.1 Vero cytotoxins (VTs)

Konowalchuk et al. (1977 & 1978) first described at least three distinct E.coli VTs, two of these being from strains isolated from humans and a third from strains isolated from pigs. Emerging evidence has since indicated that VTs are a family of structurally and functionally related cytotoxins.

The types discovered to date are:

**VT1**

VT1 has been shown to be immunologically very similar to shiga toxin (ShT) of Shigella dysenteriae type 1. The isoelectric point, subunit structure and biological activities are also similar with both toxins showing complete cross neutralisation (O'Brien & LaVeck, 1983).

**VT2**

VT2 possesses the same biological activities as VT1, both are toxic to the same cell lines, cause paralysis and death in mice, and fluid accumulation in rabbit ileal loop (Strockbine et al., 1986).

VT2, however, is antigenically distinct, it is not neutralised by polyclonal antiserum to ShT or VT1 (Strockbine et al., 1988).

**VTe**

VTe, also known as Oedema disease principle or EDP (Lingwood & Thomson, 1987) has been found in pigs with oedema disease (ED) which attacks thriving pigs about one week after weaning.
VTe is antigenically similar to VT2, being neutralised with antiserum to VT2, but not to VT1. However, VTe is only cytotoxic to Vero cells, and has no effect on HeLa cells (Marques et al., 1987). This difference in biological activity on tissue culture cells has been one of the criteria commonly used to differentiate between VT2 & VTe in vitro. More recently, however, toxins displaying similar biological properties to VTe have been associated with human disease (Oku et al., 1989; Gannon et al., 1990; Schmitt et al., 1991). These toxins can be distinguished on the basis of gene sequence and have been termed VT2-variants (Ito et al., 1990; Schmitt et al., 1991) and VTe-variants (Gannon et al., 1990).

1.8.1.1 Toxin structure and mode of action

Both VT1 and VT2 have been purified to homogeneity and like ShT both are made up of one active "A" subunit, linked to multiple copies, most likely five, of "B" binding subunits (O'Brien & Holmes, 1987). The VT1 holotoxin is reported to have a relative molecular weight of 70 KDa; the molecular weight for the A and B subunits having been calculated as 32.211 KDa and 7.690 KDa respectively (Jackson et al., 1987a). The VT2 holotoxin is slightly larger, the molecular weight for the A and B subunits being 33.135 and 7.817 KDa respectively (Yutsudo et al., 1987). The A subunit in both VT1 and VT2 is enzymatically active and like the A chain of ShT is responsible for inhibiting protein synthesis through the catalytic inactivation of the 60 S ribosomal RNA in mammalian cells by cleaving the N-glycosidic bond at adenine 4324 in the 28 S ribosomal ribonucleic acid (rRNA). This enzymatic activity of VTs and ShT is the same as that of ricin, a potent cytotoxic protein produced by the castor bean Ricinus communis (Endo et al., 1987 and 1988; Igarashi et al., 1987).
The B subunits are thought to mediate specific receptor binding, cytotoxic specificity and extracellular localisation of the holotoxin (Jackson et al., 1990). The receptors for VT1, VT2 and ShT have been identified and are the same glycolipid globotriosyl ceramide "Gb3" (Lindberg et al., 1987; Lingwood et al., 1987; Waddell et al., 1988). In contrast, VTe binds minimally to Gb3 and binds with high affinity to globotetraosyl ceramide (Gb4) (De Grandis et al., 1989). It has therefore been suggested that the effect of ShT and VTs in humans and animal models reflects the relative toxin binding affinity as well as differential tissue distribution, cell surface concentration of receptors and sensitivity of the cells to the toxin (Boyd and Lingwood, 1989; Samuel et al., 1990). The mode of action of the toxin is illustrated in figure (1.7).

1.8.1.2 Genetics of Verotoxin production

The genes controlling the production of VT are bacteriophage encoded in several E. coli strains and was first demonstrated in strain H19 of serotype O26:H11 (Scotland et al., 1983a). VTEC strains which produce both VT1 and VT2 are lysogenised by two distinct phages. O157:H7 strains, 933 for example, contains two toxin converting phages, 933J which produces VT1 and 933W which carries the VT2 genes (Strockbine et al., 1986). The complete DNA sequence for the VT1 and VT2 genes, and their regulatory elements, have been reported (Calderwood et al., 1987; Jackson et al., 1987 a). The nucleotide sequence of VT1 is identical to that of shiga toxin (ShT), apart from 3 nucleotides in the A subunit which result in a single amino acid difference (Strockbine et al., 1988). By contrast, VT1 genes share only 57-60% nucleotide sequence homology and 55-57% deduced amino acid sequence homology with those of VT2 (Jackson et al., 1987 b) (table 1.2).
After intestinal colonisation and the release of the toxin, the B subunits bind to specific receptors on the cell surface and the A subunit is internalised and proteolytically cleaved to an A' fragment. The latter has N-glycosidase activity and inactivates S ribosomal subunits by cleaving the N-glycosidic bond at nucleotide residue 4324 in 28 S ribosomal RNA. This inhibits protein synthesis and leads to cell death.
Table 1.2 Comparison of the processed ShT/VT1, VT2 and VTe subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Mol.weight</th>
<th>Nucleotides-Number</th>
<th>Amino acids</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShT/VT1</td>
<td>32,225/32,211</td>
<td>879</td>
<td>296</td>
<td>11.1</td>
</tr>
<tr>
<td>VT2</td>
<td>33,135</td>
<td>888</td>
<td>293</td>
<td>9.8</td>
</tr>
<tr>
<td>VTe</td>
<td>33,050</td>
<td>891</td>
<td>297</td>
<td>8.7</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShT/VT1</td>
<td>7,690</td>
<td>207</td>
<td>69</td>
<td>5.9</td>
</tr>
<tr>
<td>VT2</td>
<td>7,817</td>
<td>210</td>
<td>70</td>
<td>5.4</td>
</tr>
<tr>
<td>VTe</td>
<td>7,565</td>
<td>204</td>
<td>68</td>
<td>10.2</td>
</tr>
</tbody>
</table>

1.8.1.3 Iron mediated regulation of toxin production

Iron is essential for the growth of most bacteria and thus specific mechanisms for its acquisition are present in most microbes. In mammalian hosts, iron is sequestrated by iron binding proteins and as a result there is little free iron available in contrast to the comparably high levels of free iron present in the environment.

Production of shiga toxin has long been known to be increased when cells are grown in medium with a low iron concentration. Similarly, iron chelation has been reported to dramatically increase transcription of the VT1 genes and hence the concentration in the extra cellular medium (De Grandis et al., 1987). However, iron was found not to suppress VT2
production (Sung et al., 1990) which indicates that the regulation of VT2 synthesis is different from that of shiga toxin and VT1.

1.8.1.4 Mitomycin C induction of Verotoxins

The mechanism whereby mitomycin C increases VT1 and VT2 production is thought to be via the induction of bacteriophage, resulting in an increase in the copy number of toxin genes (Acheson et al., 1990). In contrast, mitomycin C has no effect on toxin production by Shigella dysenteriae type 1, strain 60R, in which the toxin gene is not bacteriophage associated.

Another known inducer of phage replication, ultra violet irradiation, has been found to increase both phage and toxin production (Acheson et al., 1991).

1.8.1.5 The role of calcium uptake in toxin internalisation

Calcium plays a regulatory role in all living organisms, and it is known that the activity of bacterial toxins such as shiga toxin (Sandvig and Brown, 1987) is affected in the presence of drugs which block calcium transport across membranes. It appears that a calcium flux through naturally occurring channels is necessary for the internalisation of the A subunit into the cytosol. This hypothesis is supported by the fact that Verapamil, a licensed calcium channel-blocker, prevents the cell-bound A subunit of VT1 from entering Vero cells (Edelman et al., 1988). It is, therefore, only effective before internalisation of the toxin.
1.9 Bacterial adherence to mucosal surfaces

1.9.1 Why study bacterial adherence?

Although marine microbiologists have been aware for a long time that bacteria must stick to surfaces in order to avoid being swept away by moving streams of water (Zobell, 1943) the study of the mechanism of bacterial adherence did not receive much attention until about 20 years ago. This was largely because it became widely recognised that adherence must be an important early event in the pathogenesis of bacterial infection in animals and humans (Gibbons, 1977). Furthermore, the identification of the adhesive molecules on the bacterial surface (adhesins) as well as those on the host cell membrane (receptors), has been given particular attention due to their importance in the design of new strategies for the prevention of serious bacterial infections (Beachey, 1981). For example:

- Application of purified adhesin or receptor materials or their analogues as competitive inhibitors of bacterial adherence.
- Administration of sub-lethal concentrations of antibiotics that suppress the formation and expression of bacterial adhesins.
- Development of vaccines against bacterial surface components involved in adhesion to mucosal surfaces.

1.9.2 Infectivity versus adherence

Is bacterial adherence a prerequisite for bacterial infectivity? To answer this question many approaches have been used to study the relationship between adherence and infection.
In one approach, laboratory bacterial strains that vary in their ability to adhere to certain host cells in vitro have been assayed for their ability to produce infections in laboratory animals and human volunteers. Results of these studies have provided convincing evidence that adhesion to intestinal mucosa is an essential virulence factor in *E. coli* strains that cause diarrhoea.

Satterwhite *et al.* (1978) demonstrated that a nonadherent mutant of *E. coli* which lacked adhering ability and colonising factor, but retained its ability to produce enterotoxins, was unable to induce diarrhoea in human volunteers. Furthermore, the nonadherent derivative was shed in the stools of infected volunteers for much shorter intervals than was the parent adherent strain.

Do adherent bacteria have advantages over nonadherent bacteria in their ability to cause tissue damage? Bacteria adhering to tissue cells gain survival advantages over nonadherent bacteria by resisting the cleansing effects of mucosal secretions and peristaltic flow, adherence therefore, prevents the rapid loss of the organisms from the gut. In addition, it now appears that adherent bacteria are significantly more toxic toward the host cells (Ofek & Beachy, 1980; Ofek *et al*., 1990). The organisms that become attached to intestinal epithelial cells are able to deliver their toxin molecules in higher concentrations to the receptors in the cell membrane. The close association also prevents loss of toxin activity due to degradation by enzymes and chemicals in the extracellular and mucosal fluids. Another advantage is that the already enhanced toxicity can escape neutralisation by a number of inhibitors, including antibiotics.

These studies strongly suggest that bacteria must be able to attach to the intestinal epithelial cells in order to deliver the diarrhoea producing toxin effectively.
1.9.3 Patterns of bacterial adherence

The property of adherence of *E.coli* to HeLa or HEp-2 cells has been used to identify *E.coli* strains associated with diarrhoea. Three distinct patterns of adherence have been described:

a) **Localised adherence (LA)**

*E.coli* form micro colonies on localised areas of the surface of tissue culture cells. The LA group includes many *E.coli* strains with the enteropathogenic (EPEC) serotype, called class I EPEC (Levine 1987). These serotypes possess a 50 to 70 MDa plasmid that mediates LA via EPEC adherence factor (EAF) (Scaletsky *et al*., 1984; Nataro *et al*., 1985).

Class I EPEC produce attaching and effacing (A/E) lesions on eukaryotic cells (Tzipori *et al*., 1989). However, Knutton *et al.* (1987) provided evidence that LA to HeLa cells and A/E adherence are two genetically distinct phenomenon. Recent studies demonstrated that the A/E phenomenon is caused by a chromosomal gene (*eae*) encoding a 94 KDa membrane protein (Eae) (Jerse *et al*., 1990; 1991).

b) **Diffuse adherence (DA)**

Diffuse adherence is characterised by uniform distribution of *E.coli* over the entire cell surface.

The DA group includes such *E.coli* strains as class II EPEC (Levine, 1987) and strains detected with a DNA probe constructed from a chromosomal sequence for a pilus adhesin (F1845) (Bilge *et al*., 1989). So far, these strains have failed to demonstrate significant pathogenicity (Tacket *et al*., 1990). Consequently, the association of this type of
adherence with diarrhoea has remained unclear (Levine et al., 1988; Cravioto et al., 1991)

c) Enteroaggregative adherence pattern (EA)

Bacteria are seen to form "stacked brick" aggregates attaching to epithelial cells and usually to the (glass) surfaces between tissue culture cells.

*E. coli* strains exhibiting the aggregative pattern are referred to as enteroadherent-aggregative *E. coli* (EA-AggEC). They are a newly described category of *E. coli* associated with persistent infant diarrhoea (Vial et al., 1988).

Recent studies suggest that EAAggEC may be a large-bowel pathogen which colonises the colon by a fimbrially mediated adhesion mechanism (Knutton et al., 1992).

Both LA and DA which have been described in studies of adherence of EPEC serotypes to HEp-2 cells, are also exhibited by VTEC O157:H7 (Sherman, 1987; and this study, chapter 3).

The capacity of *E. coli* O157:H7 to adhere to epithelial cells under different physiological conditions was investigated in this project.

### 1.10 Adherence of VTEC O157:H7 to epithelial cells

Apparently, not all VT-producing *E. coli* are pathogenic, probably, because some strains produce only low levels of toxin, but more importantly, some strains do not possess the specific attachment factors that enable them to colonise mucosal surfaces (Pai et al., 1986).

The ability to adhere to intestinal epithelial cells is an important virulence factor of non-invasive pathogens. VTEC adhere to the
intestinal mucosa of infected animals in the attaching-effacing (A/E) manner, which is characteristic of EPEC strains (Moon et al., 1983). However, VTEC produce lesions in the large intestine while EPEC primarily colonise the small intestine (Karmali, 1989). In this mode of adherence, bacteria destroy the brush border of the epithelial cells and closely adhere to the apical surface, causing cupping and pedestal formation by the cell membrane (Fig.1.1). Ultrastructural studies by Knutton et al., (1989) have demonstrated the accumulation of actin beneath the attachment site and this characteristic has been exploited in the detection of bacteria which attach in an A/E manner.

1.10.1 Genetics of VTEC O157:H7 adherence

Adherence of VTEC has been linked to the possession of a 60 MDa plasmid that encodes a novel fimbrial antigen (Karch et al., 1987). However, the significance of this plasmid in the pathogenesis of VTEC is controversial, as some non-fimbriated E.coli O157:H7 can adhere to epithelial cells in vitro (Sherman et al., 1987). Tzipori et al. (1987) failed to find an association between the possession of a 60 MDa plasmid and virulence of O157:H7 strains in gnotobiotic piglets. Furthermore, analysis of genes which hybridised with a probe derived from the 60 MDa plasmid (Levine et al., 1987) indicated that the ability to produce A/E lesions was not plasmid encoded (Hall et al., 1990). Studies using plasmid-cured VTEC strains suggest that the plasmid is involved in initial adherence to the brush border but is not required for A/E activity (Tzipori et al., 1989). The plasmid encoded adhesion may be involved in tissue specificity of VTEC for human epithelial cells (Jerse and Kaper, 1991).
In EPEC strains A/E activity is dependent upon a 94 KDa membrane protein encoded by the chromosomal eae gene (Jerse et al., 1991). Homologous sequences found in VTEC strains indicate that a chromosomal gene could be responsible for VTEC A/E adherence (Jerse et al., 1991). This evidence is supported by the finding that a 94 KDa outer membrane protein competitively inhibits adherence of O157:H7 to epithelial cells (Sherman et al., 1991).

1.10.2 Intestinal colonisation by VTEC

Bacterial association with host mucosal surfaces is a complex process, this complexity resulting from the influence of a large variety of intervening factors and involving a large number of steps which include:

- Chemotactic attraction of motile bacteria to the surface of the mucus gel which is secreted by specialised cells of the mucosa and forms a layer covering the epithelium.
- Penetration of, and trapping within, the mucus gel.
- Adhesion to receptors in the mucus gel.
- Adhesion to receptors on the epithelial cell surface and multiplication.

A bacterium must therefore proceed sequentially through a number of different reactions, some of which promote or retard its' progress in approaching the epithelial cell surface (Freter, 1981). Non-specific mechanisms of adhesion which depend on such forces as the difference in surface charge, or hydrophobic interaction, have been considered as preliminary events which subsequently promote a more specific mechanism of adhesion.
Specific adherence results from the mutual recognition and interaction of surface structures from both the bacterial cell (adhesin) and the host cell (receptor).

Unlike the extensive studies on the identification of the adhesins on the surface of pathogenic organisms, limited studies have been published on the isolation and characterisation of receptors on the host epithelial cells.

1.10.3 Cell surface components and adherence

Since the cell wall is the outermost part of the bacterial cell, it is essential that this part of the cell adapts to aid the survival of the pathogens in the host.

The cell envelope of Gram-negative bacteria is much more complex than that of Gram-positive organisms. The cytoplasmic membrane of Gram-positive bacteria is surrounded by a thick cell wall composed of peptidoglycan and teichoic acid. In the case of Gram-negative bacteria however, the periplasmic space separates the cytoplasmic membrane from a narrow layer of peptidoglycan, which is itself surrounded by an outer membrane consisting of phospholipids, proteins and lipopolysaccharide (LPS). Figure (1.7) presents a schematic structure of the *E. coli* envelope.

Cell surface components such as fimbriae, outer membrane proteins (OMP), LPS and flagella have been characterised as bacterial adhesins on the cell surface of pathogenic Gram-negative bacteria (Peterson and Quie, 1981).

The structure of each cell surface component and its possible role in mediating bacterial adherence to the host cell, are discussed below.
1.10.3.1 Fimbriae (Pili)

Fimbriae are short, straight non-flagellar proteinaceous filaments, which have been implicated in adhesion of many *E.coli* strains. The term pili is often reserved for the sex pili "the filamentous appendages involved in the conjugative transfer of DNA".

Fimbriated forms of *E.coli* are able to adhere to diverse cellular substrates including erythrocytes, which have complex surface components in common with other tissues. Agglutination of erythrocytes by bacteria has been used as an indicator of fimbriation (Duguid *et al.*, 1955).

Fimbriae have been divided into two major categories, the division being based upon the effect of D-mannose on the haemagglutination (HA) reaction.

Mannose sensitive HA associated with type-I fimbriae was found to be inhibited in the presence of mannose due to the fact that these fimbriae use D-mannose or D-mannose containing glycoprotein molecules as receptors (Duguid & Old, 1980), consequently these fimbriae are classified as mannose-sensitive (MSHA).

Mannose resistant (P-fimbriae) are the second major class of fimbriae which are defined by their ability to stick to glycolipid receptors exposed on a variety of host cells, mainly on the surface of epithelial cells lining the urinary tract. Thus, strains of *E.coli* that express P-fimbriae are likely to produce infections within the kidney "Pyelonephritis" (Korhonen *et al.*, 1982). The P-fimbriae associated HA remains active in the presence of D-mannose, consequently they are described as mannose-resistant, MRHA.
Fimbriation has been shown to be important in the attachment of several strains of *E. coli* to various eukaryotic cell surfaces and has been well documented for ETEC strains (table 1.3).

**Table 1.3  Fimbrial Colonisation Factors identified in human ETEC**

<table>
<thead>
<tr>
<th>Colonisation factors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA/I</td>
<td>Evans <em>et al.</em>, 1975</td>
</tr>
<tr>
<td>CFA/II</td>
<td>Evans and Evans, 1978</td>
</tr>
<tr>
<td>(CS1, CS2 &amp; CS3)</td>
<td>Smyth, 1982; Cravioto <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>CFA/III</td>
<td>Honda <em>et al.</em>, 1984</td>
</tr>
<tr>
<td>PCFO159</td>
<td>Tacket <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>PCF8775</td>
<td>Thomas <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>(CS4, CS5 &amp; CS6)</td>
<td>Thomas <em>et al.</em>, 1985</td>
</tr>
<tr>
<td></td>
<td>Heuzenroeder <em>et al.</em>, 1990</td>
</tr>
</tbody>
</table>

Adhesins associated with diarrhoea include colonisation factor antigens CFA/I, CFA/II which consist of three fimbrial cell surface (CS) antigens (CS1, CS2, and CS3), CFA/III, putative colonisation factor PCFO159, and PCF8775 which also consisted of three antigens two of which CS4 & CS5 are fimbrial. CS6 has no demonstrable fimbrial structure.

Colonisation by ETEC is mediated by specific types of fimbriae which appear to determine host specificity. Adherence factors, K88 and K99, are found on ETEC strains pathogenic for animals, while the colonisation factor antigens, CFA/I and CFA/II are found on ETEC strains which are human pathogens (Evans and Evans, 1978).
Much is known about ETEC colonisation of the intestinal mucosa, but little is known about the adhesive mechanism of EPEC, although these strains have been recognised as a cause of infantile enteritis for over forty years (Scotland, 1983).

Certain EPEC strains have been reported to possess fimbriae which cause MRHA (Evans et al., 1980) while others have indicated that adherence of EPEC to HEp-2 cells does not depend on the expression of fimbrial adhesins (Scotland et al., 1983).

Two isolates of EPEC serogroup O86 were reported by Pal and Ghose (1990) to express MRHA and HEp-2 and HeLa cell adherence factors. Both factors were identified as cell surface proteins which are probably non-fimbrial in origin and are encoded by the same bacterial plasmid.

All published results indicate that EPEC strains express their adherence potential through factors other than fimbriae.

Fimbriae have been reported to mediate the initial attachment of VTEC to mucosal surfaces. However, results of studies concerning adherence of VTEC to different tissue culture cell lines, and the role of fimbriae in adherence, are variable and conflicting. All isolates of *E.coli* O157:H7 appear to harbour a plasmid of approximately 60 MDa which mediates fimbrial antigens that promote attachment to epithelial cells (Karch et al., 1987). In contrast, most of the recent adherence studies have shown that the plasmidless derivative of *E.coli* O157:H7 strains adhered to human epithelial cells in tissue culture (section 1.10.1).

A possible explanation for these conflicting results in the production of fimbriae could be due to the assumption that the expression of a particular fimbrial type in a given strain, under a given set of conditions, is determined by a complex series of interactions, subject to phase
variation or regulatory factors such as temperature or the growth regime (Evans et al., 1977).

1.10.3.2 Flagella

Flagella are filamentous protein appendages that protrude from the cell surface of many bacteria and are composed of repeating protein subunits termed flagellin, and a proximal basal body which anchors the flagellar filament to the bacterial cell surface by a series of hook-associated proteins.

Flagella confer motility to the organism. As a consequence of motility, flagella may permit increase bacterial penetration of the mucous gel which overlies the lining of the gastro-intestinal tract and thus, promote increased colonisation by enteric pathogens at the mucosal surface. In addition to virulence associated with the motility of bacteria, certain flagella may also function as bacterial attachment factors, or adhesins, that promote binding of pathogenic organisms to receptors on the surface of enterocytes or colonocytes.

The functional role of H7 flagella in promoting virulence of *E.coli* O157:H7 infection in humans has not yet been defined. Sherman and Soni (1988) showed that H7 flagella are not important in adherence to human epithelial cells as the attachment of *E.coli* O157:H7 to tissue culture cells was not inhibited by isolated H7 flagellar antigen. Nevertheless, H7 antigen appears to be an important factor in the pathogenesis of human disease since nonmotile, aflagellar *E.coli* strains of the O157 serogroup are much less frequently isolated from stool specimens of patients than flagellated strains.
1.10.3.3 Lipopolysaccharide (LPS)

LPS is a unique constituent of the Gram-negative cell envelope which carries the "O" antigen specificity of the bacterium. It is also responsible for the endotoxic activity of the cell. LPS is composed of three parts, the proximal, hydrophobic lipid A region, the distal hydrophilic O antigen, and the core region which connects the two. Each region has distinctive composition, biosynthesis and biological function.

The polysaccharide side chain is the serologically dominant part of the molecule responsible for the O antigenic specificity. The lipid A region consists of a single backbone structure of 6 or 7 saturated fatty acid chains. It is responsible for the toxic properties and the potent biological effects associated with LPS. Mutants incapable of synthesising lipid A cannot be isolated since the loss of this component is lethal.

Linked to the lipid A is the core oligosaccharide region. This is formed of 10 sugar units which project outward, and join the O specific side chain. Loss of the more proximal part of the core results in increased sensitivity to a wide range of hydrophobic compounds including dyes, antibiotics, bile salts, other detergents and mutagens. Consequently this region must be important in maintaining the outer membrane as an effective barrier to the external environment.

The O antigen side chain consists of repeating oligosaccharide units, often containing rare sugars, and can vary in both size and complexity. Loss of the O antigen (rough mutant) causes loss of virulence, suggesting that this region is important in host-parasite interactions.

Since LPS is the most prominent part of the outer membrane it is likely that it has great influence on cell surface interactions. However, studies
on the role of outer membrane components that mediate binding of *E. coli* O157:H7 to HEp-2 cells have indicated that constituents of the outer membranes other than the LPS mediate attachment of the organism to epithelial cells *in vitro* (Sherman and Soni, 1988).

### 1.10.3.4 Outer Membrane Proteins (OMPs)

The *E. coli* outer membrane has a characteristic protein composition which differs from that of the cytoplasmic membrane. These proteins perform a variety of functions for the cell, some are involved in specific transport systems, others maintain the structural integrity of the outer membrane and a few have enzymatic functions.

While more than 20 different polypeptides from the outer membrane of Gram-negative bacteria can be identified, the number of the "major" OMPs is relatively small, varying from 4 to 6. However, more recent studies have revealed the presence of a number of "minor" proteins as well (Nikaido and Vaara, 1987).

One of the major proteins is the murein lipoprotein, about one third of this protein present in the outer membrane is covalently linked to the peptidoglycan layer and the rest is found as free protein (Nikaido and Vaara, 1987).

Mutants with deletion of the lipoprotein structural gene have unstable cell envelopes, therefore its' main function is thought to be stabilisation of the outer membrane peptidoglycan complex.

Other major proteins are the "porins" with molecular weights ranging from 33 to 38 KDa. Porins produce relatively non-specific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane. They are the most abundant type of outer membrane
protein in *E. coli*, and the total amount of protein present is relatively constant (Nikaido and Vaara, 1987).

Omp "A" has a similar molecular weight to the porins (35-36 KDa), but behaves very differently upon solubilization in sodium dodecyl sulphate (SDS) at low temperatures. Omp A is almost as abundant as porins and may be involved with transport across the membrane, however, it is unlikely that it forms pores by itself. The function of this protein may be to stabilise the membrane, possibly in conjunction with lipoprotein.

Minor proteins are present in a low number of copies per cell. However, they may be present in higher concentrations under specific physiological conditions. Several of these have now been shown to be involved in the high affinity transport of specific nutrients. The LamB protein for example, which is involved in maltose uptake, may be present in comparable amount to porins when fully induced.

There are a number of minor outer membrane proteins that are involved in the transport of various chelates of ferric ions. These proteins appear to be induced by iron starvation and sometimes they become major proteins under these conditions.

In addition, minor proteins also serve as receptors for a range of bacteriophages and colicins.

A feature of OMPs is that their expression is variable and depends on the growth environment e.g. medium composition, pH, temperature, aeration and growth phase.

OMPs are important in pathogenesis of many diseases as the antibodies in most vaccines are commonly raised against them.

Although fimbriae and LPS have been proposed previously as attachment factors more recently attention has been focused upon the OMPs and their possible role as adherence factors. For example attachment of VTEC O157:H7 to human epithelial cells has been studied in great
detail, and numerous published data indicate that OMPs mediate binding of VTEC O157:H7 to the host cell (Sherman et al., 1991).

1.11 Diagnosis of VTEC infection

VTEC infection can be diagnosed on the basis of isolation of the organism, detection of free VT, or the presence of neutralising antibodies in the patients' serum. VT can be detected as free toxin (FVT) in stool filtrates, or can be released from cells by use of compounds which alter the permeability of the cell envelope, such as polymixin B, and subsequently assayed.

In addition to the problem of detecting relatively low numbers of VTEC in a mixed flora, it is necessary to test faeces within 4 days of the onset of symptoms as VTEC are rapidly cleared from the stool of patients. Often by the time HUS develops one or two weeks after the onset of symptoms, the organism is no longer present in faeces. The major obstacle in the large scale epidemiological investigation of the incidence of VTEC in diarrhoeal diseases is the lack of a rapid, simple and specific test to detect VTEC and their toxins. To date the most important and sensitive diagnostic method of VTEC infection is the detection of FVT (Karmali et al., 1985b).

1.11.1 Isolation and identification of VTEC

Although many VTEC serotypes are associated with human diseases, the predominant serogroup, O157, accounts for the vast majority of VTEC identified in human infections. Fortunately, VTEC of serogroup O157, unlike 95% of E.coli strains do not ferment sorbitol in 24 hours. A
modified MacConkey agar containing sorbitol instead of lactose (SMAC agar) has been recommended as the preferred culture medium to screen faecal specimens for non-sorbitol fermenting (NSF) colonies of *E. coli*. Specific typing of O157:H7 can be achieved through slide agglutination using O157 and H7 antisera (Kleanthous *et al.*, 1988).

A fluorescein labelled, polyclonal, affinity-purified goat antibody to *E. coli* O157:H7 has been developed that enables rapid direct immunofluorescence identification of *E. coli* O157:H7 isolated from faecal specimens or SMAC agar (Tison, 1990).

These routine screening procedures are clearly limited since VTEC strains other than O157:H7, which are able to ferment sorbitol will not be detected. One method used to screen colony sweeps for VT production is by releasing the toxin with polymixin B. This method ensures that all VTEC can be detected, not just O157:H7. VT released in this method has been assayed by standard Vero cell assay (Karmali *et al.*, 1985) or more rapidly by an ELISA based on the VT cell receptor, globotriosyl ceramide (Gb3).

DNA probes for VT genes provide a sensitive and specific method for the detection of VTEC of all serotypes. However, this technique is available in only a few laboratories and does not indicate expression of the toxin.

### 1.11.2 Detection of free Verotoxins

Tests for free VT (FVT) in filtrates of faeces from patients with VTEC infections are of particular value when VTEC serogroups other than O157 are involved, or when VTEC have not been isolated on primary culture because of low numbers. Free cytotoxin in filtrates produce a characteristic cytopathic effect on Vero cell monolayers within 48-72
hours and the presence of VT can be confirmed by neutralisation tests with specific VT antibodies.

Maniar et al. (1990) have shown that the use of Vero cell suspensions are as reliable as the use of Vero cell monolayers, and provide detection of VT 24-48 hours earlier. However, tissue culture facilities may not be available in many clinical laboratories, especially those in the developing countries. Moreover, these assays are slow, expensive and labour intensive. Therefore they are unsuitable for the screening of large numbers of bacterial isolates for epidemiological purposes.

A less expensive and more rapid method for detecting VT from supernatant or cell extracts is an enzyme linked immunosorbent assay (ELISA), which is specific but not as sensitive as the Vero cell assay (Kongmuang et al., 1987).

Downes et al. (1989) developed two sandwich ELISAs that detect VT1 and VT2 using toxin specific capture monoclonal antibodies and polyclonal antisera to VT1 and VT2, again this technique is not as sensitive as the cell culture cytotoxicity assay being able to detect only those strains producing high to moderate levels of VT.

1.11.3 Detection of neutralising antibodies

Patients with O157 infections usually produce high titre serum antibodies to the LPS of O157:H7 (Chart et al., 1991).

Screening of sera from patients with HC and HUS is recommended when culture methods for E.coli O157 are negative.

It is possible to look for neutralising antibody to VT using a modification of the receptor enzyme linked immunosorbant assay (Neut RELISA). This method measures the ability of antibodies in the patients sera to inhibit the binding of a known concentration of VT, to the VT
receptors (Gb3) (Boulanger et al., 1990). A positive result confirms a VT infection, and this procedure allows detection of VTEC other than O157:H7.

1.11.4 DNA probes in VT diagnosis

DNA probes have been used to detect the presence of genes coding for VT and for the lysogenic phages which carry these genes (Serwatana et al., 1988; Bettelhiem, 1990).

A DNA probe for the detection of the 60 MDa plasmid typically carried by O157:H7 that promotes epithelial cell attachment of VTEC has been prepared by Levine et al. (1987). However, because plasmids may be lost during isolation, the probe does not detect VTEC isolates that no longer carry the 60 MDa plasmid.

Specific DNA probes have been developed to identify DNA sequences that code for VT1 and VT2. Gene probing is sensitive, fast, and specific with the ability to examine a large number of test samples at one time. DNA probes however, do not give an indication of the level of toxin expression, which has been shown to be an important factor in pathogenesis (Marques et al., 1986).

Gene probing can be performed with ease and has the necessary sensitivity for the screening of food and environmental samples, and for the detection of organisms carrying the VT genes (Samadpour et al., 1990).

A number of oligonucleotide probes have been synthesised to enable the detection of the different types of VT by DNA hybridisation. Pollard et al. (1990) designed a set of four synthetic oligonucleotide probes, derived from the sequences of the VT1 and VT2 genes, and used these in
a polymerase chain reaction (PCR) amplification procedure to detect these genes in enteric pathogens.

The PCR technique is relatively simple and its enhanced sensitivity and specificity should enable rapid screening for the presence of VTEC in clinical specimens and food samples. The ability to analyse specimens without the isolation of specific organisms should facilitate rapid diagnosis and treatment.

Gene probes are a useful diagnostic technique, but they are not, as yet, a routine diagnostic procedure in most clinical laboratories.

1.12 Control of VTEC infection

1.12.1 Prevention

In order to develop intervention measures for the control of VTEC infections, it is essential to obtain more information concerning the common vehicle, source, and reservoirs of infection and to identify the possible risk factors.

In the case of other diarrhoeagenic *E. coli* infections (EPEC, ETEC and EIEC) the human intestinal tract is considered to be the major reservoir. In contrast, reservoirs of *E. coli* O157:H7 are the intestinal tract of cattle, and perhaps other animals used in food production. It would appear therefore, that VTEC O157:H7 is a greater problem as a food-borne disease than EPEC, ETEC or EIEC.

The elimination of VTEC from domestic animals is unrealistic, therefore, control measures must include the education of food workers about safe food handling techniques and proper personal hygiene; the use of good food manufacturing practice in the processing of food and proper heating of food before consumption. Also the public must be
aware of the risk factors involved in consuming unpasteurised milk and under cooked meat products.
The use of irradiation could also cut down transmission of VTEC and other food-born pathogens to humans (Karmali et al., 1989).

1.12.2 Environmental factors

It is known that VTEC are discharged in the faeces of infected patients, therefore they are likely to prevail in sewage. Today sewage treatment plants dump large quantities of sewage sludge onto agricultural land in many countries. Contamination of agricultural products could also occur directly from animal faeces containing $E. coli$ O157:H7. Untreated human sewage should not be used to fertilise vegetables and crops, likewise, untreated surface water should not be used to clean food processing equipment and food contact surfaces.

1.12.3 Vaccination

No vaccine is available for immunisation against the potentially fatal VTEC associated infections.
If future studies show that naturally acquired VTEC infection leads to long-term immunity, then, active immunisation would be an option to prevent the serious complications of HUS associated with VTEC infection.
Boyd et al. (1991) developed a vaccine candidate against the effect of shiga toxin and VT1 in humans by immunising rabbits with VT1 B subunit. Sera were obtained with a high neutralising titre against VT1, however, no activity against VT2 was observed in Vero cell assays.
In an attempt to develop an effective vaccine against Oedema Disease (ED), Gordon et al. (1992) used oligonucleotide specific site-directed mutagenesis to reduce the enzymatic activity of VTe. Pigs vaccinated with this mutant toxin developed antibodies 21 days post injection and their tissues were free of ED lesions. There are a considerable number of studies suggesting the use of bacterial adhesins as potential vaccine candidates which would competitively inhibit bacterial adherence to intestinal mucosa. Several purified fimbrial vaccines, prepared from diarrhoeagenic strains of *E. coli*, have been evaluated in farm animals. These include the K88, K99, and 987 fimbriae (Rutter and Jones, 1973). These animal studies hold great promise for trials of vaccines in humans against some pathogens whose adhesins have been identified. However, to date there are no commercially available vaccines.

1.12.4 Treatment

At present the treatment of VTEC infection is primarily supportive, with management of the complication of the infection, such as fluid and electrolyte disturbances, anaemia, renal failure and hypertension. The role of antibiotics in the treatment of VTEC infection is uncertain. Observation from previous outbreaks have suggested that the use of antibiotics before the onset of symptoms increases the risk of acquiring infection. Also, by the time laboratory investigation has verified the presence of *E. coli* O157:H7 and antibiotic treatment has begun -typically about 7 days after the onset of symptoms- it is likely the pathogen is no longer present. Consequently, antibiotic treatment would be ineffective. Sandvig and Brown (1987) have shown that Verapamil, a licensed calcium channel blocker, could inhibit the cytotoxicity of shiga toxin in
vitro, whether the drug may have clinical applications remains to be seen.
Anti-motility agents could have been a possibility for treatment, however, it is now known these allow more time for toxin absorption (Cimolai et al., 1990).
1.13 Aims of the project

One of the goals in the study of infectious disease is the prevention of disease. To find the ways by which microbial infections can be prevented there is an obvious need to understand the host-pathogen interactions at both the cellular and the molecular levels, from the primary events to the final outcome.

The mechanism of pathogenicity of E.coli O157:H7 has not been fully elucidated but important virulence factors have been identified:-
- The production of one or two Verotoxins which target the endothelial cells lining the small blood vessels, mainly in the colon and the kidney.
- The adherence of these organisms to the epithelial cells of the colonic mucosa.

This project was initiated to study the virulence factors of E.coli O157:H7 since this serotype is by far the most common in human infections.

Recent studies have indicated that the attachment of bacteria to the mucosal surface is the essential first event in the pathogenesis of most infectious diseases. Furthermore, it has been reported that the adherence of O157:H7 may, by itself, be sufficient to cause disease (Tzipori et al., 1987). Therefore, most of the work has been focused on understanding the mechanisms of bacterial adherence to epithelial cells.

The main objectives of this study were to:-
1. Examine the bacterial adherence to the host epithelial cells under various in vitro conditions which are as close as possible to those in vivo.
2. Examine the expression of bacterial cell surface components which have been implicated as adherence factors in response to the same variable conditions. This is of particular importance since it is now
well recognised that the expression of antigens in bacteria obtained directly from the infected host may be quite different to those expressed \textit{in vitro} (Smith, 1990).

3. Probe the biochemical nature of the cell surface components which mediate bacterial binding to the host cells (adhesins).

4. Characterise the molecules on the host cell membrane which recognise the bacterial adhesins and to which they bind (receptors).

5. Use competitive inhibitors of bacterial adherence to mucosal surfaces such as purified adhesins, receptors, or antibodies to specific cell surface components.

Studies on the adherence mechanisms and its' inhibition may well have a practical outcome which in the long run may be used clinically to prevent bacterial infections by blocking the binding of bacteria to the host tissues.
CHAPTER 2

MATERIAL AND METHODS
CHAPTER 2

Material and methods

2.1 Bacterial strains

The bacterial strains used in this study are listed in table 2.1

Table 2.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>ATCC35150</td>
<td>Wells (1983)</td>
</tr>
<tr>
<td>“R: Reference strain”</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O26:H11</td>
<td>NCTC 8781</td>
<td>PHLS Manchester</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>Warwick Hospital</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>University of Warwick</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7L</td>
<td>George Eliot Hospital</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>“L: Localised adherence”</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>George Eliot Hospital</td>
<td>Clinical isolate</td>
</tr>
</tbody>
</table>

2.2 Media

All media, unless otherwise stated, were sterilised by autoclaving at 121°C for 15 minutes. Solid media were prepared by the addition of 1.5% weight by volume (w/v) Difco Bacto Agar
2.2.1 Penassay broth "Antibiotic Medium 3" (DIFCO)
- Bacto Beef extracts 1.5 g
- Bacto Yeast extracts 1.5 g
- Bacto Peptone 5 g
- Sodium Chloride 3.5 g
- Bacto Dextrose 1 g
- Dipotassium Phosphate 3.68 g
- Monopotassium Phosphate 1.32 g
(pH 7.4)

2.2.2 Nutrient Broth (OXOID)
- Lab-Lemco powder (L29) 1 g
- Yeast extracts (Oxoid L20) 2 g
- Peptone (L37) 5 g
- Sodium Chloride 5 g
(pH 7.4)

2.2.3 Muller Hinton Broth (OXOID)
- Meat infusion 6 g
- Casein Hydrolysate 17.5 g
- Starch 1.5 g
(pH 7)

2.2.4 Luria-Bertani (LB) Medium
- Bacto Tryptone (Difco) 10 g
- Yeast extracts (Oxoid) 5 g
- Sodium Chloride 10 g
(pH 7.5)
2.2.5 Iron limited media
For growth under low iron conditions, media was supplemented with either, ovotransferrin (conalbumin) 0.5 mg ml⁻¹ final concentration in the medium with 0.71 M sterile sodium bicarbonate as described by Stevenson and Griffiths (1985), or 0.2 mM 2,2-dipyridyl (Sigma) from a 0.1 M 50% (v/v) ethanol:water stock, as described by Calderwood and Mekalanos (1987).

2.2.6 Agar
Nutrient agar (Oxoid) was used for routine growth and maintenance.

2.3 Maintenance of cultures
To avoid laboratory attenuation of the organisms, exponential phase cultures were stored at -20°C in 15% (v/v) glycerol. Organisms were streaked onto nutrient agar from the frozen stocks once a month to avoid prolonged subculture.

2.4 Growth conditions
Strains were routinely grown at 37°C in a Gallenkemp Orbital Shaker (250 rpm). Liquid cultures were propagated overnight by inoculating 10 ml media in 25 ml universal bottles, with one colony from an agar plate. A variety of glass culture vessels were employed with capacities ranging from 25 ml to 2 litres.

Anaerobic growth conditions for bacterial cultures were provided by static incubation at 37°C in a specially made 250 ml glass flasks, with a side tube containing saturated solution of pyrogallol to absorb oxygen. Nitrogen gas was also bubbled through the medium via hypodermic needles, immediately after bacterial inoculation.
2.5 Spectrophotometric determinations
Routine determinations of culture optical density (OD) were performed using a Shandon double beam UV 150-02 spectrophotometer. Plastic cuvettes with a 1 cm light path were routinely used. Culture turbidity was determined at a wavelength of 600nm.

2.6 Viable count determination
Routine determinations of culture viability were performed by plating 100 μl of serial dilutions in sterile phosphate buffered saline (PBS) onto agar plates. After overnight incubation at 37°C, colony counts were made and the colony forming units per ml (CFU ml⁻¹) determined.

2.7 Serological identification of *E.coli O157:H7*
A latex agglutination test (Oxoid Diagnostic Reagents) was used as described by the manufacturer to determine whether the *E.coli* clinical isolates (table 2.1) belonged to the O157 serogroup. In addition, 10μl of a single colony of the clinical isolate, resuspended in 100 μl of sterile PBS, was mixed on a slide by tilting with 10 μl of *E.coli* H7 antiserum (Difco). Agglutination with the latter was observed when small clumps of cells became visible. In both tests a negative control of *E.coli* K12 and positive control of *E.coli* O157:H7R (ATCC 35150) were used to provide comparisons.

2.8 Light microscopy
Light microscopy was carried out using a Leitz Dialux 22/22 EB microscope with phase contrast optics and fitted with a Leitz Vario Orthomat 2 automatic microscope camera. Photomicrographs were taken on
Kodak T-max 100 film which was developed in Kodak Detkol developer for 5 min at 20 °C and fixed in Kodak Unifix fixer. Prints on Kodak Veribrom paper were developed with Ilford contrast FF developer and Kodak Unifix fixer.

2.8.1 Staining of tissue culture cells
Tissue culture cells were stained for light microscopy using Diff-Quik stains (Baxter Dade AG, Switzerland). Cells on slides or coverslips were air dried, fixed in methanol, and dipped in solution I (Eosin G, in phosphate buffer) 5 x 1 second, then dipped in solution II (Thiazine dye, in phosphate buffer) 5 x 1 second, and excess stain was allowed to drain after each dip. Cells were then washed with distilled water, air dried and mounted in DPX mounting medium (Agar Scientific LTD).

2.8.2 Staining of human colonic tissues
Tissues were fixed in phosphate-buffered 4% formaldehyde, embedded in paraffin wax, sectioned (5µm) and stained with haematoxylin/eosin stain as follows:
Sections were washed with water, then stained with haematoxylin for 10 minutes. After another wash for 2 minutes, sections were differentiated in 1% hydrochloric acid in 70% ethanol for 15 seconds, washed in water for 5 minutes, and counterstained with 1% Eosin for 3 minutes. Sections were then washed briefly in tap water and rapidly dehydrated in 70% ethanol 5 seconds, neat ethanol 20 and 30 seconds. Finally, sections were rinsed in xylene and mounted in DPX (Agar Scientific LTD).
2.9 Transmission electron microscopy

2.9.1 Negative staining
Samples were examined after negative staining with phosphotungastic acid in the following manner. A drop of cell suspension was placed on a formvar coated grid (Agar 100 segment mesh; 3.05 mm diameter) for 30 - 60 sec, then the excess liquid was removed with a strip of filter paper. The grid was allowed to dry and the sample negatively stained by placing a drop of 1% (w/v) phosphotungastic acid (pH7) onto the grid and removing it immediately with a strip of filter paper. Specimens were examined using a Joel JEM-100S transmission electron microscope at an accelerating voltage of 80 KV. Photographs were taken using Kodak 4489 Estar thick base electron microscope film which was developed in Kodak D19 developer and fixed in Kodak fixer according to the manufacturers instructions.

2.9.2 Immunogold Labelling
The bacterial suspension was washed in PBS and 10 µl placed on a carbon coated grid and allowed to partially dry under a light bulb for 2-3 minutes. Excess fluid was removed using a strip of filter paper and the grids were immediately placed face down on a suitable dilution of antisera in a 1% (w/v) bovine serum albumin (Sigma) and 1% (v/v) Tween 20 (Sigma) for 15 minutes. The grids were removed, washed thoroughly in 1% (w/v) bovine serum albumin and 1% (v/v) Tween 20 and placed on a drop of diluted (1:30) gold anti-rabbit conjugate for 15 minutes. The grids were washed thoroughly again and negatively stained with 1% (w/v) ammonium molybdate.
2.9.3 Thin sectioning of human colonic tissue

For thin sectioning, the colonic segments were washed with PBS and cut into small pieces, approximately (2 mm³). Specimens were fixed in 2.5% gluteraldehyde in PBS at 4°C for 2 hours, rinsed with PBS and postfixed in 1% osmium tetroxide in distilled water for 1 hour. After another rinse with PBS, specimens were dehydrated through a graded series of ethanol solutions (20%-90%) for 10 minutes each, absolute alcohol (x2) 15 minutes, propylene oxide (x2) 15 minutes each. Propylene oxide was then removed and replaced with embedding medium "epoxy resin" (Agar Scientific LTD), consisting of [Araldite CY 212 (10 ml) mixed with an equal volume of the hardener DDSA, the setting of the mixture was speeded up by the use of an amine accelerator DMO-30 (0.4 ml), and the hardness of the final block was controlled by the addition of a plasticizer, dibutyl phthalate (0.5-1 ml)].

Embedding of the specimens was carried out in polythene capsules (Agar Scientific LTD) which were dried for 30 minutes at 60°C before use. The capsules were filled with warm (60°C) bubble-free resin and the specimens were transferred to the capsules (one piece per capsule) and allowed to sink to the bottom. Polymerisation of the mixture then proceeded overnight at 60°C.

Thin sections were cut with a (Reichert Om U2) microtome, and stained with 25% uranyl acetate in methanol for 10 minutes at room temperature.

2.10 Scanning electron microscopy (SEM)

For SEM analysis of the adherence mechanism, HeLa cell monolayers were grown on 13 mm round glass coverslips, and human mucosal specimens were cut into 0.5-cm² pieces prior to the adherence experiments.
Following the adherence assays, all samples were washed several times in PBS, fixed in 2.5% (v/v) gluteraldehyde in PBS for 30 minutes at room temperature, washed with water for 5 min., and subsequently postfixed in 1% osmium tetroxide for 15 min., washed with water and dehydrated through an ethanol series (10%; 25%; 50%; 70%; and 90%) to 2 changes of absolute ethanol for 5-10 min. in each concentration. Ethanol was gradually replaced with amyl acetate (1:3, 1:1, 3:1), and finally to neat amyl acetate for 10-15 minutes. Samples were then transferred under amyl acetate to the cooled critical point drier. Amyl acetate was replaced with liquid carbon dioxide, after which the temperature was increased to above the critical point so that the carbon dioxide evaporated. After critical point drying the samples were mounted on aluminium pin stubs (Agar) and splutter coated with gold for 120 seconds. The surface was examined using a Joel JSM T330A scanning electron microscope at an accelerating voltage of 15 KV.

2.11 Cytotoxicity Assays

2.11.1 Maintenance of tissue Culture cells

Vero cells and HeLa cells were grown in 260 ml tissue culture flasks (Nunclon) in complete growth medium consisting of Medium 199 (Flow) supplemented with 10% foetal calf serum "FCS" (Gibco), 2 mM L-glutamine (Gibco), penicillin (50 units ml⁻¹) and streptomycin (50 mg ml⁻¹) (Sigma). Confluent monolayers were removed with trypsin-EDTA and resuspended at 2x10⁵ cells ml⁻¹ in growth medium. Cells were inoculated into 24-well plastic tissue culture plates (0.2 ml of cell suspension per well). The cells were allowed to reach confluency by incubation at 37°C in an atmosphere of 5% CO₂ in air for 3 days before use. The growth medium
was then replaced with maintenance medium, which was the same as growth medium except that it contained only 5% FCS. The monolayers were then ready to be used in the cytotoxicity assays.

2.11.2 Vero cell assay
Bacterial cultures were grown overnight in 10 ml of Penassay broth at 37°C. Cells were harvested by centrifugation at 10,000 g for 10 minutes and the supernatant was passed through a 22 μm membrane filter (Millipore filters). The filtrate was then diluted (1:10, 1:100 and 1:1000) in growth medium, 100 μl aliquots of each dilution, and of the undiluted filtrate, were added to the Vero cell monolayers. The plates were incubated at 37°C for four days in an atmosphere of 5% CO₂ in air. Vero cells were checked daily for cytotoxic effect under an inverted light microscope, negative controls, containing uninoculated broth, were included in each experiment.

2.13 Culture of human endothelial cells
Endothelial cells were obtained from human umbilical cord veins by an adaptation of the method of C. Jones, 1989 Queen Elizabeth Hospital, Birmingham, (personal communication). A sterile technique was utilised in all manipulations of the cord. The cord was served from the placenta soon after birth, placed in a sterile container filled with medium 199 (Flow) and antibiotics (100 units ml⁻¹ penicillin, 100mg ml⁻¹ streptomycin "Sigma") and held at 4°C until processing. Maximal storage time was 24 hours. The cord was inspected, wiped clean with disinfectant, and all areas with clamp marks were cut off. The umbilical vein was cannulated at both ends with a specially made glass cannula connected to a polyethylene tube, and the cannulas were secured by clamping the cord over them with
polyethylene ties. The vein was perfused with 100 ml sterile PBS to wash out the blood. 10 ml of 0.1% collagenase in PBS (Sigma type 1A) was injected slowly into the vein, and the polyethylene tubing was clamped shut at both ends. The umbilical cord, wrapped in foil, was incubated at 37°C for 15-20 minutes.

After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 20 ml of complete culture medium (medium 199, antibiotics, 20% foetal calf serum, 100 µg ml⁻¹ heparin "Sigma", 100 µg ml⁻¹ L-glutamine, and endothelial cell growth factor "Sigma" 15µg/ml). The cells were sedimented at 500 g for 5 minutes, the pellet was resuspended and washed once in the same medium. The cells were cultured in the complete culture medium, in 75 ml tissue culture flasks pretreated with 1% gelatine (Sigma). The flasks were incubated at 37°C under 5% CO₂ in air with a complete change of culture medium twice a week.

2.14 Adherence assays

2.14.1 Bacterial adherence to HeLa cells

These assays were run in duplicate, one quantitative, and the other assay for microscopic examination. HeLa cells were grown overnight to 50% confluent monolayers in 12-well tissue culture plates as described in section (2.11.1) with glass coverslips in half of the wells. Cells were then washed 3 times in PBS (pH 7.0) and 2x10⁸ of washed bacterial samples suspended in 2 ml growth medium (without antibiotics) was added to the HeLa cells in each well. The tissue culture plates were incubated at 37°C for 3 hours. The culture medium was then removed and the HeLa cells were
washed 6 times in PBS to remove loosely attached bacteria. Cells on the glass coverslips were examined under oil immersion light microscopy after fixation and staining. HeLa cells with adherent bacteria on the plastic tissue culture wells were removed from the plate with 0.5 ml 0.25% trypsin, and HeLa cells were lysed with 0.5 ml BSA (0.1% in water). After vortexing, lysates were serially diluted tenfold in sterile PBS, and plated out in duplicate to enumerate the number of viable bacteria adherent to the HeLa cells.

In all assays 1% D-mannose (Sigma) was included to prevent attachment due to type 1 fimbriae, which may be expressed by the bacteria.

2.14.2 Adherence to human buccal epithelial cells (BEC)

The method was a modification of that described by Burke and Axon (1987). BEC were obtained by gently scraping the buccal mucosa with a sterile spatula and suspending in PBS (pH 7.2). They were then washed and harvested by centrifugation 3 times and resuspended in PBS containing 1% D-mannose. BEC suspension (0.5 ml) was added to 0.5ml of washed bacterial sample at a concentration of about $10^8$ ml$^{-1}$. After 30 minutes incubation on a rotary roller at room temperature, any nonadherent bacteria were removed by washing over a 5 μm membrane filter with PBS. An impression smear to a clean glass slide was then air dried, fixed in methanol and stained for microscopic examination.

2.14.3 Adherence to human colonic tissue

Intestinal specimens were obtained from patients undergoing surgery for carcinoma of the colon at George Eliot Hospital (Nuneaton). Normal tissue was excised from the healthy ends of the surgically removed segment.
The colonic segment was opened immediately, the mucosal side (from the muscularis mucosa to the mucosal epithelium) was retained, and the remaining tissue was discarded. The mucosa was washed several times with cold (4°C) PBS. A slice of the mucosa was immediately used for the adherence experiments. In some experiments, part of the mucus coat covering the mucosal surface was carefully removed with soft tissue paper. In other experiments, the mucosa was fixed with 10% formalin and maintained at 4°C.

Bacterial adherence was evaluated by incubating the mucosal segment with a sample of bacterial suspension in medium RPM1640-HEPES (GIBCO) containing 10% foetal calf serum, 2mM L-glutamine and 1% D-mannose, at 37°C for 2 hours. After incubation the mucosa was washed four times in PBS, and processed for either light or electron microscopy.

2.14.4 Adherence to isolated human colonocytes
Human colonocytes were isolated from the surgically removed colonic mucosa (2.14.3) by two methods:
- Treatment of the mucosa with EDTA-buffer (0.096 M NaCl, 0.008 M KH2PO4, 0.0056 M Na2HPO4, 0.0015 M KCl, and 0.01 M EDTA) pH 6.8 for 5 minutes at room temperature with gentle shaking.
- Gentle scraping of the mucosa with the edge of a microscope slide.
Released colonocytes were sedimented at 100g for 1 minute, and suspended in fresh medium RPM1640-HEPES. Adherence assays were performed by incubating colonocytes with washed bacterial sample in the same medium at 37°C for 30 minutes on a rotary mixer. Nonadherent bacteria were removed by repeated washing and centrifugation at 100 g for 1 minute, the pellet
which contained colonocytes with attached bacteria was resuspended in 0.5 ml PBS. Samples were examined by placing a drop on a glass microscopic slides, air dried, fixed and stained for light microscopy.

2.14.5 Adherence assay under anaerobic conditions
Adherence of anaerobically grown *E.coli* O157:H7 to HeLa cells was carried out in 12-well tissue culture plates as described in section (2.14.1). The plates were incubated at 37°C in an anaerobic jar containing a gas generating kit (Oxoid, anaerobic system BR 38) with an (Oxoid) indicator strip.

2.15 Haemagglutination of red blood cells by *E.coli* O157:H7
The erythrocyte agglutination technique was used to identify fimbriation of *E.coli* O157:H7. Human, horse and sheep red blood cells (RBC) were separated from blood by low-speed centrifugation, washed twice with 0.15M saline (NaCl), suspended in 3% (v/v) PBS and stored at 4°C. Bacterial cells were washed in PBS, and mixed with an equal volume of the RBC suspension on a glass slide at room temperature for 1 minute, placed thereafter on ice and with intermittent manual rotation, observed after a further 2 minutes. Positive reactions were recorded when small clumps of cells became visible.

2.16 Preparation of antisera
Rabbit antisera were prepared with bacterial outer membrane extracts from aerobically and anaerobically grown bacteria. Rabbits were immunised with 1mg outer membrane extracts intravenously, 3 times at 3 week intervals.
The rabbits were bled from a marginal ear vein on the second week after each injection, 10 ml of blood was collected at each time. Blood was allowed to clot for 30-60 minutes at 37°C, then stored overnight at 4°C to allow the clot to contract before serum was removed using a Pasteur pipette. Any remaining insoluble material was removed by centrifugation at 2000g for 15 minutes. Serum was stored in 1ml aliquots at -20°C. Preimmune serum was obtained by bleeding the rabbits in the same way, before immunisation with the purified antigens. Rats were immunised in the same way, except that each rat was injected with 200 μg of the outer membrane extracts subcutaneously.

2.17 Passive Haemagglutination Assay
Saline-extracted antigens were diluted 1:10 in PBS (pH7.0) and incubated for 60 minutes at 37°C with an equal volume of a 1% suspension of sheep red blood cells (RBCs), previously washed in PBS. The sensitized RBCs were centrifuged, washed three times in PBS, and resuspended in PBS to give a 0.5% RBCs suspension. A two-fold dilution of antibodies to O157:H7 were made in a 96-well microtitre tray (25 μl per well). 25 μl sensitized RBCs were added to each well, and the plates were shaken gently to mix the contents of each well. After incubation for 60 minutes at 37°C, haemagglutination was observed by eye. The titre was recorded as the highest dilution of antiserum to give agglutination of senseitized RBCs. Pre-immune serum was used in negative control assays.
2.18 Adhesion inhibition assays

2.18.1 Inhibition by rabbit antisera
The ability of specific antisera to inhibit adhesion was assessed. All assays were performed in triplicate in 12-well tissue culture plates. Adherence studies were carried out as described in section (2.14.1).
Rabbit antisera prepared against whole O157:H7 cells (Sue Colby, Warwick university, personal communication), bacterial outer membrane extracts (section 2.16), H7-flagella and O-157 antisera (Difco laboratories) were employed in these assays. All antisera were used at various dilutions from 1:5 to 1:50. Pre-immune serum was included in each assay and results were expressed as a percentage of bacteria adherent to HeLa cells in the absence of serum (control).

2.18.2 Inhibition by outer membrane extracts and lipopolysaccharides
The effect of outer membrane proteins "OMPs" (section 2.19) and isolated lipopolysaccharides "LPS" from *E.coli* O157:H7 (section 2.22) on bacterial adherence to epithelial cells was determined as follows:-
Adherence assay to HeLa cells was performed as described in section (2.14.1), 150µg of each preparation (OMPs, LPS) was incubated with HeLa cells for 1 hour at 37°C prior to their use in the adherence assay. Results were expressed as the percentage of the number of bacteria adherent to HeLa cells in the control assay, without inhibitors.
2.18.3 Inhibition by trypsin treated OMPs

Outer membranes from *E. coli* O157:H7 (500μg) were incubated with trypsin (50 μg; Sigma) and proteinase K (10 μg; Sigma) overnight at 37°C. Treated outer membranes were then used as inhibitors in the HeLa cell assay (section 2.14.1) for quantitation of bacterial adherence.

2.19 Isolation of bacterial outer membrane by detergent solubilization

The method of Achtman et al. (1983) was adapted as follows. Cells were grown in 100 ml volumes of culture media to the mid exponential or stationary phase of growth and harvested by centrifugation at 1,000 g for 10 minutes. The pellet was re suspended in 10 ml 10 mM Tris-HCl (pH8) and the cells were broken by sonication (80 seconds, 50% cycle, 4 separate bursts with 30 seconds cooling periods). The unbroken cells were removed by centrifugation at 1,000 g for 20 min at 4°C. The resulting supernatant was centrifuged at 25,000 g for 60 min at 4°C. The pellet, containing the outer membrane, was resuspended in 150 μl distilled water and stored in 50μl aliquots at -20°C.

When required 50 μl samples were thawed and extracted with 8 volumes (400 μl) of detergent solution containing [1.67% (w/v final concentration) of Sarkosyl and 11.1 mM (final concentration) Tris pH 7.6] at room temperature for 20 minutes. The insoluble outer membranes were pelleted at 25,000 g for 90 minutes at 20°C. The pellet was resuspended in 50 μl of electrophoresis sample buffer [0.0625M Tris pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 5% (v/v) mercaptoethanol] for electrophoresis, or in 50 μl of distilled water for protein determination.
2.20 Determination of protein concentration

Protein concentrations were determined using the Folin phenol reagent as described by Lowry et al. (1951), with bovine serum albumen (Sigma) as standard. The following solutions were prepared:

A. 5% (w/v) sodium carbonate (NaCO₃)
B. 1% (w/v) copper sulphate (CuSO₄)
C. 2% (w/v) sodium potassium tartrate [NaK(CHOHCOO)₂]
D. Folin Ciocalteau (Fison) diluted 1:1 with distilled water
E. 1M sodium hydroxide (NaOH)

Samples were diluted in 0.5 ml distilled water in acid washed test tubes, then 0.5 ml of 1M NaOH was added and the samples boiled for 5 min and cooled. To 50 ml of solution A, 1 ml of solution B and 1 ml of solution C were added, 2.5 ml of this mixture was added to each sample and incubated for 10 min at room temperature. The samples were vortexed and 0.5 ml of solution D was added. After thorough vortexing the samples were allowed to stand at room temperature for 30 minutes for the development of colour. The optical density of the samples was then measured at a wavelength of 750 nm against a reagent blank containing 0.5 ml of distilled water. To assess the protein content, a series of standard protein solutions (bovine serum albumin) of known concentrations (0-1 mg) were assayed in parallel. The optical densities of the standard solutions were used to draw a calibration curve from which the protein concentrations of the samples were extrapolated.
2.21 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Polyacrylamide gels were poured between two glass plates that were held apart by spacers. Gel plates were assembled according to the manufacturer instructions (KLB Biotechnology). SDS-PAGE was performed using a discontinuous buffer system (Laemmli, 1970) including the ionic detergent sodium dodecyl sulphate (SDS) to dissociate proteins into their individual polypeptide subunits. In addition to linear gels, both gradient and two dimensional gels (O'Farrell, 1975) were used when resolution of the protein bands in the linear gels was insufficient.

2.21.1 Linear SDS-PAGE

The following stock solutions were prepared:

**Buffer A**

- 0.75M Tris-HCl pH 8.8
- 0.2 % (w/v) SDS

**Buffer B**

- 0.25M Tris-HCl pH 6.8
- 0.2 % (w/v) SDS

**Acrylamide Stock**

- 44g Acrylamide
- 0.8g Biscrylamide

Made up to

- 100 ml with distilled water
Ammonium persulphate (AMPS) 1% (w/v) made immediately before use

Reservoir buffer 0.025M Tris
0.129M glycine
0.1% (w/v) SDS

Gels with a variety of polyacrylamide concentrations were used in this study depending upon the nature of the samples. Table 2.2 shows the volumes of the above stock solutions required to give a range of acrylamide for the resolving gel, similarly Table 2.3 shows the volumes required for a range of stacking gels.

Table 2.2 Volumes of stock solutions required to give various concentrations of acrylamide in the resolving gel.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Volume (ml) to give acrylamide concentrations of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15%</td>
</tr>
<tr>
<td>Buffer A</td>
<td>27.0</td>
</tr>
<tr>
<td>Acrylamide stock</td>
<td>18.4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.2</td>
</tr>
<tr>
<td>AMPS</td>
<td>1.9</td>
</tr>
<tr>
<td>TEMED</td>
<td>100μl</td>
</tr>
</tbody>
</table>

TEMED - NNN' tetramethylethylenediamine
Aproximately 25 ml of resolving gel mixture was poured between the glass plates avoiding trapping air bubbles. The gel mixture was overlaid with water saturated butanol to exclude oxygen, which inhibits polymerisation, and to ensure flat surface.

The gel was allowed to polymerise for 30-60 minutes at room temperature. When set, the butanol was washed off using distilled water and the stacking gel poured on top of the resolving gel with a slot former (comb) inserted into the stacking gel, care was taken not to allow air bubbles to become trapped under the comb teeth.

After the stacking gel had polymerised, the removal of the slot former produced wells which were rinsed immediately with distilled water prior to sample loading. The gel was placed in the electrophoresis tank containing reservoir buffer ready for sample loading. The upper reservoir was fastened into position and partially filled with reservoir buffer. The samples were then loaded (see section 2.21.4 for sample preparation) before filling the upper reservoir completely.

The gel was run at 8 mA for 16 hours or 30 mA for 3 hours until the bromophenol blue dye front reached the bottom of the gel. Gels were removed and stained accordingly.
Table 2.3 Volumes of stock solution required to give various concentrations of acrylamide in the stacking gel.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Volume (ml) to give acrylamide concentrations of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5%</td>
</tr>
<tr>
<td>Buffer B</td>
<td>10.0</td>
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<tr>
<td>Acrylamide stock</td>
<td>3.3</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.7</td>
</tr>
<tr>
<td>AMPS</td>
<td>0.5</td>
</tr>
<tr>
<td>TEMED</td>
<td>40μl</td>
</tr>
</tbody>
</table>

2.21.2 Two dimensional O'Farrell gels

In this gel system proteins were separated by their isoelectric points in the first dimension and according to molecular weight in the second dimension for obtaining maximal resolution of proteins in complex mixtures (O'Farrell, 1975).

A stable pH gradient was formed using commercial ampholines and the proteins electrophoresed until their net charge was zero and therefore migration ceased. However, as the original method of O'Farrell (1975) does not allow for the effects of detergent solubilization on the charge distribution of the protein, the modified method of Ames and Nikado (1976) was used. This involves membrane solubilization with SDS and then first dimension electrophoresis in the presence of the non ionic detergent Nonidet P-40 (NP-40) and urea to remove the SDS bound to the protein.
The following solutions were prepared:

(i) 30\% (w/v) acrylamide stock for isoelectric focusing
Acrylamide  28.38 g
Bisacrylamide  1.62 g
Made up to 100 ml with distilled water

(ii) Isoelectric focusing gel mixture (for 10 tubes)
Urea  5.5 g
30\% Acrylamide stock  1.33 ml
10\% (w/v) NP40  2.0 ml
Distilled water  1.97 ml
Ampholines pH 5-7  0.4 ml
Ampholines pH 3.5 - 10  0.1 ml
Warmed to dissolve, 7 \( \mu l \) TEMED and 10 \( \mu l \) 10\% (w/v) AMPS added just before pouring.

(iii) Sample dilution buffer (10 ml)
Urea  5.5 g
Ampholines pH 5-7  0.4 ml
Ampholines pH 3.5-10  0.1 ml
Mercaptoethanol  0.5 ml
NP-40  0.8 ml
Distilled water to  10.0 ml
(iv) Laemmil sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris - HCl pH 6.8</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>SDS (w/v)</td>
<td>2 %</td>
</tr>
<tr>
<td>Mercaptoethanol (v/v)</td>
<td>5 %</td>
</tr>
<tr>
<td>Glycerol (v/v)</td>
<td>10 %</td>
</tr>
<tr>
<td>Bromophenol blue (w/v)</td>
<td>0.05 %</td>
</tr>
</tbody>
</table>

The isoelectric focusing gels were made in 150 mm (length) x 4 mm (internal diameter) tubes which had been cleaned by boiling in 0.1M HCl for 30 minutes, followed by thorough rinsing in distilled water and a final rinse in ethanol. The bottom of the tubes were covered with two layers of parafilm and the gel mixture added using a long form pasteur pipette to give a gel length of 10 cm. This was performed at a constant temperature (37°C) to prevent crystallisation of the urea and to avoid the gel setting too rapidly. The gel was overlaid with 8M urea and allowed to set for 1-2 hours. The urea overlay was removed and replaced with 20μl of sample dilution buffer which was overlaid with distilled water. After 2 hours the water and the buffer were removed and a fresh 20 μl of sample dilution buffer added and overlaid with 0.02M NaOH.

The parafilm was carefully removed and the tubes were mounted into the lower reservoir of the electrophoresis tank (containing 0.01M phosphoric acid) at an angle to prevent air bubbles being trapped at the bottom of the tubes. The upper reservoir was filled with 0.02M NaOH. The tube gels were pre run at 200V for 15 minutes, 300V for 30 minutes and then 400 V for 30 minutes to form the isoelectric gradient. The sodium hydroxide and the sample dilution buffer were removed from the upper reservoir and the tubes. The samples (see section 2.24,4.2 for sample preparation) were
loaded and overlaid with 10μl of a solution containing (5M urea, 0.4% (v/v) ampholines pH 5-7 and 0.1% (v/v) ampholines pH 3.5-10 in 10 ml distilled water). The remainder of the tubes and the reservoir were refilled with 0.02M NaOH and the proteins focused by electrophoresis at 400V for 16 hours followed by 800V for 1 hour.

After electrophoresis gels were extruded from the tubes by pressure from a water filled syringe into a screw topped tube containing 5 ml Laemmli sample buffer and allowed to equilibrate for 1 hour after which the gels were immediately loaded onto the second dimension or stored frozen at (-20°C).

The second dimensional gels consisted of linear gels prepared as described in section (2.21.1) except that the slot former was not used in the stacking gel, which was poured to 0.5 cm below the top edge of the gel plates and overlaid with water saturated butanol. Once the stacking gel had set the butanol was washed off with distilled water. The space at the top of the gel was filled with hot 1.0% (w/v) agarose in Laemmli sample buffer plus bromophenol blue. The tube gel was carefully placed on top of the agarose and gently pushed into the agarose to ensure that no air bubbles were trapped. Agarose was used to seal the tube gel into place. A small well was made next to one end of the tube gel for the molecular weight standards. The gel was run at 14 mA for 16 hours at room temperature and then removed, fixed and stained accordingly.

2.21.3 10-30% (w/v) gradient gels

The gel solutions were prepared as follows:
1. 30% (w/v) acrylamide mixture - 20 ml
   - Low bisacrylamide stock 10 ml
   - 75% (v/v) glycerol 7.3 ml
   - Resolving gel buffer stock 2.5 ml
   - 10% (w/v) SDS 0.2 ml
   - TEMED 4.0 μl
   Immediately before casting 40 μl of ammonium persulphate was added.

2. 10% (w/v) acrylamide mixture - 50 ml
   - High bisacrylamide stock 8.3 ml
   - Distilled water 34.9 ml
   - Resolving gel buffer stock 6.25 ml
   - 10% (w/v) SDS 0.5 ml
   - TEMED 10.0 μl
   Immediately before casting 100 μl of ammonium persulphate was added.

3. Stacking gel mixture - 10 ml
   - Stacking gel acrylamide stock 3.0 ml
   - Distilled water 4.4 ml
   - Stacking gel buffer 2.4 ml
   - 10% (w/v) SDS 0.1 ml
   - TEMED 5.0 μl
   Immediately before casting 100 μl of ammonium persulphate was added.

4. Resolving gel buffer pH 8.8
   - Tris 36.6 g
   - Concentrated HCl 4.13 ml
5. **Stacking gel buffer pH 6.8**
   - Tris: 5.98 g
   - Concentrated HCl: 4.13 ml
   - Distilled water to: 100 ml

6. **60% High biscrylamide stock**
   - Acrylamide: 60 g
   - Bisacrylamide: 1.6 g
   - Made up to 100 ml with distilled water.

7. **60% low bisacrylamide stock**
   - Acrylamide: 60 g
   - Bisacrylamide: 0.3 g
   - Made up to 100 ml with distilled water.

8. **Stacking gel acrylamide stock**
   - Acrylamide: 10 g
   - Bisacrylamide: 0.5 g
   - Made up to 100 ml with distilled water.

9. **Reservoir buffer 5x stock**
   - Tris: 30.2 g
   - Glycine: 144 g
   - Distilled water to: 1000 ml
10. Reservoir buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x stock</td>
<td></td>
<td>200 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td></td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

To cast a gradient gel, 10 ml of 30% (w/v) acrylamide mixture was placed in the mixing chamber, and the 10% acrylamide mixture was pumped into the mixing chamber at a steady rate using a peristaltic pump at the same time as the mixed acrylamide was pumped from the mixing chamber to the glass plate sandwich. Since both rates of pumping were the same, the volume in the mixing chamber remained constant. Adequate mixing was ensured by using a magnetic stirrer in the mixing chamber. After pouring the gel mixture, it was overlaid with water-saturated butanol and allowed to polymerise. Once set, the butanol was removed and a stacking gel set on top of the resolving gel as in section (2.21.1).

2.21.4 Preparation of samples

2.21.4.1 Linear SDS-PAGE

The amount of protein samples loaded was adjusted depending upon the protein concentration of the samples. For gels stained by Coomassie Blue and for Western blot analysis 50-100 μg of protein was loaded per track, whilst for the more sensitive silver stain 5-10 μg of protein was loaded.

Samples were denatured by adding electrophoresis sample buffer (0.0625M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue) and boiling for 5 min. Denatured samples were loaded into the wells in the linear gel using a
Hamilton glass syringe (either 0.1 ml or 0.01 ml) which was thoroughly rinsed between samples.
Protein molecular weight markers of size range 14.4 - 94.0 KDa (Pharmacia) were reconstituted according to the manufacturer's instructions. Bromophenol blue was added and 3 µl routinely run on SDS-PAGE.

2.21.4.2 Two dimensional Isoelectric focusing gels
Due to problems with smearing in the second dimension it was necessary to use an acetone precipitation step to remove the lipid from the samples. The pellet obtained at the end of the outer membrane preparation was resuspended in 100 µl of acetone at -20°C instead of electrophoresis buffer. This was microfuged for 5 min, the pellet dried under a stream of nitrogen, resuspended in Laemmli sample dilution buffer without tracer dye and solubilized by heating at 75°C for 2 minutes. Two volumes of sample dilution buffer were added to SDS solubilized sample to remove SDS bound protein. These samples were kept frozen at -20°C until use.

2.22 Lipopolysaccharide (LPS) extraction

2.22.1 Method I. Phenol/water extraction of LPS
LPS was extracted from dried cells according to Westphal and Jann (1965). 2g of freeze-dried *E.coli* O157:H7 cells were resuspended in 100 ml of distilled water at 67°C. 100 ml of 91% phenol (w/v) preheated to 67°C were added, and the mixture stirred continuously at 67°C for 20 minutes. The homogenate was cooled in iced water and was continuously stirred for 5 minutes before centrifugation at 3,000 g at room temperature for 20
minutes. The upper aqueous phase was carefully removed and kept at room temperature, before the lower phenolic phase was reheated at 67°C. 75 ml of distilled water, preheated to 67°C, were added to the phenolic phase, and after mixing and centrifugation as before, the two phases were separated. The aqueous phases were pooled, and alongside the remaining phenol phase, were extensively dialysed against running water to remove the phenol. Dialysis was over 2-3 days for the combined aqueous phases and up to 1 week for the phenol phase. Cell debris was removed from the dialysed phenol phase by centrifugation at 2,000 g for 20 minutes. The volume of each phase was reduced to approximately 30 ml by rotary evaporation at room temperature. The concentrated extracts were centrifuged at 105,000 g, at 4°C for 4 hours in a 60 Ti Beckmann rotor. The lipopolysaccharide pellet from each phase was resuspended in water with a glass homogeniser and recentrifuged twice as above. After the final centrifugation the pellets were freeze dried.

2.22.2 Method II Proteinase K-digestion of outer membrane extracts

LPS of *E.coli* O157:H7 was prepared from the bacterial outer membrane extracts according to the method described by Hitchcock and Brown (1983). Outer membrane sample (prepared as described in section 2.19) at 2 mg ml\(^{-1}\) was added to an equal volume of 2x sample buffer (0.25M Tris-HCl pH 8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (v/v) mercaptoethanol, 0.005% (w/v) bromophenol blue). After boiling for 5 minutes, 25 μg of proteinase K (Sigma) was added to 100 μg of protein by addition of the appropriate volume of a stock solution of Proteinase K at 2.5 mg ml\(^{-1}\) in 1x
sample buffer. The sample was heated at 60 °C for 1 hour and stored at (-20 °C) until use.

2.23 SDS-PAGE of lipopolysaccharide

The LPS samples were separated using linear polyacrylamide gels prepared as described in section 2.21.1 except that 4M urea was added to the 15% resolving gel. Aliquots of samples were electrophoresed at 30 mA for 3 hours. Smooth LPS from *Escherichia coli* O111:B4 (Sigma) was used as a control, generally 20 µl of a 10 mg ml⁻¹ solution in 1x sample buffer was used.

2.24 Staining of polyacrylamide gels

2.24.1 Coomassie blue stain

Gels were immersed in the staining solution [45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) Coomassie blue R250] for 3-4 hours and then destained in 45% (v/v) methanol with 10% (v/v) glacial acetic acid until background colouration was at a minimum.

2.24.2 Silver staining of proteins

Silver staining of proteins is approximately 10-20 times more sensitive than Coomassie blue staining, requiring a minimum of 5-10 µg protein per track. The method of Wray et al. (1981) was used. Gels were soaked in 50% (v/v) methanol for a minimum of 8 hours with three changes. 1.6 g of silver nitrate was dissolved in 8 ml of distilled water and slowly added with
shaking to a solution containing 42 ml of 0.36% (w/v) NaOH and 2.5 ml of ammonia solution. The volume was made up to 200 ml with distilled water and then used to stain the gel for 15 min. After 2 washes in distilled water for 5 min each, the gel was soaked in developer (2.5 ml 1% (w/v) citric acid, 0.4 ml formaldehyde made up to 500 ml with distilled water) until the bands appeared. The reaction was stopped with a solution containing 10% (w/v) glacial acetic acid and 40% (v/v) methanol.

2.24.3 Silver staining for LPS

The method described by Hitchcock and Brown (1985) was used. The gel was soaked in 200 ml 25% (v/v) isopropanol in 7% (v/v) glacial acetic acid and then oxidised for 5 min in 150 ml distilled water with 1.05g periodic acid and 4 ml 25% (v/v) isopropanol in 7% (v/v) glacial acetic acid (made up immediately before use). After eight 30 min washes, each with 200 ml distilled water the gel was silver stained for 10 min in a solution containing [28 ml 0.1M NaOH, 1 ml concentrated (29.4%) ammonium hydroxide, 5 ml 20% (w/v) silver nitrate and 115 ml distilled water] made up just before use with constant stirring. The gel was washed again (four 10 minutes washes) in 200 ml distilled water and then soaked in developer which contained [50 mg citric acid and 0.5 ml formaldehyde in 1000 ml distilled water] made up just before use at an optimum temperature of 25°C, below which staining of protein as well as LPS occur. Once the bands developed sufficiently (takes 10-20 min) the gel was soaked in a stop solution containing [200 ml distilled water and 10% (v/v) glacial acetic acid] for 1 hour. After a final wash in 200 ml distilled water the gel was stored in an air-tight container with a small amount of water to prevent desiccation.
2.25 Photography of gels

Stained gels were routinely photographed using an Olympus camera with Kodak Tmax 100 (ASA 100) film.

2.26 Radioactive labelling of E.coli O157:H7

Radiolabelled bacteria were prepared according to the method of Conway et al. (1990). E.coli O157:H7 was grown overnight at 37°C in Muller Hinton broth. Cultures were diluted 1:20 in fresh Muller Hinton broth containing 5 μCi of [methyl-1, 2-3H] thymidine (117 Ci/mmol; Amersham International) per ml. Cells were allowed to grow to an OD of 0.5 (4x10^8 cells ml^{-1}) as a standing culture at 37°C, then centrifuged at 3,000 g for 5 minutes at 5°C, washed once in 10 ml of HEPES-Hanks buffer (pH 7.4) and centrifuged again as described above. The final pellet was suspended in 10 ml of HEAPS-Hanks buffer (pH 7.4) at room temperature, ready for use in the adherence assays.

2.27 Western Blotting

2.27.1 Transfer of proteins to nitrocellulose

Proteins separated by denaturing polyacryamide gels (SDS-PAGE section 2.21.1) were transferred to nitrocellulose using the method of Towbin et al. (1979). After electrophoresis the stacking gel was removed and the gel was soaked in Western Transfer Buffer "WTB" [25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol] for 10-15 min. A nitrocellulose filter was
cut to the size of the gel, socked in WTB and carefully placed on top of the gel ensuring that all the air bubbles had been removed. The gel and filter were placed between 4 pieces of filter paper soaked in WTB. The proteins were transferred to the nitrocellulose at 40 V for 3 hours at 4°C. After transfer the nitrocellulose was stained to visualise the protein bands by washing for 10 minutes in Ponceau S [0.5%(w/v) in 5% (v/v) trichloroacetic acid]. The position of the slots and protein markers was noted and the stain was removed by washing the filter in 20 ml PBS for 10 minutes. The filter was then washed in 10 ml PBS containing 2% (w/v) dry milk (Marvel) for a minimum of 1 hour to allow protein to bind nonspecifically to the nitrocellulose. The PBS/dry milk was replaced with a fresh 10 ml volume of PBS with 2% (w/v) dry milk containing primary antibody (rabbit antisera) at an appropriate dilution (1:200-1:500) and incubated overnight at room temperature with gentle shaking. The filter was then washed three times for 10 min each time in 10 ml PBS containing 0.1% (v/v) Tween 20.

2.27.2 Detection of antigen-antibody complexes using horse radish peroxidase colour reaction.

Peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as the secondary antibody and 4-chloro-1-naphthol (Sigma) as the colour reagent. After the washes in PBS with 0.1% (v/v) Tween 20, the filter was transferred to fresh PBS with 0.1% (v/v) Tween 20 (10 ml) containing a 1:300 dilution of secondary antibody and incubated at room temperature for 1-2 hours with gentle shaking. After two 10 minute washes in 10 ml PBS with 0.1% (v/v) Tween 20 and two washes in 10 ml PBS, The filter was
transferred to the staining solution. This solution consisted of [0.06% (w/v) 4-chloro-1-naphthol in 20% (v/v) methanol which was mixed to an equal volume of a solution containing 3% (w/v) NaCl, 2% (v/v) 1M Tris-HCl pH 7.5, 0.1% (v/v) hydrogen peroxide just before use]. Before the reaction reached completion the filter was washed in distilled water (3 changes over 30 min), air dried, photographed and stored in the dark.

2.28 Preparation of HeLa cell outer membranes

The method of Tomassini and Colonno (1986) was followed with some modifications. HeLa cell monolayers (5x10^7) were treated with 50mM EDTA in PBS for 10 minutes at 37°C to detach cells. Cells were pooled by centrifugation at 1,500 g and then washed 3 times in PBS. Washed cells were incubated with 5 ml lysis buffer containing [0.25M sucrose, 1mM PMSF "phenylmethanesulfonyl fluoride" (Sigma) and 10 mM Tris-HCl pH 7.6] for 15 minutes on ice, and disrupted by Dounce homogenisation. Cell debris and nuclei were removed by pelleting at 1,000 g for 5 minutes at 4°C and membranes were pelleted from the resulting supernatant at 100,000 g for 1 hour at 4°C. Pellet containing the membrane fraction was resuspended in 2 ml [10mM Tris-HCl pH 7.6] and stored at 0°C.

2.29 Adhesion inhibition by HeLa cell OMPs

Overnight cultures of *E.coli* O157:H7 were centerifuged at 1,000 g for 10 minutes, washed once with PBS and resuspended in growth medium 199. Bacterial cells (2x10^8) were incubated with 50µg of HeLa cell outer membrane extracts (section 2.28) for 1 hour at 37°C.
Adherence assays were performed in 12-well tissue culture plates as described in section (2.17.1). HeLa cells in half the wells were incubated with the bacteria treated with HeLa cell OMPs, while the other half of the wells were incubated with a comparable number of untreated bacterial cell "control" for 3 hours at 37°C. The number of adherent "treated bacteria" was expressed as a percentage of the number of adherent control cells.
CHAPTER 3
Demonstration of Virulence Factors of VTEC O157:H7

3.1 Introduction

One of the important components involved in the expression of bacterial pathogenicity and the development of symptoms of disease is the virulence of the causative organisms. Pathogenic organisms depend for their virulence on, amongst others, the possession of certain antigenic characters which dictate their reaction with the host cells and the host defence systems. Deletion of these factors results in either reduction in, or loss of, virulence.

*E.coli* O157:H7 strains do not elaborate the heat-labile or heat-stable enterotoxins produced by enterotoxigenic *E.coli* and they are not invasive to epithelial cells (Wells *et al.*, 1983). However, the pathogenic mechanism of *E.coli* O157:H7 may involve two virulence factors among others not yet understood:-

- the production of Vero cell cytotoxins (VTs) which play a vital role in the pathogenesis of disease associated with VTEC infection.
- intestinal colonisation which is facilitated by both adherence and attachment-effacement factors.

Adherence of enteric pathogens to mucosal surfaces is an important primary step in bacterial colonisation and for toxin delivery. Indeed, with experimental animals, intestinal colonisation may be sufficient to cause diarrhoea in the absence of Verocytotoxins (Tzipori *et al.*, 1988, 1989).

The first objective of the studies carried out in this chapter was to demonstrate that strains of VTEC O157:H7 used in this study do possess
the two virulence factors (VTs production and adherence to epithelial cells). The second objective was to develop an in vitro models which included human tissues to investigate the virulence factors of these human pathogens.

Culture filtrates of the all O157 strains examined induced cytotoxic effects on Vero and HeLa cell monolayers in tissue culture. Adherence of these strains to different human epithelial cell lines in tissue culture, buccal epithelial cells (BEC), isolated colonocytes, and colonic biopsy was demonstrated by both light and electron microscopy.

3.2. Results and Discussion

3.2.1 Cytotoxicity assay

Four VTEC strains, O157:H7 (ATCC35150), and three O157:H7 clinical isolates from local hospitals were assayed with O26:H11 (EPEC strain) and the non pathogenic E. coli K12 serving as the negative controls. Sterile culture filtrate of these strains was added to Vero and HeLa cell monolayers in 24 well tissue culture plates at different concentrations and the cytopathic effect checked daily over four days (Section 2.11.2).

All VTEC strains exhibited an equivalent toxicity to Vero and HeLa cells (Fig 3.1). Progressive morphological changes were observed, cells looked shrivelled or rounded, and subsequently detached from the monolayer becoming free in the medium. The cytotoxic effect advanced with time and with higher concentrations of the culture filtrate, with the maximum effect being obtained in four days (Fig. 3.2) when most of the Vero cells were completely detached and the culture plates were visibly clear after staining. Neither of the non-VTEC strains tested, K12 and O26:H11 had any effect on Vero cells.
The cytotoxic effect of O157:H7 culture filtrate on Vero cell monolayers advanced with time the maximum effect being obtained in 4 days. Magnification, x400.
These observations correlate with those of Konowalchuk and colleagues, (1977) who showed that the culture filtrate of some E. coli strains isolated from patients with diarrhoea produced a cytotoxic effect on Vero cells in tissue culture.

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**Figure 3.2  Time course of cytotoxicity assay**

Undiluted culture filtrate of O157:H7 induced cytotoxic effects in at least 90% of the Vero cell monolayers within 24 hours. The cytotoxicity of the diluted filtrates increased with time and concentration.

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Since the primary target cells of the toxins *in vivo* are the endothelial cells of the capillary blood vessels in the kidney and the gut, cultured
human endothelial cells would appear to be more suitable for cytotoxicity assays. The human umbilical vein endothelial cell (HUVEC) system represents therefore, an attractive in vitro model for the study of the pathogenesis of diseases associated with VTEC infection. However, our attempts to grow the endothelial cells lining the umbilical vein in tissue culture to confluent monolayers (section 2.13) for subsequent toxin assays were time consuming and not very practical for a long term project, consequently this cell system was not considered further.

3.2.2 Adherence of VTEC O157:H7 to epithelial cells:

A wide variety of adherence assays were examined in this study with two major factors being considered in selecting the method to be used: - the availability of the host cells.
- the reliability of the technique used to separate eukaryotic cells with attached bacteria from the non adherent bacteria.
The tissue culture method using human epithelial cells to evaluate the mechanisms of adherence of E.coli achieved adequate separation, but required tissue culture facilities that frequently are not available in bacteriological laboratories.
Scrapping cells from the oral mucosa, "buccal epithelial cells" (BEC) can provide many cells from a wide variety of donors which have close association with the gastrointestinal tract, but separation of cells with adherent bacteria from non adherent bacteria by either centrifugation or filtration was difficult to achieve.
Excised tissue, "human colonic segment," gave adequate separation after a few washes, and represents the ideal in vitro model to study the adherence properties of E.coli O157:H7, but unfortunately this tissue was limited in its availability.

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3.2.2.1 Tissue culture assays

Two cell lines, HeLa cells (human carcinoma of the cervix cells), HEp-2 cells (human carcinoma of the larynx cells), were selected from the many well established transformed lines which are commercially available as cell lines of epithelial origin. Since VTEC O157:H7 colonise the large bowel, CaCo-2 cells, a differentiated human colon carcinoma cell line were also studied, human normal colonic cells (CCD-33 Co) were included to represent fibroblast cells. Monolayers of HeLa and HEp-2 cells were grown on glass coverslips as described in (section 2.14.1) and incubated with washed suspensions of the bacterial test strains. Nonadherent bacteria were removed by washing, and coverslips subsequently processed either for light microscopy (section 2.8.1) or scanning electron microscopy (SEM) as in (section 2.10).

All *E.coli* O157:H7 strains examined and the EPEC strain O26:H11 attached to the cells of epithelial origin (HeLa, HEp-2, and CaCo-2 cells). The CaCo-2 cell line has been previously used to examine the adherence of EPEC strains and a limited number of VTEC strains (Knutton *et al.*, 1989), who reported that the two VTEC strains they examined did not attach to CaCo-2 cells, contrary to the data in this study.

In marked contrast, the cells of fibroblast origin (CCD-3 cells) provided no adherence sites for the bacterial strains examined (Fig 3.3). These findings indicate that these strains bind to specific receptors on the epithelial cell surface.

The non pathogenic *E.coli* K12 did not attach to any of the cell lines used (Fig 3.4), therefore, it was used as control strain in this study.
Figure 3.3  Adherence of *E.coli O157:H7* to tissue culture cells.

VTEC O157:H7 binding to cells of epithelial origin "HeLa cells" (A), but not cells of fibroblast origin "CCD-33Co cell" (B). Magnification, x900.
Figure 3.4 Adherence of *E. coli* O157:H7 and *E. coli* K12 to HeLa cells.

VTEC O157:H7 attached to HeLa cells in tissue culture (A). Magnification, x700. *E. coli* K12 did not attach to HeLa cells (B) or any of the cell lines used. Magnification, x800.
3.2.2.2. Patterns of adherence

Two distinct patterns of adherence were demonstrated in the HeLa cell assays:-
Localised adherence (LA) occurred with one strain only, O157:H7L, where bacteria attached to localised areas of the eukaryotic cell surface, while the other strains O157:H7R and O157:H7 clinical isolates, showed a diffuse pattern of adherence (DA) where bacteria were seen covering the whole surface of the HeLa cell (Fig 3.5).
These results support the findings of Sherman et al. (1987) who reported that VTEC O157:H7 strains exhibit LA and/or DA to HEp-2 cells and Henle 407 gut-derived epithelial cells in tissue culture.

The use of tissue culture (HeLa and HEp-2) cell adhesion assays has led to the identification of further putative classes of diarrhoeagenic E.coli. The aggregative pattern of adherence, where bacteria are seen in "stacked brick" aggregates attaching to the epithelial cells, was characteristic of E.coli strains (enteroaggregative E.coli) which had been associated with persistent diarrhoea in infants (Vial et al., 1988). Recently, another category, termed diffusely adhering E.coli (DAEC) was identified as putative cause of diarrhoea in children in Mexico (Giron et al., 1991). More recent studies on DAEC (Jallat et al., 1993) indicated that the genes involved in diffuse adhesion to HEp-2 cells were located on conjugative R plasmids in strains that did not hybridise with the previously described DA DNA probes.

The full significance of these adherence patterns has yet to be elucidated, in particular the assessment of the true biological significance of localised or diffuse adherence in bacterial pathogenicity.
Figure 3.5 Patterns of adherence

3.2.2.3. Buccal epithelial cell (BEC) assay

Although HeLa cell assays are commonly used to study bacterial adhesion, they have theoretical disadvantages in studying gut flora. HeLa cells are derived from a neoplastic cell line originally isolated from a non-intestinal source. For this reason BEC which may express receptors similar to those of the intestinal cells, seemed more suitable for the adherence assays. BECs were obtained by gently scraping the buccal mucosa with a sterile spatula and suspending them in phosphate buffered saline (PBS), prior to their use in the binding assays as described in (section 2.14.2). Bacteria were clearly seen to be adhering to BECs (Fig.3.6). However, since it is difficult to obtain comparable numbers of cells in repeat experiments BECs were not considered for the subsequent comparative or quantitative adherence assays performed in this study. Furthermore, the fact that these cells are dead, must be taken into consideration. Consequently there was a need to move to a cellular system that corresponded more to the *in vivo* situation to understand the sequence of events that lead to intestinal colonisation by this pathogen.

**Figure 3.6 Adherence of *E.coli* O157:H7 to buccal epithelial cells.**

Magnification, x700
3.2.2.4 Human biopsy assays

An *in vitro* adherence assay was developed using healthy colonic mucosa obtained from surgical resection specimens from patients undergoing surgery for colonic carcinoma. The intestinal segment was immediately opened, the mucosal side was saved, while the bulk of tissue at the serosal side was discarded. The mucosa was washed several times with ice-cold PBS and used immediately for adherence experiments as described in (section 2.14.3). In these experiments native mucosa specimens were incubated with bacterial cells at 37°C for a maximum 2 hours, as a longer incubation (of mucosa itself) resulted in the gradual destruction of the mucosal epithelium. Therefore, in subsequent experiments the mucosa was fixed in 10% formalin and maintained at 4°C prior to their exposure to the bacterial suspension.

Microscopic observation of the colonic tissue revealed that the bacteria were located in the mucus covering the epithelial cells with no apparent adhesion to the mucosal cells (Fig 3.7). This observation indicated that the mucus gel on the epithelial surface presents a barrier to bacterial colonisation of the colonic mucosa. However, little is known about the function of intestinal mucus in the pathogenesis of diarrhoeal disease, and its' role in protecting against or facilitating bacterial colonisation is unclear.

In other experiments, the adherence of VTEC O157:H7 was tested by using isolated colonocytes from native colonic specimen by gently scrapping the mucosal surface with a slide as described in section (2.14.4), cells were then washed a few times with PBS prior to their incubation with the *E. coli* O157:H7. Results (Fig. 3.8) demonstrated bacteria attaching to the brush border of the colonocytes.
Figure 3.7 Adherence of *E. coli* O157:H7 to human colonic biopsy.

Histologic (A) and transmission electron microscopic (B) examination of sections of human colonic mucosa treated with *E. coli* O157:H7. Most bacteria were prevented from binding to the epithelial cell surface by the mucus layer. Magnifications, A: x1000, B: x6000.
Figure 3.8  Adherence of VTEC O157:H7 to the brush border of human colonocyte. Magnification, x700.

In subsequent assays the mucosa was gently scraped with a rubber spatula to remove the adherent mucus layer, care being taken to avoid damaging the underlying epithelium. Removal of intestinal mucus by this method was found to be more efficient in removing most of the mucus and preserving the brush border of the epithelial cells, than the use of enzymes such as hyaluronidase, and led to increased bacterial adherence to the colonic mucosa (Fig 3.9).

Figure 3.9  Scanning electron micrograph showing adherence of

*E. coli* O157:H7 to human colonic mucosa. Magnification, x10,000.
3.3 Summary and overview

These findings demonstrate that the four *E.coli* O157:H7 isolates under study possess the two virulence factors implicated in the pathogenesis of diseases associated with VTEC infection (production of VTs and adherence to epithelial cells). Since human tissues present the best model to understand human disease, the prime purpose of the studies described in this chapter has been to provide an *in vitro* model which included human tissues, to study virulence factors of *E.coli* O157:H7. However, the main disadvantage encountered when working with human materials, was infrequent availability of operation specimens which meant that the reported data was based on a smaller number of experiments than optimally required.

Therefore, it was concluded that, HeLa, and HEp-2 cells were more reliable cell lines to examine the bacterial-epithelial cell interactions *in vitro*.

This study has focused on bacterial adherence rather than toxin production, largely because it became apparent from the results of the different assays performed, and from the large number of papers published during the last few years, that bacterial adherence is the more active area of study in the field of pathogenesis of most infectious diseases.

Furthermore, an understanding of the adhesive mechanism of VTEC may lead the way to the development of an anti-adhesin vaccine.
CHAPTER 4

Cell surface components as bacterial adhesins

4.1 Introduction

Adherence of many enteric pathogens to mucosal surfaces in the gastrointestinal tract is a critical step in the pathogenesis of diarrhoeal disease (Beachey, 1981). *E.coli* O157:H7 adheres to human epithelial cells in tissue culture (Sherman et al., 1987; this study chapter 3) and to the intestine of orally infected rabbits (Pai et al., 1986). However, the bacterial attachment factors, or adhesins, that mediate adherence of *E.coli* O157:H7 to mucosal epithelium have not been clearly defined. Cell surface antigens including fimbriae, outer membrane proteins (OMP), lipopolysaccaride (LPS) and flagella have been identified as bacterial adhesins on other pathogens (Beachey, 1981; Achtman et al., 1983).

Published studies have yielded variable and conflicting results concerning the characterisation of bacterial adhesins and in particular the importance of fimbriae in the adherence process. Karch et al. (1987) reported that a 60 megadalton (MDa) plasmid mediated fimbrial antigens which allowed bacterial attachment to Henle 407 intestinal cells but not to HEp-2 cells in tissue culture. In contrast, a quantitative adherence assay has shown that the plasmidless derivative of an *E.coli* O157:H7 strain adheres to Henle 407 cells three times better than the parent strain (Junkins and Doyle, 1989).
Another study indicated that only one of five *E.coli* O157:H7 strains possessing the 60 MDa plasmid was fimbriated (type 1), yet all strains adhered to both Henle 407 and HEp-2 cells (Sherman *et al.*, 1987). Adherence of *E.coli* O157:H7 to HEp-2 cells was subsequently reported to be mediated by constituents of the outer membrane, but not LPS or the H7 flagella (Sherman and Soni, 1988). More recent investigations have revealed the involvement of membrane proteins in the adherence mechanism (Sherman *et al.*, 1991).

In this study the role of cell surface components in the binding of *E.coli* O157:H7 to HeLa cells has been investigated using a series of comparative quantitative adhesion-inhibition assays. We also used morphological and serological techniques to characterise the outer membrane proteins, H7 flagellar and O157 antigens on the cell surface of VTEC O157:H7 by transmission electron microscopy (TEM). Outer-membrane extracts from VTEC O157 strains, an EPEC O26:H11 strain and the nonadherent *E.coli* K12 were analysed by gel electrophoresis (SDS-PAGE).

In summary, the findings of the ultrastructural studies of the bacterial cell envelope indicated that the adherence factors are nonfimbrial, since fimbriae were not expressed by any of the VTEC strains under study. The results of the adhesion inhibition assays suggested that flagella are less likely to be involved in the adherence process, and the final conclusion from the biochemical studies and the SDS-PAGE analysis was that outer membrane proteins (OMPs) are the likely candidates for the adherence factor(s).
4.2 Results and Discussion

4.2.1 Ultrastructural study of O157:H7 surface components

*E.coli* O157:H7 strains expressing adherence to HeLa cells in tissue culture were examined by electron microscopy, using negative staining and immunogold labelling techniques, which allowed different surface antigens to be identified.

4.2.1.1 Fimbriae

Negative staining of the four VTEC strains under study (section 2.9.1) failed to visualise any fimbriation by transmission electron microscopy. To confirm this observation the haemagglutination (HA) ability of these strains was studied (section 2.15) since erythrocyte HA has been successfully used to identify fimbriated *E.coli* (Duguid *et al.*, 1955). Human, horse and sheep erythrocytes were tested for agglutination with O157:H7 strains from the liquid and solid bacterial cultures used in the HeLa cell adherence assays. None of the VTEC strains showed agglutination of any erythrocytes indicating the lack of fimbrial expression by O157:H7. The same negative results were obtained after repeated bacterial subculture on different growth media (Nutrient broth, Penassay, Luria-Bertani and Muller-Hinton media), and remained negative regardless of the variation of bacterial growth phase. Furthermore, the presence of type-1 fimbriae which are known to bind to mannose containing receptors "mannose sensitive binding" (Duguid and Old, 1980) was investigated. Quantitative Adherence assays (section 2.14.1) performed in the presence and absence of 1% mannose (Figure
4.1) indicated that *E. coli* O157:H7 binding to HeLa cells was not inhibited by the addition of mannose (mannose resistant binding).

**Figure 4.1 Effect of mannose on adherence**

The presence of mannose did not inhibit the adherence of *E. coli* O157:H7 to HeLa cells.

Sherman *et al.* (1987) used five VTEC strains to study the surface properties of O157:H7, they found that mannose sensitive type-I fimbriae were expressed by only one strain (designated CL-49), while the other strains (CL-56) were nonfimbriated. However, all strains bound to epithelial cells in tissue culture.
The structure of the mannose-resistant adhesins in nonfimbriated strains remains to be discovered, possibly these adhesins are diffuse non-filamentous surface substances or, possibly, fibrillar structures too fragile to be detected under the experimental conditions used in this study.

These findings together with the HA results and the TEM observations indicated that the \textit{E.coli} O157:H7 strains studied, do not possess fimbriae, nevertheless, they are able to attach to epithelial cells \textit{in vitro}. The existence of nonfimbrial adhesins is not surprising, since adhesion of several strains of enteropathogenic \textit{E.coli} to HEp-2 cells has been shown to be independent of fimbriation (Scotland, 1983). The data presented in this thesis supports the studies by Sherman \textit{et al.}(1987, 1988) which suggested that some non-fimbriated \textit{E.coli} O157:H7 strains can adhere to HEp-2 cells in tissue culture.

4.2.1.2 Flagella

Under the growth conditions used in this study, \textit{E.coli} O157:H7 was motile when examined by phase-contrast microscopy. The flagella were characterised by transmission electron microscopy after negative staining and immunogold labelling (Fig 4.2) with H7 flagellar antiserum being used as the source of the primary antibody (section 2.9.2).

The functional role of H7 flagella in promoting virulence of \textit{E.coli} O157:H7 infections in humans has not been defined. Flagella confer motility which may permit penetration of the surface mucus gel and thus increase bacterial colonisation of the intestinal mucosa. However, certain flagella may also function as bacterial attachment factors that promote binding of the organism to epithelial cells (Newell and McBride,1985). Construction of mutants that do not carry flagellar
filaments would establish more definitely the significance of flagella in adherence.
The possible role of H7 antigen(s) as a binding factor has been investigated in this study (section 4.2.2.1).

Figure 4.2 Transmission electron micrographs of H7 flagella.

A- negative staining of E.coli O157:H7. B and C- immunogold labelling of flagella after incubation of O157:H7 with rabbit anti-H7 antiserum followed by immunogold conjugated to goat anti-rabbit immunoglobulin G. Gold particles are clustered along the length of the flagellar filament. Magnifications. A,B: x8,000. C: x30,000.
4.2.1.3 Outer membrane proteins (OMPs)

OMPs were demonstrated on the cell surface of VTEC O157:H7 by the immunogold labelling technique as described in section (2.9.2). Antibodies to OMPs were obtained from rabbit antiserum raised against outer membrane extracts of *E.coli* O157:H7 as described in section (2.16). OMPs appeared as clusters of gold particles in irregular patches on the cell surface (Fig 4.3). To prevent nonspecific labelling, 1% bovine serum and 1% Tween-20 were added to the wash solution. At present, there is little data available in the literature with which to compare the data on immunogold labelling of the OMPs. However, evidence has accumulated during the last decade to show that OMPs may be involved in mediating bacterial adherence to epithelial cells (Sherman *et al.*, 1991). This hypothesis was studied in detail in this project.

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**Figure 4.3 Transmission electron micrograph of OMPs**

Immunogold labelling of OMPs after incubation of *E.coli* O157:H7 with rabbit anti-OMPs antiserum followed by immunogold conjugated to goat anti-rabbit immunoglobulin G. Gold particles clustered in irregular patches. Magnification, x20,000.
4.2.1.4 Lipopolysaccarides (LPS)

Hyperimmune serum for the O-157 antigen used in the immune electron microscopy techniques demonstrated that the antibodies recognised LPS, notably the O antigen. Since LPS are the major component of the bacterial outer membrane, *E.coli* O157:H7 appeared to be uniformly coated with gold labelled particles (Fig.4.4).

The role of O-157 antiserum and purified LPS in inhibiting adherence of VTEC O157:H7 to tissue culture cells has been evaluated in sections (4.2.2.1 and 4.2.2.2).

**Figure 4.4 Transmission electron micrograph of LPS**

Immunogold labelling of LPS after incubation of *E.coli* O157:H7 with rabbit anti-O157 antiserum followed by immunogold conjugated to goat anti-rabbit immunoglobulin G. Gold particles uniformly coated the bacterial cell surface.  
A: 10nm colloidal Gold used. Magnification, x15,000.  
B: 20nm colloidal Gold used. Magnification, x20,000.
4.2.2 Adhesion Inhibition Assays

Adhesion inhibition assays were used to evaluate the role of H7 flagella, LPS, and OMPs as potential bacterial adhesins. Comparison was made between viable bacterial counts of adherent bacteria obtained from triplicate experimental sets (with inhibitors) and from those of the control sets (without inhibitors).

4.2.2.1 Role of rabbit antisera as inhibitors

Adherence of *E.coli* O157:H7 to HeLa cells and HEp-2 cells was quantified in the presence and absence of homologous rabbit antisera raised against, whole cells, outer membrane proteins (OMP) lipopolysaccharide (LPS) and the H7 flagella. Pre-immune rabbit serum was also included in all assays, which induced a 2-10% reduction in the number of adherent cells due presumably to nonspecific inhibition (section 2.18.1).

Data are expressed as a percentage of the number of adherent organisms in the absence of putative inhibitors.

Antisera raised to whole cell O157:H7 reduced bacterial adherence to 21%, while H7 flagellar antiserum blocked bacterial adherence to 37%.

More significant inhibition of the adherence of O157:H7 to HeLa cells was observed with antisera raised against the O157 antigen and the OMP, where binding of *E.coli* was only 3.7% and 2.5% respectively of the level of adherence observed in the absence of antisera (Fig.4.5).

All antisera, used in various dilutions from 1:5 to 1:50, inhibited adherence in a concentration dependent manner. HeLa cells were substituted for HEp-2 cells in these assays with similar results.
These findings indicate that the role of the H7 flagellar antiserum as an inhibitor of adherence is the least significant. Other studies (Sherman and Soni, 1988) have similarly demonstrated that the H7 antiserum induced a minimal degree of inhibition and purified H7 flagellin did not inhibit adherence of *E. coli* O157:H7 to human epithelial cells. Clearly any role flagella have in pathogenesis is not at the attachment level. However, the marked adhesion-inhibition caused by both LPS and OMP antisera strongly suggests their possible role as attachment factors.

### 4.2.2.2 Role of purified LPS and OMP as inhibitors

To support the findings obtained with LPS and OMP antisera, the capacity of these respective antigens to inhibit bacterial adherence was investigated.
Outer membrane extracts from *E. coli* O157:H7R, prepared by the standard sarcosine extraction method (section 2.19), and incubated with HeLa cell monolayers prior to their use in the adherence assays (section 2.18.2), exhibited a reduction in the number of adherent bacteria to 45% of the adherence level of the control assay without inhibitors.

Lipopolysaccharides isolated by phenol-water extraction from the same strains (section 2.22.1), and used at the same concentration as the OMP (150µg), caused much less inhibition i.e. 90% of the bacterial cells remained attached to the HeLa cells.

Figure (4.6) shows that LPS extracts caused a slight but insignificant reduction in the number of adherent bacteria. Similar findings were observed by Sherman and Soni (1988) who reported that LPS antigens did not inhibit binding of *E. coli* O157:H7 to epithelial cells in tissue culture.

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**Figure 4.6 Adhesion inhibition by purified OMPs and LPS**

![Bar chart showing adhesion inhibition by purified OMPs and LPS.](image)

Binding of VTEC O157:H7 to HeLa cells in the presence of purified OMPs (45% of the bacteria remained attached) and LPS (90% of bacteria still binding to HeLa cells)
In other experiments pretreatment of HeLa cells with higher concentrations of OMP (300μg) completely inhibited bacterial adherence to HeLa cells (data not shown). Therefore, OMPs were investigated further for their possible role as bacterial attachment factors.

4.2.2.3 Role of trypsin-treated OMP as inhibitors

Aiming at probing the biochemical nature of the bacterial outer membrane extracts which promote binding of VTEC O157:H7 to HeLa cells, these extracts were incubated with trypsin overnight prior to their use as inhibitors (section 2.18.3). Trypsin-treated OMPs failed to demonstrate any inhibitory effect on the adherence of O157:H7 to HeLa cells as shown in (Fig.4.7). Trypsin was replaced by proteinase K in these assays with similar results.

Figure 4.7 Effect of trypsin treated OMPs on adherence

Outer membrane extracts from *E.coli* O157:H7 inhibited bacterial adherence significantly. Trypsin treatment of these extracts reversed this effect.
These findings indicate that the adherence factors of *E.coli* O157:H7 are most probably proteinaceous in nature. Recent data published by Sherman *et al.* (1991) support this hypothesis. They demonstrated that antiserum raised against a 94 KDa membrane protein induced maximal inhibition of bacterial adherence and abolished the attaching-effacing binding of *E.coli* O157:H7 to epithelial cells.

### 4.2.3 SDS-PAGE analysis of outer membrane proteins

Outer membrane extracts from all *E.coli* O157 strains studied in the adherence assays, the O26:H11 EPEC strain which demonstrated adherence to HeLa cells (section 3.2.2.1) and the nonadherent *E.coli* K12 strain were analysed by gel electrophoresis as described in (section 2.21.1).

The protein profiles (Fig.4.8) obtained by SDS-PAGE analysis showed that all extracts gave a similar pattern of major OMPs. However, a distinct protein band of apparent molecular weight (18 KDa) was absent from the protein profile of *E.coli* K12 extracts. Since all *E.coli* O157 strains and *E.coli* O26:H11 were shown to be adherent to HeLa cells earlier in this study and they all possess the same protein (18 KDa), while *E.coli* K12 is the only nonadherent strain and lacks this protein, it seems that this protein may be involved in promoting the adherence of these *E.coli* strains to epithelial cells. The use of immune serum to this 18 KDa protein in inhibition assays would establish more clearly the significance of this protein in adherence. (Time did not permit these experiments to be carried out).
Figure 4.8 Outer membrane proteins of *E.coli* strains separated by 15% SDS-PAGE and visualised with silver staining.

Lane A, reference proteins with molecular weight in kilodaltons shown on the left. Lanes B,C,D and E are different isolates of O157:H7. Lane F O157:H7R. Lane G *E.coli* O26:H11 and lane H *E.coli* K12. Arrow indicates the location of a major protein which is missing in lane H.
4.3 Summary and overview

The production of cell surface components is known to be sensitive to growth conditions and absolute standardisation is difficult to achieve. Thus *in vitro* bacterial adhesion assays may give variable results. However, these studies have shown that under carefully standardised conditions, the relative level of adherence was fairly stable and reproducible.

The ultrastructural approach was used to provide direct evidence of cell surface antigens that could mediate binding of the bacteria to epithelial cells *in vitro*.

The results of the preliminary characterisation of cell surface components indicated that the *E.coli* O157:H7 strains studied are not fimbriated. However, these strains have been shown to be capable of binding to tissue culture cells. Therefore, further studies were focused upon the identification of nonfimbrial cell surface antigens which could serve as bacterial adhesin(s).

Flagella were clearly characterised by negative staining and immunoelectron microscopy. However, the results of the quantitative adhesion-inhibition assays have indicated that the role of H7 antigen as an adherence factor is insignificant, if any.

On the other hand, the role of the LPS and the OMPs as possible attachment factors seems more likely due to the prominent inhibition of bacterial adherence caused by immune sera to both antigens. Subsequent assays comparing the inhibition of adherence by purified OMPs and LPS indicated that, outer membrane extracts were more effective as competitive inhibitors than isolated LPS.
These results suggest that LPS may, in part, be involved but the OMPs seem to have the major role in mediating attachment of *E.coli* O157:H7 to epithelial cells.

Reviewing these data, it appears that *E.coli* O157:H7 expresses its adherence potential through cell surface constituents other than fimbriae, flagella, or LPS.

Conclusions drawn from the biochemical studies and the SDS-PAGE analysis of the OMPs suggest that protein constituents of the bacterial outer membrane are the likely candidates for the adherence factor(s).

Further studies were therefore focused upon the protein moieties of the bacterial outer membrane.
CHAPTER 5
CHAPTER 5

Physiology of bacterial adherence

5.1 Introduction

Little information is available from human cases to assess directly the nature, site, or mechanism of intestinal colonisation by VTEC O157:H7. If adherence data obtained in vitro are to be related to bacterial pathogenicity in vivo it is important to examine bacterial-epithelial cell interactions in as close to the in vivo situation as possible. To achieve this it is also important to understand the physiology of the gastrointestinal tract, mainly the large intestine, as the colonic mucosa is the target site for bacterial colonisation.

5.1.1 Human intestinal physiology

Intestinal physiology and host defence mechanisms play an important role in preventing the bacterial flora over-running the host and in determining the infective dose of the enteric pathogens. Gastric acid is undoubtedly important in reducing the number of bacterial pathogens entering the small intestine. This is mainly achieved by the low pH of the gastric juice, which in a fasting stomach is normally less than 3.0, thus, organisms ingested with food are largely killed by the germicidal action of the gastric acid. In the small intestine the digestive pancreatic juice and bile are secreted, neutralising the gastric acid. In addition, bile-salts have detergent action
and are able to disrupt the cell wall and membrane of some bacteria. The flow-rate of gut contents (peristalsis) also contributes to the control of bacterial colonisation. This is greatest at the top of the small intestine where microbial multiplication usually does not exceed the rate at which organisms are removed, while movement in the colon is effectively reduced so the relative stasis allows bacterial numbers to rise rapidly.

The colon has been compared to a continuous culture fermentor with the dietary residue as culture medium in which the microflora remains constant, a change in diet will result in an alteration in the composition of the medium, which in turn will affect bacterial growth. Diet is therefore probably extremely important in determining the quantitative and qualitative characteristics of the intestinal flora.

Adhesion of the indigenous normal flora to specific receptors on the intestinal mucosa has been suggested as an important mechanism by which pathogens are excluded. The indigenous microflora reflects the essentially anaerobic environment in the colon, and consists mainly of anaerobic or facultatively anaerobic micro-organisms (Savage, 1986). Therefore, pathogenic bacteria reaching the colon must adapt to an anaerobic environment, and it is under these conditions that the colonisation of intestinal epithelial cells occurs.

Most of the surfaces of the gastrointestinal tract are covered by a mucus layer. This mucus is proposed to have a number of functions, including cytoprotection of the stomach, lubrication of the intestinal contents, and protection against infection. Mucus proteins (mucins) are the macromolecules forming the mucus gel and are very rich in carbohydrates, which occur as oligosaccharides. Theoretically, the diversity of mucin
oligosaccharides should provide multiple receptors for bacterial adhesins and thus, mimic or compete with epithelial membrane receptor sites. Although mucin binding of bacteria may be protective for the host in some instances, there is little evidence to support this role. Indeed, in many cases adherence to mucin may facilitate bacterial colonisation (Cohen, 1985). Therefore, the role of intestinal mucus in protecting against, or facilitating bacterial adherence, is not clear and relatively few studies have been undertaken to examine the mechanism of bacterial binding to the intestinal mucus.

Pathogenic *E. coli* act by a variety of mechanisms to overcome the host defences and disturb the intestinal physiology in order to attach to the colonic mucosal cells, and produce diarrhoea. The adaptation to a low iron environment is particularly important for commensal and pathogenic organisms, since the amount of iron that is readily available to bacteria in body fluids is extremely small. This is because extra-cellular iron is attached to high affinity iron binding glycoproteins, transferrin in serum and lymph, and lactoferrin in external secretions and milk, both of which bind iron tightly and ensure that no free iron is available to invading bacteria.

This chapter deals with the influence of variable physiological conditions, e.g. pH values, oxygen limitation, iron restriction, bacterial growth medium, and bacterial growth phase, on the adherence of VTEC O157:H7 to epithelial cells. Marked differences in the number of adherent bacteria were noticed under different environmental conditions. Organisms harvested during the stationary phase, from aerobic, low iron, and low pH (2-4) cultures, demonstrated lower binding capacity compared to the same
O157:H7 strains harvested during the exponential growth phase from anaerobic, iron-replete culture media of higher pH (6-7). It is now well-recognised that the expression of antigens by bacteria obtained directly from the infected host may be quite different to those expressed in vitro (Smith, 1990), in particular, a number of outer membrane proteins (OMPs) are induced in various bacteria growing in vivo which are not expressed in standard laboratory media (Griffiths et al., 1983). The effect of different in vitro growth conditions on the outer membranes of E.coli O157:H7 was investigated by electrophoresis (SDS-PAGE) and significant variations in the expression of certain OMPs were observed when growth conditions were altered.

5.2 Results and Discussion

5.2.1 Influence of growth media on the adherence of E.coli O157:H7 to epithelial cells

The aim of the initial studies in this chapter was to achieve constant and reproducible culture conditions that optimised bacterial adherence to HeLa cells, and so form a basis for subsequent comparative quantitative adherence assays. Several growth media including, Penassay "antibiotic medium 3", nutrient broth, Muller Hinton broth, and Luria-Bertani medium, have been used to grow VTEC strain O157:H7R under the same cultural conditions. Samples of equal optical density (OD) were taken from these cultures and incubated with HeLa cell monolayers under similar test conditions for 3 hours as described in section (2.14.1). After repeated washing to remove
nonadherent bacteria, HeLa cells were lysed and attached bacteria were serially diluted and cultured for enumeration by plate counting. These experiments were repeated several times, included attempts to reduce the differences between the number of the bacterial cells added to each well of the tissue culture plates, as well as the number of the HeLa cells per well. The results of 4 triplicate experiments (Fig.5.1), showed considerable differences in the viable counts of bacterial cells adherent to HeLa cells. Organisms grown in Muller-Hinton medium prior to their use in the adherence assay demonstrated maximal adherence capacity.

**Figure 5.1  Effect of growth media on O157:H7 adherence to HeLa cells.**

Maximal adherence was achieved by growing O157:H7 in Muller-Hinton medium. Adherence is expressed as mean number ± standard deviations of adherent bacteria in three assays for each medium.

These differences point out the possible correlation between the growth media constituents (table 5.1) and the adherence ability of VTEC O157:H7 to HeLa cells.
Table 5.1 Composition of culture media used to grow O157:H7

<table>
<thead>
<tr>
<th>Medium Description</th>
<th>Components</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penassay broth &quot;Antibiotic Medium 3&quot; (DIFCO)</td>
<td>Bacto Beef extracts</td>
<td>1.5 g</td>
</tr>
<tr>
<td></td>
<td>Bacto Yeast extracts</td>
<td>1.5 g</td>
</tr>
<tr>
<td></td>
<td>Bacto Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>3.5 g</td>
</tr>
<tr>
<td></td>
<td>Bacto Dextrose</td>
<td>1 g</td>
</tr>
<tr>
<td></td>
<td>Dipotassium Phosphate</td>
<td>3.68 g</td>
</tr>
<tr>
<td></td>
<td>Monopotassium Phosphate</td>
<td>1.32 g</td>
</tr>
<tr>
<td></td>
<td>Nutrient Broth (OXOID)</td>
<td>(pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>Lab-Lemco powder (L29)</td>
<td>1 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extracts (Oxoid L20)</td>
<td>2 g</td>
</tr>
<tr>
<td></td>
<td>Peptone (L37)</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Muller Hinton Broth (OXOID)</td>
<td>(pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>Meat infusion</td>
<td>6 g</td>
</tr>
<tr>
<td></td>
<td>Casein Hydrolysate</td>
<td>17.5 g</td>
</tr>
<tr>
<td></td>
<td>Starch</td>
<td>1.5 g</td>
</tr>
<tr>
<td></td>
<td>(pH 7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Luria-Bertani Medium</td>
<td>(pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>Bacto Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extracts</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>10 g</td>
</tr>
</tbody>
</table>

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It is known, however, that the production of various bacterial extracellular structures responsible for adherence can be enhanced or decreased when organisms are cultured in different growth media. Chan and Bruce (1982) reported that yeast nitrogen base decreases the production of both fimbriae and expolysaccharides by uropathogens so decreasing the mean adherence of uropathogenic *E.coli* to epithelial cells.

The nutrient substrates available for *E.coli* in the colon depend to a large extent on the host- or diet-derived substances. Mucopolysaccharides from the mucosa and undigested food particles (starch, pectins and cellulose) provide the primary carbohydrate polymers. Whether "natural food" has any influence on the adherence of VTEC O157:H7 to mucosal surfaces is unknown.

Reiter and Brown (1976) reported that agglutination of red blood cells by *E.coli* K88 and K99 antigens can be inhibited by milk fat globules or fat globule membranes, to the extent that milk fat globule membrane inhibitory activity may extend to other adhesive pathogens as well. Since most bacterial adhesins appear to react with carbohydrate receptors on mammalian cell surfaces (Jones, 1977), it was assumed that foodstuffs containing sugars, polysaccharides, glycoproteins or glycolipids, will antagonise the adhesion of bacteria in the gastrointestinal tract by competitive inhibition. Obviously, bacteria bound to cell debris of dietary products passing through the gut are likely to be diverted from association with mucosal epithelium. Recent studies carried out by Cravioto *et al.* (1991) demonstrated that the protective effect of breast milk against EPEC-associated diarrhoea was due to its' antiadhesive capacity, which is due to the presence of many factors including secretory IgA (sIgA) and oligosaccharides. sIgA purified from colostrum and breast milk inhibited
the localised adherence of EPEC to HEp-2 cells and was found to respond to a 94-KDa plasmid encoded OMP implicated as the EPEC adherence factor by Levine et al. (1985). An oligosaccharide-enriched fraction from these samples also inhibited the attachment of EPEC. However, attachment of isolates with diffuse or aggregative adherence was not inhibited. The addition of such milk components to cows' milk preparations for children who cannot be breast-fed could help as a vehicle for protection against enteric infections in infants (Cravioto et al., 1991).

Although nutrient-broth is generally used for the growth of *E.coli*, in this study Muller-Hinton medium was more satisfactory and promoted maximum adherence, consequently it was selected to grow VTEC O157:H7 for the subsequent quantitative adherence assays.

### 5.2.2 Influence of bacterial growth phase on adherence

The prime purpose of these experiments was to determine at which growth point a culture should be harvested to achieve maximal adherence. Quantitative assays were therefore performed to monitor bacterial adherence during the growth cycle (section 2.14.1). There was a gradual increase in the binding of *E.coli* O157:H7 to HeLa cells during the exponential growth phase, reaching a maximum rate near the end of this phase Figure (5.2). As the culture entered the stationary phase there appeared to be a decline in the number of adherent bacteria, which remained at a low level through the next 20 hours. This observation could be due to the fact that the transition time between the exponential and the stationary phase involves a period of unbalanced
growth, during which the various cellular components are synthesised at an unequal rate.

**Figure 5.2  Effect of bacterial growth phase on O157:H7 adherence to HeLa cells.**

Actively growing bacteria in the exponential phase are more adherent to HeLa cells than those in the stationary phase. The mean is given for triplicate assays.

Since the expression of most adhesins is a regulated process dependent on the phase of growth, and conditions of culture (Jones and Isaacson, 1983), this decline in the adherence capacity of *E. coli* O157:H7 may be due to the collapse of adhesin production when exponential growth is arrested.

The biosynthesis of K88, K99 and F41 fibrillar adhesins by enterotoxigenic *E. coli* strains has been shown to be dependent on the growth phase of the cultures (Jacobs and Graaf, 1985). An increase in these adhesins was
observed during exponential growth with production being arrested in the stationary phase.

To investigate these attachment variations further, outer membranes were extracted from *E. coli* O157:H7 in the exponential and stationary phases by precipitation of bacterial sonicates in Sarkosyl, as described in section (2.19). Protein profiles (Fig. 5.3) obtained by gel electrophoresis (SDS-PAGE) demonstrated differences in the low molecular weight (MW) proteins. Two bands of an apparent MW of 14 and 17 KDa, expressed by the exponential phase extract were not present in the stationary phase. On the other hand, two protein bands of estimated MW 15 and 18 KDa were present in the stationary phase profile but absent from that of the exponential phase.

Variations in OMP profiles at different stages of the growth cycle probably reflect changes in nutrient availability at the different growth stages, however, changes due to differences in growth rate *per se* cannot be discounted (Brown and Williams, 1985).

The correlation between the expression of the 17 KDa protein band, with the high adherence capacity of VTEC O157:H7 to HeLa cells, indicates that this protein may be involved in promoting bacterial adherence. However, the expression of the 18 KDa protein in the stationary phase only correlated with an obvious drop in the number of bacteria adherent to HeLa cells, and may be of equal significance in terms of adherence.

### 5.2.3 Influence of pH values on adherence

Since *E. coli* O157:H7 shares an oral route of infection with other enteric pathogens, and produces pathological lesions in the large intestine it means
that these organisms have the ability to survive the pH changes from the high acidity in the stomach to the colonic pH of 6-7.

Figure 5.3 SDS-PAGE of outer membrane extracts from VTEC O157:H7 in the stationary and exponential phase of growth.

Lane A- Molecular mass standards in kilodaltons.
Lane B- OMPs profile of VTEC O157:H7 in the stationary phase of growth.
Lane C- OMPs profile of VTEC O157:H7 in the exponential phase of growth.
Arrows indicate to the proteins which exhibited variations in expression.
To investigate this phenomenon *in vitro*, a series of quantitative adherence assays were performed under the same conditions as described in section (2.14.1), except for the pH of the incubation media which was adjusted between 2 to 8. The viable counts of adherent organisms (figure 5.4) showed very low numbers of VTEC O157:H7 attaching to HeLa cells when incubated at pH 2 and 3, while the binding activity of the same strain increased considerably at pH values of 6 and 7. The correlation of the minimal adherence with the pH values of the stomach and proximal intestine (2-3), and the maximal adherence with the pH of the colon (6-7), may explain why bacteria permanently colonise the large intestine, while samples from the proximal intestine contain few if any cultivable bacteria, and perhaps explains why only an achlorhydric "non-acidic" stomach is usually heavily colonised by bacteria.

**Figure 5.4** Effect of pH on bacterial adherence.

Minimum adherence demonstrated when the binding assays were performed at pH2 and maximum adherence obtained at pH6.
To investigate whether these quantitative differences were attributable to changes in the outer membrane constituents, outer membranes extracted from *E. coli* O157:H7 grown under cultural conditions similar to the adherence test conditions (pH 2, 4, 6 and 8), were examined by SDS-PAGE (figure 5.5). Profiles of 3 prominent proteins were similar in all extracts. However, other protein bands of estimated MW 18, 33, and 43 KDa were lightly expressed in the profile of pH 2 extracts, and became more abundant in the extracts from pH 4 medium, these protein bands were not observed in the preparations from pH 6 and pH 8 media. Since the synthesis of these proteins did not promote adherence, it seems that they are less likely to be involved in mediating attachment of *E. coli* O157:H7 to HeLa cells, and the reverse could be true, i.e. it is possible they have an inhibitory effect on the adherence of these organisms to epithelial cells.

Although the concept of pathogenic organisms adapting to changeable environments within the host is an important one, there are few reported examples. The effect of pH variations on the attachment of VTEC O157:H7 to epithelial cells and on OMPs expression has not, to the best of our knowledge, been reported previously.

### 5.2.4 Influence of iron-availability on bacterial adherence

Iron is essential for the growth of most bacteria and thus, specific mechanisms for its' acquisition are present in most microbes. The human host presents an environment in which the availability of iron is strictly limited.
Figure 5.5  SDS-PAGE of outer membrane extracts from O157:H7 grown in different pH media.

OMP profiles of VTEC O157:H7 grown in media of pH 2, 4, 6 and 8.
Lane A- molecular mass standards in kilodalton.
Arrows indicate to the proteins which exhibited variation in expression.
In order to assess the influence of iron limitation on bacterial adherence, comparative, quantitative adherence assays were performed using E.coli O157:H7 strains, which were cultivated in the presence and absence of the iron-chelator ovotransferrin (conalbumin) prior to their use in the adherence assays. Another set of experiments were performed using the iron-chelator 2,2’ dipyridyl as described in section (2.2.5). Results of both sets of assays indicated that adherence activity of VTEC O157:H7 to HeLa cells was considerably reduced in response to iron limitation (Fig.5.6).

Figure 5.6  Effect of low iron on bacterial adherence

Iron limited growth conditions induced lower adherence capacity of O157:H7 to HeLa cells.

These results contrast to the findings of Sherman et al.(1991), who suggested that the presence or absence of iron-chelator had no influence on the binding of E.coli O157:H7 strain CL-56, a representative nonfimbriated
\textit{E. coli} O157:H7 strain (Sherman \textit{et al.}, 1987) to HEp-2 cells. Nevertheless, they reported that iron-restriction induced the expression of new outer membrane proteins (85, 52 and 28 KDa) when outer membrane extracts were analysed by gel electrophoresis.

In this study, protein SDS-PAGE profiles (Fig. 5.7) of the outer membranes of \textit{E. coli} O157:H7 grown in iron limited medium, demonstrated the expression of two protein bands of apparent MW 72 and 79 KDa in response to iron deprivation.

Another observation, the repression of a protein band estimated to have a MW of 69 KDa in the same preparation, correlated with the drop in the number of adherent bacteria. Thus, this protein may have a possible role in mediating the attachment of O157:H7 to epithelial cells. However, additional studies are required to investigate if any of the iron-regulated outer membrane proteins mediate bacterial attachment to host cells.

Iron chelation has been reported to increase transcription of VT1 in the extracellular medium. VT2 production, on the other hand, was not determined by iron levels in the media (De Grandis \textit{et al.}, 1987; Sung \textit{et al.}, 1990). However, unlike the extensive studies on the role of iron on VT production, there is relatively little information on the effect of iron limitation on the adherence of VTEC O157:H7 to epithelial cells.
5.2.5 Influence of anaerobiosis on bacterial adherence

The microflora of the large intestine consists mainly of anaerobic and facultatively anaerobic organisms generating a strictly anaerobic
environment. Thus, when pathogenic bacteria reach the colon, they must adapt to an anaerobic environment to carry out essential metabolic activities, including colonisation of the intestinal mucosa. The role of anaerobiosis as a possible controlling factor in the adherence of VTEC O157:H7 to epithelial cells was therefore examined.

Another series of comparative adherence assays was performed using *E. coli* strains grown under aerobic conditions and the same strains grown under oxygen-limited conditions (section 2.4). Strains used in these assays included the reference strain O157:H7R and two clinical isolates of *E. coli* O157:H7L and O157:H7D.

Anaerobic growth of these strains resulted in substantially lower cell yields than aerobic growth, furthermore, anaerobic cultures were observed to reach stationary phase much earlier (Fig. 5.8).

**Figure 5.8 Growth curve of *E. coli* O157:H7 under aerobic and anaerobic conditions.**

![Growth curve of *E. coli* O157:H7 under aerobic and anaerobic conditions.](image)

Anaerobic conditions reduced the final cell density of O157:H7, in comparison to the aerobic conditions.
Comparable numbers of bacterial cells from both aerobic and anaerobic cultures were added to comparable numbers of HeLa cell monolayers in each case. Bacterial cells were allowed to attach to HeLa cells for 3 hours under aerobic conditions as described in section (2.14.1). After repeated washing to remove nonadherent bacteria, HeLa cells were lysed and attached bacteria were serially diluted and cultured for enumeration by plate counting. These assays were carried out in triplicate, and the mean values of the number of adherent organisms are given in Fig. 5.9. These data indicate that the number of the anaerobically grown O157:H7 attached to HeLa cells is nearly twice that of the same strains grown aerobically. However, when the 3 hours incubation of the anaerobically grown bacteria with HeLa cells was also held under anaerobic conditions (section 2.14.5) the number of adherent bacteria increased to more than five times that of the same strains grown and incubated under aerobic conditions (Fig. 5.10). HeLa cells have been substituted for by HEp-2 cells in these assays with similar results.

Radiolabelling of aerobic and anaerobic O157:H7 strains prior to their use in the adherence assays was also performed, as described in section (2.26). Adherent bacteria were released by 5% sodium dodecyl sulphate (SDS) and the level of radioactivity determined by scintillation counting. The number of adherent organisms quantitated by this method, gave very close results to the numbers obtained by the plate counting method (anaerobic:aerobic cells adherence ratios).

In each assay, a set of HeLa cell monolayers grown on coverslips were treated with aerobic and anaerobic O157:H7 in the same way as for the quantitative assays, after washing, fixation, and staining as described in section (2.8.1), the coverslips were examined by light microscopy.
Figure 5.9 Effect of anaerobic growth on bacterial adherence.

Growth of VTEC O157:H7 under anaerobic conditions gave an increase in the number of adherent bacteria nearly twice that from aerobically grown cells. Radiolabelling and plate counting methods gave similar results.

Figure 5.10 Effect of anaerobic growth and incubation on adherence.

Growth of bacteria under anaerobic conditions, followed by incubation with HeLa cells under anaerobic conditions increased adherence of anaerobic cells to nearly five times the adherence of aerobic cells. Radiolabelling and plate counting methods gave similar results.
In all experiments, microscopic observations confirmed the quantitative findings.

These results are difficult to explain since the molecular mechanism involved in the adherence of VTEC O157:H7 to epithelial cells is not yet understood and there are no publication pertinent to this phenomenon. However, in one study carried out by Sherman et al. (1991), it was reported that the growth of VTEC O157:H7 strain CL-56 under strict anaerobic conditions, did not alter the number of organisms adherent to HEp-2 cells, (data were not shown and incubation conditions were not reported). However, similar studies on Salmonella typhimurium uptake by epithelial cells demonstrated that anaerobically grown cultures showed on average an association with epithelial cells more than 10 times greater than that shown by aerobically grown cultures (Schiemann and Shope, 1991).

The apparent increase in the binding of O157:H7 to HeLa cells under anaerobic conditions may be associated with changes in the bacterial envelope structures induced by anaerobiosis.

To explore this possibility, the influence of oxygen limitation on cell envelope components that have been implicated as possible adherence factors was investigated.

5.2.5.1 Lipopolysaccharides

LPS was extracted from aerobic and anaerobic cultures of VTEC O157:H7 by proteinase-K digestion of bacterial outer membrane extracts, as described in section (2.22.2), and separated by electrophoresis (SDS-PAGE) (section 2.23). Profiles obtained after silver staining (section
2.24.3) of the gels (Fig. 5.11) revealed no obvious structural differences between the distinctive ladder-like pattern of the two preparations. Therefore, the increase in binding activity of anaerobically grown O157:H7 is less likely to be due to LPS alterations. In general, structural changes in LPS significantly alter the interaction between Gram-negative pathogens and the target host cells. For example, rough mutants of *Salmonella typhimurium* are phagocytosed and killed more efficiently than the wild type by phagocytes (Makela and Stocker, 1984). LPS analysis of other Gram-negative bacteria (*Pasteurella haemolytica*) carried out by Davies *et al.* (1992) demonstrated that the LPS profile of the cells grown anaerobically differed from that from cells grown aerobically. However, in the absence of any obvious structural changes in LPS profiles of VTEC O157:H7, it was concluded that LPS are less likely to be involved in mediating adherence of O157:H7 to epithelial cells, this conclusion supports the findings of adherence studies carried out by Sue Colby (1992) who demonstrated that the binding capacity of two LPS mutants from VTEC O157:H7 to mouse colonic mucus, and to HeLa cells was not altered when compared to the wild-type O157:H7. Furthermore, earlier studies by other investigators, has also shown that LPS of O157:H7 is unlikely to be an attachment factor (Sherman and Soni, 1988).

5.2.5.2 **Fimbriae**

Since fimbriae have been shown to be important in promoting attachment of some VTEC strains (Karch *et al.*, 1987), and the expression of fimbriae is known to be sensitive to growth conditions, it was important to determine whether the increased adherence capacity of anaerobically grown bacteria is
associated with the expression of fimbriae. Thus, VTEC O157:H7 strains grown anaerobically were examined by electron microscopy after negative staining (section 2.9.1). However, fimbriae were not observed on any bacterial strains grown either in liquid or solid media.

Figure 5.11 Lipopolysaccharide of O157:H7 separated by 15% PAGE and visualised with silver staining.

Lane A- control LPS from E.coli O111 (Sigma). Lane B- LPS from VTEC O157:H7 grown aerobically. Lane C- LPS from the same VTEC strain when grown anaerobically. No significant differences between lane B and lane C.
5.2.5.3 Outer Membrane Proteins (OMPs)

The influence of anaerobiosis on cell wall components was investigated further by comparison of the outer membrane extracts from *E. coli* O157:H7 grown aerobically with extracts from the same strains grown under oxygen limited conditions. Protein profiles obtained by linear SDS-PAGE analysis (Fig. 5.12) demonstrated clearly the expression of protein bands of estimated MW 18, 24, and 33 KDa under aerobic growth conditions only, indicating that these proteins were repressed under anaerobic growth conditions. This observation suggests that increased adherence of anaerobically grown O157:H7 may result from the repression of OMPs, rather than from the synthesis of new proteins.

To obtain better separation of the OMPs, these preparations were analysed further by 10%-30% gradient gel electrophoresis as described in section (2.21.3), which gave clearer resolution of bands of higher MW. Figure 5.13 shows a prominent protein band of estimated MW 72 KDa expressed under aerobic conditions, in addition to the bands previously demonstrated in the linear gels. The 18 KDa and 33 KDa bands were also expressed under low pH conditions (section 5.2.3). Furthermore, the 18 KDa protein was expressed in OMPs of O157:H7 in the stationary phase (section 5.2.2).

The association of these two proteins with lower binding activity once again supports the hypothesis that they are not involved in the adherence process or, they could act as anti-adherence factors. The same explanation applies to the 72 KDa protein band which was shown previously (section 5.2.4) to be expressed under iron limitation and correlated with the lower adherence capacity of O157:H7.
Figure 5.12  OMPs of aerobic and anaerobic O157:H7 separated by 15% linear SDS-PAGE.

Lane A- Molecular sizes of the protein standards in kilodaltons.  
Lane B- OMP profiles of aerobically grown VTEC O157:H7. Lane C- OMPs extracted from the same VTEC strain when grown under anaerobic conditions.  
Arrows indicate to the proteins which exhibited variation in expression.
Figure 5.13  OMPs of aerobic and anaerobic O157:H7 separated by 10%-30% gradient gel electrophoresis.

Lane A- OMP profiles of aerobically grown VTEC O157:H7  Lane B- OMPs extracted from the same VTEC strain when grown under anaerobic conditions.  Arrows indicate to the proteins which exhibited variation in expression.  Molecular mass markers in kilodaltons shown on the left.
However, the gradient gels also demonstrated the expression of low MW outer membrane proteins under anaerobic growth conditions, one distinct band of apparent MW of 15 KDa and a diffuse protein band of 10-12 KDa MW.

Since a large number of proteins were observed in each preparation, two-dimensional SDS-PAGE techniques were employed (section 2.2.1.2) to obtain maximal resolution of these proteins. After silver staining (Fig.5.14) several proteins could be recognised as being specific to, or mainly synthesised under, aerobic growth conditions. Some changes in protein profiles were only quantitative, (care was taken to load the same amount of protein on each gel), however, two prominent proteins with an approximate MW of 43 and 92 KDa were present in the aerobic cells only. The 43 KDa protein was also expressed under low pH (2-4) growth conditions, and was associated with lower numbers of adherent organisms (section 5.2.3).

The 15 KDa protein demonstrated in the gradient gel was present in the second dimensional electrophoresis of the anaerobic cell extracts.

These differences in the expression of O157:H7 OMPs, have not been described previously, however, differences have been described for S. typhimurium (Schiemann and Shope, 1991) where anaerobic growth conditions resulted in the repression of one major OMP of estimated MW 24.6 KDa, a second protein of 22.9 KDa, and a smaller protein estimated MW 9.8 KDa appeared to be slightly repressed.

It may be that the true explanation of increased attachment by anaerobically grown E.coli O157:H7 is not simply a consequence of one factor or the other but a combination of repression of anti-adherence proteins and the synthesis of adherence promoting proteins.
Silver stained two-dimensional gel electrophoresis of OMPs from VTEC O157:H7 grown under aerobic (A) and anaerobic (B) conditions. The horizontal axis represents a pH range of 3.5 to 10 and the vertical axis is the molecular mass in kilodaltons. The arrows indicate the most prominent proteins which exhibited variation in expression.
To facilitate comparison/characterisation of the various proteins immune sera were raised in rabbits against OMPs extracted from VTEC O157:H7 grown aerobically and anaerobically as described in section (2.16). The presence of antibodies was confirmed by passive haemagglutination tests (section 2.17). These antibodies were used in two further approaches to investigate the role of anaerobiosis on VTEC adherence to epithelial cells, immunoblotting, and adhesion inhibition assays.

5.2.5.4 Immunoblotting

Two identical Western blots were prepared from aerobic and anaerobic OMP preparations, by SDS-PAGE analysis as before, the antigens being transferred from the unstained gel to nitrocellulose. One of the blots was then reacted with antiserum raised against the aerobic cell OMPs. The other blot was reacted with antiserum against the anaerobic cells OMPs (section 2.27). The results of these blots are shown in figure 5.15. As can be seen by comparing the two blots, both antisera reacted with all the proteins separated by SDS-PAGE from both aerobic and anaerobic cell extracts. This would appear to indicate either cross-reactivity or nonspecific binding. Thus, this approach did not help in identifying the differences in protein expression induced by oxygen limitation. Attempts to raise monospecific polyclonal antibodies to individual proteins were hampered by restrictions on using rabbits at the time (due to infection), however, the alternative use of rats for this purpose was not successful for unknown reasons, thus, these attempts were not considered further.
Western blots of OMPs to nitrocellulose Lanes A- OMPs of aerobically grown VTEC O157:H7. Lanes B- OMPs of the same VTEC strain grown anaerobically. Identical gels were immunoblotted with, I- antiserum to the OMPs of aerobic cells, and II- with antiserum to OMPs of anaerobically grown cells. Both antisera reacted with OMPs expressed under both aerobic and anaerobic conditions. Numbers in the middle indicate molecular mass in kilodaltons.
5.2.5.5 Adhesion inhibition assays

Since rabbit antiserum raised against OMPs of aerobically grown O157:H7 was shown to cause significant inhibition of bacterial adherence to tissue culture cells earlier in this study (section 4.2.2.1), comparison was made with the inhibition induced by antiserum raised against the OMPs of anaerobically grown O157:H7 cells (section 2.18.1).

Two sets of adhesion inhibition assays were performed, each set included three duplicate experiments. In the first set (assay I) HeLa cell monolayers were incubated with aerobically grown bacteria, under aerobic conditions, the first experiment used as a control (without inhibitor), in the second experiment, antiserum to the aerobic cells OMPs was used as an inhibitor and in the third experiment antiserum to the anaerobic cells OMPs was used as inhibitor.

In the second set of experiments (assay II) the same steps were followed, except that the bacterial cells were grown under oxygen limited conditions, and their incubation with HeLa cells was also under anaerobic conditions.

Bacterial binding to HeLa cells was quantified in the culture media alone, and in the presence of the first and second antisera. Figure 5.16 shows once again the higher attachment rate of anaerobically grown bacteria to HeLa cells, comparing the two control experiments there is marked inhibition of adherence by both antisera under both aerobic and anaerobic conditions. Thus, there was no significant difference in the inhibition of attachment by the antiserum to the aerobic OMPs (93.4%) and the antiserum to the anaerobic OMPs (93.2% of the control levels), suggesting that the antisera are probably nonspecific, and that antibodies to the different proteins
identified in the SDS-PAGE analysis were not raised, either because these proteins are poorly immunogenic or that they were not expressed in sufficient quantities to stimulate antibody production in the rabbits.

**Figure 5.16  Adhesion inhibition assays.**

<table>
<thead>
<tr>
<th>assay type</th>
<th>number of adherent bacteria x10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>control</td>
</tr>
<tr>
<td>inhibitor 1</td>
<td>inhibitor 1</td>
</tr>
<tr>
<td>inhibitor 2</td>
<td>inhibitor 2</td>
</tr>
</tbody>
</table>

**Assay I: Aerobic**
- control: 25
- inhibitor 1: 1.5
- inhibitor 2: 1.8

**Assay II: Anaerobic**
- control: 82
- inhibitor 1: 5
- inhibitor 2: 6

*Control: no inhibitor.*  
*Inhibitor 1: antiserum to aerobic cells OMPs*  
*Inhibitor 2: antiserum to anaerobic cells OMPs*

No significant difference in the inhibition of bacterial binding to HeLa cells by the aerobic and anaerobic rabbit antisera was demonstrated.
5.3 Summary and Overview

It appears that bacteria may rely on more than one specific mechanism to achieve colonisation of the intestinal mucosa, or may, in response to a particular environmental condition, synthesise a specific adhesin which mediates bacterial binding to mucosal surfaces. Moreover, bacterial-mucosal interactions are complicated and modified in vivo by other intervening events that are not reproduced in the currently popular in vitro tests for bacterial adhesion. Consequently when a bacterial pathogen multiplies in vivo it can be quite different from the same strain grown in laboratory media. Therefore, it is difficult to correlate bacterial adherence, as measured in vitro, with the presumed pathogenic potential of bacteria that infect the human body.

In the course of these studies quantitative adherence differences have been observed in response to variable cultural and environmental factors. The reasons for these changes are unknown, but clearly these factors have some influence on the expression of the adhesin molecules. However, not much is known about the synthesis and expression of bacterial adhesins and very limited studies have been reported on the influence of variable environmental conditions on bacterial adherence to epithelial surfaces.

Since the cell envelope is the prime structure through which bacteria interact with a potentially changeable environment, and data presented in this study (chapter 4) indicated that outer membrane proteins are possible adherence factors; comparison was made between the SDS-PAGE patterns of outer membrane extracts from VTEC O157:H7 grown under the same
variable conditions applied to the adherence assays. Comparison was based on the most prominent bands expressed. Although there may not be direct evidence from these observations, this study has highlighted the importance of examining the OMP profiles of cells grown under different conditions, and shed some light on the mechanisms of bacterial adherence. Therefore, a better understanding of these mechanisms should help in finding ways by which adherence can be prevented. One example of a possible clinical applications perhaps, by altering the pH of the colonic environment the adhesion of *E. coli* O157:H7 may be prevented. Such an approach has been previously suggested by Taylor-Robinson (1981) for preventing urinary tract infection i.e. changing the pH of the urine.

Another preventive approach may be by controlling the type of food that high-risk people consume as some O157:H7 strains could become host- or food-adapted (Ostroff *et al.*, 1989). If so, an example would be milk-specific strains associated with illness in children. The protective role of breast milk against infantile diarrhoea associated with ShT or VT was studied by Newburg and Ashkenazi (1992), who reported that the glycolipids of milk consist mainly of galactocerebroside, lactosylceramide, Gb3 and Gb4. Gb3 and Gb4 are known to be the natural receptors for the VT family, more significantly Gb3 in human milk is biologically active and capable of binding to ShT. Thus, it could contribute to the protection of breast fed infants to ShT and VTs.

The effects of diet on bacterial flora has been the subject of active investigation since the late 1960s. The hypothesis was that the intestinal bacteria are able to produce carcinogens, and that this process may be modified by diet. Although diet is regarded as one of the most important
factors controlling the intestinal flora in man, direct evidence is very
difficult to obtain and no clear results linking particular bacteria with
particular items of diet have emerged.
The availability of iron can, in some circumstances have an influence on the
clinical outcome of infections, since the abnormal presence of freely
available iron in vivo would be expected to increase the rate of bacterial
multiplication and to tip the balance in favour of the invading pathogen
(Griffiths, 1993). Moreover, in the case of VTEC O157:H7 infection, iron
may increase virulence of these organisms probably by increasing their
binding to the colonic mucosa. Iron restriction of E.coli O157:H7 strains
used in this study induced a reduction in the number of adherent bacteria to
HeLa cells.
Oxygen-limitation significantly affected the final cell density of the three
VTEC strains examined and the binding capacity of these strains to HeLa
cells. Since anaerobiosis is an environmental condition known to resemble
more closely that usually found in host tissues, the high level of adherence
under anaerobic conditions could well give O157:H7 a selective advantage
in an environment such as the colon. Therefore, this observation was
investigated in more detail. Under anaerobic conditions, no fimbrial
expression was apparent and no significant differences occurred in the
expression of LPS. However, significant differences were apparent in the
OMPs profiles and data presented have characterised the expression and
repression of certain proteins in the outer membrane extracts with anaerobic
growth. Such changes may also occur in vivo, therefore, additional studies
are required to assess fully the consequences of these changes upon the cell
interactions with its environment and the host cells.
Despite the foregoing data, not much is known about bacterial adherence mechanisms since this phenomenon consists of complex sequential reactions, and the overall outcome of one reaction depends very much on other reactions occurring before and after. The variations, derived from factors such as laboratory manipulations, source of isolation, phase and condition of growth and number of laboratory passages, may or may not be pertinent in vivo. However, the study of how one phenomenon affects another is very important and can give a clearer insight into the bacterial characteristics associated with virulence and pathogenicity.
CHAPTER 6

HeLa cell receptors for VTEC O157:H7

6.1 Introduction

Ever since bacteria were recognised as causing infectious disease a century ago, investigators have been interested in understanding how they do so. However, the study of bacterial pathogens is complicated by the role of the host. Thus, to comprehend the mechanisms of disease, one must also understand the host, host response, and the resulting set of interactions between pathogen and host (Eisenstein, 1987).

Since the initial cell-cell interaction is a surface phenomenon, investigators have been searching for molecules on the surface of the prokaryotic and eukaryotic cells that might attract and bind each other in a specific way. Unlike the extensive studies on the identification of the adhesins on the surface of pathogenic organisms, limited studies have been reported on identifying the constituents of the receptors on the host epithelial cells.

Epithelial cells cover all surfaces of animals and humans, and therefore, represent the first cells that the colonising organisms encounter. However, the mucosal surfaces of the gastrointestinal, respiratory, and urinary tracts are constantly bathed in mucus and other secretions which are rich in glycoproteins and glycolipids that are analogues to epithelial cell receptors for various bacterial adhesins (Beachey, 1981).

Although it is clear that the initial association involves the interaction of the pathogen with the mucus, the role of mucus receptors in the
infectious process is unclear, similarly, the origin of such receptors and their relation to epithelial brush borders remain to be elucidated (Metcalfe et al., 1991).

Carbohydrate structures acting as receptors for bacterial adhesins have been found on glycolipids as well as on glycoproteins (Lindahl and Carlsted, 1990). However, the biochemical nature of VTEC O157:H7 specific receptors has not been clearly identified.

Binding studies carried out by Sajjan and Forstner (1990) using purified rat intestinal mucin revealed that the mucin-binding sites for type-1 piliated O157:H7 strain CL-49 are located on N-linked oligosaccharide of the 118 KDa link glycopeptide region of the mucin.

The main objective of the studies carried out in this chapter was to characterise the HeLa cell receptors for the non piliated strain of VTEC O157:H7R used in this study. Outer membrane extracts from HeLa cells were found to inhibit bacterial adherence significantly and two low molecular weight proteins from these extracts reacted with the bacterial outer membrane proteins which were shown earlier in this study to be involved in the adherence process (adhesins). Both findings suggest that protein constituents of the HeLa cell outer membranes are acting as receptors for the bacterial adhesins.

6.2 Results and Discussion

6.2.1 Surface morphology of HeLa cells

Scanning electron microscopy showed that the HeLa cells used in this study display numerous surface microvilli. Closer examination of several
samples of HeLa cells after incubation with VTEC O157:H7 for 3 hours (section 2.14.1) indicated that these bacteria entangled with these microvilli (Figure 6.1). These data suggest that the receptor molecules for O157:H7 are located on the surface microfilaments.

Figure 6.1 Scanning electron microscopy of VTEC O157:H7 adherence to HeLa cells.

Magnification
x 2,000.

Magnification
x 5,000.
Studies on the role of microfilaments in HeLa cells following the phenomenon of localised adherence of enteropathogenic *E. coli* (EPEC) indicated that microvilli are the membrane structures responsible for the recognition step and that they participate in the initial binding of the cell to the bacteria (Monteiro da Silva *et al.*, 1989).

### 6.2.2 Effect of HeLa cell outer membranes on adherence

Outer membrane proteins (OMPs) were extracted from confluently growing HeLa cell monolayers as described in section (2.28). The effect of coincubation of *E. coli* O157:H7 with HeLa cell OMPs on the adherence of the bacteria to HeLa cell was examined. Two sets of triplicate quantitative binding assays were performed. Comparable numbers of the same bacterial strain, O157:H7R, were incubated with HeLa cell monolayers under the same test conditions as described in section (2.29). The bacterial cells in the first test were treated with HeLa cell outer membrane extracts for 1 hour at 37°C prior to their use in the adherence assays, while the second binding assay was run in the absence of putative inhibitor (control). After 3 hours incubation at 37°C there was a significant difference between the adherence values for treated bacterial cells and non treated cells. Figure (6.2) demonstrates inhibition in the binding capacity of VTEC O157:H7 treated with HeLa cell OMPs to 46% of the number of untreated cells.

The competitive inhibition induced by HeLa cell outer membrane extracts provided preliminary evidence as to the possible role of these extracts in mediating bacterial adherence to HeLa cells.
Figure 6.2  Role of HeLa cell outer membrane proteins as an inhibitor of bacterial adherence.

Pretreatment of VTEC O157:H7 with purified HeLa cell OMPs caused reduction in the binding of bacteria to 46% of the control assay.

6.2.3 Immunoblotting

To determine whether a specific constituent of the HeLa cell outer membrane extract was capable of acting as a receptor for the O157:H7 adhesins OMPs were separated by gel electrophoresis (Fig. 6.3). The proteins (antigens) were transferred to a nitrocellulose membrane and overlaid with purified VTEC O157:H7 OMPs for 2 hours at room temperature. The membrane was subsequently treated with rabbit antiserum raised against the bacterial OMPs followed by peroxidase conjugated goat anti-rabbit IgG to detect the antigen-antibody complex (section 2.27). The result of this blot (Figure 6.4) showed that the bacterial OMPs (potential adhesins) reacted with two HeLa cell OMPs of estimated
molecular weight 18 to 19 KDa. These proteins are therefore likely candidates for receptor activity.

**Figure 6.3** SDS-PAGE of HeLa cell OMPs

SDS-PAGE analysis of HeLa cell OMPs (13.5%). Protein markers are shown in lane 1.

**Figure 6.4** Western blot analysis

Immunoblot of SDS-PAGE (13.8%) of HeLa cell OMPs with VTEC O157:H7 OMPs. The arrows indicate the position of 18-19 KDa "receptor" proteins.
This is the first direct demonstration of bacterial OMPs binding to the host OMPs. Most bacterial adherence studies have utilised crude mucus or partially purified preparations of mucin for characterisation of specific receptors. Mannose sensitive type 1 fimbriae of *E.coli*, which interact with mannose or mannose like residues on various eukaryotic cells, have been particularly well studied. However, very few studies have been undertaken to characterise the receptors for *E.coli* O157:H7. Purified rat intestinal mucin has been used as a model mucin in binding studies carried out by Sajjan and Forstner (1990 a). They used eight VTEC isolates and it was found that one strain of serotype O157:H7 (CL-49) expressed type 1 (mannose-sensitive) fimbriae and bound avidly to mucin. Since the only component of intestinal mucin that contains mannose is the 118 KDa putative link glycopeptide, subsequent studies provided evidence that the intestinal mucin bears oligomannosyl receptors for type 1 fimbriae and that these receptors are located on N-linked oligosaccharides of the 118 KDa link glycopeptide region of the mucin (Sajjan and Forstner, 1990 b).

Specific receptors for several other adhesins have been identified e.g. a 60 KDa glycoprotein isolated and purified from human saliva was found to interact with type 1 fimbriae and blocked the ability of type 1 fimbriated *E.coli* to attach to human buccal epithelial cells (Babu et al., 1986 b). They demonstrated that the glycoprotein interacts with type 1 fimbriae through its' carbohydrate moiety, however, in earlier studies (Babu et al., 1986 a) it was shown that the same glycoprotein interacted with *Streptococcus mutans* through the protein moiety, thus, the
glycoprotein has the ability to interact with different species of bacteria by different mechanisms.

The binding sites for K99-fimbriae of enterotoxigenic *E. coli* which colonise the small intestine of neonatal pigs, calves, and lambs were identified as mucin glycopeptides in the mucus layer of the pig small intestine (Lindahl and Carlstedt, 1990).

Laux et al., (1986) have shown that the receptors to K88-fimbriae present in mouse small intestinal mucus, are glycoproteins. More recent data presented by Metcalfe et al. (1991) suggested that the receptor for the K88ab fimbrial adhesin present in porcine small intestine mucus is a 40 to 42 KDa glycoprotein. The knowledge of the receptor structures is highly desirable for understanding the pathogenesis of infectious diseases, and is of potential importance in the design of new strategies for prevention of infection aimed at the inhibition of cell adhesion (Rosenstein et al., 1988).

### 6.3 Summary and overview

Unlike the extensive studies on the binding of toxins and hormones to cell membranes, there is relatively little information on the nature of receptors for the *E. coli* adhesins. The receptors for the Gram-negative bacteria are in general composed of carbohydrates (Beachey, 1981). The receptors for type 1 fimbriated *E. coli* appear to contain mannose residues, that is, mannose alone is capable of blocking the binding of these organisms to the host cells (mannose-sensitive) (Ofek et al., 1977). Mannose-resistant adhesins of *E. coli* have received little attention, however, one of the clearly defined receptor structures for a mannose
resistant adhesin is that for the P fimbriae of uropathogenic E. coli which has been shown to be a digalactose component common to the glycosphingolipids of the human P blood group antigens (Leffler and Svanborg Eden, 1980).

The prevalence of mannose-sensitive adhesins among E. coli O157:H7 strains, and the potential role of type 1 fimbriae in the pathogenesis of HC and HUS, remains unknown.

The E. coli O157:H7R strain studied in this chapter was shown to be nonfimbriated, and the addition of mannose did not inhibit bacterial binding to epithelial cells (chapter 4). These findings indicate that mannose is less likely to be involved in the binding process of VTEC O157:H7 to epithelial cell. However, the HeLa cell outer membrane protein bands demonstrated in the Western blot as potential receptors, are most likely glycoproteins. Further characterisation and sequencing of the receptor proteins is essential and will inevitably aid in understanding the pathogenesis of diseases associated with VTEC infection. However, because of the time limiting factor these studies had to stop at this stage.
CHAPTER 7
CHAPTER 7

Overall conclusions

VTEC are a recently recognised group of pathogenic *E.coli*. The pathogenesis of haemorrhagic colitis and haemolytic uremic syndrome, which have been associated with VTEC O157:H7 infection, is still unclear. To investigate the potential virulence attributes of these strains, it was demonstrated that the O157:H7 isolates under study possess two virulence factors, the production of Verotoxin in their culture filtrate and are capable of binding to a wide variety of epithelial cells in tissue culture. A diffuse pattern of adherence was observed with the exception of one strain which demonstrated a localised form of adherence.

The life threatening sequel of infections with O157:H7 make volunteer studies to investigate this human pathogen unlikely, therefore initial efforts in this study were focused on the development of an *in vitro* model utilising human tissues to investigate the virulence factors of VTEC O157:H7. However, human biopsy specimens which were difficult to acquire and gave fluctuating results because of the different donors, they were therefore replaced by a continuous cell line. Nevertheless, the use of native colonic segment in the adherence experiments demonstrated clearly the role of the mucus gel covering the epithelial surface as a barrier to bacterial colonisation. The role of intestinal mucus in protecting against, or facilitating bacterial adherence, is still controversial. HeLa and HEp-2 cell monolayers proved to be the most satisfactory cell lines for the development of quantitative, comparative adherence assays.
To study the optimal conditions for adherence, and to obtain data from an *in vitro* experiments which have more *in vivo* significance, adherence assays were performed under variable physiological conditions - growth media, growth phase, pH, iron-restriction and oxygen-limitation. *E. coli* O157:H7 in the exponential growth phase were shown to be more adherent to HeLa cells than cells in the stationary phase, and considerable differences in the number of adherent cells were observed when bacteria were grown in different culture media prior to their use in the binding assays. The same strains of O157:H7 were shown to survive in media of pH 2-3 (human stomach pH), but maximal adherence ability was expressed at pH 6-7 (human large intestine pH).

Both iron and oxygen limitation induced a reduction in the final cell density. Low-iron medium was shown to reduce the number of bacteria attaching to HeLa cells, while anaerobiosis induced a marked increase in the binding capacity of VTEC O157:H7 to HeLa cells, which appeared to increase further (approximately five times) when anaerobically grown bacteria were incubated with HeLa cells under anaerobic conditions.

These observations might be important *in vivo* where levels of free iron are very limited and activities of the intestinal microflora makes the large intestine environment strictly anaerobic.

It was concluded from these findings that bacteria may, in response to a particular environmental condition, synthesise a specific adhesin which mediates bacterial binding to mucosal surfaces. When more specific knowledge has accumulated concerning the physiological and biochemical mechanisms of bacterial adherence, it may become possible to achieve simple prophylactic methods against infection with VTEC, for example the
possible addition to diet of an agent which inhibits the \textit{in vivo} synthesis of adhesins, or it may be possible to enrich food with antiadhesive substances.

In order to probe the biochemical nature of bacterial cell surface components which have been implicated as colonisation factors (fimbriae, flagella, LPS and outer membranes), an ultrastructural approach using negative staining and immunogold labelling techniques of O157:H7 allowed the identification of H-7 flagella, O-157 antigen of the LPS and the outer membrane proteins (OMPs). However, an exhaustive search with the electron microscopy did not reveal any fimbriation, in addition, VTEC strains failed to agglutinate RBCs from different sources indicating that these strains are nonfimbriated. It was therefore, concluded that these strains express adherence potential through nonfimbrial adhesins.

The second approach to characterise the adhesins was through the use of competitive inhibitors of bacterial adherence which included, purified LPS, OMPs and antibodies specific to the H-7 flagella, O-157 antigen and OMPs.

The highest degree of inhibition was obtained with the purified OMPs and antibodies to them. Purified LPS and the antibodies specific to the O-157 antigen induced less significant inhibition of adherence, while the H-7 flagella antibodies were the least effective in blocking the binding of VTEC O157:H7 to HeLa cells. From these findings, it appeared that OMPs are the likely candidates to be involved in mediating bacterial adherence. Trypsin treatment and SDS-PAGE analysis of outer membrane extracts indicated that protein constituents of the bacterial outer membranes are the potential colonisation factor(s). Animal colonisation factors have been used in
vaccine preparation and it has been suggested that human colonisation factors could also be used in a similar fashion.
Protein profiles of outer membranes extracted from the same O157:H7 strain grown under different culture conditions demonstrated the expression and repression of certain protein bands when bacteria were harvested at different phases of growth, variable pH values, low iron concentrations and when grown under anaerobic conditions. These findings could explain the observed quantitative differences in the adherence capacity of E.coli O157:H7 to HeLa cells under variable physiological conditions (chapter 5).

To characterise the host cell receptors, HeLa cell membranes were studied. Bacterial cells were shown by scanning electron microscopy to attach to microfilaments on the HeLa cell surface which are thought to carry the specific receptors for the bacterial adhesins.
Purified HeLa cell outer membranes were found to induce considerable inhibition of bacterial adherence (46% of the control test). Immunoblotting of the HeLa cell OMPs with the bacterial OMPs revealed two protein bands on the HeLa cell surface that are potential receptors for the O157:H7 adhesins. Further characterisation of these proteins will inevitably aid in understanding the pathogenesis of VTEC infections, however this was not possible in the context of this study due to the time constraints.

Future studies on the regulation of the adhesins and receptors at the genetic and environmental levels, should provide a practical outcome, of new strategies for the prevention of infection aimed at inhibition of bacterial adherence to the host tissues.
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