STUDIES ON THE ADHESIVE FACTORS OF PATHOGENIC STRAINS OF *Escherichia coli* ISOLATED FROM MAN.

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by

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ABSTRACT

The present study was designed to answer a number of questions related to the presence and function of known or suspected adhesive factors in strains of E.coli.

A total of 808 E.coli strains isolated from intestinal and extraintestinal sources in man were tested for the ability to agglutinate human, calf and guinea-pig RBC in the presence and absence of D-mannose, and for the presence of CFA/I and CFA/II by slide agglutination and immunodiffusion techniques using specific antisera.

The biochemical and serological characteristics of CFA/I and CFA/II positive strains were examined, and related to the genetic control of these adhesive factors. The morphological characteristics of these fimbriate antigens was investigated by electron microscopy studies.

Strains that did not possess CFA/I or CFA/II, but were able to cause MRHA of human and/or calf RBC, were further examined by serological methods for the presence of other adhesive factors.

An in vitro assay using HEp-2 tissue culture cells was developed to test the adhesive ability of strains of E.coli able and unable to cause MRHA or MSHA.

The relationship between the biochemical and genetic characteristics of the E.coli strains and their ability to adhere to HEp-2 cells was also investigated.

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3. Smith, H. R., Cravioto, A., Willshaw, G. A., McConnell, M. M., Scotland, S. M., Gross, R. J., Rowe, B.: A plasmid coding for the production of colonisation factor antigen I and heat-stable enterotoxin in strains of Escherichia coli of serogroup O78.

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I. INTRODUCTION

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Ia. Importance of infectious diarrhoea

Diarrhoeal disease in infants and adults is an illness characterized by four or more abnormal bowel movements within 24 hours. A liquid or semi-liquid consistency of the stools or the presence of blood and mucus are criteria that independently or jointly characterize them as abnormal.

Although infectious agents are still the major cause of diarrhoea, congenital or acquired enzymatic and metabolic defects (Holmberg, Perheentupa and Pasternack, 1977; Dodge, Hamdi and Walker, 1977) and hormone secreting tumours (Rambaud and Matuchansky, 1974) can be a frequent cause of the disease. For this reason the basic treatment of diarrhoea is symptomatic. Fast oral or parenteral replacement of water and electrolytes lost through vomit and stools will generally be sufficient to limit the disease. Infectious diarrhoea lasts usually three to five days and the search for a specific causative agent has low therapeutic value but important epidemiological significance.

If adequate and opportune treatment was offered to every patient with diarrhoea, the mortality rates due to the disease would be extremely low. Unfortunately, the high incidence of diarrhoea in developing countries puts a great strain on the resources of these countries and makes treatment inaccessible to large sections of the population. Diarrhoea and its complications are therefore the most frequent causes of death in children under five years of age throughout the Third World (Puffer and Serrano,

1973).

Prospective studies on the incidence and causes of diarrhoea have been carried out in several parts of the world (Cramblett, Azimi and Haynes, 1971; Mata and Urrutia, 1971; Black et al., 1980). From the figures obtained in these studies one can calculate a world average of around 2000 million cases of diarrhoea among small children of Asia, Africa and Latin America each year (Puffer and Serrano, 1973). According to figures of the Pan American Health Organisation (PAHO) approximately 147,000 people die annually in Latin America alone because of diarrhoea and its more frequent complication, third degree oedematous malnutrition, commonly called Kwashiorkor (PAHO Publ., 1974), but perhaps more important than the mortality figures are the long term effects caused by diarrhoea and severe malnutrition on the physical growth and mental development of children surviving from these diseases (Cravioto et al., 1966; Scrimshaw, Taylor and Gordon, 1968).

Experience in the developed industrial world has shown that reduction in the incidence of infectious diseases is directly related to improvement in environmental sanitation. This has led developing nations to deploy large quantities of their health resources to supply their urban and rural communities with adequate water supplies and waste disposal units (Feecham et al., 1978). It is hoped that these long term projects will eventually have a positive effect on the general welfare of the population. Recent evidence, however, offers little optimism in the short term prospects. This has encouraged scientific groups throughout the world to look for more immediate ways of

controlling diarrhoeal infectious diseases. Two general lines of research have been followed for this purpose; studies on the pathogenic mechanisms of the causative agents, and studies on how to increase the resistance of the host to infection through the use of vaccines.

The cholera pandemics and the study of organisms causing diarrhoea in travellers to developing or tropical countries have also stimulated the interest of many research groups in recent years. This latter subject has greater economical repercussions than public health importance, when compared with diarrhoeal morbidity and mortality figures in small children of developing countries. However, since both the traveller and the local population might be infected by the same organisms, any insight into the pathogenic mechanisms and control of these agents could be beneficial for both groups.

Infectious diarrhoea in travellers and communities throughout the world can be caused by a wide variety of organisms. Viruses, bacteria, protozoa, fungi and parasites have been incriminated as aetiological agents of the disease (Steinhoff, 1980; Keller et al., 1977; Krogstad, Spencer and Healy, 1978; Stevens and Roberts-Thomson, 1978). With a growing list of possible intestinal pathogens, the number of cases of diarrhoea in which an associated agent is not isolated is becoming increasingly smaller. In most studies, however, there is always a number of cases in which no specific pathogen can be found. This, of course, depends on the thoroughness of the investigations being carried out, on the awareness of the researchers of the emergence of new pathogens, and the recognition of new pathogenic

mechanisms in known agents. Comprehensive studies on the aetiology of diarrhoeal disease are useful to both epidemiologists and health workers. To the former they indicate the areas in which preventive measures might have a greater impact, while to the latter they provide correlation with clinical symptomatology and a more adequate approach to individual treatment. Nevertheless, epidemiological studies are not sufficient to prove an association between isolation of specific microbes and diarrhoeal disease. Since the intestinal tract of all animal species is colonised with a great variety of microorganisms, putative pathogenic agents must be shown to possess special mechanisms which allow them to survive in adverse environments, enabling them to cause disease in the host. In the last four decades the development of new laboratory techniques, the use of animal models and human volunteer studies, have contributed greatly to the differentiation between normal and abnormal gut flora. The knowledge obtained from these studies permits the deployment of efforts and resources towards the control of this latter group.

Ib. Intestinal colonisation studies

Although most studies on the aetiology of diarrhoea are not comprehensive enough to identify an infectious agent in every case, certain organisms are isolated with consistent high frequency. One of these organisms is Escherichia coli (E.coli). A normal inhabitant of the intestinal tract, E.coli has also been associated with a variety of pathological conditions in man and animals (Rowe, 1979).

Colonisation with E.coli starts shortly after birth (Gordon and Pesti, 1971). As is well known, the gastrointestinal tract is sterile in the normal foetus up to the time of delivery (Drasar and Hill, 1974). During normal birth the foetus picks up microbes from the vaginal canal and the external genitalia of the mother, as well as from other environmental sources to which it is exposed (Savage, 1977). Many of these organisms are not able to colonise the intestinal mucosa and are excreted in the meconium. Other microbes are able to reproduce in the gut lumen and colonise parts of the gastro-intestinal (GI) tract. The type of organisms forming this initial faecal flora depends greatly on the characteristics of the host and its type of diet. The bacterial flora in the first months of life can change rapidly until all possible habitats in the GI tract are colonised by permanent communities. The sequence of colonisation is dependent on the species and the age of the animals studied.

The maintenance of a balance in the GI ecosystem depends largely on the interaction between the host, its diet and environment, and the various microbial types. Given stability

in the animal's health, diet and environment, only ageing should influence the composition of the biota. A dramatic change in any of these three factors will bring about an imbalance in the composition of the microbial flora and the possibility of colonisation of certain areas of the intestine by autochthonous microbes that have left their ecological niche or by allochthonous transient organisms, both of which may be capable of producing disease.

Ic. *E.coli* as an intestinal pathogen

E.coli is a facultative anaerobic Gram negative rod that grows rapidly at temperatures between 20 to 40 C in common bacteriological media such as MacConkey agar or blood-agar. Most strains have the ability to ferment glucose, lactose and other sugars to produce carbon dioxide and hydrogen in equal quantities and to produce catalase (Joblik and Smith, 1972).

The serologic structure of *E.coli* is usually defined by three different antigenic classes:

1) A somatic 'O' antigen that is resistant to inactivation by temperatures of 100 C.

2) A capsular 'K' antigen that masks the agglutinability of the 'O' antigen and can be inactivated by temperatures of 100 to 121 C.

3) The flagellar 'H' antigen present only in motile strains and also inactivated by temperatures of 100 C.

The WHO International Centre for Escherichia in Copenhagen recognizes at present 164 'O' groups, 100 'K' and 56 'H' antigens (Orskov *et al.*, 1977).

The ability of *E.coli* to cause cholera-like diarrhoea was recognised by clinicians in the latter part of the 19th century. Severe watery-diarrhoea in infants and children was called 'cholera infantum', although it could be distinguished from classical Asiatic cholera by clinical symptoms and epidemiology. Mortality rates in children during outbreaks of the disease in the warm season of the year could be similar to those for cholera.

Epidemiological studies from different parts of the world had shown a definite association between the isolation of certain types of E.coli and epidemics of diarrhoea in children. Bray (1945) in Britain, and Varela, Aguirre and Carillo (1946) in Mexico isolated a strain of E.coli from children with diarrhoea. The British called it Bacterium coli var neapolitanum, a variety of E.coli first isolated from patients with diarrhoea in Naples. The Mexicans showed that the strain had an 'O' antigen cross-reacting with surface antigens of Salmonella adelaide, and called it E.coli gomez.

General acceptance of E.coli as a major cause of human diarrhoeal disease came in 1947 when Kauffmann published a serotyping scheme for these strains. Giles, Sangster and Smith (1949) studied an epidemic of gastroenteritis in children of Aberdeen, Scotland. The only pathogens isolated in this study were two strains of E.coli, which they called Aberdeen alpha and Aberdeen beta. Two years later, Taylor, Powell and Wright (1949) described other outbreaks of diarrhoea caused by a strain of E.coli called D433, similar to the one described by Giles' group. Kauffmann and DuPont (1950) provided further evidence on the relationship between E.coli and diarrhoea by the study of new outbreaks, but more important perhaps, as described above, by being able to show that the E.coli strains which had been isolated in different parts of the world belonged to only two serotypes. Bacterium coli var neapolitanum, E.coli gomez, Aberdeen alpha and Taylor's D433 were all serotype O111:K58 (Kauffmann and DuPont, 1950; Olarte and Varela, 1952) while

Aberdeen beta and similar strains isolated afterwards in other parts of the world belonged to serogroup 055 (Smith, 1953; Buttiaux et al., 1956; Ewing, Tatum and Davis, 1957; Lie Kian Jal et al., 1960).

In the next 15 years the strains of E.coli associated with infant diarrhoea were found in O groups 26, 44, 55, 86, 111, 114, 119, 125, 126, 127, 128, 142 (Taylor, 1961; Rowe, 1979). These strains became known as Enteropathogenic E.coli (EPEC). The development of commercially prepared poly- and monovalent antisera from these serotypes has permitted routine laboratories everywhere to detect these EPEC serotypes. In a few centres, however, E.coli has been studied with a complete serotyping scheme using not only 'O' antisera but also 'K' and 'H' determinations. The results obtained by these laboratories showed that EPEC strains belonged to just a few O:H combinations (Rowe, 1979).

For many years veterinarians had known of the association between scours in calves and piglets and isolation of E.coli. With the Kauffman (1947) serotyping scheme available, researchers were able to describe the E.coli serogroups most commonly associated with diarrhoea in the different animal species. Bokhari and Ørskov (1952) serotyped a large quantity of E.coli strains isolated in the 1940s by Wramby in Sweden. They found that scours in calves was caused by a few E.coli O-groups, the most common being 078, followed by 015, 08, 09, 045 and 026. Studies in other European countries during the 1950s and 1960s confirmed these results (Rees, 1958a; Dam, 1960).

In pigs, isolation of E.coli was associated with oedema

disease and swine enteritis. Ewing, Tatum and Davis (1958) found that most strains of E.coli causing oedema disease in the United States and Ireland belonged to O-groups 138 and 139. Sojka, Lloyd and Sweeney (1960) studying strains isolated in England confirmed these results, and reported that swine enteritis was not associated with these O-types but with those of groups 08, 0138 and 0141. Outbreaks of lamb scours in Australia and England showed isolation of E.coli 024 and 078 (Roberts, 1958; Rees, 1958b). Most of the strains isolated in these studies did not belong to E.coli serotypes commonly associated with human diarrhoea.

Research was then directed towards animals that had greater contact with humans, such as house pets. Studies in healthy cats and dogs showed isolation of human E.coli serotypes in 12 to 15% but transmission to humans was never proven (Mian, 1959; Mackel et al., 1960). More recently several groups have studied transmission of E.coli to humans through the handling of infected animal carcasses. Only one out of 14 healthy handlers of chicken carcasses infected with an antibiotic-resistant E.coli was shown to be excreting the same serotype of E.coli as the one found in the infected chicken carcasses (Linton et al., 1977).

These results do not support the classification of diarrhoea caused by E.coli in humans as a zoonosis. Epidemiological evidence from outbreaks and the high inoculum of E.coli needed to cause diarrhoea in human volunteers (10^8 - 10^{10} bacteria/ml) point to transmission from man to man through contaminated food and water (DuPont et al., 1971; Levine et al., 1977). Poor community sanitary conditions permit a high degree of faecal

contamination that, coupled with lack of adequate water supplies, increases the risk of bacterial proliferation in food and kitchen utensils. Ingestion of food prepared in these conditions is the main cause of diarrhoea in non-immune individuals (Ewing, 1962; Rowe, 1974; Feecham et al., 1978).

Controversy on the role of EPEC strains in diarrhoea started with their discovery and has continued until the present time. Since the initial studies on diarrhoea caused by EPEC were based on retrospective evidence, researchers in the 1950s carried out prospective case-control studies to obtain more sound epidemiological information. Results obtained from these studies were far from conclusive. From a well controlled study of infants under one year of age Ørskov, Ørskov and Paerrlgaard (1956) concluded that the same serotypes occurred with equal frequency in healthy and sick babies. Gamble and Rowson (1957) isolated similar EPEC serotypes from symptomatic and asymptomatic children during the same period of time. The carrier state of EPEC strains in healthy individuals varied from 4.7 to 50% in the different studies. However, since these investigations were carried out in hospitalized patients suffering from sporadic diarrhoea, they did not invalidate the findings from the earlier studies.

Initial epidemiological observations were complemented by studies on the ability of EPEC strains isolated from children with and without diarrhoea to cause diarrhoea in human adult volunteers. EPEC strains isolated from infants with diarrhoea were fed to healthy adult volunteers by Kirby, Hall and Coackly (1950), by Neter and Shumway (1950), and by June, Ferguson and

Warfel (1953). Diarrhoea was induced in the majority of volunteers fed EPEC strains isolated from infants with diarrhoea. EPEC strains isolated from healthy infants had no effect. Koya, Kosakai and Fukasawa (1954) showed that only adult volunteers with duodenal isolation of the challenge strain developed diarrhoea, indicating that only strains able to colonise the upper intestine were pathogenic.

Epidemics of diarrhoea caused by EPEC strains were further reported in Europe and North America in the 1960s and 1970s. Most of these outbreaks were in nurseries and although adults were found to be excreting the epidemic strain, only the infants developed diarrhoea (Love et al., 1972; Kennedy et al., 1973; Hone et al., 1973). Outbreaks of EPEC diarrhoea in adults are rare, probably due to protective immunity developed by early contact in life with these strains. Common source outbreaks due to contaminated food or water sources have been the cause of adult EPEC diarrhoea in recent years (Schroeder et al., 1968; Vernon, 1969).

The E.coli serotyping scheme developed by Kauffmann was also useful to investigate the role of some E.coli strains causing "dysentery-like" disease. Ewing and Gravatti (1947) studied cases of dysentery in American troops in the Mediterranean in which, instead of a shigella strain, E.coli 0124 was the only pathogen isolated. Dysentery is a colonic infection manifested by frequent bowel movements of semiliquid stools with mucous and blood. Hobbs, Thomas and Taylor (1949) investigated an outbreak of dysentery-like diarrhoea caused by an E.coli 0124. This same strain was isolated in a multi-state

epidemic of dysentery in the United States due to contaminated French cheese (Marier et al., 1973). Lanyi et al. (1959) have also reported a large dysentery outbreak in Hungary due to water supplies contaminated with E.coli 0124.

Outbreaks and sporadic cases of dysentery-diarrhoea caused by E.coli 0164 have been reported in Australia, Britain and Israel (Riley, 1968; Rowe, Gross and Woodroof, 1977; Shmilovitz, Kretzer and Levy, 1974).

E.coli O-groups 28ac, 112ac, 136, 143, 144 and 152 have also been associated with sporadic cases of dysentery-like disease (Rowe, 1979).

Hobbs, Thomas and Taylor (1949) were able to cause dysentery-like disease by feeding E.coli 0124 to human adult volunteers. The pathogenicity of these strains was further confirmed by demonstrating epithelial invasion of the large intestine in a guinea-pig model by the same E.coli strains that were able to cause dysentery-like disease in human adult volunteers (DuPont et al., 1971). These groups of shigella-like E.coli were therefore called enteroinvasive E.coli (EIEC).

Diarrhoea in travellers from developed countries to tropical or developing countries has been known for a long time and given humorous titles such as "Montezuma's revenge" or "turista". Rowe, Taylor and Bettelheim (1970) reported that approximately 50% of British soldiers stationed in Sharjah who developed diarrhoea showed isolation of an E.coli of serotype 0148:H28. DuPont et al. (1971) found this same serotype of E.coli in American soldiers fighting in Vietnam who developed diarrhoea.

DuPont et al. (1971) showed that two strains of E.coli isolated from American soldiers in Vietnam were able to dilate rabbit intestinal loops without invading the mucosa. These strains belonged to serotypes O6:H16 (B2C) and O148:H28 (B7A). These strains produced diarrhoea in human volunteers when challenged orally with 10^8 and 10^{10} bacteria in 45 ml of milk. The lower dose produced only mild diarrhoea in half of the volunteers, while the higher one produced severe diarrhoea in all of them. A four-fold or greater antibody rise to the 'O' antigen of the challenge strain was found in 50% of those given 10^8 bacteria and 80% of those given 10^{10} bacteria. Non-pathogenic E.coli used as controls were not able to dilate rabbit gut loops or cause diarrhoea in volunteers even with doses of 10^{10} bacteria. Other human volunteer studies using ETEC strains have confirmed the pathogenicity of these organisms (Levine et al., 1977; Satterwhite et al., 1978).

The best controlled study of TD was carried out by Merson et al. (1976) in a group of physicians and their families journeying to a congress in Mexico City. This study showed that the single most common pathogen isolated from TD cases was E.coli. Other studies in Brazil, Central America, Kenya and Cyprus have given similar results (Shore et al., 1974; Sack et al., 1977; Bäck et al., 1978).

Most E.coli strains isolated from cases of travellers' diarrhoea (TD) have been found to produce enterotoxins. These enterotoxin producing organisms cause secretion of water and ions in the upper small intestine, resulting in a cholera-like watery diarrhoea.

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Isolation of enterotoxigenic E.coli (ETEC) has also been reported in common source outbreaks involving children and adults in developed countries (Gorbach and Khurana, 1972; Kudoh et al., 1977; Ryder et al., 1976; Rosenberg et al., 1977), and in longitudinal community studies in developing countries (Nalin et al., 1975; Sack et al., 1977; Merson et al., 1980).

It is generally accepted nowadays that there are three distinct groups of E.coli strains: enteropathogenic E.coli (EPEC), enterotoxigenic E.coli (ETEC) and enteroinvasive E.coli (EIEC). Each one of these groups is able to cause intestinal disease by a different pathogenic mechanism, and is associated with a restricted number of E.coli serotypes (Table 1).

Id. Enteropathogenic E.coli (EPEC)

In 1915 Violle and Crendiropoulo described a test in ligated rabbit loops to test the pathogenicity of Vibrio cholerae (V.cholerae). De and Chatterjee utilized this technique in 1953 to test the ability of strains of E.coli to cause active intestinal secretion and histopathological changes in the rabbit intestinal mucosa. Results with this test showed that only E.coli strains isolated from children with diarrhoea caused dilation of the ligated loops due to a mucopurulent exudate. Strains belonging to EPEC serotypes but isolated from healthy infants did not dilate intestinal loops. Taylor, Maltby and Payne (1958) obtained similar results with E.coli strains isolated from English babies with and without diarrhoea. The number of organisms found in positive and negative loops were the same, indicating that the results obtained were not related to failure of the strains from babies without diarrhoea to multiply after injection into the loops. The conclusion of all these studies was that E.coli strains from patients with diarrhoea produced a toxic substance able to cause intestinal secretion and a mucopurulent exudate. The presence of necrosis and ulcerative enteritis in loops a few hours after inoculation gave support to the hypothesis. Purification and characterisation of the toxic substance produced by these pathogenic strains of E.coli in gut loops developed slowly over the next 25 years.

Strains of E.coli causing scours in animals gave inconsistent results when tested in ligated rabbit gut loops. While some strains gave positive results others, although pathogenic in the original host and able to multiply in the test

system, were consistently negative (Taylor, Wilkins and Payne, 1961). However, these strains were able to cause fluid secretion in intestinal segments of the original host. Thus, for example, strains of E.coli isolated in pigs with diarrhoea could be negative in rabbit gut loops but positive in pig gut loops. This finding suggested a high but not complete host specificity. Many E.coli strains isolated from humans, as already stated, were positive in rabbit gut loops. The use of live cultures in most of these studies left open the possibility that the fluid accumulation was due to invasion of the mucosa and production of toxaemia.

In 1971 Smith and Linggood reported for the first time the dilation of rabbit gut loops using cell-free lysates from an E.coli strain isolated from an infant with diarrhoea. This strain of E.coli, called H19, belonged to serotype O26:K60:H11. The ability of this strain to dilate gut loops could be transferred to an E.coli K12 laboratory strain. This characteristic was thus thought to be plasmid-mediated. The toxic substance produced by H19 was heat-labile and the plasmid controlling it was called Ent$^+$ for enterotoxin production. The transmissible character of other Ent$^+$ plasmids in E.coli isolated from pigs with diarrhoea had been reported earlier by Smith and Halls (1968).

The Ent$^+$ character in H19 was thought to be similar to cholera toxin because of the positive rabbit gut loop results obtained. This prompted other research groups to test EPEC strains in assays originally developed for cholera toxin (CT). These assays, as will be described in detail later, were based on the ability to activate adenylate cyclase by CT. EPEC strains

however, were found to give negative results in these tests, suggesting that they were either non-pathogenic or that they produced a "toxin" with a different mechanism of action to that of CT (DuPont et al., 1971; Gross, Scotland, Rowe, 1976). These results rekindled the controversy on the role of EPEC strains in infant diarrhoea.

Studies carried out in the 1940s and 1950s had to be repeated with recently isolated EPEC strains from well characterized nursery outbreaks of diarrhoea in the United Kingdom to confirm that they were pathogenic. Gross, Scotland and Rowe (1976) demonstrated that recently isolated EPEC strains from nursery outbreaks in the U.K. did not produce enterotoxins as detected by tests based on the increase of cAMP or cGMP. However, these EPEC strains were capable of producing intestinal secretion in an in vivo perfusion method using rat jejunum, developed by Klipstein et al. (1978), and to produce diarrhoea in healthy adult volunteers (Levine et al., 1978). These studies confirmed that EPEC strains were capable of causing diarrhoeal disease in humans through mechanisms that have just started to be characterised. The search for and study of these organisms is justified by their high rate of isolation in infants with diarrhoea in all parts of the world.

Ie. Enterotoxigenic E.coli (ETEC)

Although it was thought at first that enterotoxigenicity was unique to cholera, later studies have shown that other pathogens causing similar diarrhoea also produce intestinal exotoxins. Craig (1971) proposed the term 'enterotoxic enteropathies' for those acute diarrhoeal diseases of man or animals in which fluid losses into the gut are caused by an exotoxin, called enterotoxin, elaborated by the causative microbe; the microorganisms are able to multiply in the gut lumen and attach to the epithelial cells without invading them or spreading to other tissues.

On the basis of this functional classification only three infections fulfil the criteria of enterotoxic enteropathies: Clostridium perfringens type A food poisoning, diarrhoea associated with enterotoxigenic producing E.coli, and cholera (Craig, 1971). More recently, however, exotoxin production in vitro has been reported by strains of shigellae, Vibrio parahaemolyticus, non-typhoid salmonellae, Bacillus cereus and food poisoning staphylococcus (Keush and Jacewicz, 1977; Ketyi et al., 1979; Hornick, 1979). The pathogenic role of these "toxins" in diarrhoeal disease remains to be clarified.

The demonstration by De (1959) that cell-free culture filtrates of cholera vibrios caused fluid accumulation in ligated segments of rabbit ileum opened up a whole new field in diarrhoeal disease research. This cholera enterotoxin (CT) was antigenic and inactivated by heating at 60 C for 30 min. Antiserum against CT prepared in rabbits was able to neutralize the gut-loop reaction produced by the enterotoxin and prevent

diarrhoea in rabbits infected with virulent cholera strains. To test whether the enterotoxin found in culture filtrates was the same factor causing cholera in man, Craig (1965) tested stool filtrates from bacteriologically confirmed cases of cholera using a vascular permeability test in rabbit skin. With this test, he found that only filtrates from stools obtained in the first days of the disease caused an increase in vascular skin permeability in rabbits. Stools obtained later did not. Serum obtained from individuals with and without cholera diarrhoea was able to neutralize these effects. The stool filtrates used by Craig showed similar characteristics to those obtained in cultures of V.cholerae by De et al. (1956), thus confirming that CT produced in vitro was similar to that causing disease in vivo.

Once it was accepted that V.cholerae produced an enterotoxin which gave reproducible results in several tests, researchers became interested in its mechanism of action. The first step, however, was to obtain a purified preparation that maintained its specific activity in rabbit gut-loops and skin permeability tests. Finkelstein and Lo Spalluto (1969) reported the purification and characterization of CT. Cholera toxin is a simple protein without appreciable contents of lipid or carbohydrate.

The cholera toxin has a molecular weight of 82,000 to 84,000, as determined by ultracentrifugation. The molecule consists of two subunits called A and B. The two subunits readily dissociate using sodium dodecyl sulphate (SDS) gel electrophoresis (Lönnroth and Holmgren, 1973). The A subunit has an approximate molecular weight of 28,000 and reduction and

carbomethylation with thiols cleave the subunit into two peptides (A_1 and A_2). The B subunit has a composite molecular weight of approximately 56,000. Heating in SDS dissociates this subunit into six separate units of approximately 9,000 molecular weight. The complete CT molecule is composed of six B subunits which form the attachment region of the toxin to its cell receptor, and one A subunit which forms the active toxigenic portion. The intestinal receptor for CT is the ganglioside GM_1 (van Heyningen et al., 1971; Holmgren, 1973). Once bound to the epithelial cell the A_1 fraction of the A subunit activates the adenylate cyclase system increasing the intracellular level of cAMP and causing hypersecretion of chloride and bicarbonate (Field, 1979). For this purpose the cholera toxin requires oxidized nicotinamide adenine nucleotide (NAD^+), a generating system for guanosine triphosphate (GTP) and a 42,000 dalton cytosol protein (Gill and Richardson, 1980). Using unpurified CT preparations, Greenough, Pierce and Vaughn (1970) had shown that the enterotoxin activated fat cell lipase and Schafer et al. (1970) demonstrated an increase in cAMP levels in the rabbit intestinal mucosa after treatment with CT.

The discovery that CT activated adenylate cyclase was used to develop new in vitro assays for its detection, less cumbersome and expensive than rabbit gut loops. Donta, King and Sloper (1973) observed that CT stimulated steroidogenesis and altered the morphology of a mouse adrenal tumor (Y1) cell line. Guerrant et al. (1974) showed that CT stimulated the production of cAMP and caused elongation of Chinese hamster

ovary (CHO) cells. Bourne, Coffino and Tomkins (1975) reported that CT could be detected by its cytotoxic effect on a lymphosarcoma cell line.

These tissue culture tests have also been used to detect other enterotoxins produced by strains of salmonella (Peterson and Sandefur, 1979), shigella (Keusch and Donta, 1975), Vibrio parahaemolyticus (Honda, et al., 1976) and others. These assays, however, have been mostly used to detect and study the enterotoxins produced by E.coli.

Ie. 1. *E.coli* heat-labile (LT) enterotoxin

Strains of *E.coli* isolated from patients with severe watery-diarrhoea, in which *V.cholerae* could not be isolated, were shown by De, Bhattacharya and Sarkar (1956) to produce dilation when injected live into rabbit gut loops. Taylor, Wilkins and Payne (1961) later reported that strains not belonging to EPEC serotypes but isolated from patients with diarrhoea were able to cause secretion in rabbit gut loops. Smith and Halls (1967) clarified these previous investigations by showing that the 'toxigenic' factor produced by *E.coli* isolated from pigs with diarrhoea was present in the cell-free culture supernatant. DuPont *et al.* (1971) demonstrated that this heat-labile (LT) enterotoxin produced by ETEC strains isolated from humans with 'cholera-like' disease was similar biochemically and antigenically to cholera toxin.

Before *E.coli* LT was purified, its biological activity was studied by using cell-free culture filtrates from enterotoxigenic *E.coli* (ETEC) strains (Nalin, Battacharjee and Richardson, 1974; Sack, 1975). These filtrates were shown to cause fluid accumulation in ligated rabbit intestinal segments, to be antigenic, non-dialyzable, precipitated with 40% ammonium sulphate, and inactivated by heating at 60 C for 30 min (Gyles, 1971; Sack, 1975). Antiserum prepared in rabbits against CT had a partial neutralizing effect on LT, while rabbit anti-LT antiserum had no neutralizing effect on CT (Smith and Gyles, 1970; Pierce, 1973).

When *E.coli* LT was also shown to activate adenylate cyclase and to increase cAMP levels by Kantor, Tao and Gorbach (1974)

using rabbit intestinal mucosa, and by Evans et al. (1972) and Donta and Smith (1974) using the Y1 adrenal cell assay, its detection was greatly enhanced by the use of tissue culture assays developed for CT. Appart from Y1 adrenal cells, Chinese hamster ovary cells (CHO) and African green monkey kidney cells (VERO) were found to show a cytotonic response to LT (Konowalchuk and Speirs, 1979). Of these three cell lines CHO cells were found to be the most sensitive.

Knowledge on the antigenicity of LT and its immunological cross-reactivity with CT have been used to develop easier and more accessible assays than the tissue culture tests for the detection of these enterotoxins. They include the lysis inhibition test (LIT) (Evans and Evans, 1977) which uses the inhibition of haemolysis of LT-sensitized sheep red blood cells (RBC) by LT positive ETEC strains. LT-sensitized sheep RBC had also been used to detect enterotoxin production in a radial passive immune haemolysis assay (Bramucci and Holmes 1978) and to detect anti-LT antibodies by passive immune haemolysis in acute and convalescent sera from patients with diarrhoea (Evans and Evans, 1977).

Poly-vinyl microfilter trays pre-coated with burro anti-cholera serum have been used to detect LT and CT by solid-phase radioimmuno-assay (RIA) and by enzyme-linked immunosorbent assay (ELISA) (Greenberg et al., 1977; Yolken et al., 1977).

A coagglutination technique using Protein-A containing staphylococci coated with specific CT-antiserum has also been used with good results by Brill, Wasilauskas and Richardson (1979) to detect LT positive ETEC.

Other assays for LT and CT based on the inhibition of platelet aggregation by activation of an adenylate cyclase-dependent system (Fumarola et al., 1976), the use of human lung embryo fibroblasts in tissue culture (Holzel, 1979) or mouse foot pads (Ketyi et al., 1978b) do not offer any advantages over the more widespread available tests.

Although cholera toxin has been purified and characterized, LT has proved difficult to purify and has not yet been fully characterized. Molecular weight estimations for purified LT enterotoxin range from 20,000 to 200,000 (Evans, Evans and Gorbach, 1974; Finkelstein et al., 1976; Schenkein et al., 1976; Dorner, Jacksche and Stöckl, 1977).

Although some of the reported differences between the various LT preparations may be attributed to a variation in strains or methodology, a complete explanation for these differences is likely to include a description of the mechanism of toxin release. LT is not an extracellular product of E.coli, at least under normal growth conditions (Dorner, 1975). The enterotoxin appears in the growth medium only after the logarithmic growth phase, and seems to be released with surface components of the cell.

E.coli LT shares many structural similarities with cholera toxin (Dallas, Gill and Falkow, 1979). The LT molecule is composed of at least two distinct proteins of 11,500 and 25,000, respectively, the smaller one immunologically related to the B subunit of cholera toxin (Clements and Finkelstein, 1978a). Further immunological and neutralisation studies have shown a cross-reactivity between both proteins of E.coli LT and the A

and B subunits of CT (Clements and Finkelstein, 1978b; Holmgren and Svennerholm, 1979).

Apart from the biochemical and immunological similarities between E.coli LT and CT, partially purified LT has been shown to also bind to GM_1 ganglioside (Zenser, 1974; Donta and Viner, 1975). This reaction has been utilized to develop a diagnostic GM_1-ELISA technique for identification of CT and LT (Svennerholm and Holmgren, 1978). The assay has been successfully used to test clinical ETEC isolates by Svennerholm and Holmgren (1979) and Sack et al. (1980).

Despite all these similarities between LT and CT there are important differences in the effects of these enterotoxins, the most striking being in the time-course of their effect, which is reflected in the duration of the clinical illness with which they are associated. Whereas the secretory response to cholera toxin does not become maximal until 3 hr after enterotoxin exposure in the rabbit gut loop and is sustained for more than 12 hr, the response to E.coli LT is maximal within 15 min and is terminated within a similar period after washing the enterotoxin from the mucosa (Carpenter, Curlin and Greenough, 1969; Guerrant et al., 1972). The differences in the time of onset of action of the two enterotoxins which activate the same membrane-bound enzyme could be due to differences in either the rate of enterotoxin binding to the cell, or in the rate of enterotoxin entry into the cell, or in other cellular events which transpire after enterotoxin exposure but before adenyl cyclase activation.

Once binding of LT and CT has occurred, a series of biochemical events are set in motion which results in adenylate

cyclase activation and an increase in intracellular levels of cAMP (Gill, Evans and Evans, 1976). The intestinal cells to which these enterotoxins bind is still unclear. Roggin et al. (1972) have experimental evidence of crypt cell involvement, whereas others have data involving primarily villous cells (DeJonge, 1975; Weiser and Quill, 1975). These apparently conflicting results could be due to the migration of the mucosal cells from the crypts to the top of the villi, where they are normally sloughed off as part of the normal cell turnover.

This upward migration and the irreversible binding of LT and CT to the epithelial cells could explain the prolonged effect of the enterotoxins and the inability of most pharmacologic agents, with the exception of nicotinic acid and chlorpromazine, to reverse their action once they are bound (Turjman et al., 1978: Holmgren, Lange and Lönnroth, 1978).

LT stimulation of intestinal secretion is not due to an increase in epithelial membrane permeability, increase in hydrostatic pressure differences or impairment of intestinal absorption (Gordon, Gardner and Kinzie, 1972; Pierce, 1973). Prostaglandins are not involved as mediators in the production of fluid by LT or CT (Kimberg, 1974) but other intestinal hormones such as vasoactive intestinal peptide (VIP) could cause hypersecretion in areas of the intestine not directly stimulated by the enterotoxins (Williams and Dodahwalla, 1969; Staley, Jones and Smith-Staley, 1973).

The morphology of the intestinal mucosa, as shown by human and animal studies, is not altered during cholera or ETEC disease (Elliott et al., 1970; Pastore et al., 1976). The

epithelium remains intact but there is evidence of dilated crypts and a small inflammatory response in the lamina propia. The increased secretion of electrolytes and water caused by LT or CT overwhelms the absorptive capacity of the large intestine, resulting in the typical rice-watery diarrhoea. The jejunum and proximal ileum show the greatest secretory response, decreasing distally, with the stomach and large intestine not responding to the enterotoxins (Carpenter, Curlin and Greenough, 1969).

Diarrhoea due to LT usually lasts for 2 - 3 days, whereas that caused by CT lasts for 3 - 7 days (Merson, 1977). This difference in duration could be due to a greater affinity between CT and GM ganglioside in the intestinal epithelium, or to a decrease in the rate of intestinal cell turnover. It is generally accepted that locally produced antibodies are responsible for the termination of the diarrhoea and the clearing of the bacteria from the small bowel (Pierce, 1973). However, a carrier state has been found in some patients with severe cholera, with the bacteria lodging indefinitely in the gall bladder (Wallace et al., 1967). ETEC have also been frequently found in healthy individuals (Merson, 1980). The epidemiological importance of these carrier states as reservoirs for cholera and ETEC diarrhoea in humans has started to be investigated only recently. Black et al. (1980) in an etiologic study of diarrhoea in rural Bangladeshi families have found that ETEC strains can be transmitted from healthy adult carriers to infants and children through faecal contamination of surface water and cooking utensils. This route of transmission explains the higher

incidence of diarrhoeal disease in young children and the early development of acquired immunity to these enterotoxigenic diseases in the populations of developing countries.

Ie. 2. *E.coli* heat-stable enterotoxin

In 1967 Smith and Halls reported the presence of a heat-stable exotoxin in cell-free culture filtrates obtained from *E.coli* strains isolated from pigs with diarrhoea. This toxin, called *E.coli* heat-stable (ST) enterotoxin, was shown to dilate pig and sometimes rabbit ligated intestinal segments. Although strains able to produce both ST and LT were isolated from both humans and animals, initially ST only ETEC strains were isolated from animals, especially young ones (Smith and Gyles, 1970; Gyles, 1971; Kohler, 1971). Human ETEC strains were generally found to produce both ST and LT (DuPont *et al.*, 1971; Sack, 1975). More recently, however, strains producing ST only have been implicated in cases of travellers' diarrhoea (Morris, 1976), infant diarrhoea (Ryder *et al.*, 1976; Black *et al.*, 1980), and common source outbreaks and sporadic cases of diarrhoea (Rosenberg *et al.*, 1977; Wachsmuth *et al.*, 1979). Furthermore, Levine *et al.* (1977) were able to show that an ST only ETEC strain isolated from an outbreak of diarrhoea on board a cruise-ship caused a dose-dependent watery diarrhoea when fed to adult volunteers.

ST produced by porcine ETEC strains was shown by Gyles (1971) to share many of the properties of LT. Both enterotoxins caused diarrhoea in piglets, dilated pig gut loops, and were controlled by transmissible plasmids. However, antiserum prepared in rabbits from porcine ETEC strains able to produce ST and LT were able to neutralise only the effects of LT enterotoxin in ligated pig intestinal segments (Smith and Gyles, (1970).

The biological activity of ST has been studied in rabbit and dog gut loops (Moon *et al.*, 1970; Staley *et al.*, 1974; Nalin *et al.*, 1975), and in rabbit ileal mucosa *in vitro* (Field *et al.*, 1978). In both models ST evokes a rapid and persistent secretory response which is rapidly reversed by rinsing, and does not require the presence of extracellular calcium. The changes in sodium and chloride fluxes produced by ST are similar to those produced by LT but of a somewhat smaller magnitude (Field *et al.*, 1978).

In vitro measurements of the electrical response in the ileal mucosa show that the addition of ST to the luminal side caused an increase in the potential difference and the short circuit current that are slowly reversed by removing ST from the luminal bathing solution. Increasing concentrations of ST cause corresponding increases in potential difference, and net chloride absorption is abolished under short circuit conditions. Addition of theophylline to the system causes a further increase in the potential difference already obtained with ST, and a net secretion of chloride not observed with ST alone. Theophylline, like LT, activates adenylate cyclase and causes an increase in cAMP levels *in vivo* and *in vitro* (Field, 1971; Hamilton *et al.*, 1978). This indicates that the secretory activity of ST is not mediated by adenylate cyclase. Measurement of mucosal cyclic nucleotide concentrations after stimulation with ST have shown no alterations in cAMP levels (Field, 1979). However, the concentration of cyclic guanosine monophosphate (cGMP) increased linearly over the entire range of concentrations of ST tested (Field *et al.*, 1978).

There is increasing evidence to suggest that ST abolishes net chloride absorption which increases intestinal secretion through activation of guanylate cyclase (Field et al., 1978; Hughes et al., 1978; Newsome, Burgess and Mullan, 1978). Analogues of cGMP cause intestinal fluid accumulation in rabbit intestinal loops, mimicking the effect of ST (Hughes et al., 1978).

Apart from increasing cGMP levels, ST can also neutralise the effect of alpha and beta adrenergic agonists such as norepinephrine and isoproterenol, in smooth muscle preparations (Pesti and Gordon, 1978). By this mechanism ST can prevent an increase in peristaltic motility and lengthen the easily reversible secretory activity caused by this enterotoxin.

Because ST is not antigenic and does not activate adenylate cyclase, its presence cannot be detected by assays used for LT or CT. There are, at present no available tissue culture lines reacting biochemically or morphologically to increases in cGMP. Production of ST by ETEC strains is detected by the use of other biological tests. Porcine, rabbit and dog intestinal loops have been shown to be sensitive to ST (Moon et al., 1970; Smith and Gyles, 1970; Staley et al., 1974). However, these tests are expensive and cumbersome, and only accessible to research laboratories. Dean et al. (1972) described an ST assay using infant mice. The infant mouse assay (IMA) has proven to be a reproducible and semi-quantitative assay (Giannella, 1976) although the age of the mice and environmental conditions are important factors.

Klipstein et al. (1976) have described a biological assay

for ST using *in vivo* perfusion of rat jejunum. This test seems more useful for the study of enterotoxigenic mechanisms or for the detection of other enterotoxins which give negative results in the IMA than for routine ST testing. Farkas-Himsley and Jessop (1978) have reported an assay for ST using its ability to inhibit the growth of L601 mouse fibroblasts by measuring ^{3}H-thymidine uptake. Unfortunately they have not compared their results with the IMA and their assay could be detecting a different *E.coli* heat-stable fraction to the one detected in the IMA.

Purification of ST from porcine ETEC strains by Alderete and Robertson (1978) has shown that the enterotoxin is a polypeptide with an approximate molecular weight between 4,400 and 5,100. Analysis of its amino acid content showed a high proportion of cystines and few hydrophobic amino acids. The protein is heat-stable at 100 C for 30 min. ST is stable to acid treatment but loses its activity at pH values greater than 9.0 (Kohler, 1971). ST is a poor antigen in rabbits and pigs, even when injected with Freund's complete adjuvant (Alderete and Robertson, 1978).

Conflicting results on the heat-stability of the toxin in rabbit ligated gut loops and infant mice have suggested the possibility of two distinct ST enterotoxins (Jacks and Wu, 1974; Giannella, 1976; Mullan, Burgess and Newsome, 1978). Moon and Whipp (1970) were the first to report that some ST strains were able to cause diarrhoea in pigs less than two weeks old, as well as in weaned pigs (eight - nine weeks old), whereas other ST strains could cause disease only in young animals. The

former were called Class I enteropathogens, while the latter were called Class II. Other authors have found similar differences between ST isolated from pigs, calves or lambs with diarrhoea (Smith and Linggood, 1972; Sivaswanny and Gyles, 1976). Burgess *et al.* (1978) studied two different ST preparations obtained by methanol extraction, STa which is partially heat-stable, methanol soluble, and active in infant mice and neonatal piglets, but inactive in rabbit gut loops; and STb which is heat-stable, inactive in infant mice, methanol insoluble, and active in ligated intestinal loops of weaned pigs and rabbit gut loops. Moon and Whipp's Class I strains would thus produce STa and STb, while Class II strains would produce only STb (Burgess *et al.*, 1978).

Gyles (1979) has compared the response of infant mice, ligated intestine of weaned pigs and ligated intestine of rabbits to ST produced by human, bovine and porcine ETEC strains. His results show two definite patterns; *ST1* which reacts in all three tests, and *ST2* which reacted only in the ligated weaned pig loops. All human and bovine ETEC strains tested belonged to the *ST1* pattern. Gyles also tested a number of porcine ETEC strains obtained by Moon and Whipp and classified by them as Class II (only positive for ST in piglets gut loops), and found them to be *ST1*. All porcine ST-only strains of serotype O138:K81 tested were also classified as *ST1*. Of 24 porcine $ST^{+}LT^{+}$ strains in the study, four were *ST1*, while the rest were classified as *ST2*. The porcine ST-only strains of serotypes other than O138:K81 were found to belong to the *ST2* pattern. Limitations of the infant mouse test for the

detection of ST produced by porcine strains has also been reported by Olsson and Söderlind (1980). These limitations, however, do not seem to apply to ST strains isolated from humans.

With the use of gene cloning techniques, Lathe et al. (1980) have been able to isolate the ST genes from two porcine ETEC strains. The ST coding region found in a PstI fragment of 1.05×10^6 molecular weight (MW) was examined for its ability to stimulate protein synthesis in a cell-free system. Examination of an extract produced by the cloned fragment revealed a 10,000 MW protein that reacted with ST-antiserum raised with purified ST in rabbits. Further analysis showed that a 7,000 MW fragment also reacted with the antiserum. This suggests that ST is produced as a 10,000 MW protein that is then broken down by proteolysis to an active 7,000 MW polypeptide. This active ST is in the same MW range as previously reported for other purified porcine and bovine ST preparations (Alderete and Robertson, 1978).

Staples, Asher and Giannella (1980) have purified an ST from a human ETEC strain by acetone fractionation and ion-exchange chromatography. The enterotoxin appears to be a peptide with a molecular weight of approximately 1,972, containing ten different amino acids and 18 amino acid residues. Sensitivity to reducing agents and performic acid oxidation indicates that this ST enterotoxin has one or more disulphide bridges. The presence of these disulphide bridges could explain the hydrophobicity and the heat and acid stability characteristic of the ST enterotoxin (Newsome et al., 1978; Alderete and Robertson, 1978; Lallier, Lariviere and St. Pierre, 1980).

ST can be detected in the mid-log phase of growth, and contrary to LT, its production is inhibited by glucose but not by changes in pH (Johnson, Lior and Johnson, 1978). ST is not detected in the periplasmic space or in sonicates of cells after 8 h growth. All the evidence suggests that ST is transported out of the cell as soon as it is synthesised, in contrast to LT which remains associated with the outer surface of bacteria and in the periplasmic space.

Ie. 3. Role of transmissible plasmids in enterotoxin production

The role of transmissible plasmids in the synthesis of LT and ST was first observed in porcine ETEC strains by Smith and Halls in 1968. The transfer by conjugation of these Ent$^+$ plasmids to E.coli K12 was not sufficient to make the recipient pathogenic in pigs (Smith and Halls, 1968; Smith and Linggood, 1972). The joint transfer of another plasmid coding for the synthesis of the K88 surface antigen (a colonisation factor) was necessary for the recipient to cause diarrhoea in pigs. Transmissible and nonconjugative plasmids controlling the production of LT and ST were also found in ETEC strains isolated from humans with diarrhoea (Smith and Linggood, 1972; Wachsmuth, Falkow and Ryder, 1976). Initial studies showed that E.coli strains isolated from humans carrying a conjugative Ent$^+$ plasmid encoding for LT and ST always transferred the ability to produce both enterotoxins to the recipients. Analysis of partially purified plasmid preparations from Ent$^+$ recipient strains showed the presence of a single plasmid of approximately 60 x 10^6 which conferred the ability to produce LT and ST.

In a study of LT$^+$ST$^+$ strains of E.coli serogroup 078 isolated from humans, McConnell et al. (1980) found that LT and ST production were coded by separate plasmids. The LT plasmids investigated by these authors were all fi$^+$ and belonged to the F-incompatibility complex, but could be subdivided into three main types when sensitivity to E.coli phages, molecular size and incompatibility reactions were compared. When these plasmids were examined by restriction enzyme digestion and DNA-reassociation, Willshaw et al. (1980) showed that they were all related, but

the degree of relationship supported the three subdivisions obtained previously.

The large size of LT plasmids makes the study of the structure and expression of a single gene difficult. So, Dallas and Falkow (1974) have isolated the LT-DNA region on a 5.8×10^6 dalton DNA fragment from a porcine Ent$^+$ plasmid and inserted it, by molecular cloning, on to the carrier plasmid pBR322. Dallas Gill and Falkow (1979), using this cloned plasmid, introduced deletion mutations of various lengths into the LT-DNA region and into the adjacent DNA sequences of the carrier plasmid. Analysis of proteins produced by these mutants in minicells showed that the maximum size of the LT-DNA region was 1.2×10^6. This mass of DNA was sufficient to code for a protein of about 600 amino acids which, assuming that the average size of an amino acid is 100 daltons, would make the largest, single LT molecule approximately 60,000 daltons.

The 1.2×10^6 fragment was able to encode for two proteins of 11,500 daltons and 25,500 daltons (Dallas and Falkow, 1979). Dallas, Gill and Falkow (1979) were able to map the two cistrons encoding each of these two LT proteins. Mutations in the adjacent DNA region of the LT-DNA fragment showed that these two proteins probably share the same promoter, with the 25,500 protein-encoding cistron proximal to it.

Production of ST in both porcine and human strains has also been shown to be plasmid mediated (Gyles, So and Falkow, 1974; Wachsmuth, Falkow and Ryder, 1976). However, unlike ST$^+$ LT$^+$ plasmids, Ent plasmids that encode only for ST production show considerable heterogeneity. Their molecular

size ranges from 21 to 86 x 10^6 (Gyles, 1971), with a G + C content between 0.41 and 0.50. DNA sequence homology studies show that ST plasmids are generally not related to each other or to other plasmids found in ETEC strains (Gyles, So and Falkow, 1974).

So *et al.* (1976) have isolated the structural genes coding for ST biosynthesis in an 8.9 kilobase (5.7 x 10^6) DNA fragment from a calf ETEC *Ent*$^+$ plasmid. Smaller fractions from this 8.9 kilobase fragment, obtained by restriction enzymes, have shown that the ST genes are contained in a transposon flanked by *IS1* inverted repeats (So, Heffron and McCarthy, 1979).

Silva, Maas and Gyles (1978) have also reported the presence of inverted repeats bounding an area that contains genes coding for ST and LT in a porcine ETEC strain. The demonstration that pathogenic determinants, other than antibiotic resistance, can be carried by transposable elements has great importance in the epidemiology of ETEC disease.

The availability of highly purified ST preparations and the production of ST by cloned DNA will be useful in the near future to develop *in vitro* techniques, such as radioimmunoassay or ELISA, for the detection of ST. With these assays it will be possible to establish if there is more than one ST, and to learn more about its mechanism of action and pathogenicity.

Ie. 3. *E. coli* cytotoxin on VERO cells (VT)

Some strains of *E. coli* have been shown to produce a 'toxin' which causes morphological changes in green African monkey-kidney cells (VERO) (Konowalchuk, Speirs and Stavric, 1977). Strains producing VERO cytotoxin (VT) do not generally produce ST or LT, and are therefore usually negative in Y1 and CHO cells, and in the infant mouse test. *E. coli* LT and cholera toxin can also cause morphological changes in VERO cells, but this response is cytotonic, and not cytotoxic as the one produced by VT. The VT is a heat-labile toxin with a molecular weight between 10,000 and 30,000 (Konowalchuk *et al.*, 1978). Isoelectric focusing studies show two components, one with a pI of 7.2 and another with a pI of 6.8. The first has a molecular weight of 28,000, and is able to produce slight fluid accumulation in rabbit gut loops and a cytotoxic effect on VERO cells. VT production was at first reported in *E. coli* strains belonging to EPEC O-groups 18, 26, 111, 126 and 128. In a more extensive study of 253 EPEC strains from infants with diarrhoea, Scotland, Day and Rowe (1980) found that of the strains they examined only those in O-groups 26 and 128 were able to produce VT. Moreover, in these two O-groups only those strains belonging to serotypes O26:H11 and O128:H2 were found to produce VT.

Both Konowalchuk *et al.* and Scotland *et al.* included in their studies a strain of *E. coli* called H19, of serotype O26:H11, which produced VT but not ST or LT. This same strain was

reported by Smith and Linggood (1971) as producer of a heat-labile enterotoxin (*Ent*$^+$) causing fluid accumulation in rabbit gut loops. The *Ent*$^+$ ability in H19 could be transferred to *E.coli* K12, and the K12 (*Ent*$^+$) recipients were shown to give a positive rabbit gut loop test (Smith and Linggood, 1971). Scotland *et al.* (1980) reported that one of Smith and Linggood's K12 (*Ent*$^+$) derivatives from H19 was able to produce VT but not LT or ST. Scotland *et al.* were also able to transfer the VT character from H19 to *E.coli* K12 with a frequency of 0.01 per recipient cell in 24 h.

It seems evident from these studies with *E.coli* H19 that the heat- labile *Ent*$^+$ character described by Smith and Linggood (1971) is not an adenylate cyclase-activating LT but a cytotoxin similar or identical to the VT found by Konowalchuk, Speirs and Stavric (1977) and Scotland, Day and Rowe (1980). However, further serological and genetic studies are needed to characterise this cytotoxin and to differentiate it from other *E.coli* enterotoxins. Because the production of VT is restricted to a few *E.coli* serotypes, its pathogenic role in diarrhoeal disease, if any, remains to be clarified.

If. Enteroinvasive E.coli

Dysentery is a disease caused by invasion and multiplication of bacteria or protozoa in the epithelial cells and mesenteric lymph nodes of the large intestine. Protozoal dysentery is caused by species of amoebae. Bacterial dysentery can be caused by strains of shigellae and E.coli. The incidence of bacillary and amoebic dysentery is highest in developing countries where the sanitation is deficient, but the disease is found world-wide.

The acceptance of E.coli strains as a cause of bacillary dysentery came as the result of aetiological studies of bloody-diarrhoea in which strains of E.coli but not of shigellae were isolated (Ewing and Gravatti, 1947; Hobbs, Thomas and Taylor, 1947; Marier et al., 1973). The enteroinvasive strains of E.coli share many biochemical and serological reactions with shigellae strains, which make the E.coli strains atypical. For this reason, routine laboratories often report them wrongly as strains of shigellae. Enteroinvasive E.coli (EIEC) strains are usually non-motile, anaerogenic and late- or non-lactose fermenters (Rowe, Gross and Woodroof, 1977; Silva, Toledo and Trabulsi, 1980). Correct identification often requires additional biochemical tests like lysine decarboxylase and utilization of sodium citrate and sodium acetate (Rowe, 1979). Several investigations have reported the presence of identical O antigens between E.coli 0124 and Shigella dysenteriae 3, between E.coli 0112ac and Shigella boydii 15, between E.coli 0149 and Shigella boydii 1, and between E.coli 0159 and Shigella dysenteriae 4 (Edwards and Ewing, 1972;

Rowe, Gross and Guiney, 1976).

Clinical symptoms and duration of dysentery are identical when caused by EIEC or shigellae strains. The bacteria cause inflammation and ulceration of the intestinal mucosa of both humans (Marier et al., 1973) and animals such as guinea-pigs (DuPont et al., 1971) and monkeys (LaBrec et al., 1964; Ogawa et al., 1967). These animal models, however, are not useful for the routine testing of EIEC strains.

Serény (1955) described a biological test for invasive shigellae strains using their ability to cause an ulcerative keratoconjunctivitis in the guinea-pig eye. EIEC strains have been shown to produce this same reaction. More recently, new in vitro assays using continuous tissue-culture cell lines have been developed to test the invasive ability of strains of shigellae and EIEC (DuPont et al., 1971; Mehlman et al., 1977). These assays are less expensive and offer more possibilities for the study of invasive mechanisms in controlled laboratory conditions.

Ig. Bacterial adhesiveness

The ability of microorganisms to colonise the mucosal surfaces of their hosts is of prime importance to their survival. In order for commensal and pathogenic bacteria to obtain nutrients and to overcome antibacterial mechanisms, they must come in contact with and attach to receptors in the epithelial cells of the host. The presence of cilia in the nasal and bronchial mucous membranes, the enamel of teeth, the flow of urine or peristaltic movements of the intestine, are all defense mechanisms used by the host to prevent or reduce microbial adherence to these surfaces.

Adhesiveness has been studied in many species of pathogenic bacteria, including Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus, Pseudomonas aeruginosa, Neisseria gonorrhoea, Escherichia coli, Proteus mirabilis and Salmonella typhimurium (Smith, 1977; Arbuthnott and Smyth, 1979; Svanborg-Eden, 1978; Evans and Evans, 1979). The mechanisms by which these bacteria adhere are incompletely understood and may differ between species of the same organism (Freter and Jones, 1976). The evidence available from these studies points to a high degree of tissue- and species-specificity.

The concept of specific cell receptors has come mainly from studies of peptide hormones. Recognition between macromolecules involves the reception and uncoding of a certain signal and its translation into an effect that the cell already knows how to accomplish (Keusch, 1979). The attachment of cholera toxin B subunit to GM_1 ganglioside and the activation

of adenylate cyclase is a good example of this concept.

Susceptibility and immunity to infection have been directly related to the existence of specific cell receptors. Research into this interaction is therefore of great importance in the development of therapy or vaccines against specific infections.

Ig. 1. Type 1 pili and other mannose-sensitive adhesins

Many enteropathogenic bacteria have been shown to have non-flagellar filamentous surface protein antigens that enable them to adhere to intestinal epithelial cells. The first of these filaments was described by Houwink and van Iterson (1950), Duguid et al. (1955) and Duguid and Gillies (1957) in strains of E.coli, Shigella flexneri, Salmonella, Proteus and Enterobacter cloaceae. These filaments were called 'fimbriae'. Electron microscopy revealed that 'fimbriae' had an approximate width of 5 - 10 nm and variable length depending on the growth conditions of the bacteria. All the strains studied underwent phase mutations between a fimbriate and a non-fimbriate phase. Fimbriated cells became dominant after serial growth of the bacteria in broth, and the production of fimbriae was accompanied by the formation of a surface pellicle.

The fimbriate bacteria rapidly adhered to and agglutinated the red blood cells (RBC) of guinea-pig, fowl, horse, mouse, rabbit, sheep and man, and also cells of Candida albicans and Saccharomyces cerevisaea (Duguid and Gillies, 1957). This haemagglutination was completely inhibited by small concentrations of D-mannose, α-methyl-mannoside and yeast mannan, but not by mucin, normal serum, warming up to 55 C or pH values between 3 and 10. Specific antiserum against the fimbriae of Shigella flexneri prepared by Gillies and Duguid (1958) was able to agglutinate fimbriated strains of different species and totally inhibited the ability of these strains to cause mannose-sensitive haemagglutination (MSHA).

In a more detailed study, Brinton (1965) described several types of fimbriated appendages in Enterobacteriaceae. Those causing MSHA were called 'type 1 pili' because of their 'hair-like' morphology. This term has been more widely accepted than 'fimbriae' and will be the one used in this study.

Some strains of E.coli possess other fimbriated adhesions that are not capable of causing MSHA of guinea-pig RBC (Eshdat et al., 1978; Silverblatt, 1979; Mangan and Snyder, 1979a). These appendages have been called 'mannose-sensitive adhesins' because their function can be inhibited by the presence of D-mannose in the growth medium.

Strains of E.coli with type 1 pili have been studied for their adhesive ability. Ofek, Mirelman and Sharon (1977) reported that an E.coli B and an E.coli K12 able to produce type 1 pili were able to attach to human buccal cells by binding to mannose-specific lectins on the cell surface. This adherence was inhibited by D-mannose and its analogues. Mannose-binding activity by type 1 pili can also be measured by agglutination of mannan-containing yeast cells (Ofek and Beachey, 1978).

Binding of E.coli to buccal and yeast cells can also be inhibited by growing the strain in sublethal concentrations of streptomycin (Eisenstein, Ofek and Beachey, 1979). The streptomycin effect is reversible, requires growing organisms and is most apparent in the early log-phase. Electron microscopy has shown that this inhibitory effect is due to a decrease in the length of the type 1 pili when the bacteria

are grown in the presence of the antibiotic (Eisenstein, Beachey and Ofek, 1980).

Attachment of E.coli and Salmonella typhi to mouse peritoneal macrophages has also been shown to be mediated by binding to mannose containing residues in these cells (Bar-Shavit et al., 1977).

Type 1 pili from strains of E.coli and Shigella flexneri have been isolated and purified (Brinton, 1959; Salit and Gotschlich, 1979a). Purified type 1 pili from an E.coli K12 show a protein subunit with a molecular weight of 19,000 (McMichael and Du, 1979). They are able to cause MSHA of guinea-pig RBC by binding to mannose-like molecules on the erythrocyte surface. Isolated type 1 pili are also able to adhere to VERO cell monolayers without the need of enzymatic activity (Salit and Gotschlich, 1979b). This model of prokaryote-eukaryote cell adherence occurred optimally at pH 4-5 and could be inhibited by D-mannose and its analogues, by anti-pili antibodies and by incubation of the VERO cells with mannose-specific plant lectins. Binding of type 1 pili was not inhibited by adding glycosidase, trypsin or a protease mixture to the cell monolayer, and treatment of the VERO cells with neuraminidase caused an increase in type 1 pili adhesion. These results complement previous findings and indicate that type 1 pili are able to attach to mammalian cell surfaces by binding to mannose-containing glycoproteins.

The relationship between the presence of these mannose sensitive adhesins and pathogenicity is still unclear. A high percentage of bacterial strains possess type 1 pili and

other mannose-sensitive adhesins. Duguid (1964) studying E.coli strains isolated from humans found that 74% of them had type 1 pili and caused MSHA. Duguid also reported that some pathogenic strains of E.coli did not possess type 1 pili and others gave a haemagglutination that was not inhibited by D-mannose. The presence of type 1 pili in strains of E.coli, shigella or salmonella does not seem to be a requisite for pathogenicity (Cefalů, 1960; Duguid, 1964). Complete removal of type 1 pili by ultraviolet light irradiation in strains of E.coli reduces but does not abolish adherence of the strain to human buccal cells (Silverblatt, 1979). Nevertheless, mannose and its analogues have been found to inhibit adherence of E.coli and Proteus mirabilis to rabbit, rat and human intestinal and urinary tract epithelial cells (Duguid and Gillies, 1957; Silverblatt and Ofek, 1978) and mouse peritoneal macrophages (Bar-Shavit et al., 1977). Anti pili antibodies have also been reported to protect animals against infection with E.coli (Silverblatt and Cohen, 1979). Mannose-specific ligands are widely distributed among animal cells and probably serve as receptors for the attachment of these strains.

The role played by non-haemagglutinating mannose sensitive adhesins in the pathogenicity of the strains possessing them is still unclear. Recent evidence suggests that bacteria with type 1 pili and other mannose sensitive adhesins are more susceptible to phagocytosis and removal by defence mechanisms in the host (Silverblatt, Dreyer and Schauer, 1979; Mangan and Snyder, 1979a; Rottini et al., 1979; Ørskov, Ferencz and

Ørskov, 1980). The presence of these factors would thus seem to be a liability rather than an advantage to the bacteria, and perhaps reflects an evolutionary defence mechanism in the host developed from centuries of colonisation by the same organisms.

Ig.2. Adhesive factors and mannose-resistant haemagglutination in E.coli strains

Ig.2a. K88 adhesive factor

Strains of E.coli causing diarrhoea in pigs were shown to possess an adhesive factor called K88 (Wittig, 1965; Gossling and Rhoades, 1967). Smith and Linggood (1971) tested K88-positive and K88-negative variants of porcine strains of ETEC and demonstrated that only the K88-positive were able to colonise the anterior small intestine of pigs. Moreover, $K88^+$ ETEC strains were able to cause diarrhoea in pigs, whereas K88-negative variants which were still enterotoxigenic in rabbit gut loops were not. Jones and Rutter (1972) confirmed these results by showing that in isolated intestinal tissue both K88-positive ETEC strains and the K88 antigen extracted from them adhered to epithelial cells in the small intestine whereas K88-negative variants of these same strains did not. Furthermore, K88-positive variants did not adhere to epithelial cells in the large intestine even though present there in large numbers, thus emphasizing the specificity of the association between enteropathogen and small intestinal epithelial cells.

Further information confirming the importance of the K88 antigen in the pathogenesis of porcine diarrhoea came from immunological studies. Antiserum raised in rabbits was shown to inhibit the intestinal adhesion of K88-positive strains when fed to gnotobiotic pigs and to prevent the development of diarrhoea in these animals (Jones and Rutter, 1972).

Jones and Rutter (1974) reported that the strains of E.coli isolated from pigs with diarrhoea were able to agglutinate guinea-pig RBC. This haemagglutination was not inhibited by

the presence of D-mannose and had been previously described by Duguid and Gillies (1957). Mannose-resistant haemagglutination (MRHA) was found only in porcine E.coli strains that possessed K88. Loss of K88 from these strains was accompanied by loss of MRHA ability and loss of fimbriae surrounding the bacteria when examined by the electron microscope (Jones and Rutter, 1972). K88-positive strains grown at 18 C do not give MRHA. The haemagglutination can also be inhibited by anti-K88 rabbit antibodies.

Using isolated pig intestinal cells, Wilson and Hohmann (1974) confirmed the specificity of K88-positive adhesion and its inhibition by anti-K88 rabbit antibodies. ETEC strains isolated from humans and calves with diarrhoea were not able to adhere to pig intestinal cells and did not possess K88 antigen (Table 2).

With the development of a model of adhesion *in vitro* using isolated porcine intestinal brush border preparations, Sellwood *et al.* (1975) tried to find a specific cell receptor for the K88 antigen in the epithelial cells. Their results showed that K88-positive ETEC strains and isolated K88 antigen adhered to brush border preparations from some pigs ('positive') but not others ('negative'). Genetic and pedigree studies of 'positive' and 'negative' pigs have shown the existence of a simple dominant Mendelian inheritance of the K88 cell receptor on the brush border surface of the pig intestinal cells. The product of the adhesive gene (S) is dominant over that of the non-adhesive gene (s), with the homozygous recessive (ss) being a disease resistant animal (Gibbons *et al.*,

1977).

Gibbons, Jones and Sellwood (1975) have used MRHA of guinea-pig RBC by K88-positive strains to demonstrate the existence of a specific cell receptor for the antigen. Their studies suggested that K88-positive strains attach to the terminal β-D-galactosyl structure of the heterosaccharide side chain of a glycoprotein in the erythrocytes. More recent evidence seems to indicate that a non-ganglioside glycolipid found in the supernatant fraction of brush border preparations from 'positive' pigs functions as the K88 receptor in the epithelial cells of the intestine (Kearns and Gibbons, 1979).

Smyth et al. (1978) have studied porcine ETEC strains with a hydrophobic interaction chromotography (HIC) technique and found that only those possessing K88 antigen have surface associated hydrophobic properties. $K88^+$ and $K88^-$ bacteria grown at 18 C were negative for this property. This studies suggest that K88 fimbriae promote adherence to epithelial cells by hidrophobic bonds formed between the bacterial adhesins and the cell receptors.

The K88 antigen is a K antigen of the L variety that surrounds the surface of the bacterial cells in the form of fine filaments (Ørskov and Ørskov, 1960; Stirm et al., 1967a). These filaments are of protein nature, 0.5 to 1.5 microns in length and 70 to 110 ångstroms in diameter (Stirm et al., 1967b). Their amino acid composition is different from type 1 pili, although structurally and morphologically they are very similar (Stirm et al., 1967b; Klemm, 1979).

The K88 antigen exists in at least four serological forms,

K88ab, K88ac, K88ad and K88ad(e) (Ørskov et al., 1964; Guinée and Jansen, 1979). These different serological forms can only be distinguished by immunoelectrophoresis and immunodiffusion techniques, since all four cause MRHA of guinea-pig RBC (Cahill and Olantz, 1978; Guinée and Jansen, 1979).

The structural genes controlling the production of K88 antigen, like those coding for enterotoxin production, are carried on a plasmid (Ørskov and Ørskov, 1966). The K88 plasmid can be lost spontaneously or by 'curing' with acriflavine. Transfer of both the K88 and Ent$^+$ genes to certain E.coli strains from the normal pig intestinal flora would make it pathogenic for other pigs (Smith and Linggood, 1972). Introduction of the Ent$^+$ alone did not make the recipient E.coli pathogenic, while recipient E.coli with K88 only were able to cause mild diarrhoea in piglets.

The detection of K88 in transfer experiments was greatly enhanced when Smith and Parsell (1975) reported the joint transfer of K88 and raffinose-utilizing ability from several strains of E.coli. A more detailed and comprehensive study of K88 plasmids was carried out by Shipley, Gyles and Falkow (1978). These authors studied 22 K88-positive ETEC strains, all of which were also able to utilize raffinose (raf$^+$). Twelve out of 18 strains of phenotype K88$^+$-raf$^+$ were able to transfer both characters at a detectable frequency. Using raf$^+$ transfer as a marker, Shipley and her co-workers found a high rate of cotransfer of K88 and raf$^+$. Some recipient strains also acquired genes for enterotoxin production and antibiotic resistance with the raf$^+$ character, but no attempt was made to

quantitate these cotransfers. In only two instances was raf$^+$ transferred without K88. The K88-raf$^+$ genes were carried by a non-transmissible plasmid of approximately 40 to 50 x 10^6. Recombination between the raf$^+$-K88 plasmid and the transfer factor in some E.coli strains produced a larger single self-transmissible 90 x 10^6 plasmid (Shipley et al., 1978). A K88 plasmid has been digested with restriction enzymes to produce smaller fragments (Shipley et al., 1979). A HindIII fragment coding for K88 was cloned into the carrier plasmid pBR313, a derivative of the colicin E1 (ColE1) plasmid. At least three polypeptides have been found to be produced when this cloned fragment was introduced into minicells. K88-negative mutants failed to produce these polypeptides (Kehoe, Sellwood and Dougan, 1980).

Mooi, de Graaf and van Embden (1979) have cloned the genetic determinants of the K88 antigen from a 51 x 10^6 plasmid that also codes for the utilisation of raffinose. Digestion of the K88 plasmid pR18801 with restriction enzyme HindIII revealed a 7.7 Mdal fragment (called H2) coding for the K88 antigen. The raf$^+$ genes were found on a 4 x 10^6 SalI fragment. The K88 and raf$^+$ genes were not mapped closely; a 20 x 10^6 stretch of DNA separated them. The reason why they are both associated could not be explained from these experiments.

Detection of K88 was enhanced for these genetic investigations by the development of an ELISA test using K88ab serum to coat polyestyrene microtiter plastic trays (Mooi, de Graaf and van Embden, 1979).

Ig.2b. 987P and K99 adhesive factors

K88 mediated adhesion is only one, albeit the most frequent, of several colonisation factors found in porcine enterotoxigenic E.coli strains. A vast number of K88-negative ETEC strains have also been found to be able to colonise the anterior intestine of pigs (Bertschinger, Moon and Whipp, 1972). The lack of K88 in these strains was not due to growth conditions, since after passage through a live animal the E.coli strains were still not able to adhere to isolated porcine intestinal cells or to cause MRHA of guinea-pig RBC (Drees and Waxler, 1970). Hohman and Wilson (1975) compared the ability to colonise the intestine of conventional neonatal piglets by two porcine ETEC strains, one possessing K88 antigen, the other lacking K88. Strain 340 (O9:K35:H$^-$) which lacked K88 was able to adhere in vivo to cells in the posterior half of the small intestine of pigs but not to adhere in vitro to isolated porcine intestinal cells. Although this strain of E.coli did not proliferate in large numbers in the anterior small intestine, fluorescent antibody studies showed that while only the K88-positive strain was found adhering to the basal half of the villi in the anterior intestine, both K88-positive and K88-negative strains were found adhering to the entire villi of the posterior small intestine. Since both strains of E.coli were able to cause diarrhoea in pigs, the importance of colonisation of the anterior intestine as a necessity to cause disease was questioned by these authors. Shadow cast preparations of strain 340 under the electron microscope demonstrated the existence of an unusual shadow surrounding the bacteria suggesting an adhesive appendage. Thin

section preparations, however, did not confirm the presence of a specific structure in this area. Moon, Nagy and Isaacson (1977) were also able to detect this 'adhesive appendage' in porcine ETEC strains that were able to adhere to the intestinal mucosa of pigs *in vivo* but that did not possess the K88-antigen. When sections of intestinal epithelium with adhering K88-negative *E.coli* were examined by transmission electron microscopy they observed that the bacteria were separated from each other and from the epithelial microvilli by a peribacterial electron-translucent regions. These adhering *E.coli* were shown to possess filaments extending into these regions.

In a more extensive survey of K88-negative enterotoxigenic *E.coli* strains isolated from pigs with diarrhoea, Nagy, Moon and Isaacson (1976; 1977) found a strain which was negative for pili *in vitro* but showed a persistently high degree of piliation when grown in pig intestine *in vivo*. This ETEC strain (987) adhered to isolated intestinal epithelial cells only after growth *in vivo*. Antiserum prepared in rabbits with isolated 987 pili (987P) agglutinated not only the original strain but also several other K88-negative porcine ETEC strains. Strains of *E.coli* with 987P do not cause MRHA of pig or guinea-pig RBC and its detection can only be done by serological and immunological assays. With these techniques 987P have been shown to be chemically different from K88 and type 1 pili (Isaacson *et al.*, 1978). Fragments prepared from the ab region of the immunoglobulin G-molecule (Fab fragments) in antiserum prepared in rabbits previously vaccinated with 987P, K88 or type 1 pili, were only able to block serological agglutination

and adherence to isolated pig intestinal cells by the homologous type of pili. Porcine intestinal epithelial cells would thus seem to have different receptors for different type of pili.

987P have not been characterized chemically but indirect evidence shows them to be protein in nature (Nagy, Moon and Isaacson, 1977). Studies on the genetic control of these pili have not been reported.

Diarrhoea in newborn calves and lambs has also been shown to be dependent on the presence of adhesive antigens in the causative ETEC strains. Smith and Linggood (1972) were the first to observe that ETEC strains of different O:H serotypes isolated from calves and lambs with diarrhoea shared a 'common K antigen' (Kco). This Kco antigen was accepted as a K antigen by the WHO Collaborative Centre for Escherichia and designated K99 (Orskov et al., 1975).

Like K88, K99 is a plasmid controlled pilus antigen, of protein nature and is thermolabile (Smith and Linggood, 1972) (Table 2). The presence of K99 enabled ETEC strains to proliferate in the anterior small intestine of calves and lambs. The loss of the K99 antigen from a calf ETEC strain resulted in an organism that no longer caused diarrhoea. The re-introduction of the K99 plasmid into the same strain resulted in a virulent organism (Smith and Linggood, 1972).

K99 was commonly found in calf and lamb strains of E.coli. K99-positive ETEC strains and isolated K99 pili were reported to adhere to a calf brush border preparation, to cause MRHA of sheep, guinea-pig and horse RBC and to give a single

precipitin line in immunodiffusion tests with a specific antiserum prepared in rabbits with a K99-positive ETEC strain of different O:H serotype (Ørskov et al., 1975; Tixier and Govet, 1975; Burrows, Sellwood and Gibbons, 1976). K99-negative ETEC strains and K99-positive ETEC strains grown at 18 C did not adhere to calf brush border, gave no MRHA and were negative in immunodiffusion tests with specific K99 antiserum.

The presence of K antigens of lipopolysaccharide nature in K99-positive ETEC strains gave the colonies a mucoid appearance and hampered the detection of K99 due to abundant K polysaccharide formation (Guinée, Jansen and Agterberg, 1976). This mucoid appearance could be avoided if the ETEC strains were grown on a semi-synthetic medium at pH 7.5 (Minca medium) instead of MacConkey agar. Minca medium was shown to offer optimal growth conditions for K99 at pH 7.5 on one hand and decreased development of K polysaccharide on the other (Guinée, Jansen and Agterberg, 1976). Minca medium was later improved by substituting the glucose in the medium with 1% isovitalex (Minca-Is) (Guinée, Veldkamp and Jansen, 1977). Using this Minca-Is medium Guinée , Jansen and Agterberg (1976) were able to detect the K99 antigen in 70 out of 74 calf ETEC strains that gave dilation when injected into ligated calf intestinal loops. K99 expression also depends on growth conditions such as aeration, glucose concentration and time (Isaacson, 1980).

Unlike K99 which has been detected in pig ETEC strains, K99 has been reported in species other than calves and lambs

where it was originally described. Moon et al. (1977) found several porcine ETEC strains that possessed K99 antigen. ETEC strains from calves, lambs and pigs with K99 were shown to adhere to the epithelial mucosa of pig ileum and caused profuse diarrhoea in newborn pigs. This further proved the role of K99 in the colonisation of the anterior intestine by ETEC strains.

K99 pili were reported to adhere to isolated intestinal epithelial cells prepared from one day-old, hysterectomy-derived, colostrum-deprived piglets (Isaacson, et al., 1978). Antiserum against K99 prepared in rabbits and Fab fragments specific for K99 were able to inhibit the adhesion of K99 pili to isolated intestinal epithelial cells of piglets (Isaacson et al., 1978). Fab fragments specific for 987P and type 1 pili did not inhibit the adherence of K99 to the intestinal cells (Isaacson et al., 1978).

De Graaf, Wientjes and Klaasen-Boor (1980) have developed an ELISA test for the detection of K99. This assay uses polyestyrene microtiter plastic trays coated with immunoglobulin-G-enriched K99 serum treated with caprylic acid. Application of this K99 ELISA test showed a remarkable difference in K99 production by strains grown on different media. The same K99 positive ETEC was negative in the ELISA when grown in complex medium and positive when grown in Minca-Is medium (De Graaf, Wientjes and Klaasen-Boor, 1980). The presence of a K polysaccharide antigen did not affect the detection of K99 in the ELISA, indicating that factors other than capsular antigens interfere with the detectability of K99 by slide

agglutination. With the ELISA test K99 production was shown to be related to the O antigen carried by the ETEC strain (De Graaf, Wientjes and Klaasen-Boor, 1980). ETEC of O-group 0101 always produced more K99 than those of O-groups 8, 9 and 20. The differences in phenotypic expression of the K99 antigen were dependent on the host strain since no differences in K99 production were found after conjugational transfer of the controlling plasmid from ETEC 0101 and 08 to E.coli K12. K99 expression is most probably related to cell wall components of the host strains.

K99 has been partially purified by Isaacson (1977) using diethylaminoethyl (DEAE)-sephadexion exchange chromatography after ammonium sulphate precipitation. This DEAE-K99 was composed primarily of protein subunits of 22,500 and 29,500 daltons and had an isoelectric point of 10. The isolated pili did not cause MRHA of guinea-pig RBC. Morris, Stevens and Sojka (1977; 1978a) have also purified K99 pili by isoelectric precipitation at pH 4.0. This acid-K99 had an isoelectric point of 4.2, and the isolated K99 pili were able to cause MRHA of guinea-pig RBC. However, the K99 positive ETEC strain used by Morris et al. was not the same one used by Isaacson, although both strains possessed the same K99 plasmid. Orskov et al. (1975) have reported that some, but not all, K99-positive ETEC strains are able to cause MRHA of guinea-pig RBC.

To clarify these points Isaacson (1978) studied DEAE-K99 and acid-K99 prepared from ETEC strains from different O-groups and compared their properties. DEAE-K99 preparations

from all K99-ETEC strains caused MRHA of sheep and horse RBC, whereas acid-K99 pili gave MRHA of guinea-pig, sheep and horse RBC. A standard absorbed K99 antiserum prepared from an ETEC strain with no O-antigen (strain 1474) was able to inhibit the MRHA of horse RBC caused by both DEAE-K99 and acid-K99 pili, but had no effect on the guinea-pig MRHA caused by the acid-K99 preparation.

The acid-K99 preparation contains a second antigen with an isoelectric point of 4.2 that is responsible for the MRHA of guinea-pig RBC. This second component differs from the established K99 pili in its migration towards the anode in immuno-electrophoresis preparations. Crude and purified K99 has been shown to possess a strong positive charge and to migrate towards the cathode (Guinée, Jansen and Agterberg, 1976; Isaacson, 1977). The anionic component according to Isaacson (1978) was a non-K99 antigen extracted from some ETEC strains at 60 C and concentrated by the acid precipitation process.

Separation of the two components in strain B41 (E.coli O101:K99) by ion exchange chromatography showed that both the anionic and the cationic components gave a strong MRHA of sheep RBC (Morris, Stevens and Sojka, 1978b). However, while the MRHA by the anionic component remained stable for up to six weeks, the one caused by the cationic component was lost after 3 weeks when both were stored under the same conditions. All K99-positive ETEC strains of serogroup O101 were able to give both the anionic and the cationic component in double diffusion experiments against heterologous OK antiserum.

Anti-K99 antiserum prepared in rabbits from ETEC strains of serogroups other than 0101 was able only to inhibit the cationic precipitin line given by strains of serogroup 0101 (Morris, Stevens and Sojka, 1978; Isaacson, 1978).

Antibodies to the cationic haemagglutinin have been demonstrated in antisera to all K99-positive ETEC strains in every O-group examined (Morris, Thorns and Sojka, 1980). Antibodies to the anionic haemagglutinin have only been detected in antisera to ETEC strains of serogroups 09 and 0101. Morris et al. (1980) have been able to transfer the cationic component of K99 alone to an E.coli K12. This K12 (K99) strain gives MRHA of sheep RBC and adheres to calf brush border preparations. Growth of the strain at 18 C inhibits the MRHA caused by both of these components.

Recently Faris, Lindahl and Wadström have demonstrated that the K99 antigen adheres to a ganglioside (GM_2)-like erythrocyte receptor. The receptor is not GM_2 because adherence of K99 to erythrocytes is not inhibited by neuraminidase, which is known to block GM_2. K99 antigen could probably adhere to glycoconjugates resembling GM_2 in intestinal epithelial cells.

Kehoe, Sellwood and Dougan (1980) have studied a K99 hybrid plasmid pWD010 which consists of a 6 kilobase BamHI fragment linked to the carrier plasmid pBR322. The expression of the K99 plasmid in minicells was found to be associated with three polypeptides of similar molecular weight to those produced by K88. The role of these polypeptides in the expression of K99 is still under investigation.

Ig.2c. Colonisation factor antigens (CFA) I and II

When ETEC strains were isolated from humans with cholera-like diarrhoea and were shown to colonise the anterior intestine of human volunteers and laboratory animals, investigators became interested in the presence in these strains of colonisation factors (Drucker, Yeivin and Sacks, 1967; DuPont et al., 1971; Levine et al., 1977). A few human ETEC have been tested for K88, K99 and 987P and only one of serotype O149 was found to produce K88 (Moon et al., 1977; Guinée and Jansen, 1979; Ørskov and Ørskov, 1977).

Evans et al. (1975) reported the presence of a pilus-like surface antigen in an ETEC strain of serotype O78H11 (H-10407) isolated from an adult with cholera-like diarrhoea in Bangladesh. A small inoculum (10^5 bacteria) of H-10407 was able to proliferate rapidly in the anterior intestine of rabbits, causing diarrhoea in these animals. This strain was also able to cause dilation of ligated rabbit intestinal segments and fluid accumulation in the intestine of live infant rabbits. H-10407 was capable of producing both LT and ST enterotoxins (DuPont et al., 1971).

Evans et al. (1975) found a laboratory derivative of H-10407 that was not able to colonise the anterior intestine of rabbits or to cause dilation of rabbit gut loops. Antiserum prepared in rabbits with strain H-10407 was extensively absorbed with this non-colonising strain (called H-10407P). This absorbed antiserum was shown to agglutinate strain H-10407 but not H-10407P. The antiserum could also neutralize the colonising effect of H-10407 in the intestine of infant

rabbits and the dilation of rabbit gut loops.

Studies by electron microscopy of strains H-10407 revealed the presence of numerous, thin, pilus-like filaments 8 to 9 nm in width and considerable variation in length (Evans et al., 1975). H-10407 was grown on solid media to inhibit the production of type 1 pili before negative staining for electron microscopy. Strain H-10407P did not possess pilus-like structures surrounding the bacteria, although both H-10407 and H-10407P had easily identifiable flagella. Treatment of these preparations with absorbed H-10407 antiserum demonstrated that only pili from H-10407 were coated with antibody, while H-10407P did not react with the antiserum. These studies clearly showed that strain H-10407 possessed a pilus-like antigen associated with the ability of the strain to colonise the intestine. This pilus-like antigen was called colonisation factor antigen I (CFA/I) by Evans et al. (1975). Strain H-10407P would thus be a CFA/I-negative variant.

Because of the spontaneous loss of CFA/I, Evans et al. (1975) decided to study the plasmid content of strains H-10407 and H-10407P. With partially purified DNA preparations these authors found three distinct plasmids with approximate molecular weights of 60×10^6, 42×10^6 and 3.7×10^6 in strain H-10407. The sizes of these plasmids were confirmed by the sedimentation profile of the circular DNA of H-10407 in neutral sucrose gradient and by contour length measurements of purified plasmid DNA by electron microscopy. Gyles, So and Falkow (1974) had found very similar results when they first tested strain H-10407.

In these studies, Evans et al. (1975) demonstrated that strain H-10407P contained only two plasmid DNA forms with approximate molecular weights of 42 x 10^6 and 3.7 x 10^6. When derivatives of H-10407 with and without the 60 x 10^6 plasmid were tested for their ability to colonise the anterior intestine of rabbits, only those with the plasmid were found positive. Loss of the 60 x 10^6 plasmid from H-10407 was also accompanied by a decrease in the ability of the strain to cause rabbit gut loop dilation (Evans et al., 1975). This was due to the inability of H-10407P to colonise the rabbit small intestine, since both H-10407 and H-10407P were able to produce enterotoxin.

Apart from H-10407, CFA/I was initially only found in ETEC strains of serotype 078:H12 (Ryder et al., 1976). Ørskov and Ørskov (1977) tested 77 human ETEC strains in 12 O:H serotypes for the presence of CFA/I with a slide coagglutination test using absorbed H-10407 antiserum coupled with protein A-containing staphylococci to make the antigen-antibody reaction more specific. They found 17 ETEC strains positive for CFA/I, all 17 belonged to serotypes 078:H-, 078:H11 and 078:H12.

Ørskov and Ørskov (1977) tested the 77 human ETEC strains for MSHA and MRHA of human, ox and guinea-pig RBC. All the 078 ETEC strains possessing CFA/I were shown to cause MRHA of human RBC. Six of 17 CFA/I-positive 078 strains and four out of 12 06:H16 ETEC strains also gave MRHA of ox RBC. The four 06:H16 strains did not cause MRHA of human RBC. Three

out of six ETEC strains of serotype O25:K7:H42 were also able to cause MRHA of human RBC but these were negative for CFA/I by coagglutination. Forty-six of the 77 ETEC tested gave MSHA of guinea-pig RBC indicating the presence of type 1 pili in these strains. The two O149:K88:H10 ETEC strains tested gave MRHA of guinea-pig RBC due to the presence of the K88 antigen. These authors also showed that CFA/I positive H-10407 caused MRHA of human and calf RBC while the CFA/I negative H-10407P did not.

Using ETEC strains isolated from a group of students from the United States attending Summer courses in a Mexican university, Evans, Evans and Tjoa (1977) found that all ETEC strains causing MRHA of human RBC were CFA/I-positive. As with K99, the use of a semi-synthetic medium increased the production and detection of CFA/I. The CFA/I haemagglutinin was thermolabile at 65 C and its possession correlated with ability to adhere to infant rabbit small intestine. In this study ETEC strains of serotypes O20:H- (later corrected to O63:H- by Evans et al., 1978), O25:H? and O25:H42, besides O78H11, were shown to produce CFA/I. Loss of CFA/I in these strains was always accompanied by loss of MRHA ability, thus confirming the identity between the two.

In a survey of travellers' diarrhoea Evans et al. (1978) found that CFA/I positive ETEC strains were found in 25 out of 29 (18%) patients with diarrhoea, as compared with only two out of 11 (18%) of ETEC strains isolated from asymptomatic controls. The high percentage of CFA/I positive strains could have been due to the isolation of the same strain from several

common source outbreaks or from cross-infections between the travellers', both cases involving a few ETEC serotypes. Unfortunately, in this study no serotyping of the ETEC strains was done.

Freer et al. (1978) demonstrated the presence of CFA/I by MRHA of human RBC and bacterial agglutination with an absorbed H-10407 antiserum in five out of 89 ETEC strains isolated from infants with diarrhoea in Ethiopia. The five CFA/I-positive ETEC strains belonged to serotypes O78H- and O78H12.

These investigations have shown that CFA/I shares many characteristics with K88 and K99. CFA/I is a plasmid-controlled, thermolabile, pilus-like antigen of protein nature which enables the ETEC strains to cause MRHA of human RBC and to adhere in vivo and in vitro to rabbit intestinal epithelial cells (Table 2). Specific antiserum against CFA/I, growth of CFA/I-positive strains at 18C, and loss of a 60×10^6 plasmid in strain H-10407 inhibits or prevents all these reactions (Evans et al., 1975; Evans, Evans and Tjoa, 1977; Evans et al., 1978a).

The role of CFA/I in human diarrhoea was further studied by Satterwhite et al. (1978) using adult human volunteers. Seven healthy adults were fed 10^6 organisms of strain H-10407. Seven other volunteers received 10^6 organisms of strain H-10407P in the same way. Both strains were fed to the volunteers in a double-blind fashion. A second study was carried out seven weeks later in the same manner, except that seven and six volunteers were fed strains H-10407 and

H-10407P, respectively, at doses of 10^8 organisms.

In the first experiment one volunteer fed 10^6 organisms of H-10407 and one volunteer fed 10^6 organisms of H-10407P each had a watery stool on the 4th and 3rd day respectively. The other volunteers in each group remained symptomless. The shedding of organisms in each group was different. The organisms appeared in the faeces promptly, but while H-10407 continued to be excreted until the end of the study, strain H-10407P was shed only up to the third day after feeding the strains.

In the second experiment out of seven volunteers fed 10^8 organisms of H-10407, six (86%) developed watery diarrhoea. None of the volunteers receiving 10^8 organisms of H-10407P had diarrhoea. The shedding of the challenge strain was similar to the results found in the previous experiment.

In the first experiment only two patients who received 10^6 organisms of strain H-10407 presented a fourfold or greater rise in anti-CFA/I and anti-LT antibodies. No rise in antibodies against CFA/I or LT were found in volunteers receiving strain H-10407P.

When the volunteers were fed 10^8 organisms of strains H-10407 and H-10407P only the former showed sero-conversion but this was not consistent in all the students in the volunteer study. Two volunteers responded to the 078 and LT but not to CFA/I. One individual with the highest pre-challenge titre of anti-078 antibodies did not respond to the 078 somatic antigen but responded to LT and CFA/I. The student with the highest pre-challenge anti-LT antibodies showed no response

to LT but showed seroconversion against O78 and CFA/I. The two other volunteers responded to O78, LT and CFA/I antigens.

The CFA/I pili of strain H-10407 have been purified by Evans et al. (1979) using ammonium sulfate precipitation and DEAE sephadex column chromatography. The purified CFA/I pili have an average molecular weight of 1.6×10^6. The pili are composed of protein subunits of molecular weight 23,000 with an N-terminal value, 37% hydrophobic amino acid residues, and eleven residues of proline per molecule. The purified CFA/I pili retain their ability to cause MRHA of human and bovine RBC and their agglutinability with absorbed H-10407 antiserum. The antigen retained its affinity for rabbit small intestine epithelial cells after purification and was able to inhibit adhesion of CFA/I-positive bacteria to rabbit intestinal cells.

With this purified CFA/I preparation, Clegg, Evans and Evans (1980) have recently developed an ELISA test to quantitate the humoral response to infection by CFA/I-positive organisms. Paired sera from patients ill with diarrhoea caused by CFA/I-positive strains of ETEC showed an increase in anti-CFA/I antibodies of the immunoglobulin-G class detectable by this ELISA test.

Klemm (1979) has also described a purification method for the CFA/I fimbriae of H-10407 using SDS-polyacrylamide gel electrophoresis. This purified CFA/I preparation had a subunit molecular weight of approximately 14,500 or slightly higher value depending on whether 15% or 10% acrylamide gels were used. Amino acid composition of the CFA/I fimbrial

protein showed an approximate molecular weight of 14,500. The difference in MW between this protein and the one obtained by Clegg and his coworkers is probably due to differences in the purification methods employed. The protein had a high content of hydrophobic amino acids and contained no cysteine residues, very similar to K88 (Mooi and de Graff, 1979). Analysis of the N-terminal sequence of CFA/I demonstrated that this part of the antigen was completely different from K88 and type 1 pili (Klemm, 1979). The C-terminal sequence does not show any similarities with K88 either.

Like K88 and K99, Wadström et al. (1978) have demonstrated that CFA/I possesses surface-associated hydrophobic properties, which are not found in CFA/I-negative strains. HIC thus provides another assay to test the adherence of these piliated strains in vitro.

The attachment of CFA/I seems to be more specific than simple attraction of force fields. Searching for a specific cell receptor Faris, Lindahl and Wadström (1980) have used the ability of CFA/I to agglutinate human red blood cells to show how purified ganglioside preparations are able to inhibit bacterial adhesiveness. Their experiments show that only purified GM_2-ganglioside was able to inhibit the MRHA caused by CFA/I. N-acetylneuraminic acid and trypsin were not able to inhibit this MRHA reaction, while treatment with pronase did. This suggests that both glycolipids and glycoproteins may be involved in the adherence of CFA/I to erythrocyte receptors. Similar structures may be involved

in the adherence of this adhesive factor to epithelial intestinal cells.

A second colonisation factor antigen (CFA/II) was described by Evans and Evans (1978) in ETEC strains belonging to serogroups O6, O8 and O85. This second colonisation factor did not cross-react serologically with CFA/I, although both were found capable of causing MRHA of calf RBC. CFA/II was not able to cause MRHA of human RBC. Ørskov and Ørskov (1977) had already reported that ETEC of serogroup O6 caused MRHA of ox RBC. This haemagglutination had to be carried out at 4 C to be detectable. A rise in temperature caused the reaction to elute.

Like CFA/I, CFA/II has been found to be a thermolabile, pilus-like surface antigen that enables the bacteria to adhere to the anterior intestine and causes diarrhoea in infant rabbits (Table 2). CFA/II positive ETEC strains can be detected by slide agglutination and immunodiffusion techniques using a specific CFA/II rabbit antiserum. This antiserum can also inhibit the adherence of CFA/II positive ETEC strains to rabbit intestinal epithelial cells.

CFA/II positive ETEC strains that spontaneously lose the ability to cause MRHA of calf RBC are not agglutinated by the CFA/II antiserum and electron micrographs show that the bacteria have lost the surface-associated pili. CFA/II negative variants are unable to colonise the anterior intestine or to cause diarrhoea in infant rabbits.

Most CFA/II positive ETEC strains are able to produce ST

and LT. When CFA/II is lost spontaneously the ETEC strains generally, but not invariably, lose also the ability to produce either or both enterotoxins (Evans and Evans, 1978).

CFA/II positive ETEC strains have been isolated from well defined common source outbreaks of diarrhoea in infants and adults (Rosenberg et al., 1977; Rowe et al., 1978), and from cases of travellers' diarrhoea (Evans and Evans, 1978; Levine et al., 1980). These studies provide epidemiological evidence of the important role played by this new colonisation antigen in cases of human diarrhoea.

Most of the ETEC strains of serogroup O6 have been found to possess K antigen 15. K15 is a polysaccharide antigen, not a protein like K88 or K99. This K antigen is probably not associated with the adhesive properties in ETEC strains; however, the combination of O6 and K15 antigens in the same ETEC strain is a useful tool to characterise pathogenic ETEC strains in humans. Non-toxigenic E.coli strains have been rarely found to have both antigens (Scotland, Rowe and Gross, 1977; Kaijser, Svanborg-Edén and Wadström, 1978).

Both CFA/I and CFA/II-positive ETEC strains have been found to be antigenic in man. Deetz et al. (1979) have studied the serological response to bacterial antigens present in ETEC strains in an epidemiological study of travellers' diarrhoea. Paired sera from 60 United States students doing Summer courses in a Mexican University were tested for rises in antibody titres to eight E.coli somatic antigens (O6, O8 O15, O25, O63, O78, O111 and O159), to LT and to colonisation factor antigens I and II. Five paired sera showed a fourfold

or greater antibody rise to specific O antigens. Four of these students had been ill with diarrhoea and in three an ETEC strain had been isolated. Twelve of the 60 paired sera showed a response to the colonisation factor antigens, five to CFA/I and nine to CFA/II, with two showing rises to both antibodies. Seven out of 14 (50%) students had an antibody response to LT and to one or both of the colonisation factors. Eight out of 23 (35%) students with isolation of ETEC strains had an antibody rise to CFA. Antibody responses to LT and CFA correlated significantly with recovery of an ETEC in diarrhoeal patients.

Deneke, Thorne and Gorbach (1979) have reported that ETEC strains causing MRHA are able to adhere to human buccal cells more frequently than MRHA negative strains. Candy et al. (1978) have also studied the adherence of E.coli strains isolated from cases with diarrhoea to human buccal cells. Wadström et al. (1980) have studied the ability of ETEC strains possessing CFA/I and CFA/II to adhere to isolated human intestinal cells. These in vitro systems are useful to investigate the adhesive mechanisms present in these E.coli strains.

Strains of E.coli isolated from sources other than the intestine have also been found to cause MRHA of human RBC (Duguid, Clegg and Wilson, 1979). Minshew et al. (1978a) have studied 142 E.coli strains isolated from blood, urine, sputum and other extraintestinal sources and 29 faecal E.coli isolates, nine of which belonged to EPEC serotypes, for the production of haemolysin, colicin V, MRHA of human

and chicken RBC and for their ability to kill chick embryos (virulence). The production of colicin was similar in intestinal and extraintestinal E.coli.

The production of colicin V was demonstrated only in extraintestinal isolates. Thirteen (59%) of the extraintestinal E.coli were virulent for chicken embryos, while only two of the faecal E.coli strains were virulent. Haemolysin production was found in 85% of the extraintestinal E.coli and in only one faecal E.coli. Of the extraintestinal E.coli 31% caused MRHA of chicken RBC and 92% caused MRHA of human RBC. Only one faecal E.coli caused MRHA of human RBC and six caused MRHA of chicken RBC.

The ability of these extraintestinal strains to produce colicin V, haemolysin and MRHA probably enables them to invade epithelial tissues and cause infections outside their intestinal niche (Smith, 1963; Smith, 1974; Minshew et al., 1978b). However, the transfer of plasmids coding for colicin V and haemolysin to an E.coli K12 were not enough to make this strain virulent to chick embryos (Minshew et al., 1978b). Other factors coded by the same plasmid that codes for colicin V, such as iron sequestring proteins, have been shown to be more related to the pathogenic ability of these strains (Binns, Davies and Hardy, 1979).

The ability to cause MRHA of human or chicken RBC in these extraintestinal E.coli was not found to be controlled by plasmid DNA (Minshew et al., 1978b). The relationship between the mannose-resistant haemagglutinins in ETEC strains and in extraintestinal E.coli has not been studied.

EPEC strains have been shown to adhere to the mucosal surface of the intestine in some infants who have died of enteritis (Drucker *et al.*, 1970). The adhesive factors enabling these strains to adhere are poorly understood. McNeish *et al.* (1975) had studied EPEC, ETEC and non-pathogenic *E.coli* strains for their ability to adhere to isolated foetal intestinal tissue *in vitro*. With this assay these authors have shown that pathogenic strains adhere significantly more to the epithelial cells than non-pathogenic strains. Three of the pathogenic strains, one EPEC and two ETEC strains, used by McNeish *et al.* were able to cause MSHA of guinea-pig RBC but this ability did not correlate with the attachment to foetal intestinal cells. Only one of the human *E.coli* strains was able to adhere to brush border preparations from pigs, calves, rabbits or guinea-pigs.

The capacity of the EPEC strain, used by McNeish and his co-workers in the previous study, to adhere to human foetal intestinal cells was shown to be plasmid mediated (Williams *et al.*, 1978). Strain H19 (*E.coli* O26:K60:H11) was able to transfer to *E.coli* K12 the ability to adhere to isolated foetal intestinal cells, together with production of colicin Ib (ColIb) on a 56×10^6 plasmid. Initial experiments also showed the cotransfer of a multiple antibiotic-resistance plasmid with the adhesive ability and ColIb production. Conjugational experiments of limited duration showed that the multiple drug resistance determinant was coded by a different plasmid to the one coding for ColIb and adhesive ability. There was, however, no segregation of the adhesive determinant

from the ColIb production. Plasmid analysis of transconjugants carrying the ColIb- adherence phenotype confirmed the presence of a single 56 x 10^6-dalton plasmid. The presence of this ColIb- adhesive plasmid in other EPEC strains and its importance in the pathogenesis of human diarrhoea remains to be determined.

Ih. Scope of the present study

Epidemiological studies during the last forty years have demonstrated the importance of strains of E.coli in the aetiology of infectious diarrhoea in humans. The production of enterotoxins and the ability to adhere to or to invade epithelial cells are factors that contribute to the pathogenicity of these strains. The principal aim of this investigation was to study the presence, function and genetic control of known or suspected adhesive factors in strains of E.coli of human origin.

Although K88 and K99 had already been extensively studied when this investigation was started, the only known adhesive factor in strains of E.coli of human origin, at the time, was the colonisation factor antigen (CFA) I. The initial part of this work was therefore to survey ETEC and EPEC strains isolated from humans with diarrhoea for the presence of CFA/I.

CFA/I, as confirmed serologically, was only detected in ETEC strains able to cause MRHA of human and calf RBC. Other ETEC strains examined gave MRHA of human and/or calf RBC but did not possess CFA/I. These strains were examined serologically to determine whether other adhesive factors were present. Some ETEC strains which caused MRHA of only calf RBC were found to possess an adhesive factor identical to one described by Evans and Evans (1978) and called CFA/II. A third serologically distinct haemagglutinin (E8775) was found in ETEC strains causing MRHA of human and calf RBC that did not possess CFA/I or CFA/II. None of the EPEC strains initially tested possessed CFA/I, CFA/II or the E8775 haemagglutinin.

A large selection of strains of E.coli isolated from extraintestinal sources (blood, urine and cerebro-spinal fluid) in humans were examined for their ability to cause MRHA of human and/or calf RBC, and for the

presence of CFA/I and CFA/II. Approximately 50% of the extra-intestinal E.coli strains studied caused MRHA but did not possess either CFA/I or CFA/II.

The biochemical characteristics of CFA/I and CFA/II positive strains and laboratory-derived spontaneous mutants of these strains lacking the colonisation factors were studied. No relationship was found between the adhesive factors and any specific biochemical character. The biochemical typing of CFA/II positive strains, however, was found to be related to different serological types of the same colonisation factor.

Electron microscopy studies with negatively stained preparations of CFA/I and CFA/II positive strains and their derivatives lacking the colonisation factors showed distinct morphological differences in the number of fimbriae. Coating of the specific CFA fimbriae with specific antisera distinguished them from type 1 pili.

Loss of CFA/I was observed to be always accompanied by loss of ST production. A plasmid coding for the production of CFA/I and ST was found in several ETEC strains. Loss of this plasmid in a $CFA/I^{+}ST^{+}$ positive strain was accompanied by loss of both of these characters. A plasmid coding for CFA/I and ST was transferred for the first time during the present study from a wild-type ETEC strain of serotype O78:H12 to an E.coli K12.

Loss of CFA/II and the E8775 haemagglutinin was not found to be consistently related to loss of ST and/or LT. Loss of CFA/II and the E8775 haemagglutinin was accompanied by loss of ST and LT in some strains, while others could lose the adhesive factor and the ability to produce enterotoxins separately. Indirect evidence of a plasmid controlling the production of CFA/II and the E8775 haemagglutinin was found. Transfer of these plasmids, however, was not possible.

Since none of the EPEC strains examined possessed CFA/I, CFA/II or the E8775 haemagglutinin, a new *in vitro* assay using HEp-2 tissue culture cells was designed to test the adhesive ability of these strains. Approximately 80% of the EPEC strains studied adhered to the HEp-2 cells. The adhesive assay was found to be dependent on the concentration of bacteria used and the temperature at which the test was carried out. Substances that interfered with the production or expression of type 1 pili were not found to inhibit the adhesion to HEp-2 cells.

The adhesiveness to HEp-2 cells was not transferred by conjugation from wild-type EPEC strains to *E.coli* K12, and the character was not found to be plasmid mediated. Transfer of other plasmids that conferred the ability to produce cytotoxins, colicin or antibiotic resistance from wild-type EPEC strains to *E.coli* K12 did not enable the transconjugants to adhere to the HEp-2 cells.

The HEp-2 adhesive assay can be used for the selection and study of *E.coli* strains possessing adhesive factor(s) different from those already described, and preferentially found in EPEC strains. The assay also provides a good *in vitro* model of EPEC adhesion to study how these strains attach and colonise epithelial surfaces.

II. MATERIALS AND METHODS

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IIa. Organisms

The *E.coli* strains examined were from the culture collection of the Division of Enteric Pathogens (DEP), Colindale, where they had been maintained on Dorset egg medium at room temperature. They were identified as *E.coli* by biochemical tests (Cowan and Steele, 1965) and were serotyped using antisera for *E.coli* O groups 1 to 164 and for flagellar antigens H1 to H56 (Ørskov and Ørskov, 1975). This identification was carried out in the biochemistry and serology sections of the DEP.

IIb. Enterotoxin testing

Bacterial cultures were grown in 10 ml trypticase-soy broth (BBL) in Erlenmeyer flasks (250 ml) with shaking at 37 C for 24 h. After centrifugation sterile culture supernatants were prepared using Millipore filtration (0.45 µm). Bacterial filtrates were stored at 4C and tested for ST, LT and VT within 2 days.

IIb.1. ST testing

ST production was determined by the infant mouse test (Dean *et al.*, 1972). Four-day old mice from an inbred laboratory strain were used for the test. The animals were maintained with their mothers until they were used in the test. Two to four mice were used for each test. One drop of 2% (w/v) Pontamine blue in distilled water was added to 0.5 ml of bacterial filtrate, and 0.1 ml of the dyed filtrate was infected directly into the stomach of each animal. After

incubation for 4 h at 30 C the mice were killed with chloroform, and the intestines examined for distension before removal from the duodenum to the rectum. The intestines from each group of mice were weighed together and the ratio of gut-weight to remaining body-weight (G/B) was determined. A G/B ratio above 0.1 was considered positive and a G/B ratio below 0.08 was considered negative. Values between 0.08 and 0.1 were considered doubtful and were repeated.

IIb.2. LT testing

The tests for LT production used the Y1 (Donta, Moon and Whipp, 1974) and CHO (Guerrant et al., 1974) tissue culture systems.

The Y1 cells (ACTC) were maintained in plastic flasks (Nunc) of 250 ml at 37 C in Ham's F10 medium (Flow) with the addition of 12.5% (v/v) horse serum, 2.5% (v/v) foetal calf serum, 0.5 ml of glutamine/ 100 ml, 100 µg of penicillin/ml, 100 µg of streptomycin/ml, and 100 µg of amphotericine B/ml (all obtained from Flow Labs.). The medium was changed twice weekly and the cells passaged once every week.

For the LT test the cells were rinsed twice with phosphate buffered saline (PBS, 300 mOsmol/liter, pH 7.2) and removed from the flask with 1 ml of 0.05% (v/v) trypsin (Flow) and 0.02% (v/v) Versene (Flow). They were resuspended in the same Ham's F10 medium described above. The cells were counted in a Neubauer haemocytometer and diluted to give a final concentration of 5×10^4 cells per ml. 0.2 ml of this suspension was added to each of 96 wells of a flat bottom microtest tissue culture plate (Falcon). The plates were sealed with plastic film and incubated for four days at 37 C. The plates were examined for a continuous layer of cells in each well after the four days, with an inverted Olympus microscope model CK.

After 4 days the medium was replaced with 0.2 ml of fresh Ham's F10 medium as described above and 0.02 ml of the sterile bacterial filtrate was added to each well. Aliquots of the bacterial filtrate heated at 100 C for 15 min were also tested as controls. The microtiter plates were sealed with plastic film and incubated at 37 C for 24 h.

After this period the medium was removed, the cells were fixed with pure methanol for 5 min and stained with 20% (v/v) Giemsa (BDH) for 60 min, washed twice with distilled water and dryed.

The plates were examined by microscopy and the percentage of rounded cells was estimated. Values above 20-30% were considered positive.

A modification of the Y1 test described by Sack and Sack (1975) was used to screen large number of possible LT positive single colonies. In this "mini" Y1 test, the strains were grown in 0.5 ml of syncase medium with glucose at 37 C for 18 h without aeriation. The Y1 monolayer was prepared as above in microtiter plates. After 4 days the medium was changed and 0.2 ml of fresh F10 medium without antibiotics was added to each well. A total of 0.05 ml of the bacterial culture in syncase medium was also added to each well. After 10 min at room-temperature the medium with bacteria was removed, the monolayer was washed with PBS, and 0.2 ml of F10 medium with 100 µg of gentamicin was added to each well. The test was the continued as described above.

The CHO cells (ACTC) were maintained in 100 ml glass medical flats at 37 C in Ham's F12 medium (Flow) with 10% (v/v) foetal calf serum, 0.5 ml of glutamine/100 ml and 100 µg of penicillin, 100 µg of streptomycin and 100 µg of amphotericin B per ml (all obtained from Flow Labs). The cells were passaged twice weekly.

For the LT test the cells were rinsed twice with cold PBS pH 7.2 and removed from the glass with 1 ml of 0.25% (v/v) trypsin (Flow). They were resuspended in Hams F12 medium as described above except that the concentration of foetal calf serum was reuced to 1% (v/v). The cells were counted in a Neubauer haemocytometer and diluted to give a final concentration of 5×10^3 cells per ml. 0.05 ml of bacterial filtrate and 0.2 ml of the CHO cell suspension were added to each well of a plate for microtiter tissue culture test with flat bottoms (Falcon). The plates were sealed with plastic film and incubated at 37 C for 18 h. Aliquots of the bacterial extracts heated at 100 C for 15 min were tested simultaneously as controls.

After this incubation period the medium was removed. The cells were fixed with pure methanol for 5 min, stained with 20% (v/v) Giemsa for 60 min, washed twice with distilled water and dryed. The plates were then examined by microscopy and the percentage of elongated cells counted. More than 20% of elongated cells was considered positive.

IIb.3. Vero cytotoxin (VT) testing.

The test for VT production used African green monkey kidney cells (VERO) from a continuos line used in the Standards Reference Laboratory at Colindale. The test is a modification of the method of Konowalchuk, Speirs and Stavric (1977) by Scotland, Day and Rowe (1980). VERO cells were maintained in 100 ml glass medical flats in 199 medium (modified) with Earles salts (Flow), with 10% (v/v) foetal calf serum, 0.5 ml glutamine/100 ml, 100 µg of penicillin/ml, 100 µg of streptomycin/ml and 100 µg of amphotericin B/ml (all from Flow Labs). The

cells were passaged twice weekly.

For the VT test the cells were rinsed twice with cold Dulbeco's PBS pH 7.2 and removed from the glass with 0.25% (v/v) trypsine (Flow) and 0.02% (v/v) Versene (Flow). The cells were resuspended in 199 medium as described above and counted in a Neubauer haemocytometer. They were diluted to give a final concentration of 5×10^4 cells/ml. 0.2 ml of the cell suspension was added to each of 96 wells of a flat bottom microtiter tissue culture plate (Falcon); the plates were sealed and incubated at 37 C for 3 days.

At this time, 0.02 ml of a test filtrate was added to the wells without changing the medium in which the cells had been seeded, and reincubated at 37 C for 4 days. The medium was then removed and the cells fixed with methanol for 5 min and stained with 20% (v/v) Giemsa. After 60 min the cells were washed twice with distilled water and dryed. The plates were then examined by microscopy for any cytotoxic effect. Strain H19 (E. coli 026:K60:H11) received from Dr. H. W. Smith was used as a positive control for VT.

IIc. Haemagglutination tests

Test strains were grown overnight at 37 C on slopes of 2% (w/v) agar containing 1% Casamino acids (Difco) and 0.15% yeast extract (Difco) plus 0.005% $MgSO_4$, and 0.0005% $MnCl_2$. Evans, Evans and Tjoa (1977) have shown maximal production of the colonisation factor antigens using this CFA agar medium. The bacterial growth was suspended in 0.15M solution of NaCl (saline) to give a concentration of 2×10^9 bacterial per ml.

Fresh human group A and guinea-pig blood were obtained by venopuncture and cardiac puncture, respectively, and suspended in 0.4% (w/v) solution of sodium citrate in distilled water. Fresh calf whole blood in acid citrate dextrose was obtained from Tissue Culture Services. The bloods were washed 3 times with PBS pH 7.4 and resuspended in PBS to made a 50% (v/v) stock suspension. From this stock suspension a 3% (v/v) suspension in saline and saline with 0.5% (w/v) D-mannose was prepared for use in the haemagglutination tests. The bloods were kept at 4 C up to 7 days.

A total of 0.3 ml of the bacterial suspension and 0.3 ml of the 3% erythrocyte suspension in saline with and without mannose were mixed in a depression on a white porcelain tile. The tile was rocked by hand at room temperature for up to 10 min and haemagglutination was observed with the naked eye. If no haemagglutination was visible after 10 min, the tile was placed on ice for another 10 min and rocked intermittently. Agglutination of red blood cells in saline and saline with mannose was called mannose-resistant haemagglutination (MRHA), while agglutination of RBC in saline but not in saline with mannose was called mannose-sensitive haemagglutination (MSHA) (Duguid, Anderson and Campbell, 1966).

On a few occasions when a more quantitative result was desired, the haemagglutinations were carried out in 96 well microtiter plastic plates with rounded bottoms (Falcon). Bacterial cultures from CFA agar slopes were suspended in saline to a final concentration of 2×10^9 bacteria per ml. A 50 µl sample of the bacterial suspension was titrated in twofold steps in saline. A 50 µl portion of the 3% suspension of RBC was then added to each well. The plates were sealed and incubated at room temperature for 60 min. After this time the plates were examined for the presence of haemagglutination in a double

mirror viewer with the naked eye. The reciprocal of the highest dilution that showed haemagglutination was noted. MRHA was demonstrated using an erythrocyte suspension containing 0.5% (w/v) mannose (Jones and Rutter, 1974).

IId. Production of antiserum

Antisera were prepared in rabbits. Bacterial growth from two CFA agar slopes was resuspended in 20 ml of saline. Six hours before they were injected subcutaneously into the rabbits, the bacteria were killed with 0.1 ml of an 0.4% (v/v) formaldehyde solution. The animals were immunised subcutaneously every 5 days for 25 days. A total of 0.5 ml of the formalized bacterial suspension was injected on days 1 and 5, 1 ml on day 10, and 2 ml on days 15, 20 and 25. Five and ten days after the last injection 40 ml of blood were removed from the animals by ear vein puncture. Fifteen days after the last injection the rabbits were anaesthetized with intravenous pentobarbital and exanguinated by cardiac puncture.

The blood was allowed to clot for 4 to 8 h at room temperature and the serum was separated by centrifugation. The serum was then tested against the 164 E. coli O-groups for cross-reactions and the titer against the homologous O-group was determined. The sera were kept at -10 C in 25 ml universal glass bottles. For use the sera were thawed and kept at 4 C. To prevent bacterial contamination while in use, 0.05 ml of an 0.1% (v/v) solution of merthiolate was added to each thawed bottle.

Strain E6674 (E. coli 063:H-) was used as vaccine for the production of CFA/I antiserum. This strain was shown by immunodiffusion techniques to have an antigen identical to that described by Evans

et al. (1975) in strain H-10407 (E. coli 078:H11) and characterised as CFA/I. Specific CFA/I antiserum was prepared by absorption with strain E6674-H1, a laboratory-derived variant of E6674 lacking CFA/I.

E. coli strains E219a/69, E1392, and E4833 of serotype 06:H16, and E7463 of serotype 08:H9 were used as vaccine strains for the production of CFA/II antisera. Strain E1392 was shown by immunodiffusion techniques to have an antigen identical to that described by Evans and Evans (1978) in strain PB-176 (E. coli 06:H16) and called CFA/II. The other E. coli strains of serotype 06:H16 and serotype 08:H9 used in this study were chosen because they belonged to a different biochemical biotype to that of E1392 and showed serological differences to E1392 when tested by immunodiffusion techniques. Specific CFA/II antisera were prepared by absorption with laboratory-derived variants from these strains lacking CFA/II.

E8775 (E. coli 025:H42) was used as vaccine strain for the production of an E8775 antiserum. This strain possessed a haemagglutinin serologically different from CFA/I and CFA/II when tested by immunodiffusion. A specific E8775 antiserum was prepared by absorption with a laboratory derived variant of E8775 lacking the haemagglutinin.

IIe. Slide agglutination and double immunodiffusion techniques.

All strains to be tested for the presence of colonisation factor antigens by slide agglutination and double immunodiffusion were grown on CFA agar slopes at 37 C for 18 h. For slide agglutination, bacterial growth was suspended in saline to a final concentration of 2×10^9 bacteria per ml. Tests were carried out on a glass slide at room temperature; 0.05 ml of the bacterial suspension was mixed with 0.05 ml

antiserum and examined with the naked eye for visible agglutination.

Antigens for double immunodiffusion tests were prepared by simple saline extraction. Bacterial growth from one 15 cm CFA agar plate was suspended in 2.5 ml of saline and heated at 60 C for 20 min. Aliquots of this suspension were further heated at 100 C for 60 min (Ørskov et al., 1977). The extracts were kept at 4 C for up to 7 days. Double immunodiffusion tests were performed according to Ouchterlony (1949). Glass slides 57 x 70 mm (Gelman) were overlaid with 9 ml of 1.5% (w/v) Noble agar (Difco) in 0.06 M Veronal buffer (2.47 g of sodium barbitone, 0.154 g of calcium lactate, and 0.02 g of sodium azide per 100 ml of distilled water, pH 8.6). Wells 3 mm in diameter were routinely used with an intercentre distance of 7.5 mm. Wells were filled with 50 µl of antigen or antiserum preparations and incubated at 37 C for 18 h. Drawings of the precipitates were done after this incubation period. After a second 18 h incubation period at 37 C the final results were recorded. In some cases after 36 h of incubation the slides were washed in saline for 48 h and then in distilled water for a further 48 h period. At this time they were dried and stained with Commasie blue (5 g, Kenacid blue R (BDH); 450 ml, ethanol; 100 ml, 1 M glacial acetic acid; and 450 ml, distilled water) at 37 C for 30 min. The slides were destained with 7% (v/v) acetic acid in distilled water (Weeke, 1973).

IIf. Biotyping

Bacterial strains were tested for their ability to decarboxylate arginine, lysine or ornithine using the Moeller method and for the ability to utilise mucate and to ferment adonitol, arabinose, cellobiose, dulcitol, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sorbose,

sucrose, trehalose, and xylose. Urease production was tested in Christensen's urea slopes (Edwards and Ewing, 1972). Results of decarboxylation tests were recorded for 4 days; results of other tests were recorded for 14 days.

IIg. Tests for resistance to antibacterial drugs

Resistance to ampicillin (Ap), chloramphenicol (Cm), gentamicin (Gm), neomycin (Ne), kanamycin (Km), streptomycin (Sm) and tetracyclines (Tc) was tested for by a diffusion method on nutrient agar (Difco) plates, using strips of blotting paper (Ford's 428 Mill) 80 x 7 mm impregnated with the respective drugs and freeze-dried. The cultures were streaked at right angles to the antibiotic strips, and control sensitive and resistant cultures were included in each test. Resistance to sulphonamides and trimethoprim was detected by spotting 0.01 ml drops of a 10^{-4} dilution of a late exponential phase broth culture of the test strain on nutrient agar containing 5% (v/v) of lysed horse blood, and either 100 µg/ml of sulphathiazole (Su) or 0.5 µg/ml of trimethoprim (Tm). Sensitive and resistant controls were always included. Nalidixic acid (Nx) was also detected by this method, the nutrient agar containing 40 µg/ml of the drug (Anderson and Threlfall, 1974).

IIh. Tests for colicin and haemolysin production

Colicin production was examined by the method of Fredericq (1957). Bacterial strains were grown at 37 C overnight in nutrient broth (Difco). The strains were stabbed with a straight wire into nutrient agar (Difco) plates and incubated overnight at 37 C. After this time the bacteria were killed by inverting the nutrient agar plates on a watch glass with chloroform for 15 min. The chloroform

was allowed to evaporate at room temperature for 30 min and the plates were overlaid with soft agar containing an indicator strain (E.coli ROW). The plates were incubated overnight at 37 C and examined for circles of inhibition denoting colicin production.

Haemolysin production was tested by plating the strains on newly poured nutrient agar containing 2.5% (v/v) horse blood.

IIi. Transfer of plasmids

(i) Direct. Two methods were used for conjugation experiments. For mating experiments where a wild-type strain was used as donor, 0.1 ml of overnight cultures of the donor and the recipient bacteria were inoculated into 10 ml of Hedley-Wright broth, and the mixture was incubated for 24 h at 37 C. For subsequent transfer experiments the donor and recipient cultures were grown in nutrient broth to a density of 2×10^8 cells per ml. For overnight crosses 1 ml each of donor and recipient cultures were used. Mating mixtures were plated on MacConkey agar or Diagnostic Sensitivity Test (DST) Agar containing the appropriate drugs to select the transconjugants and to counterselect against the donor.

For transfer of plasmids coding for LT, ST and CFA/I production together or as separate markers from wild-type strains, two lac^+ E.coli K12 substrains were used as recipients: 14R519 (= J62 Nal^r) and 10R151 (= AB1157 Sm^r). The mating mixtures were plated on MacConkey agar containing nalidixic acid (40 µg/ml) or streptomycin (500 µg/ml).

For transfer of an R factor to a CFA positive strain, the donor was counter-selected against with colicin E2, and the transconjugants were selected on MacConkey agar containing the appropriate drugs.

(ii) Mobilisation. Two methods were used for plasmid mobilisation experiments. In the first method 0.1 ml of overnight cultures of a donor strain to be tested for the possession of a transfer factor and a strain of E.coli carrying a non-autotransferring resistance plasmid were inoculated into 10 ml of Hedley Wright broth. After incubation for 24 h, 0.1 ml of this mixed culture and 0.1 ml of an overnight culture of the final recipient, E.coli K12-J53-1 lac^+ Nal^r, were inoculated into 10 ml broth and incubated at 37 C for 24 h. The final culture was plated on selective media as described above.

In the second method non-autotransferring plasmids in wild-type E.coli strains were mobilised by the derepressed transfer factors F-T and T-Δ drp (McConnell et al., 1979), both of which code for tetracycline resistance and R1-19K$^-$ which codes for resistance to ampicillin, chloramphenicol, streptomycin and sulphathiazole resistance. The transfer factors were introduced into the wild-type strains after overnight crosses as described above. Transconjugants were selected on MacConkey agar containing either tetracycline 10 µg/ml or chloramphenicol 20 µg/ml and colicin E2 to eliminate the K12 donor. A transconjugant carrying the derepressed transfer factor which was checked for the character to be transferred was used in a cross with an E.coli K12 lac^-Nal^+ as described above. Transconjugants were selected on MacConkey agar containing tetracycline (10 µg/ml) or chloramphenicol (20 µg/ml) and nalidixic acid (30 µg/ml).

(iii) Fertility inhibition. Cells carrying derepressed F-like factors produce F fimbriae that act as receptors for the F-specific phages that cause visible lysis of the cultures. Factors that cause fertility inhibition (fi^+) inhibit the synthesis of F fimbriae.

Strains carrying derepressed F factors alone normally give visible lysis when spotted with the F-specific phage μ2 (Anderson and Threlfall, 1974). Transconjugants carrying other plasmids besides derepressed F factors are $\underline{fi}^+$ and are not lysed by μ2. The strains to be tested for $\underline{fi}^+$ were grown for 2 h with aeriation at 37 C and spotted on to nutrient agar (Difco). After drying 50 μl of phage μ2 at routine dilution was added to each bacterial spot. After overnight incubation at 37 C the plates were examined for visible lysis. Those $\underline{fi}^+$ (not lysed by the μ2 phage) were tested for the acquired new character(s).

IIj. Formation of CFA plasmids with drug resistance markers

To mark with antibiotic resistance a non-autotransferring CFA plasmid in a wild-type E.coli strain, R factor R1-19K$^-$, which carries the ampicillin resistance transposon Tn3 and codes also for resistance to chloramphenicol, streptomycin and sulphathiazole (Cohen, 1976) was transferred into the strain. Transconjugants were grown in drug-free medium and examined by replication for loss of drug resistance markers. CFA positive lines were examined for ampicillin resistance but that had lost resistance to chloramphenicol, streptomycin and sulphathiazole.

IIk. CFA enrichment studies

The ability of CFA positive strains to cause MRHA was used to develop an enrichment method for the detection of this character in transconjugants. Cells from a final overnight cross were washed twice in 1/4 strength Ringer's solution (Ringer) and

resuspended in 1/10 of the original volume (10 ml) in Ringer. To this suspension was added 1 ml of a 3% (v/v) suspension of human or calf washed RBC and the mixture was incubated with gentle shaking at 4 C for 1h. The mixture was then transferred into 10 ml glass centrifuge tubes and centrifuged for 10 min in am MSE super minor centrifuge at 1,000 x g. The supernatant was discarded and the RBC pellet was resuspended in 10 ml of saline. This process was repeated four times. After the last centrifugation the supernatant was discarded and the RBC pellet was resuspended in 0.5 ml of pre-warmed distilled water to lyse the RBC. The lysed cells were then plated on MacConkey agar and incubated overnight at 37 C.

Because these enrichment studied did not yield positive results a second method described by Guinée et al. (1979) was then used. The final mating mixture was washed twice in Krebs-Ringer-Tris buffer (NaCl, 7.5 g/l; KCl, 383 mg/l; Mg $SO_4 \cdot 7H_2O$, 318 mg/ml; $CaCl_2$, 305 mg/l buffered with 0.01 M Tris-HCl, pH 7.4), and resuspended in Krebs-Ringer-Tris (KRT) buffer, pH 7.4, with 0.5% (w/v) D-mannose (KRTM). To this cell suspension was added 0.5 ml of a 10% (w/v) solution of D-mannose in KRT buffer and 1 ml of a 3% (v/v) suspension of human or calf RBC in KRTM. This mixture was shaken gently at 4 C for 2 h and then overlaid on 6 ml of a 15% (w/v) Ficoll 400 (Pharmacia, Sweden) solution in KRTM in a 5 ml polyallomer tube (Beckmann). The tubes were centrifuged at 400 x g for 5 min at room temperature in am MSE Super Minor centrifuge with a swing-out rotor. They were then immediately frozen at -70 C. The bottoms of the tubes were cut off with a sterile knife and the frozen sedimented RBC were transferred into

1 ml of prewarmed saline. 0.1 ml of this suspension was plated on MacConkey agar and the plate incubated overnight at 37 C.

III. Preparation of partially purified plasmid DNA

Wild-type strains of E.coli were grown in 50 ml volumes of nutrient broth (Difco) at 37 C in a shaker at 170 - 200 rpm. Bacterial growth was measured every 2 h by colorimetric readings until late exponential phase (c. 1 x 10^9 bacteria per ml). The bacteria were then centrifuged in an MSE High Speed 18 centrifuge at 12,000 x g for 3 - 4 min. The supernatant was discarded and the bacteria kept on ice until they were resuspended in 1.66 ml of 0.05 M Tris-HCl pH 8.0 containing 25% (w/v) sucrose. The bacterial suspension was transferred to a 10 ml polycarbonate centrifuge tube (MSE) and kept at 4 C. At 5 min intervals 0.33 ml of 0.25 M Tris-HCl pH 8.0 containing 5 mg of lysozyme (w/v) per ml and 0.67 ml of 0.25 M Tris-HCl pH 8.0 containing 0.093 mg of EDTA (w/v) per ml were added. After 5 min on ice the cell suspension was lysed with 2.7 ml of a detergent mixture containing 1% Brij 58 (Sigma), 0.4% sodium deoxycholate and 0.0625 M EDTA in 0.05 M Tris-HCl pH 8.0 (Clewell and Helinski, 1969). When lysis was accomplished, the tubes were centrifuged at 48,000 x g for 30 min at 4 C in an MSE Super Speed 65 centrifuge. The supernatant, or 'cleared lysate', was carefully poured into a 50 ml MacCartney bottle. TES buffer pH 8.0 (3 ml) was added to each bottle. TES saturated phenol was then added to the bottles to make a 50% solution (v/v). The solutions were shaken and left at 4 C for 18 - 24 h (Meyers et al., 1976). At this time the

MacCartney bottles were centrifuged in an MSE Super Minor bench centrifuge at 2,000 rpm for 3 min in a swing-out rotor. The top interphase was removed and 3 - 5 ml of TES buffer pH 8.0 and 2 volumes of TES saturated phenol were added. This process was repeated until a clear interphase was obtained. The solution was then transferred into 100 ml glass centrifuge tubes containing sodium acetate to make a 3 M solution and 2 volumes of cold ethanol were added. The tubes were covered and left overnight at -10 C. The next day the tubes were centrifuged in an MSE Super Minor bench centrifuge at 2,000 x g for 3 min in a swing-out rotor and the supernatant was discarded. The precipitate was resuspended in 0.8 to 1.0 ml of TES buffer pH 8.0 with a wide mouth pasteur pipette. The sediment was transferred to a sterile plastic tube with a plastic cap (Falcon) and the preparation was immediately frozen and kept until use at -70 C.

IIm. Agarose gel electrophoresis

Plasmid DNA preparations were analysed by electrophoresis in vertical slab gels (16 x 15 x 0.4 cm) which contained 0.75% (w/v) agarose (BDH electrophoresis grade). The electrophoresis buffer, pH 8.0 to 8.2, contained 89 mM-Tris, 2.5 mM-EDTA and 89 mM-boric acid (Willshaw et al., 1979).

Crude ethanol-precipitated DNA (2 to 100 µl) prepared as described above was mixed with a solution of 60% (w/v) sucrose and 0.25% (w/v) bromophenol blue in 0.03 M-Tris-HCl pH 8.0 containing 0.05 M-NaCl and 0.005 M-EDTA. Samples loaded on to gels contained 6 to 10% (w/v) sucrose. Electrophoresis was at a constant voltage at 140 V, with a voltage gradient of 8.7 V cm^{-1}, for 3 - 4 h at

room temperature. Gels were soaked for 30 min in a solution of ethidium bromide (0.5 $\mu g/ml^{-1}$, in distilled water) and viewed under long and short wavelength ultraviolet light (Chromatome cabinet, Ultra Violet Products). DNA bands were photographed on Polaroid type 52 Land film or Kodak Tri-X, using a x7 red filter.

The molecular sizes of the plasmids present in the wild type strains were determined by reference to the migration of plasmids of known molecular weight subjected to electrophoresis on the same gel (Meyers et al., 1976). The distance between the origin and the plasmid band was measured in the photograph of the gels obtained. The distance measurements obtained were plotted on logarithmic graph paper against the known molecular size of the following plasmids examined on the same gel: pBR322, 2.6×10^6; ColE1, 4.7×10^6; SSu, 5.7×10^6; TP193, 20.2×10^6; S-a, 25.9×10^6; TP120, 31.7×10^6; RP1, 37.8×10^6; TP125, 64×10^6; and TP129, 77.6×10^6. From the calibration line obtained with these values, the molecular size of the plasmids present in the wild-type strains were calculated by extrapolation from the distances measured in the photographs of the slab gels.

IIn. HEp-2 adhesive test

The HEp-2 adhesive assay was adapted from that described by Labrec et al. (1964), which used HeLa cells for the study of bacterial invasion.

The HEp-2 cells were maintained in 250 ml plastic tissue culture flasks (Nunc) at 37 C in 1 x basal medium Eagle (1 x BME)

with Hank's salts (Flow), containing 0.35 g/l sodium bicarbonate, 0.5 ml/100 ml glutamine (Flow), 15% (v/v) foetal bovine serum (Flow), 100 µg streptomycin/ml, 100 µg/ml penicillin/ml and 100 µg amphotericin B/ml (Flow Laboratories). The HEp-2 cells were passaged twice weekly.

Bacterial strains to be tested were grown overnight at 37 C in 1% tryptone water with 1% (w/v) D-mannose added to inhibit adhesion due to type 1 pili (Salit and Gotschlich, 1977). In the initial experiments the bacterial growth was washed twice in PBS before use in the test. Since no difference was found in tests with washed bacteria or with bacteria used directly from the tryptone waters, later experiments were done according to the latter procedure. A total of 0.1 ml of washed bacterial suspension or bacterial culture was mixed with 4.9 ml of 1 x BME without antibiotics. This mixture gave a concentration of 10^7 - 10^8 bacteria per ml.

For the HEp-2 adhesive test the cells were rinsed twice with PBS and removed from the flasks with 0.25% (v/v) trypsin (Flow). The cells were resuspended in 1 x BME with antibiotics. The cells were counted in a Neubauer chamber and adjusted to a final concentration of 2 x 10^5 cells per ml. Sterile plastic tissue culture Petri dishes 30-x-10 mm (Nunc) into which four sterile rectangular coverslips had been placed, were seeded with 2 ml of the HEp-2 cell suspension and incubated at 37 C for 18 - 36 h. After this time the HEp-2 cell monolayer was rinsed three times with Hank's balanced salt solution (HBSS) and 1 ml of the bacterial suspension in 1 x BME was added to each Petri dish and incubated for 3 h at 37 C. At this time two coverslips were removed with sterile forceps and immersed in 2 ml of PBS (3 h test). The

remaining two coverslips were washed three times with HBSS and 2 ml of fresh 1 x BME without antibiotics was added. After a further 3 h incubation at 37 C the coverslips were immersed in PBS (6 h test). Immediately following the appropriate incubation period, the coverslips were washed three times with PBS, fixed in 70% (v/v) methanol for 5 min, and stained with 10% (v/v) Giemsa. The coverslips were cleared in acetone, acetone-xylene and xylene and mounted on glass slides. They were examined 18 h later under oil immersion with a light microscope. A minimum of 100 cells were counted in each cover slip and the percentage of HEp-2 cells with 5 or more bacteria was recorded. Since the results of the 3- and 6- h test were invariably identical, only the results of the latter will be considered.

(i) Effect of time. Strain H19 (*E.coli* 026:K60:H11) was used as a prototype adhesive strain. The HEp-2 adhesive assay was performed as described above, except that samples were taken at 15 and 30 min, and then every 30 min for a total of 180 min. After each appropriate time, two of the four coverslips in each Petri dish were removed with sterile forceps, immersed in PBS, fixed and stained as described above. The remaining two coverslips in each Petri dish were then reincubated at 37 C for 3 h. After this time the coverslips were washed, fixed and stained as described previously. All assays were done in duplicate and repeated on two separate occasions.

(ii) Effect of temperature. The HEp-2 adhesive assay using strain H19 was performed as described above. Inoculated Petri dishes were incubated at 4 C, 22 C, 37 C or 42 C for 3 h. The monolayers were

then washed three times with HBSS reincubated at the same temperature used before for a further 3 h period. The coverslips were then washed, fixed, stained and examined. The assays were done in duplicate and repeated on two occasions.

(iii) Effect of bacterial concentration. Strain H19 was grown overnight at 37 C in 1% tryptone water with 1% (w/v) D-mannose. A total of 0.5 ml of washed bacteria was mixed with 4.5 ml of 1 x BME without antibiotics. Tenfold serial dilutions were made from this initial suspension and 1 ml from each dilution was used to inoculate HEp-2 cell monolayers. The HEp-2 adhesive test was then carried out in the usual manner in duplicate. Bacterial counts of each dilution were made on MacConkey agar in duplicate at the time of the assay.

(iv) Effect of heat and formaldehyde. Strain H19 was grown in the usual way, washed and diluted 100 fold in 1 x BME. The suspension was heated at 60 C for 20 min before using in the HEp-2 adhesive assay as described above. Assays were done in duplicate.

A total of 0.3 ml of an 0.4% (v/v) formaldehyde solution was added to washed bacteria diluted in 1 x BME and used to inoculate HEp-2 cell monolayers in the way previously described. Assays were done in duplicate. In both experiments bacterial counts of the 'killed' bacteria were done in duplicate on MacConkey agar at the time of the adhesive test.

(v) Effect of sublethal concentrations of streptomycin

Strain H19 was inoculated into 1% tryptone water with and without 1% (w/v) D-mannose with 20, 10, 5, 2.5 and 1.25 µg/ml of streptomycin. After overnight incubation at 37 C the bacteria grown at each dilution were washed twice with PBS and used in the HEp-2 adhesive assay as described above. Bacterial counts and microtiter haemagglutination tests were done at the same time as

the adhesive assay. All determinations were done in duplicate.

In each one of the experiments described, appropriate positive and negative controls were used.

IIo. Electron microscopy (EM) studies

The strains were grown on CFA agar for 18 h at 37 C and subcultured daily for three days. After each overnight incubation the strains were tested for MRHA with human or calf RBC, and for MSHA with guinea-pig RBC. After the last passage, growth from two CFA agar slopes was suspended in 1 ml of peptone water containing 4% (v/v) formaldehyde. The suspension was diluted 1/2 in distilled water and one drop was applied to a formvar-carbon coated electron microscope grid (400 mesh) for 3 min. Excess fluid was removed by blotting with filter paper and a drop of 1% phosphotungstic acid, pH 6.4, was applied on the grid for 1 min before blotting as before.

For immune electron microscopy cultures were suspended in peptone water without formaldehyde. One drop of suspension, diluted 1/2 in distilled water, was applied to the grid as before. Excess fluid was removed by blotting and the grid was floated, sample side down on a drop of the appropriate antiserum diluted 1/30 in PBS. The grids with serum drops were incubated in a humidified chamber for 15 min at room temperature. Grids were removed from the drops, blotted, washed four times with distilled water, stained for 1 min with 3% (v/v) phosphotungstic acid and finally blotted.

Before examination in an AEI EM 801 electron microscope the grids were irradiated for 2 min each side under a high intensity

short wave ultraviolet lamp to inactivate organisms.

All the EM studies were kindly done by Dr. Anne M. Field of the Virus Reference Laboratory, Colindale.

III. RESULTS

III. RESULTS

IIIa. Distribution of MRHA, CFA/I and CFA/II in E.coli from various sources

Table 3 shows the distribution of strains of E.coli according to source of isolation and ability to give a positive MRHA of human, calf or human and calf RBC. A total of 768 E.coli strains were studied. Three hundred and twenty-five (42%) of these E.coli strains caused MRHA of human and/or calf RBC.

Of the 206 ETEC strains examined, 93 (45%), gave a positive MRHA. One (0.5%) of the 93 MRHA positive ETEC strains agglutinated human RBC only, 50 (24%) agglutinated only calf RBC, and 42 (20%) agglutinated both human and calf RBC.

Twenty-four (13%) of the 186 EPEC strains examined gave a positive MRHA. Twelve (6%) of the EPEC strains caused MRHA of human RBC, six (3%) caused MRHA of calf RBC, and seven (4%) caused MRHA of both human and calf RBC.

Of the 122 E.coli strains isolated from cerebrospinal fluid (CSF) a total of 81 (66%) cause MRHA. Sixty-two (51%) caused MRHA of human RBC only, 12 (10%) caused MRHA of calf RBC only, and seven (6%) caused MRHA of human and calf RBC.

One hundred and twenty-four E.coli strains isolated from urine samples were examined. A total of 57 (46%) of these urinary E.coli strains gave a positive MRHA. Forty-five (36%) of the 49 positive MRHA agglutinated only human RBC. Four (3%) agglutinated only calf RBC, while eight (6%) agglutinated both human and calf RBC.

Seventy-eight (60%) of the 130 E.coli strains isolated from blood samples caused MRHA. Sixty (46%) caused MRHA of human RBC, four (3%) caused MRHA of calf RBC, and 14 (11%) caused MRHA of human and calf RBC.

CFA/I and CFA/II were found in 85 (11%) of the 768 E.coli strains examined (Table 3). CFA-positive strains were restricted to MRHA-positive ETEC strains. Eighty-five of the ETEC strains that gave a positive MRHA possessed either CFA/I or CFA/II. None of the other E.coli strains studied, whether able or unable to cause MRHA, were found to possess either of the colonisation factor antigens.

IIIa. 1. Presence of MRHA, CFA/I and CFA/II in ETEC strains

Table 4 shows the human and/or calf MRHA results for the ETEC strains according to serotype, enterotoxin production and presence of colonisation factor antigens. Ninety-four (46%) of the 206 ETEC strains were able to produce both ST and LT enterotoxins, 54 (26%) produced only ST, and 58 (28%) produced only LT. Of the 93 ETEC strains that caused MRHA of human and/or calf RBC 80 (86%) produced both ST and LT, 11 (12%) produced ST only, and only two (2%) produced LT only.

Thirty-six (17%) of the 206 ETEC strains examined possessed CFA/I. Twenty-six (72%) of these CFA/I positive strains produced both ST and LT and ten (28%) produced ST only. CFA/I was not found in LT only ETEC strains.

Forty-nine (24%) of the 206 ETEC strains were found to possess CFA/II. All 49 CFA/II positive strains were able to

produce both ST and LT enterotoxins. Of the seven MRHA positive strains that did not possess either CFA/I or CFA/II, four strains produced both ST and LT, two produced LT only and one produced ST only.

The 93 MRHA positive ETEC strains belonged to 22 E.coli O:H serotypes in 13 different O-groups (Table 5). Thirty-five strains belonged to serotype O6:H16, with 33 (94%) able to agglutinate only calf RBC and two (5%) able to agglutinate both human and calf RBC. All of these 35 MRHA positive ETEC strains possessed CFA/II. One strain of serotype O7:H- was found to agglutinate human RBC only but did not possess either CFA/I or CFA/II. Ten ETEC strains of serotype O8:H9 were able to agglutinate only calf RBC. These ten strains possessed CFA/II. Two strains of serotype O15:H- were also able to agglutinate calf RBC only, but these two strains did not possess either colonisation factor antigen. Two strains of serotype O25:H42 were able to cause MRHA of human and calf RBC. These two strains were negative for CFA/I and CFA/II. Nine strains of serotype O63:H- and one of serotype O63:H12 caused MRHA of human and calf RBC. These ten strains possessed CFA/I. Seventeen strains in O-group 78 were able to agglutinate both human and calf RBC. One O78 ETEC strain was non-motile (H-), three had an H11 flagellar antigen and 13 had an H12 flagellar antigen. All ETEC strains of O-group 78 able to cause MRHA possessed CFA/I. One strain of serotype O80:H9 and two of serotype O85:H7 caused MRHA of calf RBC and possessed CFA/II. CFA/I was detected in one ETEC strain of serotype O114:H21 that was able to cause MRHA of human and calf RBC. Two ETEC strains of serotype O115:H40 gave MRHA of human and calf RBC but were negative for CFA/I or CFA/II. One ETEC strain of

serotype O115:H51 was found to agglutinate calf RBC and to possess CFA/II. Seven ETEC strains in O-group O128 were able to agglutinate human and calf RBC. One had an H7 flagellar antigen, two had an H10, two an H12, one an H20 and one was motile but with an unidentifiable H antigen. All seven MRHA positive ETEC strains were positive for CFA/I. One strain of serotype O128:H20 caused MRHA of calf RBC only but did not possess CFA/I or CFA/II. One ETEC strain of serotype O153:H12 and one autoagglutinable ('rough') strain with flagellar antigen H12 were also found to cause MRHA of human and calf RBC and to possess CFA/I.

One hundred and twenty-one (59%) of the 206 ETEC strains studied belonged to the 22 E.coli O:H serotypes in which MRHA positive strains were found. Of these 121, 93 (77%) were positive for human and/or calf MRHA, 36 (30%) possessed CFA/I, and 49 (40%) possessed CFA/II. ETEC strains with CFA/I were found in serotypes O63:H-, O63:H12, O78:H-, O78:H11, O78:H12, O114:H21, O128:H7, O128:H10, O128:H12, O128:H?, O153:H12, and ORH12 (Table 5). CFA/II positive ETEC strains were found in serotypes O6:H16, O8:H9, O80:H9, O85:H7 and O115:H51 (Table 5).

IIIa.2. Presence of MRHA, CFA/I and CFA/II in EPEC strains

One hundred and eighty-six E.coli strains belonging to EPEC serotypes were also examined for MRHA. Twenty-five (13%) out of these 186 strains caused MRHA (Table 6). Twelve (6%) strains were able to agglutinate only human RBC, six (3%)

agglutinated calf RBC only, and seven (4%) agglutinated both human and calf RBC. MRHA positive strains were found in eight out of 11 O-groups commonly associated with EPEC serotypes. None of the EPEC strains examined, whether positive or negative for MRHA, was found to possess either CFA/I or CFA/II.

IIIa.3. Presence of MRHA, CFA/I and CFA/II in extraintestinal *E.coli*

Table 7 shows the total number of extraintestinal strains tested in each *E.coli* O-group. Thirty-nine strains did not have an identifiable O-group (O?) and 28 strains were auto-agglutinable (O-Rough). Two hundred and seventy-six (74%) of the strains tested belonged to only 13 O-groups. The percentage in each O-group that gave a positive MRHA varied from greater than 75% (O16, O1, O7) to less than 25% (O83, O9). On average 57% of the 276 strains in these 15 O-groups gave positive MRHA.

Of the 122 *E.coli* strains isolated from cerebrospinal fluid (CSF) samples, a total of 81 (66%) gave MRHA (Table 8). Sixty-two (51%) of the 81 MRHA positive strains agglutinated only human RBC. Twelve (10%) strains caused MRHA of calf RBC only and seven (6%) caused MRHA of human and calf RBC. MRHA positive strains isolated from CSF were found in 14 different *E.coli* O-groups. None of the *E.coli* strains isolated from CSF able to cause MRHA was found to possess either CFA/I or CFA/II.

Fifty-seven (46%) of 124 *E.coli* strains isolated from

blood samples were able to cause MRHA (Table 9). Forty-five (36%) caused MRHA of human RBC only, four (3%) caused MRHA of calf RBC only, and eight (6%) caused MRHA of human and calf RBC. MRHA positive strains were found in 16 different O-groups. Seven of the strains examined in this group belonged to commonly accepted EPEC serotypes. Two of these seven strains cause MRHA of human RBC. None of the E.coli strains isolated from urine possessed CFA/I or CFA/II.

Of the 130 E.coli strains isolated from blood samples, 78 (60%) caused MRHA (Table 10). Sixty (46%) of the 130 strains gave MRHA of human RBC only, four (3%) caused MRHA of calf RBC only, and 14 (11%) caused MRHA of human and calf RBC. MRHA positive strains were found in 17 different E.coli O-groups. None of the MRHA positive E.coli strains isolated from blood were positive for CFA/I or CFA/II.

IIIb. CFA/I studies in E.coli of serogroup 078

A number of ETEC strains of serogroup 078 isolated in several different countries were studied in detail. In addition to enterotoxin production and the presence of CFA/I, these strains were examined for drug resistance, biochemical characteristics and plasmid content.

IIIb.1. Properties of ETEC strains of serogroup 078

Twenty-six ETEC strains of serogroup 078 were studied. Nineteen of these 26 strains were of flagellar type 12, three of flagellar type 11, three were non-motile (H-), and one of flagellar type 18 (Table 11). Twenty of the ETEC strains possessed CFA/I as detected by slide agglutination and immunodiffusion tests using a specific antiserum. All the 20 CFA/I positive strains produced ST, and 16 of them also produced LT. Of the six strains that did not possess CFA/I, one produced both ST and LT, three produced only ST, and two produced only LT. Ten of the 26 ETEC strains were resistant to one or more antibiotics, with six of them resistant to more than three antibiotics (Table 11). None of the strains were resistant to heavy metals and none produced colicin or haemolysin.

All ETEC strains of serogroup 078 fermented arabinose, glucose, glycerol, lactose, maltose, mannitol, rhamnose, sorbitol and trehalose; they all failed to ferment cellobiose, inositol, and inulin and did not decarboxylate arginine. Differences were found in the decarboxylation of lysine and

ornithine, the utilisation of mucate, and the fermentation of adonitol, dulcitol, raffinose, salicin, sorbose, sucrose, and xylose (Table 12). Nine different biotypes were recognised on the basis of these tests. The biotype of each O78 ETEC strain is given in Table 11. No specific biotype could be associated with geographic source of isolation, O:H serotype, enterotoxin production or presence of CFA/I. Spontaneous derivatives from some of the 20 ETEC strains that had lost CFA/I were also biotyped; none of these derivatives differed in any reaction from the original CFA/I positive strain.

IIIb.2. Loss of CFA/I and heat-stable enterotoxin production

Strains were investigated for loss of CFA/I by examining single colonies of cultures stored on Dorset egg medium kept at room temperature by slide agglutination using specific CFA/I antiserum and by haemagglutination. The presence or absence of CFA/I was confirmed by immunodiffusion tests. Derivatives which lacked CFA/I were then tested for enterotoxin production. Loss of CFA/I was detected in 10 strains and in all cases this was accompanied by loss of ST production. Eight of these strains were also able to produce LT, but this property was not lost with CFA/I and ST production in any of these strains. Loss of CFA/I accompanied by loss of ST was also found in CFA/I-ST positive ETEC strains of serogroups 063, 0114, 0128 and 0153.

IIIb.3. Examination of the plasmid content of the 078 *E.coli* strains

The plasmid content of several of the CFA/I positive ETEC strains was examined by agarose gel electrophoresis. Derivatives from these strains that had lost CFA/I and ST production as described above were also studied and the number and sizes of plasmids present were compared with those in the original strains. The properties and plasmid content of seven pairs of strains are given in Table 13.

A plasmid of molecular weight 56 x 10^6 was present in strain H-10407 but absent in its CFA/I derivative strain H-10407P (Figure 1). The same results were observed by Evans *et al.* (1975) who concluded that this plasmid coded for the production of CFA/I. The results of the present study indicate that this 56 x 10^6 plasmid codes for both CFA/I and ST production. The analysis of strains E9505, E7464, E7473 and E9570 also showed a single plasmid difference between CFA/I-ST positive strains and their CFA/I-ST-negative derivatives (Figure 1). The CFA/I-ST positive strains carried, respectively, plasmids of 57 x 10^6, 60 x 10^6, 61 x 10^6 and 60 x 10^6 not detected in the CFA/I-ST negative lines. Strains E6085 and E9434 from which CFA/I-ST negative derivatives were also obtained showed no clear difference between the pairs in the number and size of their plasmids. These two strains, however, also had a plasmid of approximately 60 x 10^6.

Seven other strains which were CFA/I, ST and LT positive were also examined for their plasmid content. Each of these strains carried a plasmid of molecular weight 57 - 60 x 10^6,

as well as plasmids of other sizes. No CFA/I-ST negative derivatives could be isolated from these strains.

IIIb.4. Transfer of a CFA/I-ST plasmid to E.coli K12

Direct transfer of the CFA/I-ST plasmid from several O78 ETEC strains to E.coli K12 was not successful. Co-transfer of the CFA/I-ST plasmid with an R factor was tried for this reason. R1-19K$^-$ (R factor coding for resistance to ApCmSmSu) was transferred into the wild-type ETEC strain E7473 and an R$^+$ colony was used as a donor in a cross with E.coli K12. Five out of 30 ApCmSmSu transconjugants were not lysed by phage μ2 and were CFA/I-ST positive and LT negative. These results suggest that the CFA/I-ST plasmid, which caused fertility inhibition, was transferred with R1-19K$^-$. After loss of the R factor from one transconjugant still able to produce CFA/I and ST, it was shown by gel electrophoresis that a single plasmid of molecular weight 60 x 10^6 was present. This plasmid was labelled NTP113. These experiments were carried out by Drs. Moyra McConnell, H. R. Smith and Geraldine Willshaw, of the Molecular Genetics Unit, DEP, Colindale.

IIIb.5. Electron microscopy study of the CFA/I pili

Electron microscopy (EM) studies of the CFA/I strain E9562 of serotype O78:H- and its CFA/I negative derivative showed clear differences in the percentage of fimbriae in these strains. One hundred and sixty-two (94%) out of 172 single CFA/I positive bacteria showed the presence of fimbriae, while only 15 (13%) out of 114 CFA/I negative bacteria had fimbriae surrounding their surface (Figures 2a

and 2b). All the fimbriae in these preparations had an average diameter of 6.5 nm and were of different lengths. The fimbriae in these CFA/I positive and negative variants were also antigenically different. CFA/I antiserum coated the fimbriae of the CFA/I positive variant of E9562, but not the fimbriae present in the CFA/I negative derivative of this strain (Figures 3a and 3b). Similar results were found when the E.coli K12 carrying the CFA/I-ST plasmid, NTP113, and its E.coli K12 parent strain were examined for fimbriation and the coating of fimbriae with specific CFA/I antiserum.

IIIc. CFA/II studies in ETEC strains

The presence of CFA/II was only detected in faecal E.coli strains able to produce enterotoxins. CFA/II was present in 49 (24%) of the 206 ETEC strains studied. All CFA/II positive strains caused MRHA of calf RBC but only two of these also caused MRHA of human RBC (Table 3). ETEC strains able to produce CFA/II were found in five E.coli O:H serotypes: O6:H16, O8:H9, O80:H9, O85:H7 and O115:H51 (Table 14). Three more ETEC strains, two of serotype O15:H- and one of serotype O128:H20 were able to cause MRHA of calf RBC, but immunodiffusion tests did not confirm the presence of CFA/II. Forty-five (92%) of the CFA/II positive strains belonged to E.coli serotypes O6:H16 and O8:H9. Forty-seven (96%) of the 49 CFA/II positive strains produced both ST and LT enterctoxins, one produced ST- only and two produced LT- only (Table 14). Of a total of 69 ETEC strains in the five O:H serotypes in which CFA/II positive strains were found, 51 (74%) gave MRHA of calf RBC, and 49 (71%) possessed CFA/II (Table 14).

IIIc.1. Biochemical reactions of ETEC strains of serotype O6:H16

The biochemical reactions of ETEC strains belonging to serotype O6:H16 were classified according to the biotypes described by Scotland, Gross and Rowe (1977) (Table 15). All the ETEC strains of serotype O6:H16 in the present survey utilized mucate and fermented glucose, lactose, mannitol, maltose, salicin, sorbitol, arabinose, xylose, trehalose and glycerol; they failed to ferment inositol, inulin, cellobiose,

and sucrose. On the basis of their reactions in the five remaining tests, of the 35 CFA/II positive strains, 15 belonged to biotype A, nine to biotype B, nine to biotype C and one to biotype F. One CFA/II positive strain (E9186) did not belong to any of the reported biotypes. Of the four CFA/II negative strains of serotype O6:H16, two were of biotypes B and C respectively, and two did not belong to any of the reported biotypes.

IIIc.2. Biochemical reactions of ETEC strains of serotypes O8:H9, O80:H9, O85:H7 and O115:51

ETEC strains belonging to serotype O8:H9 showed different biochemical reactions to those found in serotype O6:H16 and could not be classified in the O6 biotypes. All O8:H9 strains were able to utilize mucate and to ferment glucose, lactose, mannitol, raffinose, dulcitol, sorbitol, arabinose, rhamnose, xylose, trehalose, glycerol and maltose; they failed to ferment inositol, adonitol, inulin and cellobiose. On the basis of their reactions in the four remaining tests, five biotypes were recognised (Table 16). Seven of the ten CFA/II positive strains of serotype O8:H9 belonged to biotype I. Of the remaining three CFA/II positive strains one belonged to biotype H, one to biotype K and one to biotype L. Two of the five CFA/II negative strains were also classified in biotype I, and one each in biotypes H, K and L.

The biochemical reactions in strains belonging to serotypes O80:H9 and O85:H7 were different from each

other and could not be classified in either the O6 or the O8 biotypes. All the O115:H51 strains examined with and without CFA/II were able to utilize mucate and to ferment glucose, lactose, mannitol, raffinose, sucrose, salicin, dulcitol, sorbitol, arabinose, rhamnose, xylose, trehalose, glycerol and maltose; they failed to ferment inositol, inulin, cellobiose and sorbose.

IIIc.3. Serological reactions of CFA/II positive ETEC strains

Regardless of biotype, except in four cases, all ETEC strains that gave a positive MRHA of calf RBC only, gave a precipitin line with the E1392 CFA/II antiserum and were classified as CFA/II positive. As mentioned before, two ETEC strains of serotype O15:H- and one of serotype O128:H20 able to cause MRHA of only calf RBC did not react with this antiserum. The other positive MRHA strain that was negative for CFA/II was E2047/69 an O6:H16 ETEC strain producing ST and LT.

A clear differenc, however, was observed in the CFA/II positive strains when diffused against the E1392 antiserum. While all the strains showed a clear precipitin line half-way between the antigen and the antiserum wells, some strains also showed a second, slower precipitin line close to the antigen well. This second precipitin line was only found in some CFA/II positive strains of serotype O6:H16. When these strains were classified according to their biochemical biotype it was observed that all the strains giving the slower component were of biotype A. The strain used to prepare the CFA/II antiserum used in these initial tests, E1392, was also

of biotype A. Diffusion tests using a CFA/II antiserum prepared with strain PB-176, the prototype CFA/II positive strain used by Evans and Evans (1979), gave identical results to those obtained with E1392. Strain PB-176 is also of biotype A.

The fast component of CFA/II was also observed when the ETEC strains that caused MRHA of calf RBC were tested with CFA/II antisera prepared with strains E4833 (biotype B) and strain E219A/69 (biotype C). Once again a second, slower component was observed in some MRHA-positive ETEC strains of serotype O6:H16. However, with these antisera the strains showing the slower component were of biotypes B, C and F, but not of biotype A.

To differentiate these CFA/II components, the fast one found with all the antisera used was called C; the slower components were called A or B depending on whether it was found with antiserum prepared with a biotype A or a biotype B or C strain. Thus most CFA/II positive strains of serotype O6:H16 of biotype A possessed components A and C, while most strains of biotype B, C or F possessed components B and C.

Table 17 shows the results of the CFA/II positive and negative strains of serotype O6:H16. Fifteen of the 16 strains of biotype A showed an A and a C component. One strain E1069/66 however showed only the C component. Eighteen out of 20 strains of biotypes B, C and F possessed the B and C components. One strain E2047/69, showed only the B component. This explains why this strain was negative for CFA/II with the E1392 antiserum. Another strain E1064/72

of biotype C showed the slower component only when tested with antiserum prepared with the biotype A strain. This strain thus possessed components A and C, but not component B as would be expected.

Of the three strains that could not be classified in the O6 biotypes, two were MRHA and CFA/II negative. The other, strain E9186 showed only component C when tested with all the antisera used.

All the ETEC strains of serotype O8:H9 that gave a positive MRHA of calf RBC were shown to possess CFA/II (Table 18). These strains, however, showed only the C component when tested with antisera prepared with the O6:H16 strains of biotypes A, B and C. The C component found in O8:H9 strains was identical to the C component found in O6:H16 strains. The same C component was found in CFA/II positive strains of serotypes O6:H16 and O8:H9 when diffused against a CFA/II antiserum prepared with an O8:H9 strain (E7463). The slower components A and B were not found in any of the CFA/II positive strains of serotypes O6:H16 and O8:H9 when tested by immunodiffusion with the E7463 (O8:H9) antiserum. No relation between the O8 biochemical biotypes and the presence of the CFA/II C component was found.

The one and only ETEC strain of serotype O80:H9 and the two strains of serotype O85:H7 in the survey were able to cause MRHA of calf RBC. When these three strains were tested by immunodiffusion with antisera prepared from CFA/II positive O6:H16 strains of biotype A, B and C and from the CFA/II positive O8:H9, they showed the presence only of the

C component of CFA/II (Table 19). No A or B component of CFA/II was found in these strains.

One of the three ETEC strains of serotype O115:H51 caused MRHA of calf RBC. This strain also possessed the C component of CFA/II when diffused with antiserum from CFA/II positive O6:H16 strains of biotype A, B and C and from the CFA/II positive O8:H9 (Table 19). No A or B component of CFA/II was found in this strain.

Table 20 summarises the serological reactions in CFA/II positive ETEC strains and their CFA/II negative derivatives. At least ten single colonies of each strain and its negative derivative studied were tested for MRHA and immunodiffusion before the result was recorded.

Two ETEC strains in the survey showed the presence of a precipitin line identical with the C component of CFA/II. These strains, one of serotype O63:H30 and one of serotype O114:H49, were not able to cause MRHA of calf or human RBC. These strains remained MRHA negative after repeated passage in liquid medium ruling out the possibility of a phenotypic phase variation. On the other hand, a number of strains of serotype O8:H9 were initially found not to give MRHA but to be positive by serology with the CFA/II antiserum. When these strains were passaged repeatedly in liquid medium they became MRHA positive.

The ETEC strains of serotype O15:H- and O128:H20 that were able to cause MRHA of only calf RBC were negative for the three components of CFA/II when tested with antiserum prepared from CFA/II positive O6:H16 strains of biotypes A, B and C, and from the CFA/II positive O8:H9 strain.

IIIc.4. Loss of CFA/II and enterotoxin production in ETEC strains

Strains were investigated for loss of CFA/II by examining single colonies of cultures stored on Dorset egg medium kept at room temperature by haemagglutination with calf red blood cells. The presence or absence of CFA/II was confirmed by immunodiffusion with biotype A and biotype B CFA/II antisera. Derivatives which lacked CFA/II were then tested for enterotoxin production. CFA/II positive and negative derivatives from a single MRHA positive colony were selected for plasmid analysis after testing for enterotoxin production.

None of the MRHA negative derivatives of CFA/II positive strains of serotype O6:H16, O8:H9 and O115:H51 showed the presence of CFA/II components A, B or C.

Spontaneous loss of the ability to cause MRHA of calf RBC in eight ETEC strains of serotype O6:H16 was accompanied by the loss of components A and C in strains of biotype A, and components B and C in strains of other biotypes (Table 21). In one of these strains, E201/69, however, loss of ability to cause MRHA of calf RBC was accompanied only by loss of the B component of CFA/II. The MRHA-negative derivative of E201/69 retained the C component of CFA/II. Ten single colonies of MRHA positive and negative variants of E201/69 showed identical results.

Loss of ability to cause MRHA of calf RBC in ETEC strains of serotypes O8:H9 and O115:H51 was always accompanied by loss of the CFA/II C component (Table 21). No MRHA negative derivates could be obtained from the ETEC strains of serotypes O80:H9 and O85:H7.

Loss of CFA/II was accompanied by loss of ST production in two ETEC strains (E1069/66 and E9187) of serotype O6:H16, and one strain (E9034) of serotype O8:H9 (Table 21). Loss of CFA/II was accompanied by loss of both ST and LT production in three strains, E5470 of serotype O6:H16, E9035 of serotype O8:H9, and E8783, the only CFA/II positive strain of serotype O115:H51 (Table 21).

Loss of ST and LT production without loss of CFA/II was found in two ETEC strains of serotype O6:H16, E1392 and E9187A4, and in two strains of serotype O8:H9, E7463 and E9033 (Table 21).

Loss of CFA/II without loss of enterotoxin production was found in three strains of serotype O6:H16, two able to produce ST only, E219A/69 and E11361, and one E4833, able to produce ST and LT. Strain E201/69 able to produce ST and LT lost the B, but not the C, component of CFA/II without losing the ability to produce both enterotoxins (Table 21). No consistent pattern of loss of CFA/II and enterotoxin production was observed in the strains studied.

IIIc.5. Examination of the plasmid content of the CFA/II positive strains and their CFA/II negative derivatives

The plasmid content of several of the CFA/II positive ETEC strains was examined by agarose gel electrophoresis. Derivatives from these strains that had lost CFA/II with or without enterotoxin production were also studied. The number and sizes of plasmids present in these negative derivatives were then compared with those found in the original strains. The properties and plasmid content of 14 pairs of strains are given in Table 21.

No difference in the number and size of their plasmids was found in four CFA/II positive strains of serotype O6:H16 and their CFA/II negative derivatives (E201/69, E219A/69, E4833 and E5470) or in one CFA/II positive strain of serotype O8:H9 and its CFA/II negative derivative (E9034) (Table 21). In three of these strains,

E201/69, E219A/69 and E4833, no differences were found in the production of ST or ST and LT between the CFA/II positive strains and their CFA/II negative derivatives. Two other strains, E5470 of serotype O6:H16, and E9034 of serotype O8:H9, lost both CFA/II and enterotoxin production without any visible difference in the plasmid content of these pairs.

In strain E1069/66 of serotype O6:H16, loss of CFA/II and ST production was accompanied by loss of two plasmids, one of 55 x 10^6 molecular weight and another of 39 x 10^6 molecular weight. Ten other CFA/II negative derivatives of E1069/66 were also found to have lost ST production. No separate loss of CFA/II and ST could be found in this strain.

Loss of CFA/II in strain E1392 was accompanied by loss of two plasmids, one of 58 x 10^6 molecular weight and another of 39 x 10^6 molecular weight although neither the CFA/II positive strain or its negative derivative were able to produce ST or LT enterotoxins (Table 21). It seems interesting to note that the size of the absent plasmids in the CFA/II negative derivative of E1392 were of similar size to those in the CFA/II negative derivative of strain E1069/66.

In strain E9187 of serotype O6:H16 loss of CFA/II and ST production was accompanied by loss of a single plasmid of 68 x 10^6 molecular weight (Table 21). No plasmid difference, however, was found between strain E9187 and strain E9187A4, a CFA/II positive ST negative derivative from this strain (Table 21). Loss of CFA/II in strain 9187A4 was accompanied by loss of a single plasmid of 66 x 10^6 molecular weight.

Loss of a single plasmid of 61 x 10^6 molecular weight was

also found accompanying the loss of CFA/II in strain E11361 of serotype O6:H16 (Table 21). Both strains in this pair were able to produce ST enterotoxin.

Two strains of serotype O8:H9, E7463 and E9033, also showed a single plasmid difference when the CFA/II negative derivative was compared with the CFA/II positive subclone of the original strains in each pair (Table 21). Neither the original strain nor their CFA/II derivatives were able to produce enterotoxins. Loss of CFA/II in E7463 was accompanied by loss of a 61 x 10^6 molecular weight plasmid. Loss of CFA/II in E9033 was accompanied by the loss of a 41 x 10^6 molecular weight plasmid.

In one strain of serotype O8:H9 (E9035) and in one strain of serotype O115:H51 the loss of a single plasmid of molecular weight 60 x 10^6 and 82 x 10^6 respectively, was found accompanying the loss of CFA/II and ST and LT production (Table 21).

The molecular weight of plasmids possibly coding for the production of CFA/II in these ETEC strains varied from 40 x 10^6 to 80 x 10^6 molecular weight, with most between 60 x 10^6 and 70 x 10^6 molecular weight (Figure 3).

IIIc.6. Transfer of the CFA/II plasmid

Direct transfer by conjugation of the CFA/II plasmid between wild strains of serotypes O6:H16, O8:H9, O80:H9 and O115:H51 and E.coli K12 was not successful.

Although CFA/II positive subclones of strains E1392, E201/69, E219/69, E7463, E9187 and E11361 accepted the R factor R1-19K$^-$ (ApCmSmSu) no cotransfer of the CFA/II

plasmid was observed in forward transfers into E.coli K12 using an R^+ CFA/II positive wild strain.

Cotransfer of tetracycline resistance with the R1-19K^- plasmid was obtained in strain E9187. Agarose gel electrophoresis of the recipient tetracycline resistant E.coli K12 recipient showed that this character was coded for by a 55×10^6 molecular weight.

IIIc.7. Effect of CFA/II on the ability to acquire Ent plasmids

ETEC strains E1392-2A (O6:H16, CFA/$II^+ST^-LT^-$) and E1392-7A (O6:H16, CFA/$II^-ST^-LT^-$) were tested for their ability to accept Ent plasmids in overnight crosses. Three Ent plasmids carried by E.coli K12 $lac^- Sm^r pro^- his^- trp^-$ were employed: TP213 coding for ST, LT and ampicillin resistance, TP235 coding for LT and kanamycin resistance, and TP224 coding for ST and tetracycline resistance. Single colony recipients were checked for CFA/II production and donors for the appropriate antibiotic resistance and enterotoxin production before use. No transfer of plasmids TP224 or TP235 was detected in the recipient strains. E1392-2A accepted TP213 with a transfer rate of 4.9×10^{-5}. E1392-7A accepted TP213 with a transfer rate of 7.1×10^{-6}.

IIIc.8. Electron microscopy studies of CFA/II

The CFA/II positive and negative derivatives of strain E1392 were used for electron microscopy (EM) studies of the CFA/II fimbriae. Strain E1392 does not produce type 1 pili.

The EM Studies showed that 84 (92%) of 86 CFA/II positive bacteria examined were fimbriated. In some fields the background was full of loose fimbriae which made it difficult to determine if the bacteria were fimbriated or not. Studies with the CFA/II negative derivative showed that only 16 (7%) out of 225 bacteria examined possessed fimbriae (Figures 5a and 5b). Some loose fimbriae were also found in the background of the CFA/II negative preparation but these did not present a problem in determining if the bacteria were fimbriated or not.

When specific CFA/II antiserum was added to the EM preparations, the fimbriae in the CFA/II positive strain were found heavily coated with antibody. The few CFA/II negative bacteria found to possess fimbriae were not found to be coated with antibody when CFA/II antiserum was added to this EM preparation (Figures 6a and 6b). The fimbriae in the background of the CFA/II positive preparation were also heavily coated with antibody from the CFA/II antiserum, while those loose ones in the CFA/II negative preparation were not.

IIId. Studies of MRHA positive ETEC strains of serogroups 025, 0115 and 0158

Two ETEC strains of serotype 025:H42 and two strains of serotype 0115:H40 were found to cause MRHA of human and calf RBC. When these strains were tested for CFA/I by slide agglutination with a specific antiserum, the two 025:H42 strains were recorded as positive and the two 0115:H40 strains as negative. The presence of CFA/I, however, was not confirmed in these putative CFA/I positive 025:H42 strains when tested by immunodiffusion with the CFA/I antiserum.

The four MRHA strains of serotypes 025:H42 and 0115:H40 gave a weak positive reaction when tested by slide agglutination with the CFA/II antiserum. The four strains were, however, negative for all the CFA/II components when tested by immunodiffusion with CFA/II antisera. Non-specific bacterial agglutination was also observed when these four MRHA positive strains were tested with a variety of non-related antisera. This non-specific agglutination was more frequent in the two strains of serotype 025:H42. These four MRHA positive strains were classified as negative for CFA/I and CFA/II based on the immunodiffusion tests using CFA/I and CFA/II antisera.

IIId.1. Serological studies of MRHA positive CFA negative ETEC strains

The ETEC strains in the survey were tested by immunodiffusion using a rabbit antiserum prepared with an MRHA positive 025:H42 strain (E8775). Out of the 206 ETEC strains examined, only

the four MRHA positive strains in serotypes O25:H42 and O115:H40 gave a precipitin line with the E8775 antiserum. The MRHA positive strains possessing CFA/I and CFA/II, and the MRHA negative ETEC strains did not show any precipitin lines when tested with the E8775 antiserum. The precipitin produced by the MRHA positive strains of serotype O25:H42 with the E8775 antiserum was identical to that produced by the MRHA positive strains of serotype O115:H40 when tested with the E8775 antiserum.

Twenty-three more ETEC strains of serotype O25:H42, all able to produce both ST and LT were tested for the ability to cause MRHA of human and calf RBC. Including the two strains of serotype O25:H42 from the original ETEC survey, 21 (84%) of a total of 25 ETEC O25:H42 strains were able to cause MRHA of human and calf RBC (Table 22). These 21 MRHA positive strains gave a precipitin line when tested by immunodiffusion with the E8775 antiserum, and were all negative for CFA/I and CFA/II. None of the MRHA negative O25:H42 strains reacted with the E8775 or the two CFA antisera (Table 22).

Eleven ST-LT producing ETEC strains of serotype O115:40, including the two strains of this serotype from the original ETEC survey, were also tested for their ability to cause MRHA of human and calf RBC. Seven (64%) of the O115:H40 strains gave MRHA of human and calf RBC (Table 22). These seven strains gave a precipitin line when tested by immunodiffusion with the E8775 antiserum, but were all negative for CFA/I and CFA/II (Table 22). None of the MRHA negative O115:H40 strains were positive by immunodiffusion with the

E8775 or the two CFA antisera.

During routine MRHA testing of ETEC strains an ST-LT strain of serotype O158:H42 was found to cause MRHA of human and calf RBC. This strain was negative for CFA/I and CFA/II, but gave a precipitin line when tested by immunodiffusion with the E8775 antiserum (Table 22). Out of a total of 37 ETEC strains in serotypes O25:H42, O115:H40 and O158:H42, 29 (78%) were found to give MRHA of human and calf RBC and to react with the E8775 antiserum when tested by immunodiffusion techniques (Table 22). All these MRHA positive strains were negative for CFA/I and CFA/II.

IIId.2. Loss of the E8775 haemagglutinin and enterotoxin production

MRHA ability was lost when the strains were grown overnight at 18 C. These strains were also negative by immunodiffusion with the E8775 antiserum. When these strains were re-grown at 37 C they became MRHA positive and gave a precipitin line with the E8775 antiserum.

Loss of the E8775 haemagglutinin was investigated in the three strains initially found positive for this character, by testing single colonies from cultures kept at room temperature on Dorset egg medium by haemagglutination with human and calf RBC. The absence of the E8775 haemagglutinin was confirmed by immunodiffusion with E8775 antiserum. Strains that had lost MRHA ability were then tested for enterotoxin production.

In the three ETEC strains examined, two of serotype O25:H42 and one of serotype O115:H40, the loss of MRHA ability was accompanied by loss of the precipitin line in the diffusion studies with the E8775 antiserum (Table 23). The two O25:H42 strains lost the E8775 haemagglutinin without losing the ability to produce ST and LT enterotoxins. In both strains, however, testing of more single colonies showed that the E8775 haemagglutinin and enterotoxin production could be lost separately. Joint loss of ST and

LT was always found when the single colonies studied lost the ability to produce enterotoxins.

The MRHA positive and negative derivatives of strain E8780 (O115:H40) were found to have lost the ability to produce LT but not ST enterotoxin when compared with the original strain which was able to produce both ST and LT. Loss of the E8775 haemagglutinin in this strain was not accompanied by loss of ST production (Table 23).

IIId.3. Examination of the plasmid content of ETEC strains of serotypes O25:H42 and O115:H40

The plasmid content of several strains possessing the E8775 haemagglutinin was analysed by agarose gel electrophoresis. Strains that had lost the E8775 haemagglutinin were also examined and their plasmid content was compared with that in the original strain. The properties and plasmid sizes of these strains are given in Table 23.

In three pairs of strains, two of serotype O25:H42 (E8774 and E8775) and one of serotype O115:H40 (E8780), the loss of the E8775 haemagglutinin was not accompanied by the loss of a specific plasmid (Table 23). The MRHA positive and negative variants in each pair showed a similar plasmid content. The two O25:H42 pairs had two large plasmids of approximately 55×10^6 and 52×10^6 Mdal. The two strains in each pair were able to produce ST and LT enterotoxins. The MRHA positive and negative pair from strain E8780 were both able to produce only ST and possessed a single large plasmid of 88 to 90×10^6 Mdal.

Examination of a spontaneous variant of E8774 (E8774A) that had lost the ability to produce both ST and LT enterotoxins but not the E8775 haemagglutinin showed a similar plasmid content to that of the original E8774 strain. Spontaneous loss of the E8775 haemagglutinin from this E8774A was accompanied by loss of a 56×10^6 molecular size plasmid when compared with the enterotoxin negative parent strain.

Transfer of the ability to cause MRHA from these strains into E.coli K12, either directly or by mobilisation, was not successful.

IIIe. Adhesive factors in EPEC strains

Twenty-four (13%) of the 186 enteropathogenic E.coli (EPEC) studied were able to cause MRHA of human and/or calf RBC (Table 3). None of the MRHA positive or negative EPEC strains possessed CFA/I, CFA/II or the E8775 haemagglutinin when tested by immunodiffusion with specific antisera.

Preliminary results obtained with an in vitro assay to detect bacterial adherence to HEp-2 cells showed that EPEC strains had a greater affinity for receptors in these tissue culture cells than ETEC strains and strains of E.coli isolated from extra-intestinal sources.

In the HEp-2 assay, adhesive and non-adhesive strains could easily be differentiated (Figure 7). Strains which were considered positive adhered to at least 40% of the HEp-2 cells, while non-adhering strains attached to less than 10%. Intermediate results were not found.

IIIe.1. Factors affecting the HEp-2 adhesive test

Preliminary results showed that EPEC strain H19 (O26:K60:H11) adhered consistently to HEp-2 cells. In order to standardize the HEp-2 adhesive test, the effect of time and temperature during the duration of the test were examined.

(i) Effect of time. Figure 8 shows the percentage of HEp-2 cells with adhering bacteria in relation to time of incubation in minutes. Monolayers of HEp-2 cells were inoculated simultaneously; samples were then removed at 30 min intervals. The monolayers were then rinsed, fixed and stained. The results in this section are

presented as percentage of bacterial adhesion to HEp-2 cells in relation to total contact time between bacteria and cells in minutes.

There was no visible adhesion of strain H19 to HEp-2 cells when the total contact time was 30 min. Total contact of 60 min allowed bacteria to attach to 2 - 3% of the cells. With a total contact time of 90 min a sharp increase in the percentage of bacterial adhesion to over 20% was found. The percentage of adhesion continued to increase and peaked to approximately 70% when total contact time was 120 min. This percentage of adhesion remained unchanged when total contact time was 150 and 180 min. When total contact time was continued beyond 3 h the bacterial concentration and the acidity of the medium affected the HEp-2 cells and made them come off the coverslips.

(ii) Effect of bacterial concentration in tissue culture medium. The second part of Figure 8 shows the increase in the concentration of bacteria during the time of incubation. The bacteria grew slowly during the first 120 min to reach approximately 3×10^7 bacteria/ml. After 150 min of incubation time there was a dramatic increase in the concentration to approximately 3×10^8 bacteria/ml. This concentration was maintained for the final 30 min of incubation time to complete a total of 180 min. As can be seen by reading both graphs simultaneously, the percentage of adhesion seems dependent on the concentration of bacteria during the incubation of the test.

Table 24 shows the effect of increasing the initial concentration of bacteria used on the percentage of adhesion to the HEp-2 cells at the end of the 6 h test. Initial concentrations

of 8×10^5 to 8×10^6 bacteria/ml gave a low percentage of adhesion in the assay. Concentrations between 1×10^7 and 8×10^7 bacteria/ml gave intermediate results, which were not found with concentrations of 10^8 bacteria/ml used in this survey. Initial concentrations between 2×10^8 and 3×10^8 bacteria/ml gave a percentage of adhesion between 70% and 80%. All the HEp-2 cells were found dead floating in the medium when initial concentrations above 7×10^8 bacteria/ml were used.

These results confirm the findings shown in Figure 8. The percentage of adhesion in the HEp-2 assay was more dependent on the concentration of bacteria obtained during the test than on the length of time that the bacteria were in contact with the HEp-2 cells.

(iii) Effect of temperature. The effect of temperature on the HEp-2 adhesive assay was studied by carrying out the test at 4 C, 22 C, 37 C and 42 C. At the end of the 6 h test no adhesion was observed when the incubation time was 4 C or 22 C. The percentage of adhesion at 37 C was approximately 70%. Few HEp-2 cells remained attached to the coverslips after incubation at 42 C for 6 h, probably due to a rapid acidification of the medium.

There was no attachment of bacteria that had been incubated at 60 C for 15 min from cultures with a concentration of 10^8 bacteria/ml. These same results were found when the bacteria were formalized before use in the HEp-2 assay.

IIIe.2. Effect of type 1 pili inhibitors on the adhesion of EPEC strains to HEp-2 cells

Since most of the E.coli strains used in this study possessed type 1 pili, as detected by MSHA of guinea-pig RBC, the effect of these appendages on the adhesion to HEp-2 cells was investigated. These studies were carried out using strain H19 which is HEp-2 adhesive and possesses type 1 pili.

(i) Effect of mannose. H19 causing a strong MSHA of guinea-pig RBC was grown overnight in tryptone water at 37 C with and without the addition of 1% D(+) Mannose. Bacteria grown with and without mannose were tested simultaneously in the HEp-2 adhesive assay as previously described. When examined, the bacteria grown without mannose were found adhering indiscriminately to both the HEp-2 cells and the glass surface of the coverslip between the cells. In contrast, the bacteria grown with mannose were found adhering to the HEp-2 cells but not to the glass spaces in the coverslips.

(ii) Effect of phenotypic variations of type 1 pili. The second experiment consisted of subculturing strain H19 at 37 C on CFA medium every 24 h for four days. After each passage the strain was tested for MSHA of guinea pig RBC to detect the presence of type 1 pili. After four days the culture became invariably MSHA-negative. An MSHA-negative culture of H19 was then tested in the HEp-2 test using as control a strain of H19 giving a strong MSHA of guinea-pig RBC. Both variants of H19 grown in tryptone water with 1% mannose were found adhering to the HEp-2 cells, although the MSHA negative culture did have a slightly lower percentage of adhesion (56% to 58%). This difference, however, was not statistically significant.

(iii) Effect of sublethal concentrations of streptomycin. Sublethal concentrations of streptomycin have been shown to inhibit the production and size of type 1 pili (Eisenstein et al., 1979). Strain H19 which is streptomycin sensitive was grown in 1% tryptone water with tenfold serial dilutions of the antibiotic. Bacterial growth was only found when concentrations of streptomycin in the medium were of 2.5 µg/ml or less (Table 25). Bacteria grown under these conditions were then tested simultaneously for MSHA of guinea-pig RBC in microtiter plates (see section IIc.) and for HEp-2 adhesion. The MSHA titer decreased rapidly from 1:32 to 1:4 as the concentration of streptomycin in the medium increased, confirming a phenotypic inhibition of type 1 pili in these bacteria. This inhibition of the expression of type 1 pili, however, did not affect the adhesion of H19 to HEp-2 cells as compared to bacteria grown without streptomycin and giving a high MSHA titer (Table 25).

IIIe.3. Ability to adhere to HEp-2 cells by faecal strains of E.coli

A total of 248 strains of E.coli were tested for their ability to adhere to HEp-2 cells. These strains were divided into five groups (Table 26). Group A consisted of 51 EPEC strains which were isolated from faeces of patients involved in outbreaks of diarrhoeal disease in the United Kingdom, Australia and Ireland (Table 27).

The 135 EPEC strains in Group B were isolated from the faeces of patients involved in sporadic cases of diarrhoea in different countries. At least six strains from each of the commonly associated EPEC O-groups were selected for study (Table 28).

Group C consisted of 22 ETEC strains isolated in several countries from the faeces of patients with diarrhoea (Table 29). All 22 produced ST and 19 also produced LT. Fourteen

of these 22 strains produced CFA/I and two produced CFA/II. They were selected because of the presence of these colonisation factors from the large group of ETEC strains used in this study.

Group D consisted of 17 E.coli strains which were isolated from outbreaks of diarrhoeal disease in the United Kingdom (Table 30). The evidence from serotyping and epidemiology suggested that these serotypes were the epidemic strains, although they did not belong to EPEC serotypes and were not enterotoxigenic.

The 23 strains in Group E were isolated in the United Kingdom from children and adults without diarrhoea (Table 26). These strains were non-toxigenic and did not belong to EPEC serotypes.

The occurrence of adhesive factors among the different groups of E.coli strains is summarized in Table 26. The incidence of HEp-2 adhesion was significantly higher among EPEC strains from outbreaks and sporadic cases of diarrhoea than among the other three groups studied ($p < 0.001$ by χ^2 test). There was no significant difference between the occurrence of HEp-2 adhesion between the two groups of EPEC strains (A and B) ($p > 0.3$ by χ^2 test).

Of the 51 strains belonging to traditional EPEC strains from outbreaks of diarrhoea, none of the strains caused MRHA of human or calf RBC, and none possessed CFA/I or CFA/II as judged by slide agglutination and immunodiffusion with specific antisera (Table 27). Forty-one strains (80%) adhered to HEp-2 cells and 31 of these also possessed type 1 pili ten strains adhered to HEp-2 cells but did not possess type 1 pili.

Two strains did not adhere to HEp-2 cells and did not possess type 1 pili, while eight strains that did not adhere to HEp-2 cells nevertheless possessed type 1 pili. In all the EPEC outbreaks, some or all of the strains adhered to HEp-2 cells (Table 27).

One hundred and three EPEC strains (76%) of the 135 isolated from sporadic cases of diarrhoea adhered to HEp-2 cells. Ability to cause MRHA of human and/or calf RBC was found in 25 (19%) of the 135 strains, but none possessed CFA/I or CFA/II by slide agglutination or immunodiffusion with specific antisera (Table 26). A total of 118 strains (87%) possessed type 1 pili. Of the 135 EPEC strains, 92 adhered to HEp-2 cells and possessed type 1 pili, 11 adhered to HEp-2 cells but did not possess type 1 pili, 26 did not adhere to HEp-2 cells but possessed type 1 pili and 16 neither adhered to HEp-2 cells nor possessed type 1 pili.

Of the 22 ETEC strains, 16 (73%) gave a positive MRHA with human and calf RBC. The presence of CFA/I was confirmed in 14 of these strains by slide agglutination and immunodiffusion using a specific antisera (Table 29). The other two strains causing MRHA of human and calf RBC, both of serotype O25:H42, possessed the E8775 haemagglutinin (see section IIId) but not CFA/I or CFA/II. Two (9%) of the ETEC strains caused MRHA of calf RBC but not human RBC. Both of these strains were of serotype O6:H16 and were positive for CFA/II by immunodiffusion with specific antiserum. Type 1 pili were found in 17 (77%) of the ETEC studied. In spite of the high incidence of CFA/I and II, and the E8775 haemagglutinin in these ETEC strains, only three gave a positive result in the

HEp-2 assay (Table 29).

Among the 17 strains which were neither EPEC nor ETEC but were the possible causative agents in outbreaks of diarrhoeal disease, only two strains, both belonging to serotype O149:H-, gave a positive human MRHA (Table 30). None of the 17 strains were able to cause MRHA of calf RBC and none possessed either CFA/I or CFA/II by slide agglutination or immunodiffusion with specific antisera. Type 1 pili were found in 13 strains (76%). Five (29%) of the 17 were positive in the HEp-2 adhesion assay. Strains which adhered to HEp-2 cells were found in only three of the five outbreaks studied (Table 30).

Of the 23 E.coli strains from subjects without diarrhoea, four (17%) gave a positive human MRHA, and two (9%) gave a positive calf MRHA. None of the 23 strains were positive for CFA/I or CFA/II by slide agglutination or immunodiffusion using specific antisera. Twelve strains (52%) had type 1 pili and four (17%) were positive in the HEp-2 adhesive assay (Table 26).

IIIc.4. Characteristics of EPEC strains able and unable to adhere to HEp-2 cells

Table 31 shows the frequency of MRHA of human and/or calf RBC in EPEC strains able and unable to adhere to HEp-2 cells. Only 24 (14%) of the 186 EPEC strains examined caused MRHA of human and/or calf RBC. Nine (6%) of 142 EPEC strains adhering

to HEp-2 cells caused MRHA of human RBC only, while only one (2%) of 44 EPEC strains unable to adhere to HEp-2 cells caused MRHA of human RBC only. Four HEp-2 adhesive EPEC strains (3%), and two non HEp-2 adhesive strains (5%) caused MRHA of calf RBC only. Four (3%) of EPEC strains able to adhere to HEp-2 cells and four (9%) of EPEC strains unable to adhere to HEp-2 cells caused MRHA of human and calf RBC. There were significantly more EPEC strains unable to adhere to HEp-2 cells causing MRHA of human and calf RBC ($p < 0.05$ by χ^2 test). The frequency of MRHA of only human or only calf RBC in EPEC strains able and unable to adhere to HEp-2 cells was not significantly different.

One hundred and two (72%) of the 142 EPEC strains adhering to HEp-2 cells possessed type 1 pili (Table 31). Thirty (68%) of the 44 EPEC strains unable to adhere to HEp-2 cells also possessed type 1 pili (Table 31). The number of EPEC strains able and unable to adhere to HEp-2 cells that possessed type 1 pili was not significantly different ($p > 0.9$ by χ^2 test).

No significant differences were found in the number of HEp-2 adhesive and non-adhesive EPEC strains producing VERO cytotoxin ($p > 0.25$ by χ^2 test) or causing urea hydrolysis ($p > 0.1$ by χ^2 test) (Table 32).

EPEC strains able to cause MRHA of human and/or calf RBC did not differ significantly in their ability to adhere to HEp-2 cells, to produce type 1 pili, or to produce urea hydrolysis from EPEC strains unable to cause MRHA, when compared by χ^2 test (Table 33). The number of EPEC strains producing VERO cytotoxin was significantly higher in the strains

unable to cause MRHA ($p < 0.05$ by χ^2 test). The incidence of HEp-2 adhesion, production of type 1 pili and urease was similar in EPEC strains able to produce VERO cytotoxin when compared with EPEC strains unable to produce VT, (Table 34).

III e.5. Phenotypic characteristics of EPEC strains of serogroup 026.

Because of the high incidence of VERO cytotoxin production and HEp-2 adhesion in EPEC strains of serogroup 026, the phenotypic and genetic characteristics of strains of this serogroup were examined in more detail.

A total of 68 strains studied belonged to serogroup 026 in the EPEC strains examined. Twenty-two of these strains were non-motile (H-), 44 had flagellar antigen 11, and one each a flagellar antigen 32 and 46 (Table 35). Twenty (91%) of the 22 EPEC strains of serotype 026:H-, 39 (89%) of the 44 strains of serotype 026:H11, and one strain of serotype 026:H32 adhered to HEp-2 cells.

Twenty-one (95%) of the 22 strains of serotype 026:H-, 38 (86%) of the 44 strains of serotype 026:H11 and the one strain of serotype 026:H32 possessed type 1 pili. Thirty-two (73%) of the 44 strains of serotype 026:H11 were able to produce VT, while only two (9%) of the 22 strains of serotype 026:H- and neither strain of serotype 026:H32 or 026:H46 were found to produce VT (Table 35).

There were significantly more strains able to produce VERO cytotoxin in strains of serotype 026:H11 than in the other 026 serotypes ($p < 0.001$ by χ^2 test).

Seven (32%) of the 22 strains of serotype 026:H-, six (14%) of the 44 strains of serotype 026:H11 and the one strain of serotype 026:H32 were able to produce urea hydrolysis (Table 35). The difference in the ability to cause urea hydrolysis in the different 026 serotypes did not reach statistical significance ($p < 0.1$ by χ^2 test).

All the 22 strains of serotype 026:H- and the one strain of serotype 026:H32 were able to ferment sucrose (Table 35). The 44 strains of serotype 026:H11 were also all able to ferment sucrose. The only strain of serotype 026:H46 was unable to adhere to HEp-2 cells or to produce type 1 pili and VT. This strain was also unable to utilise urea and to ferment sucrose (Table 35).

IIIe.6. Examination of the plasmid content in EPEC strains of serotype 026:H11, and loss of HEp-2 adhesion

Loss of HEp-2 adhesion was analysed by testing single colonies of HEp-2 adhesive EPEC strains in the assay described. Loss of ability to adhere to HEp-2 cells was not found in 100 single colonies of strain H19 or in a minimum of 10 single colonies each of serotypes 026:H11, 0114:H2 and 0128:H2. The ability to adhere to HEp-2 cells was not found either in 20 single colonies of strain H19 "cured" with ethidium bromide or acridine orange. These strains were obtained from Dr. Sylvia Scotland.

Seven EPEC strains of serotype 026:H11 able to produce VT and to adhere to HEp-2 cells were examined for their plasmid content by electrophoresis. Five of these strains were also able to produce colicin. All 7 strains examined possessed a large plasmid of approximately 56 - 60 x 10^6 molecular weight, and several smaller plasmids. Since these results did not clarify if the HEp-2 adhesive ability was plasmid-controlled, as can be seen in Table 36, a more extensive study of the plasmid content of subclones of strain H19 was carried out.

Table 37 shows the phenotypic characteristics of strain H19 (E. coli 026:K60:H11). H19 adhered to HEp-2 cells, was able to produce colicin and VT and was resistant to streptomycin, sulphathiazole and tetracycline (SmSuTc).

Analysis of partially purified plasmid DNA from H19 by agarose gel electrophoresis revealed the presence of three large plasmids of 61 x 10^6, 58 x 10^6 and 52 x 10^6 molecular weight, and two small plasmids of approximately 4.5 x 10^6 and 2.5 x 10^6 molecular weight. Four derivatives of H19 were also examined. Strains G241, G243 and G246 were spontaneous segregants of H19. Strains G241 and G243 had lost the ability to produce colicin but maintained their ability to produce VT and were SmSuTc resistant. The two strains adhered to HEp-2 cells. Strain G246 had lost the resistance to SmSuTc but maintained its ability to produce VT and colicin. This strain also adhered to HEp-2 cells.

The percentage of adhesion to HEp-2 cells for strain H19 was 68%, for strain G241 was 65%, for strain G243 was 71% and for strain G246 was 62%. The differences in the percentage of adhesion in these strains was not statistically significant. The

ability of these strains to adhere to HEp-2 cells was not related to the ability to produce colicin or drug resistance.

Analysis of the plasmid content of these H19 derivatives was compared with that in the original H19 strain (E1426/65). Strains G241 and G243, which had lost the ability to produce colicin, showed the presence of two large plasmids of molecular weight 58 x 10^6 and 51 x 10^6, and a small plasmid of 4.5 x 10^6 molecular weight (Table 37). These two strains had lost the 61 x 10^6 molecular weight plasmid present in the original H19.

Strain G246, which had lost the SmSuTc resistance, showed the presence of two large plasmid of molecular weight 60 x 10^6 and 57 x 10^6, and two small plasmids of approximately 4.5 and 2.5 x 10^6 molecular weight. This strain had lost the 52 x 10^6 molecular weight plasmid present in the original H19 strain. H19 and its spontaneous derivatives showed the presence of a plasmid of 60 x 10^6 molecular weight coding for the production of colicin and a plasmid of 52 x 10^6 molecular weight coding for the SmSuTc resistance. The 58 x 10^6 molecular weight plasmid and the two small plasmids of 4.5 and 2.5 x 10^6 did not code for a phenotypically defined character.

Strain G245 was a derivative of strain G243 after treatment with ethidium bromide that had lost the ability to produce colicin and resistance to antibiotics. Strain G245 adhered to HEp-2 cells but the percentage of adherence was only 20% compared with 71% found in the parent strain G243. The difference in the percentage of adhesion between G243 and G245 was highly significant ($p < 0.001$ by X^2 test).

Analysis of the plasmid DNA content of strain G245 showed the presence of one large plasmid of molecular weight 55 x 10^6 and two smaller plasmids, one of 13 x 10^6 and another of 4.5 x 10^6 molecular weights. In strain G245 the 51 x 10^6 and 13 x 10^6 molecular weight plasmids could be derived from the 58 x 10^6 plasmid present in H19. Neither H19 nor its derivatives were found to possess a similar 13 x 10^6 molecular weight plasmid.

IIIe.7. Examination of the phenotypic characteristics and plasmid content of *E. coli* K12 transconjugants from crosses with strain H19

Table 38 shows the phenotypic characteristics of transconjugants obtained from crosses with strain H19. The ability to produce VT, colicin and drug resistance was acquired by strain G208 (*E. coli* K12-J53). Analysis of the plasmid DNA content of strain G208 revealed the acquisition of three large plasmids of 59 x 10^6, 55 x 10^6, and 51 x 10^6 molecular weight, and one small plasmid of 4.5 x 10^6 molecular weight. Neither strain G208 nor its K12 parent strain were able to adhere to HEp-2 cells.

Strain G209 is an *E. coli* K12 transconjugant that acquired the ability to produce colicin and drug resistance from H19. Analysis of the plasmid DNA of strain G209 showed the presence of two large plasmids only, one of 57 x 10^6 molecular weight and one of 51 x 10^6 molecular weight (Table 38). Strain G209 was not found to adhere to HEp-2 cells.

Strain G221 was a K12 transconjugant that had acquired only the ability to produce VT from H19. No plasmids were found in this strain when analysed by electrophoresis. Strain G221 was

was not found to adhere to HEp-2cells (Table 38).

Table 39 shows the phenotypic characteristics of the H19 strain (E3787/76) used by Smith and Linggood (1972) in their work on the *Ent*$^+$ plasmid. Strain E3787/76 was able to adhere to HEp-2 cells, to produce colicin and VT and was resistant to streptomycin, sulphathiazole and tetracycline. Analysis of the plasmid DNA from this strain revealed the presence of two large plasmids of 59 x 10^6 and 54 x 10^6 molecular weight and two small plasmids of approximately 4.5 and 2.5 x 10^6 molecular weight.

Transconjugants obtained from crosses between E3787/76 and *E. coli* K12-(711) showed that acquisition of the ability to produce colicin and VT and drug resistance was accompanied by the acquisition of two plasmids of 61 x 10^6 and 55 x 10^6 molecular weight. Strain G166, a K12 transconjugant which had acquired only the SmSuTc resistance, showed the presence of a single plasmid of 46 x 10^6 molecular weight. Strain G167 a K12 transconjugant that had acquired the ability to produce colicin and drug resistance from E3787/76, acquired two plasmids of 61 x 10^6 and 54 x 10^6 molecular weight. Loss of the drug resistance in strain G167 and in strain G168, a K12 transconjugant that had acquired the ability to produce VT, was accompanied by the loss of the 52 x 10^6 molecular weight plasmid. None of the K12 transconjugants from crosses with strain E3787/76 were able to adhere to HEp-2 cells (Table 39).

IV. DISCUSSION

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IVa. Implication of the findings

The ability of strains of E.coli to agglutinate RBC from different animal species has been correlated with the presence of adhesive factors on the bacterial surface (Duguid and Gillies, 1957; Ørskov and Ørskov, 1977; Evans, Evans and Tjoa, 1977). In the present study strains of E.coli isolated from intestinal and extra-intestinal sources in man were tested for the ability to cause haemagglutination of human, calf and guinea-pig RBC in the presence and absence of D-mannose. Strains of E.coli causing MSHA of guinea-pig RBC were classified as possessing type 1 pili (Duguid and Gillies, 1957; Brinton, 1959; Salit and Gotschlich, 1977a). Those causing MRHA were tested serologically by slide agglutination and immuno-diffusion for the presence of CFA/I and CFA/II.

As previously reported the ability to cause MRHA of human and/or calf RBC was found to be widespread in strains of E.coli from intestinal and extraintestinal sources (Duguid, et al., 1955; Minshew et al., 1978; Duguid, Clegg and Wilson, 1979). The percentage of strains causing MRHA was similar in intestinal and extraintestinal E.coli. In the intestinal group the percentage of ETEC strains causing MRHA was three times higher than in EPEC strains. Similar findings have recently been found by Evans et al. (1980). This higher incidence of MRHA in ETEC strains is probably due to the presence of CFA/I and CFA/II. These two fimbrial antigens are known to cause MRHA of human and/or calf RBC (Ørskov and Ørskov, 1977; Evans and Evans, 1978). Ability to cause MRHA was also found in EPEC strains and in E.coli strains isolated from extraintestinal sources. These strains, however, did not possess either CFA/I

or CFA/II.

(i) CFA/I. Of the 206 ETEC strains examined 17% possessed CFA/I. CFA/I$^+$ strains caused MRHA of human and calf RBC and were found in 11 different O:H combinations in five different E.coli O-groups. The ability to cause MRHA of human and calf RBC was also found in other ETEC strains not possessing CFA/I. In addition, certain non-enterotoxigenic E.coli strains isolated from intestinal sources and belonging to the same serotypes as ETEC strains possessing CFA/I were also able to cause MRHA of human and calf RBC but were negative for CFA/I. Therefore the ability to cause MRHA of human and calf RBC correlated with the presence of CFA/I only in ETEC strains.

The reasons for the prevalence of CFA/I among ETEC strains of certain serotypes are not known. The presence of genes coding for CFA/I and ST production on a single plasmid, as found in this study, could be one possible explanation. Loss of CFA/I together with ST production has also been found by Evans and Evans (1978). Reis et al. (1980) have recently reported the transfer of a plasmid coding for CFA/I and ST production from an ETEC strain of serotype O128ac:H12 to an E.coli K12 strain. This plasmid has similar molecular weight and genetic characteristics to the one found in ETEC strains of serogroup O78. It would be interesting to study whether these plasmids encoding CFA/I and ST are all related philogenetically or if they form a more heterogenous group.

Genes coding for the production of ST in an ETEC strain isolated from a calf with diarrhoea was found on a transposon flanked by repeats of insertion sequence 1 (IS1)(So, Heffron and

McCarthy, 1979). The ST encoded by this transposon was positive in the infant mouse test and in pig gut loops (ST1). This type of ST is similar to that produced by ST positive ETEC strains isolated from humans (So and McCarthy, 1980). In the first paper by these authors it was suggested that transposition may result in the ST genes being acquired by a plasmid which also determines adherence to the intestinal mucosa and it was stated that one such plasmid had been identified. Isaacson, Moon and Schneider (1978) have reported that all K99 positive ETEC strains from calves also produce ST, but the genes coding for these characters have not been found on the same plasmid.

Since both toxin production and adhesive ability are necessary for an ETEC strain to cause diarrhoea (Smith and Linggood, 1972) the presence of a single plasmid coding for both of these characters would definitely increase the pathogenic ability of the strain. Other combinations of plasmid-coded genes have been described in wild-type E.coli strains. Plasmids coding for enterotoxin production and antibiotic resistance have been identified in E.coli strains isolated in humans and animals (Gyles, Palchaudhuri and Maas, 1977; Scotland et al., 1979). Further studies will probably reveal the presence of other recombinant plasmids which affect the pathogenicity of E.coli strains.

Negatively stained preparations of a CFA/I^+ ETEC strain and its CFA/I^- derivative examined by electron microscopy showed that both types of cells possessed fimbriae. Morphologically the fimbriae in these two preparations were very similar. There were, however, five times more fimbriated bacteria in the CFA/I^+ ETEC preparation than in the CFA/I^- one. Immune electron microscopy further

differentiated the two preparations when it was shown that only the CFA/I fimbriae were coated by the specific antiserum. These results are similar to those found by Evans et al. (1975). Wadström et al. (1978) have also measured CFA/I fimbriae and found diameters of up to 9 nm which were not found in this study or in the one by Evans et al.

Two methods for the purification of CFA/I fimbriae from strain H-10407 have recently been published (Evans et al., 1979; Klemm, 1979). Although these authors used the same ETEC strain they obtained preparations with very different molecular weights. This was probably due to the different purification methods employed and to the presence of contaminants such as other types of fimbriae in their preparations. Evans et al. claimed that the production of type 1 pili is inhibited in H-10407 by growing the strain on CFA agar. The electron microscopy results in this study do not confirm this statement. After repeated subculturing on CFA agar 9 - 13% of cells in the CFA/I$^-$ ETEC preparation showed the presence of fimbriae which had not been coated by the CFA/II antiserum. Since CFA/I, type 1 pili and other fimbriae are similar in morphology it is possible to assume that at least 10% of the cells in the CFA/I$^+$ preparation also possessed type 1 pili and other fimbriae different to CFA/I. None of these preparations, however, caused MSHA of guinea-pig RBC, probably due to insensitivity of the test. Clarification of many of these differences will probably come from the purification of CFA/I fimbriae produced by laboratory strains of E.coli that are genetically unable to produce flagella, type 1 pili or other types of fimbriae into which the CFA/I-ST plasmid has been transferred.

(ii) CFA/II. A second colonisation factor antigen (CFA/II) was first reported as a single fimbrial antigen, different from CFA/I, found in ETEC strains of serogroups O6, O8, O80 and O85 (Evans and Evans, 1978). CFA/II, like CFA/I, was not expressed when the strains were grown at 18 C and was found associated with a specific MRHA pattern. These findings were confirmed in the present study. CFA/II was detected in ETEC strains of the same serogroups as those reported by Evans and Evans and in one ETEC strain of serotype O115:H51. All CFA/II$^+$ strains were able to cause MRHA of calf RBC. This haemagglutination pattern, however, was not sufficient to characterize ETEC strains as CFA/II$^+$ since two of these strains also caused MRHA of human RBC, a haemagglutination pattern normally found related to CFA/I (Evans et al., 1980). Furthermore, other ETEC strains not possessing CFA/II were found to cause MRHA of calf but not human RBC.

A large number of non-toxigenic strains of E.coli isolated from intestinal sources and belonging to the same serogroups as those of ETEC strains possessing CFA/II were also tested for MRHA of human and calf RBC. A number of these non-toxigenic strains were shown to cause MRHA of human and/or calf RBC, but not to possess CFA/II. Screening for the presence of CFA/II can be done by haemagglutination of calf RBC but serological methods must then be employed in order to confirm its presence.

Almost all CFA/II$^+$ strains in this and other surveys have been found to be ETEC strains of serotypes O6:H16 and O8:H9 (Evans and Evans, 1977; Smyth et al., 1979). The reasons for this are not known. It could be related to patterns of acceptance and loss of plasmids encoding for enterotoxin production and adhesive ability

in strains of these serotypes. To test this hypothesis a strain of E.coli of serotype O6:H16 (E1392) that had lost the ability to produce ST and LT but not CFA/II and a CFA/II$^-$ derivative from this strain were used as recipients for three Ent plasmids, one coding for ST, one for LT and a third for ST and LT. Only the plasmid coding for ST and LT was accepted by the CFA/II$^+$ strain and its CFA/II$^-$ derivative. The rate of transfer for this ST-LT plasmid was similar for both strains. When these same three plasmids were used in transfer experiments using as recipients non-toxigenic CFA/II$^-$ E.coli strains of serogroup O6 that were non-motile or that had flagellar antigens other than H16, the three Ent plasmids were accepted with similar transfer rates by these strains. These initial results could explain in part why most ETEC strains of serotype O6:H16 are producers of both ST and LT, but does not explain why E.coli strains of serogroup O6 with flagellar antigens other than H16 have not been found to be toxigenic (Ørskov et al., 1976; Scotland, Gross and Rowe, 1977; Evans and Evans, 1978).

CFA/II$^+$ strains of serotype O6:H16 were found to produce a stronger MRHA of calf RBC than CFA/II$^+$ strains of other serotypes. Repeated subculturing of these latter strains in liquid medium to increase the expression of the CFA/II fimbriae did not change these results. Slide agglutination and immunodiffusion studies with specific antiserum did not show differences in the production of CFA/II by strains of different serotypes. However, these serological studies were only qualitative. Differences in the production of K99 in different E.coli O-groups have recently been found using hydrophobic interaction chromatography and an enzyme-

linked immunosorbent assay (De Graaf, Wientjes and Klaasen-Boor, 1980). Similar tests would be useful to study and quantify the production of CFA/II by ETEC strains of different serotypes.

The use of bacterial agglutination to detect CFA/II was not found to be a reliable method. Some CFA/II$^+$ strains gave a non-specific agglutination with several unrelated antisera, probably due to the presence of other surface features which interfere with specific agglutination. Furthermore, the CFA/II antiserum gave a number of false positive results with other ETEC strains not possessing CFA/II. On the other hand, the use of this same antiserum in immunodiffusion studies produced reliable results when tested against crude saline extracts prepared from the strains studied. Immunodiffusion is therefore the method of choice to confirm the presence of CFA/II.

The results of this survey suggest that most CFA/II$^+$ strains of serotype O6:H16 possess three antigenically distinct components, a fast diffusing one (component C) which was found in all except one CFA/II$^+$ strain, and two very similar slower diffusing ones (components A and B) which were only found in CFA/II$^+$ strains of serotype O6:H16 when they were tested against antiserum prepared with a strain of serotype O6:H16 of the same biotype.

If one accepts that all ETEC strains which cause MRHA of calf RBC and give a precipitin line when diffused against CFA/II antiserum possess CFA/II, then the C component which is found in almost all of these strains would be considered the adhesin responsible for adherence to rabbit intestinal epithelium and the agglutinin causing MRHA of calf RBC. In this study, however, two ETEC strains unable to cause MRHA were found to produce

a precipitin line identical with the C component of CFA/II when tested by immunodiffusion with CFA/II antisera. These results could be due to the presence of other surface features present in these strains which cross-react with the CFA/II antiserum but are not CFA/II. Similar results have been obtained by Smyth (1980) who has suggested that in CFA/II$^+$ strains producing two serologically distinct components the C component, which he calls coli-surface antigen (CS) 3, is the CFA/II adhesin enabling the bacteria to colonise the intestine of rabbits (Evans and Evans, 1978), while the A and B components, which he calls CS1 and CS2 respectively, are the mannose-resistant (MR) haemagglutinin.

In favour of this hypothesis would be the results obtained with strain E201/69, an ETEC strain which caused MRHA and produced the B and C components of CFA/II. Loss of MRHA ability in this strain was accompanied by loss of the B but not the C component of CFA/II. It would seem that in this strain the B component is the MR haemagglutinin. There was, however, another strain of serotype O6:H16 which caused MRHA of calf RBC but produced only the C component of CFA/II after repeated testing and staining of the immunodiffusion plates with Comassie blue to detect the presence of other precipitin lines. It would appear that in this strain (E1069/66) the C component is both the adhesin and the haemagglutinin since loss of the C component was accompanied by loss of both characters.

These results are similar to those reported by Morris, Stevens and Sojka (1978) in studies with a K99 antigen extract from strain 341 (E.coli O101:K99). This K99 extract is composed of two antigenically distinct components, a slowly diffusing anionic

component and a faster diffusing cationic component. In subsequent studies Morris, Thorne and Sojka (1980) have shown that both anionic and cationic components caused MRHA of horse RBC. All $K99^+$ strains were found to possess the cationic component, but only $K99^+$ strains of serogroups 09 and 0101 were also found to possess the anionic component. The two K99 antigens have been shown to mediate adherence to calf brush borders. Immunoelectrophoresis analysis of purified CFA/II fimbriae from strains producing one or both of these CFA/II serological components would be useful for the characterisation of these antigens. It would then be necessary to complement serological analysis with studies of colonisation in animals and feeding experiments in humans in order to test the biological significance of these CFA/II components and their importance in the pathogenesis of diarrhoea.

Loss of CFA/II was not consistently accompanied by loss of enterotoxin production. In some strains loss of CFA/II was accompanied by loss of ST and/or LT, while in other strains CFA/II or enterotoxin production could be lost separately. Some CFA/II subclones were compared by plasmid analysis with CFA/II^- variants derived from them. When such variants did not differ from the parent strain in their ability to produce enterotoxins there were no clear differences in the number and sizes of their plasmids. However, plasmid analysis of other pairs of CFA/II^+ subclones and their CFA/II^- derivatives did show clear differences in the number and sizes of their plasmids. In strain E9187 loss of CFA/II and ST production was accompanied by loss of a single plasmid of 68×10^6 molecular weight, but analysis of a CFA/II^+ST^- derivative from this strain did not show any difference in its plasmid content when compared with the original

strain. Analysis of further derivatives that had lost CFA/II showed once again the loss of a single plasmid of 66 x 10^6 molecular weight. These results suggest that in strain E9187 CFA/II and ST production could be encoded by two separate plasmids of very similar molecular weight, with one obscuring the presence of the other. A second explanation could be the presence of small mutations in a single plasmid coding for CFA/II and ST production which blocks the message for the production of the latter in the CFA/II$^+$ST$^-$ derivative. This could be possible if the production of ST was encoded by a transposon similar to the one described by So and McCarthey (1980) which could be easily lost. Loss of CFA/II in the ETEC subclones studied was accompanied by loss of single plasmids of 40 x 10^6, 60 x 10^6, and 80 x 10^6 molecular weight. Loss of ST or ST and LT was also found when the single plasmid lost was above 60 x 10^6 molecular weight. These findings would indicate that if the production of CFA/II in these strains is indeed a plasmid-mediated characteristic, the size of the plasmids coding for it are very heterogenous in molecular weight, which would make them very different from those coding for CFA/I. Unfortunately, transfer of these putative CFA/II plasmids was not possible even after employing an enrichment method to increase the detection of positive transconjugants. Recently Penaranda et al. (1980) have been able to transfer a plasmid of 60 x 10^6 molecular weight coding for CFA/II, ST and LT production, from a wild-type ETEC strain to an E. coli K12 strain by cotransformation. In the same study two other CFA/II$^+$ST$^+$LT$^+$ and their CFA/II$^-$ST$^-$LT$^-$

derivatives, were shown to have a single plasmid difference when analysed by electrophoresis. One strain was of serotype O6:H-, the other of serotype O85:H7. The plasmid apparently coding for CFA/II and enterotoxin production in the O6:H- strain was of 65×10^6 molecular weight and of 72×10^6 in the one of serotype O85:H7. This study also demonstrates that plasmids coding for CFA/II are of heterogenous molecular weight.

Negatively stained preparations of a CFA/II$^+$ strain and its CFA/II$^-$ variant showed very clear morphological differences. The CFA/II$^+$ bacteria were heavily fimbriated while the CFA/II$^-$ bacteria were completely nude. Since this CFA/II$^+$ strain does not possess type 1 pili, as detected by MSHA of guinea-pig RBC, the fimbriae produced by these bacteria were probably CFA/II. Immune electron microscopy studies showed that the fimbriae produced by the CFA/II$^+$ cells were coated by the specific antiserum. These CFA/II fimbriae were similar in size and diameter to those produced by CFA/I$^+$ strains and indistinguishable morphologically from type 1 pili.

The study of CFA/II$^+$ strains has shown that the antigen is more complex in its serological expression and in its relationship with enterotoxin production than CFA/I. ETEC strains of serotype O6:H16 appear to possess two different types of the CFA/II antigen. The role played by these antigens in human diarrhoea remains to be determined.

(iii) E8775 haemagglutinin. ETEC strains of serotype O25:H42 able to cause MRHA of human and calf RBC were initially found negative

for CFA/I when tested by immunodiffusion with a specific antiserum. Ørskov and Ørskov (1977) reported similar findings with ETEC strains of this serotype causing MRHA. All strains in this survey causing MRHA were retested for CFA/I only by bacterial agglutination in a subsequent investigation. MRHA positive ETEC strains of serotype O25:H42 that had initially been clasified as CFA/I$^-$ by immunodiffusion were found to agglutinate with the specific CFA/I antiserum and were therefore reclassified as CFA/I$^+$. Because of a high incidence of non-specific bacterial agglutination giving false positive results found when the ETEC strains in this survey were tested for CFA/II, it was decided to use only immunodiffusion assays to confirm the presence of CFA/I and CFA/II. As in the initial study, the MRHA positive strains of serotype O25:H42 were not found to possess CFA/I or CFA/II when tested by immunodiffusion with the appropiate antisera in subsequent tests.

Other ETEC strains in the study were also found to cause MRHA of human and calf RBC but not to possess CFA/I or CFA/II. These strains belonged to serotype O115:H40. Immunodiffusion tests with an antiserum prepared from an MRHA positive strain of serotype O25:H42 (E8775) and absorbed with an MRHA negative derivative of this strain, showed that these CFA/I$^-$, MRHA positive strains shared an identical serological antigen. This antigen was present only in MRHA positive strains but not in their MRHA negative derivatives. Other MRHA positive strains of serotypes O25:H42 and O115:H40 able to produce enterotoxins, showed the presence of the same antigen when tested by immunodiffusion to that found in the MRHA positive strains of these serotypes tested initially. These other strains

were also negative for CFA/I and CFA/II. During routine testing of ETEC strains for MRHA ability one strain of serotype 0158:H42 was found to possess an antigen identical to that present in the MRHA positive strain E8775 strain. This 0158:H42 strain also caused MRHA of human and calf RBC.

ETEC strains of serotypes 025:H42, 0115:H40 and 0158:H42 which did not cause MRHA were not found to react with the E8775 antiserum. Certain non-toxigenic E. coli strains isolated from intestinal sources and belonging to serogroups 025, 0115 and 0158 were able to cause MRHA of human and calf RBC but these strains were not found to react with the absorbed E8775 antiserum either. These findings suggest that the antigen detected with the E8775 antiserum is related to the presence of a mannose-resistant haemagglutinin in these ETEC strains. For identification this haemagglutinin was called E8775.

The E8775 haemagglutinin was shown to share many similarities with both CFA/I and CFA/II. It was found only in MRHA positive ETEC strains belonging to a few E. coli serotypes. The antigen was not formed at 18 C. Loss of the antigen during storage at room temperature in some strains was accompanied by loss of enterotoxin production. In other strains, however, the E8775 haemagglutinin was lost separately from the ability to produce LT with or without ST.

Plasmid analysis of an MRHA negative derivative of strain E8774 (E. coli 025:H42) showed the loss of a single plasmid of 56×10^6 molecular weight when compared with the MRHA positive original strain. This difference was observed only after

the MRHA positive strain had lost the ability to produce ST and LT enterotoxins. Another two MRHA negative strains that had lost the E8775 haemagglutinin showed no differences in the number and sizes of their plasmids when compared with their respective E8775 haemagglutinin positive original strains. One of these two strains was E8775 with which the antiserum against the haemagglutinin was prepared. These pairs of strains, however, were still able to produce either ST or ST and LT enterotoxins. The results of the plasmid analysis of these strains are similar to those found with the CFA/II$^+$ strain E9187 described above.

Neither the ability to cause MRHA nor enterotoxin production was transferred directly or by mobilisation from strains with the E8775 haemagglutinin to E. coli K12. Indirect evidence from the plasmid analysis studies, however, suggests that this haemagglutinin could be another plasmid-mediated adhesive factor in ETEC strains. Evans and Evans (1978) have characterized their MRHA positive ETEC strains of serotype O25:H42 as CFA/I$^+$, mainly on the basis of bacterial agglutination tests. The presence of fimbriae, other than type 1 pili, on the bacterial surface can cause non-specific agglutination and a "rough" appearance of pathogenic bacteria when grown in liquid medium (Lopez-Alvarez and Gyles, 1980). Immunodiffusion studies must therefore be used to confirm the presence of these factors.

ETEC strains of serogroup O115 were found to possess two different haemagglutinins, albeit, in two different O:H combinations. CFA/II was found in strains of serotype O115:H51 and

the E8775-type in strains of serotype O115:H40. ETEC strains causing MRHA in other serogroups were found to possess either one or none of the serological types of haemagglutinins so far described.

Epidemiological evidence supports the role played by ETEC strains of the same serotypes as those found in this study to possess the E8775 haemagglutinin in cases of acute diarrhoea (Merson et al., 1979; Wachsmuth et al., 1979; Merson et al., 1980a). Further studies are now indicated to show that strains possessing the E8775 haemagglutinin but not derivatives that have lost the ability to cause MRHA are able to colonise the intestine and cause diarrhoea in animals and humans. The results of these investigations will determine whether the E8775 haemagglutinin is a new colonisation factor antigen.

(iv) Adhesive factors in EPEC strains. Strains of E. coli belonging to the traditional infantile enteropathogenic serotypes (EPEC) have been the cause of numerous outbreaks of infantile enteritis in different countries (Taylor, 1961; Guerrant et al., 1972; Gurwith et al., 1978; Rowe, 1979). Most EPEC strains do not produce enterotoxins detected by the tests widely used for the identification of ETEC strains (Gross, Scotland and Rowe, 1976). Nevertheless, a number of these strains have been shown to elaborate toxic substances which cause fluid secretion in a rat gut perfusion model (Klipstein et al., 1978), and their ability to cause diarrhoea has been confirmed by feeding experiments with adult volunteers (Levine et al., 1978). The occurrence of colonisation

factors, as distinct from type 1 pili, has not been reported among non-enterotoxigenic EPEC strains. In the present study, EPEC strains isolated during outbreaks of enteritis and from sporadic cases of diarrhoea were tested for the presence of adhesive factors by means of haemagglutination and serological tests. They were also tested for their ability to adhere to HEp-2 cells in tissue culture. ETEC strains and E.coli strains from the faeces of subjects without diarrhoea were also examined in this in vitro HEp-2 tissue culture assay.

Approximately 80% of the EPEC strains studied, whether from outbreaks or sporadic cases of enteritis, were found to adhere to HEp-2 cells. The bacterial adhesion demonstrated by this method was not due to the presence of CFA/I, CFA/II or the E8775 haemagglutinin, none of which were found in the EPEC strains examined in this survey. A few ETEC strains were also found to adhere to HEp-2 cells. However, the incidence of HEp-2 adhesive strains among ETEC strains and among non-EPEC, non-ETEC strains from outbreaks of diarrhoea was similar to that found among control strains obtained from individuals without diarrhoea.

Adhesion to HEp-2 cells was not due to type 1 pili or to other mannose-resistant haemagglutinins which were found in 13% of the EPEC strains in the survey. HEp-2 adhesion was not related to VERO cytotoxin (VT) or urease production, both frequently found characters in EPEC strains especially of serogroup 026 (Scotland, Day and Rowe, 1980; Wachsmuth, Davis and Allen, 1979).

HEp-2 adhesive strains were found in all the EPEC outbreaks and in all the traditional EPEC serotypes studied. In the case of strains isolated after 1972, almost all the strains were adhesive,

whereas non-adhesive strains were found more frequently in earlier isolates. It was initially thought that these results could be due to loss of the adhesive factor during the storage of these strains and it was therefore suggested that HEp-2 adhesiveness could be plasmid-mediated.

Williams et al. (1978) have reported adhesion of the human EPEC strain H19 (E.coli 026:K60:H11) to human foetal small intestine cells mediated by a specific plasmid also coding for the production of colicin Ib. In this study strain H19 has been shown to adhere consistently to the HEp-2 cells. However, following the conjugal transfer of the ColIb plasmid from strain H19, the E.coli K12 transconjugants that acquired ColIb were not HEp-2 adhesive. Furthermore, many of the HEp-2 adhesive EPEC strains in the present survey were non-colicinogenic. E.coli K12 transconjugants that had acquired other plasmids from strain H19 were not HEp-2 adhesive either. Examination of spontaneous or "cured" mutants of H19 confirmed that adhesion to HEp-2 cells was not mediated by any of the plasmids present in this strain. Treatment with ethidium bromide significantly reduced the percentage of adherence to HEp-2 cells but was not able to make the strains non-adhesive. Transfer experiments using other HEp-2 adhesive wild EPEC strains gave the same results as those with H19. These findings suggest that adhesion to HEp-2 cells is not a plasmid-mediated characteristic and may be controlled by the bacterial chromosome. Genetic control of this HEp-2 adhesion may therefore be similar to that found in strains of V.cholerae which adhere to isolated rabbit brush borders and human RBC (Jones, Abrams and Freter, 1976). Adhesion of V.cholerae strains was found to be dependent on temperature, rate of contact,

initial concentration of bacteria used and motility (Jones and Freter, 1976). The genetic study of spontaneous or "cured" mutants of EPEC strains able and unable to adhere to HEp-2 cells and genetic complementation studies, such as those used to investigate the control of type 1 pili production (Swaney et al., 1977) will probably help to define where and how this HEp-2 adhesive ability is controlled.

Other enteropathogenic strains of E.coli have been shown to possess adhesive ability. Cantey and Blake (1977) have studied a strain of E.coli of serotype O15:H- (RDEC-1) which adheres to the intestinal epithelial cells of rabbits and causes diarrhoea in these animals without invading the mucosal cell or producing ST or LT enterotoxins. Strain RDEC-2 is species specific, heavily colonising the intestine of rabbits but not of rats or guinea-pigs and causing diarrhoea only in the first (Cheney et al., 1980). Adhesive experiments using isolated rabbit brush borders in vitro have also shown that strain RDEC-1 has greater affinity for ileal epithelial cells than for jejunal epithelial cells (Cheney et al., 1980). Like some EPEC strains, RDEC-1 seems to produce a cytotoxin which destroys the epithelial brush border and allows the bacteria to come into close contact with the epithelial intestinal cells; in some cases the bacteria have been found to penetrate the epithelial cells, but never beyond the lamina propria (Takeuchi et al., 1978).

Recently, Ulshen and Rollo (1980) have described the case of an infant with severe enteritis probably caused by a strain of E.coli isolated as the putative pathogen from duodenal fluid. This strain of E.coli of serotype O125ac:H21 did not produce ST or LT enterotoxins and was not invasive. Biopsies of the duodenal

and colonic mucosa of this infant revealed histologic and ultrastructural changes very similar to those found in rabbits with enterocolitis caused by strain RDEC-1. Although some dissimilarities between the human and the animal disease were also found, these could probably be explained by species differences and differences in the stage of development of the enteritis when the biopsies were taken. Strain RDEC-1 has been shown to adhere to hydrophobic columns which the E.coli O125ac:H21 isolated from this case did not; the importance of this difference is not known.

Other E.coli strains belonging to traditional EPEC serotypes have also been shown to be adhesive by in vivo and in vitro assays. Using a fluorescent periodic acid-Schiff reaction, Khavkin et al. (1980) have found that a non-enterotoxigenic E.coli strain of serotype O26:K60:H11 was able to cause extensive brush border lesions and mucous cell hyperproduction in rabbit intestinal epithelium. This strain penetrated some epithelial cells but did not invade the lamina propria. An ETEC strain of serotype O148:H28 (B7A) able to produce ST and LT enterotoxins was also examined in this study. This strain caused fluid secretion and restricted brush border damage but was not found inside the epithelial cells. In post-mortem studies of infants that died from severe enteritis caused by EPEC strains, Boyd (1978) has reported extensive brush border damage and bacterial invasion of the intestinal epithelial cells. In these cases, however, no correlation was made between the bacteria found inside the intestinal epithelial cells and the EPEC strain isolated while the patient was alive. Although the histological findings described by Boyd could have been due to post-mortem degeneration, they are similar to those found in the other EPEC studies mentioned

before.

A number of *in vitro* adhesive assays using mammalian or tissue culture cells have been described. Human cells obtained from the oral mucous membranes, the skin, the intestine or the urinary tract have been used to detect adhesiveness of different organisms (Sugarman and Donta, 1979a; McNeish *et al.*, 1975; Wadström *et al.*, 1979; Svanborg-Eden, 1978). HeLa, HEp-2, Intestine-407, Y1, CHO and VERO tissue culture cell lines have also been used for the same purpose (Chiarini and Giammanco, 1978; Andreyeva, Khalitova and Davydova, 1977; Hartley, Robbins and Richmond, 1978; Sugarman and Donta, 1979a). In some studies only one or two well characterized strains have been tested, whereas in others a large number of poorly characterized strains have been examined. In both cases the results have been difficult to interpret and to relate to events *in vivo*. Nevertheless, these assays have been able to show that the adhesive process is complex with both bacteria and mammalian cells playing an active role.

In the present study a large number of biochemically and serologically characterized EPEC strains have been shown to adhere *in vitro* to HEp-2 cells. These strains seem to have greater affinity for receptors in these HEp-2 cells than other pathogenic and non-pathogenic *E.coli* strains. Although the assay may have little relevance to situations *in vivo*, it provides a good *in vitro* model to test the adhesive ability of these EPEC strains. The best conditions for HEp-2 adherence were found to be similar to those described for the production of diarrhoea by EPEC strains in animals and humans (Koya, Kosakai and Fukasawa, 1954; Taylor, 1961; Levine *et al.*, 1978). The HEp-2 assay can also be used for the

selection and study of E.coli strains possessing an adhesive factor different from those already described and preferentially found in EPEC strains. The results of this interaction may be important in vivo, and the use of this model could facilitate our understanding of the mechanisms underlying the specificity of adherence. Initial serological studies demonstrate that adhesion to HEp-2 cells is due to several antigenically distinguishable factors. Studies to characterize these different adhesive factors and their genetic control are now in progress. Sugarman and Donta (1979a) have suggested that bacteria could be "typed" according to which cell line(s) they best adhere and that such typing could be of potential epidemiological value. The results in this study support this proposition.

IVb. Future applications of the findings

Strains of E.coli have been shown to possess a variety of pathogenic mechanisms that enable them to cause diarrhoeal disease in humans. As mentioned in the introduction to this study, recent investigations on the ability of E.coli strains to produce enterotoxins and to invade epithelial cells have helped to elucidate the mechanism by which these bacteria cause disease in mammalian hosts. For a strain of E.coli to be able to use these pathogenic mechanisms it must be able to adhere to the epithelial cells of certain areas of the host intestine. Once attached the bacteria can multiply and compete for nutrition and survival with the local microbiota.

Most strains of E.coli that colonise humans and animals are normally non-pathogenic. They abide in the distal regions of the

ileum and the caecum where the ecological conditions are best suited for their growth and survival. Enteropathogenic strains tend to colonise the jejunum and proximal ileum, areas of fast luminal flow with great quantities of nutrients available and almost no bacterial competition (Savage, 1977).

Bacteria adhere initially to the intestinal mucous and to a layer of thin filaments radiating from the microvilli of the enterocytes, called the glycocalix, both structures rich in polysaccharide and glycoprotein (Ito, 1969). Hydrolysis of these macromolecules can provide a ready source of energy, carbon, nitrogen and sulphur, mainly through degradation by the host digestive enzymes, since few bacteria are able to produce glycosidases (Savage, 1977). The pathogens must also be able to compete successfully with the host for particular substances necessary to both, such as iron. For this purpose pathogenic bacteria have developed specific uptake systems (Binns, Davies and Hardy, 1979).

Under these conditions the bacteria can overcome the host non-immune defense mechanisms and come into contact with the cell membrane of the enterocytes. Once there, the pathogens can produce substances such as enterotoxins, that stimulate the epithelial cells to secrete water and electrolytes into the intestinal lumen.

The production of enterotoxins can be of advantage to pathogenic bacteria in several ways. They stimulate the secretion of large quantities of fluids containing free oxygen and thus create aerobic conditions which promote bacterial growth but are not found normally in the intestine (Savage, 1980). Enterotoxins have been shown to stimulate the smooth muscle of the intestine causing an increase in peristalsis (Pesti and Gordon, 1978); this would

allow the pathogenic bacteria to survive in areas of high competition by dislodging the local microflora from its niches and flushing it out of the intestine. With the onset of the resulting diarrhoea the pathogens can be shed in sufficient quantities from the intestine to contaminate the environment and from there infect other hosts.

Recent knowledge of the way by which bacteria attach to the intestinal cell has increased the number of possible strategies for the control of diarrhoeal diseases. As with other infections the bacteria causing the disease can be eliminated with antibacterial drugs. Antibiotic treatment coupled with restitution of liquids and electrolytes lost by the individual is one of the most frequently used treatments for bacterial diarrhoeas (Hirschhorn, 1980). Sack et al. (1979) have studied the effect of doxycycline - a type of tetracycline - as a prophylactic against travellers diarrhoea. In a field trial with Peace Corps volunteers in Kenya, individuals receiving the antibiotic had fewer episodes of diarrhoea than controls not receiving the drug. Merson et al. (1980b) have also shown that tetracyclines might be of use in the control of diarrhoea caused by ETEC strains since patients treated with the antibiotic shed the pathogenic bacteria for a significantly shorter time than control patients not receiving the antibiotic. Tetracyclines might be effective because they interfere with the adhesive ability of ETEC strains. Roland and Heelan (1979) have reported that this antibiotic inhibits the MRHA caused by ETEC strains possessing CFA/I. Adherence of E.coli strains and other Enterobacteriaceae to human buccal cells has also been shown to be inhibited by tetracyclines (Sugarman and Donta, 1979). Other antibiotics such as chloramphenicol and

streptomycin have also been found to inhibit E.coli adherence to human buccal cells (Sugarman and Donta, 1979b) and to mannose-receptors in epithelial cells by interfering with the expression of type 1 pili (Eisenstein, Ofek and Beachey, 1979).

The problem associated with the widespread use of these antibiotics, especially in a world-wide disease such as diarrhoea, has caused many researchers to question their use. The number of individuals involved in the reported studies has been very small to justify their use beyond people travelling for short periods of time from developed countries to tropical areas where the risk of contracting diarrhoea is high. Tetracyclines, of course, are not indicated in children and expectant mothers because of undesirable side effects. Pathogenic strains in areas with a high incidence of diarrhoea have been found to be more often resistant to one or more antibiotics than strains from areas with a low incidence of the disease (DuPont et al., 1978; Echeverria et al., 1978). More important, perhaps, is the possible occurrence of genetic recombination between plasmids encoding for antibiotic resistance and other pathogenic mechanisms, such as enterotoxin production, under selective pressure from the antibiotic in the intestine. Two such recombinant plasmids have already been reported in ETEC strains isolated from cases of diarrhoea, one from a pig (Gyles, Palchaudhuri and Maas, 1977), the other from a human (Scotland et al., 1979).

Epithelial cell receptors for adhesive factors in enteropathogenic strains of E.coli have only recently started to be studied (Kearnes and Gibbons, 1979; Faris, Lindahl and Wadström, 1980). With more information on the structure and function of these

receptors a second strategy for the control of bacterial diarrhoeas could be developed. Receptor megatherapy (Keusch, 1978) consists of supplying an excess of receptor to compete with those on the epithelial cells for the pathogenic bacteria or their products. Receptor blockade, on the other hand, consists of supplying substances that compete for the epithelial cell receptors to which pathogenic bacteria attach. This blockade prevents the interaction between bacterial products and biochemical mechanisms in the epithelial cell that causes active secretion and cause diarrhoea.

A number of methods for the purification of fimbriae isolated from pathogenic strains of E.coli have recently been published (Klemm, 1969; Evans et al., 1979; Korhonen et al., 1980). These purified fimbriae could be used to block intestinal receptors when given for the prophylaxis of diarrhoea.

Binding of strains of E.coli to epithelial cells has also been reported to be mediated by mannose-specific lectin-like substances present on the surface of the bacteria which bind to mannose-like residues on the mucosal cell (Ofek, Mirelman and Sharon, 1977). Inhibition of these receptors by externally supplied D-mannose and its analogues can provide another strategy for the control of diarrhoea. This strategy, called elution therapy, (Keusch, 1978) consists of administering an excess of the saccharide on the receptor site to dislodge or displace the bacteria. This type of therapy has been successfully used in urinary tract infections in mice caused by strains of E.coli possessing mannose-sensitive fimbriae (Aronson et al., 1979). Most of the strains of E.coli that cause intestinal infections, however, adhere to epithelial

cells via mannose-resistant fimbriae (Evans et al., 1975; Evans and Evans, 1978; this study). Therefore elution therapy using D-mannose would be of little value in cases of diarrhoea caused by these organisms.

Bacterial diarrhoeas can also be controlled by pharmacological inhibition with the use of compounds that reverse specific biochemical processes involved in the pathogenesis of the disease. At present this type of therapy is only available for diarrhoea caused by ETEC strains. Compounds such as bismuth subsalicylate and chlorpromazine have been shown to reverse the cyclic-AMP mediated intestinal secretion caused by cholera toxin and E.coli heat-labile enterotoxin in vitro (Ericsson et al., 1977; Holmgren, Lange and Lönnroth, 1978). The protection offered by these compounds is not complete and both may cause undesirable side effects in some individuals (Merson, 1980).

The most widely available treatment for bacterial diarrhoeas continues to be intravenous and/or oral replacement therapy. This type of therapy, called physiological antagonism by Keusch (1978) consists of promoting intestinal absorption of water and electrolytes by unaffected metabolic pathways similar to those used by the bacteria to cause intestinal secretion. One example of this is the use of oral glucose-electrolyte solutions in the treatment of diarrhoea to promote glucose-mediated absorption of sodium and water by the intestinal cell (Nalin et al., 1979).

Diarrhoea caused by strains of E.coli is frequently a disease of young infants and animals or of travellers to areas of lower environmental sanitation. This suggests that contact with these pathogens produces an immune response in the host that prevents repeated infections by the same or similar organisms.

Measures designed to increase the host immunity to pathogenic E.coli strains would therefore be expected to offer a further strategy for the control of diarrhoea caused by these strains.

The mucosal surface of the intestine is defended from pathogenic organisms and toxic agents by the production of intestinal antibodies secreted locally by plasma cells. When these cells come in contact with the bacterial antigens they proliferate and differentiate into antibody-secreting cells. The main secretory antibody produced by these plasma cells is secretory immunoglobulin A (sIgA) (Walker and Isselbacher, 1977). Other immunoglobulins, IgG, IgD and IgE are also present in secretions, with IgM taking the functions of sIgA when the production of the latter is deficient. Once primed these secreting plasma cells can defend the intestinal mucosa from reinfection by the same or similar organisms. Plasma cells can also "travel" from the intestine to the mammary gland and produce specific sIgA against bacterial antigens in breast milk (Roux et al., 1977). Antibodies synthesized in the lymphoid tissue of the gut can also find their way into breast milk increasing the amount of specific sIgA available (Halsey, Johnson and Cebra, 1980).

sIgA has been shown to have a bacteriostatic and a bactericidal effect. The former functions mainly as an antiadhesive mechanism probably by coating of the bacterial fimbriae and inhibiting adhesion of these organisms to epithelial cells (Williams and Gibbons, 1972). The formation of antiadhesive antibodies, however, must be preceded by adherence of the bacterial fimbriae to the intestinal mucosa in order to stimulate the antibody-producing plasma cells. Adhesion to epithelial cells by

these pathogens may be enhanced by the maintenance of the local microbiota in a repressed adhesive state by sIgA, giving the temporary colonisers, such as ETEC and EPEC strains, an advantage due to lack of competition for epithelial cell receptors (Williams and Gibbons, 1972).

Parenteral vaccination against enteric infections caused by Gram-negative bacteria has been unsatisfactory because the vaccines are frequently ineffective, protection is transient or because of side-reactions associated with endotoxins (Robbins, 1978). Oral prophylaxis with attenuated bacteria has more advantages since it follows a process similar to that of the natural infection, but there are also inherent dangers associated with the administration of live organisms. An alternative approach has been to recognise the pathogenic determinants of enteropathogenic bacteria and to stimulate the production of protective immunity to these determinants.

The potential use of antitoxic immunity in the prophylaxis of diarrhoea has been studied by several authors. Sack (1973) has immunized rabbits with culture filtrates containing E.coli LT enterotoxin and shown anti-enterotoxic activity in rabbit sera and protection in vaccinated animals against homologous enterotoxin challenge. Pierce (1977) has vaccinated rats with cholera toxoid and shown protection in these animals against diarrhoea when challenged with an E.coli LT-producing strain. Recently, Klipstein, Engert and Short (1980) have shown cross-protection between heat-labile enterotoxins produced by ETEC and EPEC strains in rats vaccinated only with the LT obtained from an ETEC strain. These studies would suggest that antitoxic

immunity does afford protection against diarrhoea. Studies in humans, however, have been less successful. Levine et al. (1979) have immunized human volunteers with an ETEC strain producing LT and ST enterotoxins and shown a rise in anti-LT antibodies in these subjects. The antibody response was transitory and only protected the volunteers against diarrhoea but not colonisation by the homologous organism. When challenged with a heterologous ETEC strain producing only LT most of the vaccinated subjects developed diarrhoea. Furthermore, anti-LT vaccination does not afford protection against diarrhoea caused by the non-antigenic heat-stable enterotoxin produced by many ETEC strains either alone or in combination with LT. The incidence of ETEC strains producing ST with or without LT is very high in some areas (Merson et al., 1980a) and vaccination against LT only would therefore be inadequate in such areas.

A second and more promising alternative for the immunoprophylaxis of diarrhoea is the use of somatic pili present on the surface of enteropathogenic bacteria as vaccines to promote anti-adhesive immunity in the host. The serological identification of these specific adhesive fimbriae and their role in the attachment of ETEC strains has already been discussed.

Rutter and Jones (1973) vaccinated pregnant gilts with partially purified K88 antigen and found a significant reduction in mortality due to diarrhoea in piglets from vaccinated mothers when challenged with a K88 positive ETEC strain. Since there is no transplacental passage of antibodies to the foetus in pregnant gilts, Rutter and Jones suggested that the presence of K88 antibodies in the colostrum had afforded passive protection to the newborn animals. Jones and Rutter (1974) subsequently

demonstrated anti-adhesive ability *in vitro* of colostrum from sows vaccinated with partially purified K88 antigen. Nagy *et al.* (1978a) showed that natural infection with K88 positive *E.coli* or the ingestion of colostrum from sows which had been vaccinated with a vaccine rich in K88, could prevent adhesion of K88 positive ETEC strains to the epithelial cells of the anterior intestine. Colostrum and milk from these immunised sows also showed a powerful bactericidal effect against ETEC strains *in vitro*.

Suckling piglets from dams that had been vaccinated parenterally with purified K99 and 987P fimbriae have also been found to be protected against enteric disease when challenged with ETEC strains possessing the homologous adhesive factors (Morgan *et al.*, 1978; Nagy *et al.*, 1978b). The protection, however, was not extended against ETEC strains carrying a heterologous type of fimbriae. Colostrum of cows vaccinated either intramuscularly or subcutaneously near the mammary gland with formalized ETEC bacteria possessing K99 has also been shown to afford protection in newborn calves challenged with the same K99-positive ETEC strain (Bagley and Call, 1979). In a recent study, Isaacson *et al.* (1980) have reported a direct correlation between levels of pilus-specific antibodies measured with a specific ELISA test in serum and colostrum of pregnant dams vaccinated with purified K99 and 987P fimbriae and the protection of their suckling piglets against fatal diarrhoea caused by ETEC strains possessing the same fimbriae as the vaccine. Colostral anti-K99 antibodies have also been reported to offer passive protection against diarrhoea caused by K99-positive ETEC strains in newborn lambs after vaccinating the pregnant ewes with cell-free

K99 antigen (Sojka, Wray and Morris, 1978).

Breast-fed infants are less likely to be colonised by enteropathogenic strains of E.coli and show a lower incidence of infectious diarrhoea than bottle-fed infants in developing areas of the world (Mata and Urrutia, 1971). Breast milk obtained from women in these areas has been shown to possess specific sIgA antibodies against the heat-labile enterotoxins produced by V.cholerae and ETEC strains (Stoliar et al., 1976) and against CFA/I (Martins Filho et al., 1980; D. G. Evans and D. Sack, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B48, p. 25). Whether the anti-diarrhoeal effect of human milk depends on the presence of these specific immunoglobulins or on the bactericidal effect of lactoferrin, lysozyme and other compounds also present in the milk remains to be determined (Bullen, 1977; Rogers and Synge, 1978).

Isolation and characterization of bacterial fimbriae that mediate adherence to epithelial cells could be of great value in the control of infections in humans caused by these pathogens. In addition to CFA/I and CFA/II, other mannose-resistant haemagglutinins have been found in ETEC and EPEC strains (Deneke, Thorne and Gorbach, 1979; Wevers et al., 1980) and in E.coli strains causing extraintestinal infections (Minshew et al., 1978; Ljung, Faris and Wadström, 1979; Ørskov, Ørskov and Birch Anderson, 1980). These mannose-resistant (MR) fimbriae are not related serologically to CFA/I and CFA/II. However, they also mediate attachment to epithelial cells, especially in the case of urinary tract infections, where their functions have been more extensively studied (Källenius and Möllby, 1979; Korhonen, Edén and Svanborg-Edén, 1980). Specific sIgA antibody against these

MR fimbriae has also been found in human breast milk (Svanborg-Edén et al., 1979). Purified MR fimbriae from these pathogens could therefore be used as vaccines to enhance passive and active immunity against intestinal and extraintestinal infections caused by E.coli possessing these antigens.

There are, however, many pathogenic strains of E.coli that do not produce MR fimbriae. These strains are able to colonise and produce intestinal and extraintestinal infections by mechanisms that are only now beginning to be recognised (Klipstein et al., 1978; Levine et al., 1980). Assays like the HEp-2 adhesive test will be useful in the identification of structures on the surface of the bacteria that mediate attachment to specific receptors in epithelial cells. Combinations of these structures could then be used as vaccines for the immunoprophylaxis of infections caused by pathogenic strains of E.coli.

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Table 1

Characteristics of types of *Escherichia coli* strains causing diarrhoea in humans

	ENTEROPATHOGENIC (EPEC)	ENTEROTOXIGENIC (ETEC)	ENTEROINVASIVE (EIEC)
Pathogenic mechanism	Probably a 'toxin'	Heat-stable and heat-labile enterotoxins	Epithelial cell invasion and multiplication
Age groups affected	Infants Adults - rarely	Infants and young children Adults (travellers)	All ages
World areas affected	Worldwide	Mainly developing and 'tropical' countries	Worldwide
Most common *E.coli* O-groups associated	26, 44, 55, 86, 111, 114, 119, 125, 126, 127, 128, 142	6, 8, 15, 25, 27, 63, 78, 148, 159	28ac, 112ac, 124, 136, 143, 144, 152, 164

Table 2

Comparison of colonisation factor antigen (CFA) I and II, K88 and K99

Adhesive factor	Associated with serotypes	Causing diarrhoea in	Protein fimbrial structure	Formed at		Plasmid determined	MRHA* of red blood cells indicated				
				18 C	37 C		Human	Calf	Guinea-pig	Sheep	Horse
CFA/I	063, 078 etc.	Humans	Yes	No	Yes	Yes	+	+	-	-	-
CFA/II	06, 08 etc.	Humans	Yes	No	Yes	?Yes	-	+	-	-	-
K88	08, 0141, 0149 etc.	Pigs	Yes	No	Yes	Yes	-	-	+	-	-
K99	08, 09, 0101 etc.	Calves, lambs, pigs	Yes	No	Yes	Yes	-	+	+/-	+	+

*Mannose-resistant haemagglutination.

Table 3

Mannose-resistant haemagglutination and presence of colonisation factor antigens in *E.coli* strains from various sources

Source of *E.coli*	Number of strains tested	Number giving MRHA* of indicated red blood cells		Number CFA/I** positive	Number CFA/II** positive
Faeces Enterotoxigenic *E.coli* (ETEC)	206	Human only	1 (0.5%)	0	0
		Calf only	50 (24%)	0	47 (23%)
		Human and calf	42 (20%)	36 (17%)	2 (1%)
		Total	93 (45%)	36 (17%)	49 (24%)
Faeces Enteropathogenic *E.coli* (EPEC)	186	Human only	12 (6%)	0	0
		Calf only	6 (3%)	0	0
		Human and calf	7 (4%)	0	0
		Total	25 (13%)	0	0
Cerebro-spinal fluid	122	Human only	62 (51%)	0	0
		Calf only	12 (10%)	0	0
		Human and calf	7 (6%)	0	0
		Total	81 (66%)	0	0
Urine	124	Human only	45 (36%)	0	0
		Calf only	4 (3%)	0	0
		Human and calf	8 (6%)	0	0
		Total	57 (46%)	0	0
Blood	130	Human only	60 (46%)	0	0
		Calf only	4 (3%)	0	0
		Human and calf	14 (11%)	0	0
		Total	78 (60%)	0	0

*Mannose-resistant haemagglutination.

**Colonisation factor antigen.

Table 4

Enterotoxigenic E.coli studied according to serotype and mannose-resistant haemagglutination (MRHA) of human and/or calf red blood cells and colonisation factor antigen (CFA) I and II

Serotype	Number of strains				MRHA Positive	CFA/I Positive	CFA/II Positive
	Total	ST^+	LT^+	ST^+ LT^+			
O1H1	1			1	0	0	0
O6H16	39		4	35	35	0	35
O7H-	1	1			1	0	0
O7H18	1		1		0	0	0
O8H-	1		1		0	0	0
O8H9	15		2	13	10	0	10
O15H-	2		2		2	0	0
O15H11	3		1	2	0	0	0
O20H11	1	1			0	0	0
O25H16	3		3		0	0	0
O25H42	2			2	2	0	0
O27H7	5	5			0	0	0
O27H20	1	1			0	0	0
O44H-	1	1			0	0	0
O60H19	1			1	0	0	0
O63H-	12		3	9	9	9	0
O63H12	2	2			1	1	0
O63H30	1			1	0	0	0
O75H10	1		1		0	0	0
O78H-	3	1	1	1	1	1	0
O78H11	3			3	3	3	0
O78H12	19	4	2	13	13	13	0
O78H18	1	1			0	0	0
O80H9	1	1			1	0	1
O85H7	2			2	2	0	2
O88H21	1		1		0	0	0
O89H-	1	1			0	0	0
O109H-	3		3		0	0	0
O114H2	1		1		0	0	0
O114H21	4	4			1	1	0
O114H49	3		3		0	0	0
O114H?	1	1			0	0	0
O115H40	2			2	2	0	0
O115H51	3			3	1	0	1
O128H7	1	1	0	0	1	1	0
O128H10	2	2			2	2	0
O128H12	2	2			2	2	0
O128H18	1	1			0	0	0
O128H20	1	1			1	0	0
O128H27	4	4			0	0	0
O128H49	7		7		0	0	0
O128H?	3	3			1	1	0
O148H28	7	5		2	0	0	0
O153H10	1	1			0	0	0
O153H12	1	1			1	1	0

Continued

Table 4 (continued)

Serotype	Number of strains				MRHA Positive	CFA/I Positive	CFA/II Positive
	Total	ST^+	LT^+	ST^+ LT^+			
O159H4	5		4	1	0	0	0
O159H20	1	1			0	0	0
O159H21	3		3		0	0	0
O159H34	3		2	1	0	0	0
O?H-	3		2	1	0	0	0
O?H7	1		1		0	0	0
O?H8	1		1		0	0	0
O?H25	1	1			0	0	0
O?H27	3	3			0	0	0
O?H31	1		1		0	0	0
O?H33	1	1			0	0	0
O?H40	3		3		0	0	0
O?H49	3		3		0	0	0
ORH-	1	1			0	0	0
ORH10	1		1		0	0	0
ORH12	1	1			1	1	0
ORH21	1			1	0	0	0
ORH27	1	1			0	0	0
ORH55	1		1		0	0	0
Total	206	54	58	94	93	36	49
		(26)	(28)	(46)	(45)	(17)	(24)

Figures in parentheses are percentages.

Table 5

Relationship between mannose-resistant haemagglutination (MRHA) of human and/or calf red blood cells (RBC) and the presence of colonisation factor antigen (CFA) I and II in enterotoxigenic E.coli (ETEC) strains by serotype.

Serotype	Total number of strains tested	Number of RBC	giving MRHA indicated	Number CFA/I positive	Number CFA/II positive
O6H16	39	H*	0	0	0
		C**	33	0	33
		H+C***	2	0	2
O7H-	1	H	1	0	0
		C	0	0	0
		H+C	0	0	0
O8H9	15	H	0	0	0
		C	10	0	10
		H+C	0	0	0
O15H-	2	H	0	0	0
		C	2	0	0
		H+C	0	0	0
O25H42	2	H	0	0	0
		C	0	0	0
		H+C	2	0	0
O63H-	12	H	0	0	0
		C	0	0	0
		H+C	9	9	0
O63H12	2	H	0	0	0
		C	0	0	0
		H+C	1	1	0
O78H-	3	H	0	0	0
		C	0	0	0
		H+C	1	1	0
O78H11	3	H	0	0	0
		C	0	0	0
		H+C	3	3	0
O78H12	19	H	0	0	0
		C	0	0	0
		H+C	13	13	0

Continued

Table 5 (continued)

Serotype	Total number of strains tested	Number giving MRHA of RBC indicated		Number CFA/I positive	Number CFA/II positive
O80H9	1	H	0	0	0
		C	1	0	1
		H+C	0	0	0
O85H7	2	H	0	0	0
		C	2	0	2
		H+C	0	0	0
O114H21	4	H	0	0	0
		C	0	0	0
		H+C	1	1	0
O115H40	2	H	0	0	0
		C	0	0	0
		H+C	2	0	0
O115H51	3	H	0	0	0
		C	1	0	1
		H+C	0	0	0
O128H7	1	H	0	0	0
		C	0	0	0
		H+C	1	1	0
O128H10	2	H	0	0	0
		C	0	0	0
		H+C	2	2	0
O128H12	2	H	0	0	0
		C	0	0	0
		H+C	2	2	0
O128H20	1	H	0	0	0
		C	1	0	0
		H+C	0	0	0
O128H?	3	H	0	0	0
		C	0	0	0
		H+C	1	1	0
O153H12	1	H	0	0	0
		C	0	0	0
		H+C	1	1	0
ORH12	1	H	0	0	0
		C	0	0	0
		H+C	1	1	0
Total	121		93 (77)†	36 (30)	49 (40)

*H - Human MRHA only
**C - Calf MRHA only
***H+C - Human and calf MRHA
†Figures in parentheses are percentages

· Table 6

Mannose-resistant haemagglutination (MRHA) of human and/or calf red blood cells by human enteropathogenic E.coli (EPEC) strains

O-group	Total number of strains tested	Total MRHA positive	Human MRHA	Calf MRHA	Human and Calf MRHA
018ac	5	2	0	0	2
026	68	11	5	4	2
055	6	1	1	0	0
0111	14	1	1	0	0
0114	12	4	2	1	1
0119	14	0	0	0	0
0125	17	1	0	0	1
0126	10	1	0	0	1
0127	14	0	0	0	0
0128	15	4	3	1	0
0142	11	0	0	0	0
Total	186	25 (13%)	12 (6%)	6 (3%)	7 (4%)

. Table 7

Extraintestinal E.coli strains by frequency of O-group and mannose-resistant haemagglutination (MRHA) of human and/or calf red blood cells

O-group	Total number of strains studied	MRHA positive	(%)
018ac	44	31	(70)
06	39	25	(64)
075	32	22	(69)
07	24	19	(79)
016	23	21	(91)
01	21	16	(76)
04	21	16	(76)
02	19	11	(58)
09	16	3	(19)
08	10	4	(40)
0101	7	2	(29)
025	6	2	(33)
083	6	2	(33)
018ab	5	0	(0)
0160	3	0	(0)
0?	39	10	(26)
O Rough	28	16	(57)
Others [a]	33	15	(45)
Total	376	215	(57)

[a] One or two isolates in 30 different O-groups.

Table 8

Mannose-resistant haemagglutination of human and/or calf red blood cells by human strains of E.coli isolated from cerebrospinal fluid

O-group	Total number of strains tested	Total MRHA positive (%)	Human MRHA	Calf MRHA	Human and Calf MRHA
01	10	7 (70)	7	0	0
02	5	3 (60)	3	0	0
04	3	3 (100)	1	0	2
06	5	3 (60)	0	1	2
07	15	13 (87)	13	0	0
08	2	0 (0)	0	0	0
011	1	1 (100)	1	0	0
015	1	0 (0)	0	0	0
016	16	15 (94)	14	0	1
018ab	1	0 (0)	0	0	0
018ac	22	15 (68)	12	3	0
020	1	0 (0)	0	0	0
075	2	1 (50)	1	0	0
077	1	0 (0)	0	0	0
078	1	0 (0)	0	0	0
083	2	2 (100)	1	1	0
098	1	1 (100)	0	1	0
0101	4	1 (25)	0	0	1
0120	2	2 (100)	2	0	0
0132	1	1 (100)	1	0	0
0?	10	3 (33)	1	2	0
OR	16	10 (63)	5	4	1
Total	122	81 (66)	62 (51)	12 (10)	7 (6)

Figures in parentheses are percentages.

Table 9

Mannose-resistant haemagglutination of human and/or calf red blood cells by human strains of *E.coli* isolated from urine

O-group	Total number of strains studied	Total MRHA positive (%)	Human MRHA	Calf MRHA	Human and Calf MRHA
01	2	2 (100)	2	0	0
02	8	3 (38)	2	1	0
04	10	7 (70)	7	0	0
05	2	0 (0)	0	0	0
06	14	8 (57)	3	3	2
07	7	5 (71)	4	0	1
08	5	2 (40)	1	0	1
09	8	1 (13)	1	0	0
011	1	0 (0)	0	0	0
012	1	0 (0)	0	0	0
015	1	1 (100)	1	0	0
016	2	2 (100)	2	0	0
018ac	12	9 (75)	5	0	4
021	1	0 (0)	0	0	0
022	2	1 (50)	1	0	0
023	1	0 (0)	0	0	0
025	1	0 (0)	0	0	0
039	2	0 (0)	0	0	0
068	1	0 (0)	0	0	0
070	1	0 (0)	0	0	0
075	12	10 (83)	10	0	0
077	1	1 (100)	1	0	0
083	2	0 (0)	0	0	0
0101	2	0 (0)	0	0	0
0105ac	1	0 (0)	0	0	0
0114	1	1 (100)	1	0	0
0118	2	1 (50)	1	0	0
0119	1	1 (100)	1	0	0
0125	1	0 (0)	0	0	0
0126	1	0 (0)	0	0	0
0127	1	0 (0)	0	0	0
0143	1	0 (0)	0	0	0
0150	1	0 (0)	0	0	0
0160	2	0 (0)	0	0	0
0?	11	1 (9)	1	0	0
OR	2	1 (50)	1	0	0
Total	124	57 (46)	45 (36)	4 (3)	8 (6)

Table 10

Mannose-resistant haemagglutination of human and/or calf red blood cells by human strains of E.coli isolated from blood

O-group	Total number of strains tested	Total MRHA positive (%)	Human MRHA	Calf MRHA	Human and Calf MRHA
01	7	7 (100)	7	0	0
02	6	5 (83)	5	0	0
04	8	6 (75)	4	0	2
06	17	14 (82)	10	1	3
07	2	1 (50)	1	0	0
08	4	2 (50)	1	0	1
09	8	2 (25)	2	0	0
015	1	0 (0)	0	0	0
016	5	4 (80)	2	0	2
017	1	0 (0)	0	0	0
018ac	12	7 (58)	4	1	2
021	1	1 (100)	1	0	0
025	5	2 (40)	2	0	0
033	1	1 (100)	0	1	0
071	1	0 (0)	0	0	0
073	2	1 (50)	1	0	0
075	14	11 (78)	11	0	0
078	1	1 (100)	1	0	0
083	2	0 (0)	0	0	0
087	1	0 (0)	0	0	0
0101	1	1 (100)	1	0	0
0103	1	0 (0)	0	0	0
0119	1	1 (100)	1	0	0
0146	1	0 (0)	0	0	0
0157	1	0 (0)	0	0	0
0160	1	0 (0)	0	0	0
O?	18	6 (33)	5	0	1
OR	7	5 (71)	1	1	3
Total	130	78 (60)	60 (46)	4 (3)	14 (11)

Table 11

Enterotoxigenic strains of Escherichia coli of serogroup 078

Strain No. [a]	H antigen	Origin	Enterotoxin production		Colonisation factor antigen CFA/I	Biotype pattern	Drug resistance [b]
			ST	LT			
H10407	11	Bangladesh c. 1971	+	+	+	1	-
E9642	11	Bangladesh	+	+	+	1	-
E9643	11	Bangladesh	+	+	+	2	SmTc
E9505	12	Bangladesh 1977	+	-	+	7	-
E9506	12	Bangladesh 1977	+	+	+	9	-
E9507	12	Bangladesh 1977	+	+	+	5	-
E9508	12	Bangladesh 1977	+	+	+	9	-
E9640	12	Bangladesh	+	+	+	5	-
E9641	12	Bangladesh	+	-	+	5	-
E5258	12	India ca. 1971	+	+	-	3	-
E6085	12	India 1976	+	+	+	6	SmSu
E9426	12	Thailand 1975	+	+	+	5	-
E9434	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9435	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9436	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9443	12	Thailand 1977	+	-	-	3	ApCmTc

Continued

Table 11 (continued)

Strain No. [a]	H antigen	Origin	Enterotoxin production		Colonisation factor antigen CFA/I	Biotype pattern	Drug resistance [b]
			ST	LT			
E9446	12	Thailand 1977	-	+	-	5	CmSmSuTc
E7464	12	South Africa 1977	+	+	+	4	-
E7473	12	South Africa 1977	+	+	+	3	SmSu
E7479	12	South Africa 1977	+	-	+	7	ApSmSuTc
E11936	12	South Africa 1978	+	+	+	4	-
E9570	12	Ethiopia 1977	+	-	+	4	-
E9060	18	Costa Rica 1977	+	-	-	5	-
E5541	-	South Africa 1976-	-	+	-	8	-
E9442	-	Thailand 1977-	+	-	-	3	Cm
E9562	-	Ethiopia 1977	+	+	+	4	-

[a] Strains were received from the following persons: H10407, E9640-9643 (Dr. D. G. Evans), E9505-E9508 (Dr. R. E. Black), E6085 (Dr. A. Varghese), E9426, 9434-9436, 9442, 9443, 9446 (Dr. M. L. Ratanasuda Phan-Urai), E9562, E9570 (Dr. T. Wadström), E7464, 7473, 7479, 11936 (Dr. H. J. Koornhof), E9060 (Dr. L. J. Mata), E5541 (Dr. A. S. Greeff).

[b] Symbols for drug resistances: Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

Table 12

Biotyping reactions of ETEC of serogroup 078

Pattern	Decarboxylation of		Mucate utilisation	Fermentation of						
	Lysine	Ornithine		Adonitol	Dulcitol	Raffinose	Salicin	Sorbose	Sucrose	Xylose
1	+	-	-	+	-	-	(+)	-	-	-
2	+	-	+	+	+	+	(+)	-	+	+
3	+	+	+	-	+	+	(+)	+	(+)	+
4	+	+	-	-	+	+	(+)	+	+	+
5	+	+	+	-	(+)	+	(+)	-	+	+
6	+	+	+	-	-	+	-	-	+	+
7	+	+	+	-	(+)	+	-	-	+	+
8	+	+	+	-	-	+	(+)	+	+	+
9	-	+	+	-	(+)	+	-	-	+	+

+, positive on day 1 or 2.

(+), delayed positive reaction.

-, negative.

Table 13

Plasmid content of enterotoxigenic E.coli strains

Strain No.	Colonisation factor antigen (CFA/I)	Enterotoxin production		Molecular weights of the plasmids (10^6)			
		ST	LT				
H10407	+	+	+	56	42	3.8	
H10407-P	-	-	+		42	3.9	
E9505	+	+	-	57	42	4.1	
	-	-	-		41	4.0	
E7464	+	+	+	60	55	34	
	-	-	+		54	34	
E7473	+	+	+	61	54	42	4.4
	-	-	+		54	42	4.3
E9570	+	+	-	60	44		
	-	-	-		43		
E6085	+	+	+	58	4.6	4.1	
	-	-	+	59	4.5	4.0	
E9434	+	+	+	76	58	<3	
	-	-	+	78	57	<3	

Table 14

Mannose-resistant haemagglutination (MRHA) of calf red blood cells and presence of colonisation factor antigen (CFA) II in enterotoxigenic E.coli (ETEC) strains

Serotype	Number of strains					
	Total	MRHA positive	CFA/II positive	Enterotoxin production[b]		
				ST	LT	ST LT
O6H16	39	35 (90)[a]	35 (90)	2		33
O8H9	15	10 (67)	10 (67)			10
O15H-	2	2 (100)	0 (0)			
O80H9	1	1 (100)	1 (100)	1		
O85H7	2	2 (100)	2 (100)			2
O115H51	5	1 (20)	1 (20)			1
Total	69	51 (74)	49 (71)	3 (6)	0	46 (94)

[a] Figures in parentheses are percentages.

[b] in CFA/II positive strains.

Table 15

Biochemical reactions of *E.coli* O6H16 [a]

Biotype	Utilisation of Mucate	Fermentation of				
		Adonitol	Dulcitol	Raffinose	Rhamnose	Sorbose
A	+	+	-	-	-	-
B	+	+	-	-	+	-
C	+	+	-	+	+	-
D	+	+	+	-	+	+
E	-	-	-	-	+	-
F	+	-	-	-	+	-
G	-	+	-	-	+	-

[a] According to Scotland, S. M., *et al.*, J. Hyg. Camb., 79: 395-403, 1977.

Table 16

Biochemical reactions of *E.coli* O8H9

	Fermentation of			
Biotype	Dulcitol	Salicin	Sorbose	Sucrose
H	+	+	+	+
I	+	-	+	-
K	+	-	+	+
L	+	+	+	-
M	-	+	-	-

Table 17

Enterotoxigenic strains of *Escherichia coli* of serotype O6H16

Strain No. [a]	Origin	Enterotoxin production		MRHA [b] of calf RBC	CFA/II [c] component			Biotype pattern	Drug resistance [d]
		ST	LT		A	B	C		
PB-176	Mexico	+	+	+	+	-	+	A	-
E1052/66	Aden	+	+	+	+	-	+	A	Su
E1069/66	Aden	+	+	+	-	-	+	A	Su
E201/69	Sharjah	+	+	+	-	+	+	C	-
E219A/69	Sharjah	+	+	+	-	+	+	C	-
E220/69	Sharjah	+	+	+	-	+	+	B	-
E2047/69	U.K.	+	+	+	-	+	-	B	-
E1064/72	Ethiopia	+	+	+	+	-	+	C	-
E651/73	U.K.	+	+	+	+	-	+	A	-
E652/73	U.K.	+	+	+	+	-	+	A	-
E653/73	U.K.	+	+	+	+	-	+	A	-
E1392	Hong Kong	+	+	+	+	-	+	A	SmSu
E2983	Canada	+	+	-	-	-	-	*	-
E4683	U.K.	+	+	+	-	+	+	C	-
E4833	Canada	+	+	+	-	+	+	B	-
E4904	Canada	+	+	-	-	-	-	D	-

Continued

Table 17 (continued)

Strain No. [a]	Origin	Enterotoxin production		MRHA [b] of calf RBC	CFA/II [c] component			Biotype pattern	Drug resistance [d]
		ST	LT		A	B	C		
E5470	U.K.	+	+	+	-	+	+	C	-
E6709	Brazil	+	+	+	-	+	+	B	-
E6710	Brazil	+	+	+	-	+	+	B	-
E7608	Canada	+	+	+	-	+	+	C	-
E7609	Canada	+	+	+	-	+	+	C	-
E8040	U.K.	+	+	+	-	+	+	C	-
E8068	Gambia	-	+	+	-	+	+	F	-
E8071	Gambia	+	+	+	+	-	+	A	-
E8207	Gambia	+	+	+	+	-	+	A	-
E8218	Gambia	+	+	+	+	-	+	A	-
E8224	Gambia	+	+	+	+	-	+	A	-
E8272	Gambia	+	+	+	-	+	+	B	-
E8292	Gambia	+	+	+	+	-	+	A	-
E9132	Mexico	+	+	+	-	+	+	B	-
E9183	Japan	+	+	+	-	+	+	C	-
E9184	Japan	+	+	+	+	-	+	A	-
E9185	Japan	+	+	-	-	-	-	*	-

Continued

Table 17 (continued)

Strain No. [a]	Origin	Enterotoxin production		MRHA [b] of calf RBC	CFA/II [c] component			Biotype pattern	Drug resistance [d]
		ST	LT		A	B	C		
E9186	Japan	+	+	+	-	-	+	*	-
E9187	Japan	+	+	+	-	+	+	B	Tc
E9188	Japan	+	+	+	+	-	+	A	-
E9644	Mexico	+	+	+	-	+	+	B	-
E11361	Australia	-	+	+	+	-	+	A	-
E13281	Sierra Leone	+	+	+	+	-	+	A	-

[a] Strains were received from the following persons: PB-176, E9183, E9644 (Dr. D. G. Evans), E1064/72 (Dr. T. Wadström), E2983, E4333, E4904, E7608, E7609 (Dr. M. Gurwith), E6709, E6710 (Dr. R. Guerrant), E8068, E8207, E8218, E8224, E8272 (Dr. T. Tyrrell), E9183-E9188 (Dr. K. Kudoh).

[b] Mannose-resistant haemagglutination of calf red blood cells.

[c] Colonisation factor antigen II.

[d] Symbols for drug resistances: Su, sulphathiazole; Sm, streptomycin; Tc, tetracycline.

* Biotype not classifiable.

Table 20

Relationship between serogroup, biotype and mannose-resistant haemagglutination with serological reactions in CFA/II positive and negative ETEC strains[1]

Serotype	Biotype	MRHA	Serological components		
			A	B	C
06H16[2]	A	+	+	-	+
		-	-	-	-
	B	+	-	+	+
		-	-	-	-
	C	+	-	+	+
		-	-	-	-
	F	+	-	+	+
		-	-	-	-
08H9		+	-	-	+
		-	-	-	-
080H9		+	-	-	+
085H7		+	-	-	+
0115H40		+	-	-	+
		-	-	-	-

[1]Results based on the MRHA and serologic reactions of at least 10 single colonies of each positive strain studied and its negative derivatives.

[2]Exceptions in these findings are recorded in separate tables.

Table 18

Enterotoxigenic strains of Escherichia coli of serogroup 08

Strain No. [a]	H-type antigen	Origin	Enterotoxin production		MRHA [b] of calf RBC	CFA/II [c] component			Biotype pattern	Drug resistance [d]
			ST	LT		A	B	C		
E4237	NM [d]	U.K.	-	+	-	-	-	-	H	-
E7463	9	S. Africa	+	+	+	-	-	+	L	-
E8785	9	Bangladesh	+	+	-	-	-	-	K	-
E8790	9	Bangladesh	+	+	-	-	-	-	M	-
E8791	9	Bangladesh	+	+	+	-	-	+	I	-
E8792	9	Bangladesh	+	+	-	-	-	-	I	-
E8794	9	Bangladesh	+	+	+	-	-	+	K	-
E9033	9	Mexico	+	+	+	-	-	+	H	pCm
E9034	9	Mexico	+	+	+	-	-	+	I	-
E9035	9	Mexico	+	+	+	-	-	+	L	-
E9135	9	Mexico	+	+	+	-	-	+	I	-
E9137	9	Mexico	+	+	+	-	-	+	K	-
E9789	9	Canada	-	+	-	-	-	-	H	-
E9813	9	Canada	-	+	-	-	-	-	I	-
E11935	9	S. Africa	+	+	+	-	-	+	I	-
E11939	9	S. Africa	+	+	+	-	-	+	H	-

[a] Strains were received from the following persons: E7463, E11935, E11939 (Dr. H. J. Koornhof), E8785-E8794 (Dr. M. H. Merson), E9033-E9135 (Dr. D. G. Evans), E9789, E9813 (Dr. M. Gurwith).

[b] Mannose-resistant haemagglutination of calf red blood cells.

[c] Colonisation factor antigen II.

[d] Symbols for drug resistances: pCm, partial chloramphenicol.

Table 19

Enterotoxigenic strains of E.coli of serotypes 08:H9, 085:H7 and 0115:H51

Strain No. [a]	Serotype	Origin	Enterotoxin production		MRHA [b] of calf RBC	CFA/II [c] components			Drug resistances [d]
			ST	LT		A	B	C	
E6670	080H9	Brazil	+	-	+	-	-	+	SuT
E5264	085H7	Vietnam	+	+	+	-	-	+	-
E9136	085H7	Mexico	+	+	+	-	-	+	-
E8782	0115H51	Bangladesh	+	+	-	-	-	-	-
E8783	0115H51	Bangladesh	+	+	+	-	-	+	-
E8784	0115H51	Bangladesh	+	+	-	-	-	-	-

[a] Strains were received from the following persons: E6670 (Dr. R. Guerrant), E5264 (Dr. R. B. Sack), E9136 (Dr. D. G. Evans), E8782-E8784 (Dr. M. H. Merson).

[b] Mannose-resistant haemagglutination of calf red blood cells.

[c] Colonisation factor antigen II.

[d] Symbols for drug resistances: Su, sulpathiazole; Tc, tetracycline.

Table 21

Plasmid content of enterotoxigenic Escherichia coli strains of serotypes O6:H16, O8:H19, O80:H9 and O115:H51

Subclones* of Strain No.	Serotype	MRHA[a] of calf RBC	CFA/II components			Enterotoxin production		Molecular weight of the plasmids (x 10^6)						
			A	B	C	ST	LT							
E1069/66	O6H16	+	+	-	+	+	-	55	41	39	28	25	3.6	
		-	-	-	-	-	-		44		29	27	3.5	
E1392	O6H16	+	+	-	+	-	-	58	39	4.4				
		-	-	-	-	-	-			4.4				
E201/69	O6H16	+	-	+	+	+	+	76	60	49	32	3.6		
		-	-	-	+	+	+	76	60	49	32	3.6		
E219A/69	O6H16	+	-	+	+	+	-	78	47.5	42	4.0	<2		
		-	-	-	-	+	-	78	47.5	42	4.0	<2		
E4833	O6H16	+	-	+	+	+	+	51						
		-	-	-	-	+	+	51						
E9187	O6H16	+	-	+	+	+	-	68	55	37	32	18	4.0	3.6
		-	-	-	-	-	-		55	37	32	21	3.8	3.6
E9187A4	O6H16	+	-	+	+	-	-	66	50	33	31	22	4.0	3.6
		-	-	-	-	-	-		50	33	30	21	4.0	3.6
E5470	O6H16	+	-	+	-	+	+	50						
		-	-	-	-	-	-	51						
E11361	O6H16	+	+	-	+	+	-	61	44	35	3.4			
		-	-	-	-	+	-		44	34	3.4			
E7463	O8H9	+	-	-	+	-	-	62	30					
		-	-	-	-	-	-		30					
E9033	O8H9	+	-	-	+	-	-	41	36					
		-	-	-	-	-	-		35					
E9034	O8H9	+	-	-	+	+	-	55	33	<3				
		-	-	-	-	-	-	55	34	<3				
E9035	O8H9	+	-	-	+	+	+	60	<3					
		+	-	-	+	+	+	60	<3					
E8783	O115H51	+	-	-	+	+	+	82	30					
		-	-	-	-	-	-		30					

[a] Mannose-resistant haemagglutination of calf red blood cells.
[b] Colonisation factor antigen.
*Single strain derivatives of the original strains.

Table 23

Plasmid content of enterotoxigenic Escherichia coli strains of serotype 025:H42 and 0115:H40 possessing the E8775 haemagglutinin and derivatives negative for this character

Subclones of Strain No.	Serotype	MRHA[a]	E8775 precipitin[b]	Enterotoxin production ST	Enterotoxin production LT	Molecular weight of plasmids (x 10^6)					
E8774	025H42	+	+	+	+	56	52	8.4	7.8	5.6	4.9
		-	-	+	+	56	52	8.0	6.4	5.8	4.6
E8774A	025H42	+	+	-	-	56	52	8.0	6.4	5.8	4.6
		-	-	-	-		52	8.0	6.4	5.8	4.6
E8775	025H42	+	+	+	+	55	54	4.3	3.4	3.1	
		-	-	+	+	55	54	6.4	3.7		
E8780	0115H40	+	+	+	-	88					
		-	-	+	-	90					

[a] Mannose-resistant haemagglutination of human and calf red blood cells.

[b] By immunodiffusion with E8775 antiserum.

Table 22

Mannose-resistant haemagglutination (MRHA) and enterotoxin production in enterotoxigenic E.coli of O-groups 25, 0115 and 0158

Serotype	Number of strains				MRHA*	Immunodiffusion tests[a]		
	Total	ST^+	LT^+	ST^+ LT^+		CFA/I positive	CFA/II positive	E8775** positive
025H42	25			25	21 (84)	0	0	21 (84)
0115H40	11			11	7 (64)	0	0	7 (64)
0158H42	1			1	1 (100)	0	0	1 (100)
Total	37			37	29 (78)	0	0	29 (78)

[a]Using specific antisera.

*MRHA of human and calf red blood cells.

**E8775 E.coli 025H42 $ST^+LT^+MRHA^+CFA/I^-CFA/II^-$.

Table 24

Effect of bacterial concentration on the ability of strain H19 (*E.coli* 026K60H11) to adhere to HEp-2 cells

Bacteria/ml	HEp-2 adhesion*
7.2×10^8	0**
2.9×10^8	87
1.8×10^8	71
8.0×10^7	45
3.0×10^7	29
1.0×10^7	25
8.0×10^6	15
3.0×10^6	4
2.0×10^6	2
8.0×10^5	.05

*Percentage of HEp-2 cells with 5 or more adherent bacteria per cell.

**All cells dead.

Table 25

Effect of streptomycin on the expression of mannose-sensitive haemagglutination of guinea-pig red blood cells and HEp-2 adhesion in strain H19 (*E.coli* 026K60H11).

Streptomycin concentration (μg/ml)	Bacteria/ml	MSHA of guinea-pig RBC[a]	HEp-2 adhesion %
20	N.G.[b]	-	0
10	N.G.	-	0
5	N.G.	-	0
2.5	4.2×10^8	4[c]	68
1.25	7.2×10^8	32	73
0	1.0×10^9	64	71

[a]Mannose-sensitive haemagglutination of RBC.

[b]No growth.

[c]Reciprocal of the highest dilution giving visible haemagglutination.

Table 26

Adhesive factors among various classes of Escherichia coli strains isolated from human faeces

Group	Source	Total No. of strains tested	No. positive in HEp-2 adhesive test (%)***	No. positive with Type 1 pili (%)#	No. giving MRHA* of human and/or calf red blood cells (%)	No. of strains with CFA/I (%)**	No. of strains with CFA/II (%)**
A	Enteropathogenic E.coli (EPEC) from outbreaks of diarrhoea	51	41 (80)‡	39 (76)	0	0	0
B	EPEC strains from sporadic cases of diarrhoea	135	103 (76)	118 (87)	24 (18)	0	0
C	Selected enterotoxigenic E.coli (ETEC) strains from cases of diarrhoea	22	3 (14)	17 (77)	16 (73)	14 (63)	2 (9)
D	Non EPEC - non ETEC strains from outbreaks of diarrhoea	17	5 (29)	13 (76)	2 (12)	0	0
E	E.coli strains from subjects without diarrhoea	23	4 (17)	12 (52)	6 (26)	0	0
	Total	248	156 (63)	199 (80)	48 (19)	14 (6)	2 (1)

*Mannose-resistant haemagglutination.

**Colonisation factor antigen.

***χ^2 22.48 D f 4 p <0.001 for groups A to E.

‡ χ^2 24.32 D f 1 p <0.001 for groups A + D vs B + C + E.

χ^2 3.23 D f 4 p >0.35 for groups A to E.

Table 27

Adhesive factors among enteropathogenic *E.coli* (Group A) isolated from outbreaks of diarrhoea

Source	Serotype	Patients affected	No. of cultures tested[a]	No. Type 1 pili (%)	No. HEp-2 adhesion (%)
Teeside 1967	0128.H2	Infants	4	3	2
Manchester 1968-69	0114.H2	Infants	4	4	3
Taunton 1969	0127.H6	Infants	4	4	2
Wakefield 1969	0119.H6	Infants	4	4	4
Glasgow 1969-71	0142-H6	Infants	4	3	2
Australia 1972	0111.H-	Adults	4	2	4
Dublin 1972	0126.H2	Infants	4	1	2
London 1972	0119.H-	Infants	4	4	4
Norwich 1973	0127.H4	Adults	4	0	3
London 1976	0125.H21	Infants	4	4	4
Sheffield 1976	0111.H12	Infants	4	3	4
Birmingham 1978	0125.H?	Infants	7	7	7
Total			51	39 (76)	41 (80)

[a]All strains tested were negative for mannose-resistant haemagglutination (MRHA) of human and calf red blood cells and colonisation factor antigens (CFA) I and II.

Table 28

Some characteristics of strains of Escherichia coli belonging to enteropathogenic O-groups isolated from cases of sporadic diarrhoea (Group B). Presence of HEp-2 adhesion and type 1 pili.

O-groups	No. of strains tested[a]	Vero cytotoxin	HEp-2 adhesion	MRHA* positive	Type 1 pili
018ac	5	0	2 (40)[b]	2 (40)	5 (100)
026	68	34 (53)	60 (88)	11 (16)	60 (88)
055	6	0	3 (50)	1 (17)	5 (83)
011	6	0	5 (83)	1 (17)	5 (83)
0114	8	0	6 (75)	4 (50)	7 (88)
0119	6	0	6 (100)	0	6 (100)
0125	6	0	4 (67)	2 (33)	6 (100)
0126	6	0	4 (67)	1 (17)	6 (100)
0127	6	0	3 (50)	0	4 (67)
0128	11	5 (45)	5 (45)	4 (36)	9 (82)
0142	7	0	5 (71)	0	7 (100)
Total	135	39 (29)	103 (76)	25 (19)	118 (87)

*Mannose-resistant haemagglutination.

[a]All strains tested were found negative for colonisation factor antigens I and II.

[b]Figures in parentheses are percentages.

Table 29

Adhesive factors among enterotoxigenic E.coli (Group C)

Serotype	Enterotoxin(s) produced		Number tested	Human and calf MRHA (%)	CFA/I positive (%)	Calf only MRHA (%)	CFA/II positive (%)	Type 1 pili (%)	HEp-2 adhesion positive (%)
O6H16	ST	LT	2	0	0	2	2	0	0
O25H42	ST	LT	2	2	0	0	0	1	0
O63H-	ST	LT	2	2	2	0	0	2	0
O63H12	ST		1	1	1	0	0	1	0
O78H11	ST	LT	2	1	1	0	0	2	0
O78H12	ST	LT	11	8	8	0	0	8	2
O128H12	ST		1	1	1	0	0	2	1
O153H12	ST		1	1	1	0	0	1	0
Total			22	16 (73)	14 (63)	2 (9)	2 (9)	17 (77)	3 (14)

ST = Heat stable

LT = Heat labile

MRHA = Mannose resistant haemagglutination

CFA = Colonisation factor antigen

Table 30

Adhesive factors among non EPEC, non ETEC strains of E.coli (Group D)

Source	Serotype	Patients affected	No. of cultures tested[a]	No. human MRHA* (%)	No. Type 1 pili (%)	No. HEp-2 adhesion positive (%)
Winchester 1967	091H7	Infants	4	0	4	1
Staines 1972	083H4	Adults	2	0	2	2
Ashford 1972	0132H12	Adults	3	0	3	0
Huddersfield 1974	06H1	Adults	4	0	2	2
London 1976	0149H-	Infants	4	2	2	0
Total			17	2 (12)	13 (76)	5 (29)

*MRHA = Mannose resistant haemagglutination.

[a]All strains tested were negative for MRHA of calf red blood cells and colonisation factor antigens (CFA) I and II.

Table 31

Number of EPEC strains able and unable to adhere to HEp-2 cells causing mannose-resistant haemagglutination (MRHA) and producing type 1 pili

HEp-2 adhesion	Total No. of strains tested	MRHA			Type 1 pili
		Human RBC*	Calf RBC	Human and calf RBC	
Positive	142	9 (6)	4 (3)	4 (3)**	102 (72)
Negative	44	1 (2)	2 (5)	4 (9)**	30 (68)
Total	186	10 (5)	6 (3)	8 (4)	132 (71)

*Red blood cells.

**X^2 4.80 Df1 $p<0.05$.

Figures in parenthesis are percentages.

Table 32

Correlation of HEp-2 adhesion with VERO cytotoxin production and urease activity in EPEC strains*

HEp-2 adhesion	Total number of strains tested	VERO cytotoxin production	Ureolytic strains
Positive	103	30 (29)	17 (17)
Negative	32	7 (22)	2 (6)
Total	135	37 (27)	19 (14)

*Enteropathogenic strains of E. coli from sporadic cases of diarrhoea only.

Figures in parentheses are percentages.

Table 33

Number of EPEC strains able and unable to cause mannose-resistant haemagglutination (MRHA) adhering to HEp-2 cells and producing VERO cytotoxin, type 1 pili and urease

MRHA*	Total No. tested	HEp-2 adhesion	VERO cytotoxin production	Type 1 pili	Ureolytic strains
Positive	24	17 (71)	2 (8)**	20 (83)	6 (25)
Negative	111	86 (77)	37 (33)**	97 (87)	13 (12)
Total	135	103 (76)	39 (29)	117 (87)	19 (14)

*Of human and/or calf red blood cells.

**X^2 4.27 Df 1 $p<0.05$.

Figures in parenthesis are percentages.

Table 34

Correlation of VERO cytotoxin production and HEp-2 adhesion, mannose-resistant haemagglutination production of type 1 pili and urease activity in EPEC strains*

VERO cytotoxin production	Total No. of strains	HEp-2 adhesion	MRHA**	Type 1 pili	Ureolytic strains
Positive	39	30 (77)	2 (5)***	33 (85)	2 (5)
Negative	96	73 (76)	22 (23)***	84 (88)	17 (18)
Total	135	103 (76)	24 (8)	117 (87)	19 (14)

*Enteropathogenic E.coli strains from sporadic cases of diarrhoea only.

**Mannose-resistant haemagglutination of human and/or calf RBC.

***χ^2 4.94 Df 1 $p<0.05$.

Figures in parentheses are percentages.

Table 35

Phenotypic characteristics of enteropathogenic E.coli (EPEC) of strains of serogroup 026

Serotype	Total No. of strains tested	HEp-2 adhesion	Type 1 pili	Vero cytotoxin production	Ureolytic strains	Sucrose fermentation
026:H-	22	20 (91)	21 (95)	2 (9)*	7 (32)**	22 (100)
026:H11	44	39 (89)	38 (86)	32 (73)*	6 (14)**	43 (98)
026:H32	1	1 (100)	1 (100)	0	1 (100)**	1 (100)
026:H46	1	0	0	0	0	0
Total	68	60 (88)	60 (88)	34 (50)	14 (21)	66 (97)

*X^2 11.9 Df 1 $p<0.001$.

**X^2 5.58 Df 2 $p<0.1$.

Table 36

Phenotypic characteristics and plasmid analysis of EPEC strains of serotype 026:H11 able to adhere to HEp-2 cells

Strain No.	VERO cytotoxin	Colicin production	Molecular size of plasmids (x 10^{-6})[a]				
E2487	+	+	58	5.0	2.3	<2	
E7320	+	+	60	4.7	<2		
E7937	+	-	62	5.8	4.7	3.0	2.6
E9612	+	+	57	5.9	4.6	2.2	<2
E9957	+	-	60	3.6	3.1	2.5	
E10311	+	+	58				
E12965	+	+	56	6.4	5.3	2.3	

[a]For plasmids <10 x 10^{-6}, the table represents an estimate of the actual number of plasmids present in the strains.

Table 37

Phenotypic characteristics and plasmid analysis of strain H19 (E.coli 026:K60:H11) and its segregants

Strain No.	HEp-2 adhesion (%)	Colicin production	Vero cytotoxin	Drug resistance[a]	Molecular size of plasmids (x 10^6)				
E1426/65 (H19)	68*	+	+	SmSuTc	61	58	52	4.5	2.5
G241[b]	65*	-	+	SmSuTc		58	51	4.5	
G243[b]	71*‡	-	+	SmSuTc		58	51	4.5	
G245[c]	20‡	-	+	-		55	13	4.5	
G246[b]	62*	+	+	-	60	57		4.5	2.5

[a]Symbols for drug resistances: Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

[b]Spontaneous segregants of H19.

[c]G243 derivative after ethidium bromide treatment.

*-X^2 0.67 Df 3 $p>0.9$ for values between H19, G241, G243 and G246.

‡-X^2 28.30 Df 1 $p<0.001$ for values between G243 and G245.

Table 38

Phenotypic characteristics and plasmid analysis of strain H19 (*E.coli* O26:K60:H11) and its *E.coli* K12 derivatives

Strain No.	HEp-2 adhesion	Colicin production	Vero cytotoxin	Drug resistance[a]	Molecular size of plasmids (x 10^6)				
E1426/65 (H19)	+	+	+	SmSuTc	61	58	52	4.5	2.5
G208[b]	-	+	+	SmSuTc	59	55	51	4.5	
G209[b]	-	+	-	SmSuTc	57		51		
G221[b]	-	-	+	-	No plasmids found				

[a]Symbols for drug resistances: Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

[b]*E.coli* K12 derivatives of strains E1426/65 (H19).

Table 39

Phenotypic characteristics and plasmid analysis of strain H19 E3787/76 (*E.coli* 026:K60:H11) and its *E.coli* K12 derivatives

Strain No.	HEp-2 adhesion (%)	Colicin production	Vero cytotoxin	Drug resistance[a]	Molecular size of plasmids (x 10^{-6})			
E3787/76[b] (H19)	65	+	+	SmSuTc	59	54	4.5	2.5
711 (Ent^+)[c]	-	+	+	SmSuTc	61	55	-	-
G166[d]	-	-	-	SmSuTc	-	46	-	-
G167[d]	-	+	-	SmSuTc	61	54	-	-
G167T[-e]	-	+	-	-	57	-	-	-
G168[d]	-	+	+	SmSuTc	59	52	-	-
G168T[-f]	-	+	-	-	59	-	-	-

[a] Symbols for drug resistances: Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

[b] Strain H19 received from Dr. H. W. Smith.

[c] *E.coli* K12 transconjugant from *E.coli* H19 (E3787/76) received from Dr. H. W. Smith.

[d] *E.coli* K12 transconjugants from *E.coli* H19 (E3787/76).

[e] Spontaneous derivative from strain G167.

[f] Spontaneous derivative from strain G168.

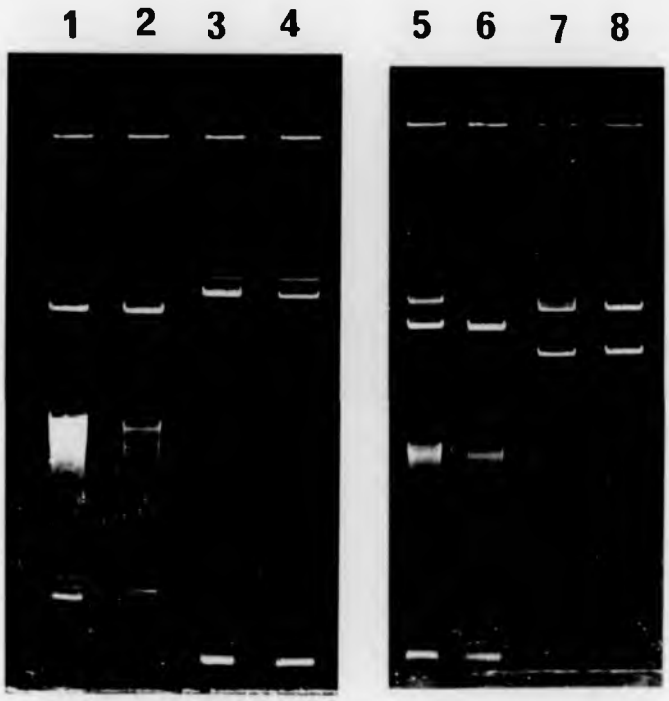


Figure 1. Agarose gel electrophoresis of partially purified plasmid DNA preparations of CFA/I^+ST^+ strains and their CFA/I^-ST^- derivatives. Electrophoresis on 0.7% agarose vertical gels. Track 1: H-10407 CFA/$I^+ST^+LT^+$; Track 2: H-10407-P CFA/$I^-ST^-LT^+$; Track 3: E9434 CFA/$I^+ST^+LT^+$; Track 4: E9434 CFA/$I^-ST^-LT^+$; Track 5: E9505 CFA/$I^+ST^+LT^-$; Track 6: E9505 CFA/$I^-ST^-LT^-$; Track 7: E7464 CFA/$I^+ST^+LT^+$; Track 8: E7464 CFA/$I^-ST^-LT^+$.

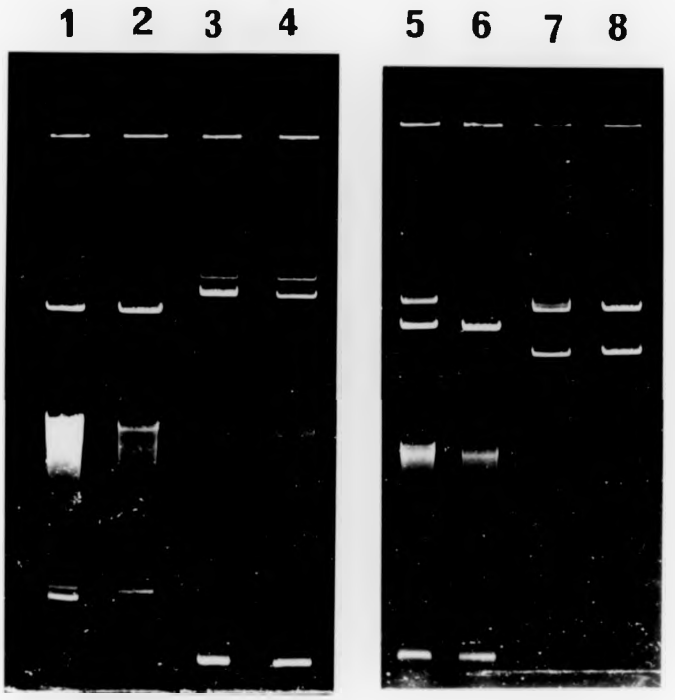


Figure 1. Agarose gel electrophoresis of partially purified plasmid DNA preparations of CFA/I$^+$ST$^+$ strains and their CFA/I$^-$ST$^-$ derivatives. Electrophoresis on 0.7% agarose vertical gels. Track 1: H-10407 CFA/I$^+$ST$^+$LT$^+$; Track 2: H-10407-P CFA/I$^-$ST$^-$LT$^+$; Track 3: E9434 CFA/I$^+$ST$^+$LT$^+$; Track 4: E9434 CFA/I$^-$ST$^-$LT$^+$; Track 5: E9505 CFA/I$^+$ST$^+$LT$^-$; Track 6: E9505 CFA/I$^-$ST$^-$LT$^-$; Track 7: E7464 CFA/I$^+$ST$^+$LT$^+$; Track 8: E7464 CFA/I$^-$ST$^-$LT$^+$.

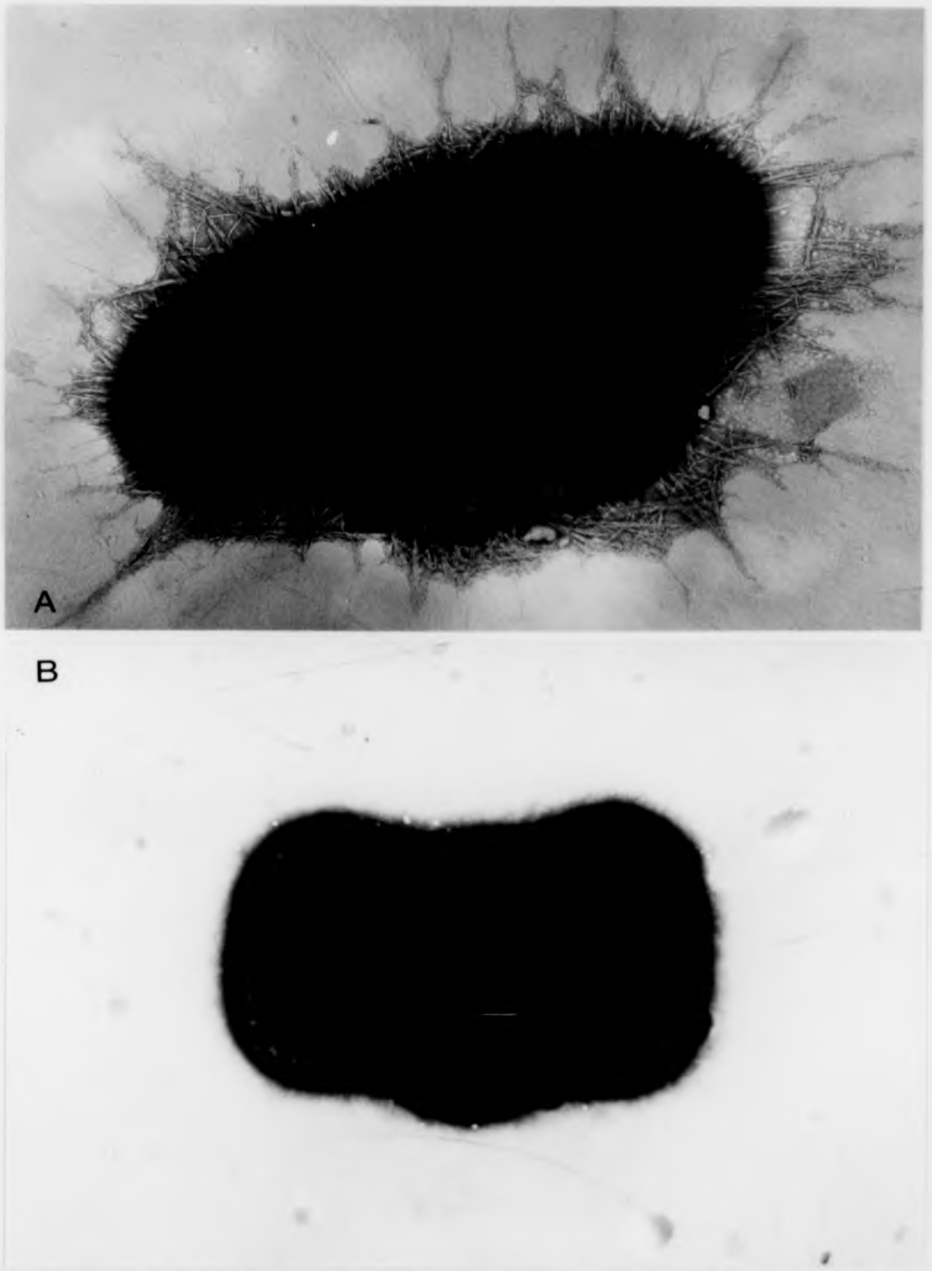


Figure 2. A) Negative-stained preparation of strain E9562 CFA/I$^+$ (*E. coli* 078:H-), magnification x 18,250. B) Similar preparation of a CFA/I$^-$ variant of E9562, magnification x 16,000.

B

Figure 2. A) Negative-stained preparation of strain E9562 CFA/I$^+$ (*E. coli* 078:H-), magnification x 18,250. B) Similar preparation of a CFA/I$^-$ variant of E9562, magnification x 16,000.

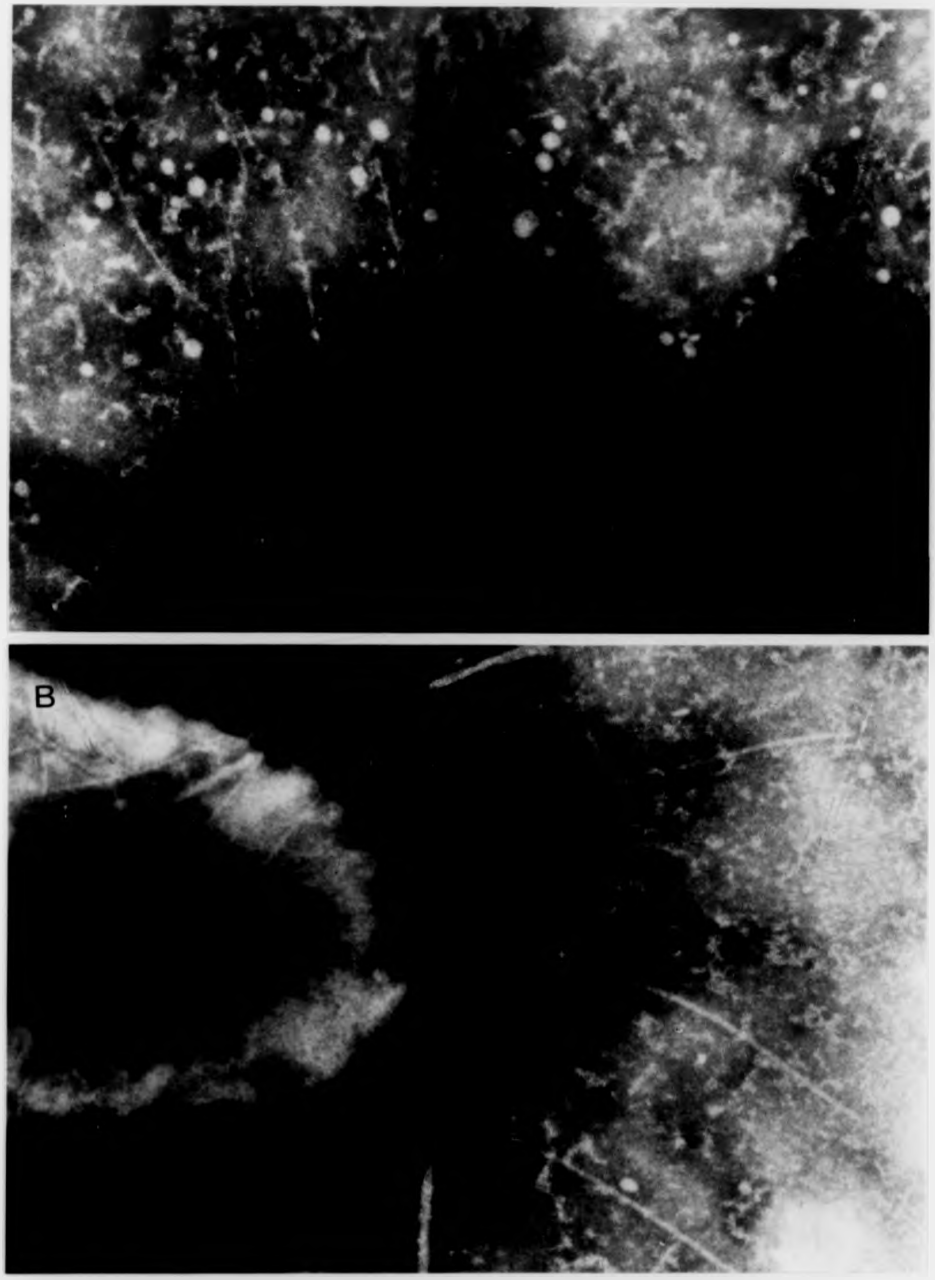


Figure 3. Immune-electron microscope studies with CFA/I antiserum. A) Strain E9562 CFA/I^{+} (E. coli 078:H-), magnification x 63,000; note fimbriae heavily coated with the antiserum. B) CFA/I^{-} variant of E9562, magnification x 63,000; note fimbriae not coated with the antiserum.

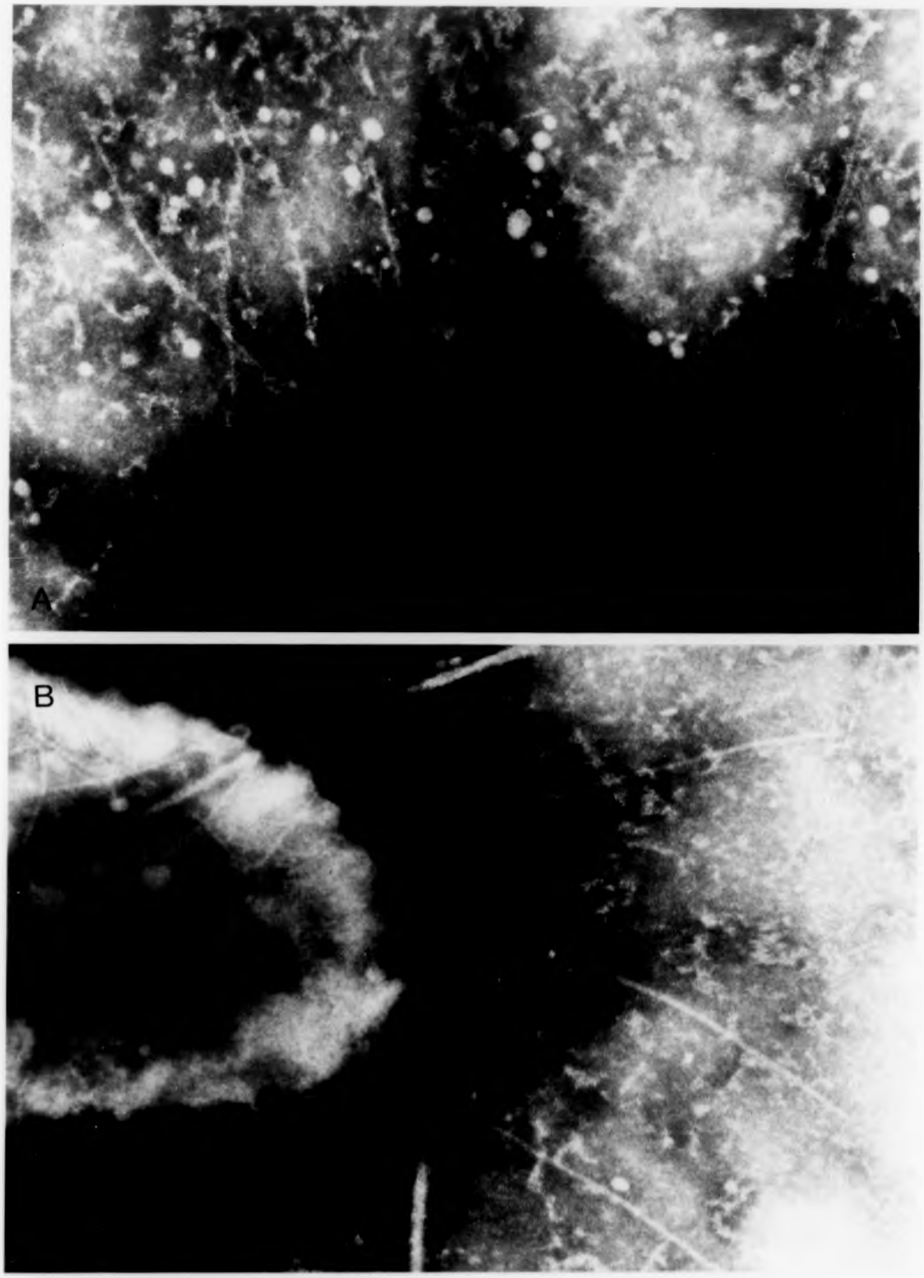


Figure 3. Immune-electron microscope studies with CFA/I antiserum. A) Strain E9562 CFA/I$^+$ (*E. coli* 078:H-), magnification x 63,000; note fimbriae heavily coated with the antiserum. B) CFA/I$^-$ variant of E9562, magnification x 63,000; note fimbriae not coated with the antiserum.

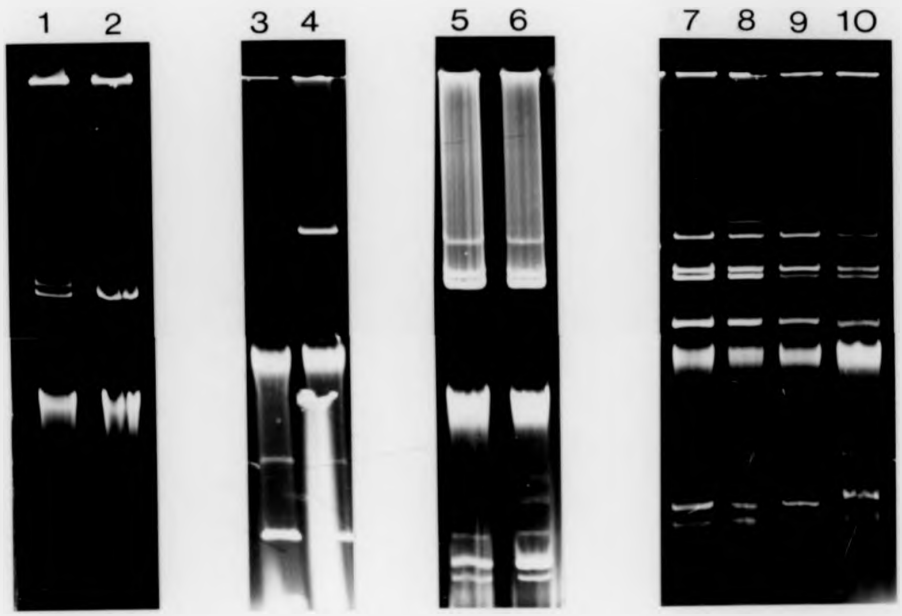


Figure 4. Agarose gel electrophoresis of partially purified plasmid DNA preparations of CFA/II$^+$ strains and their CFA/II$^-$ variants. Electrophoresis on 0.6% agarose vertical gels.

E9033 (*E. coli* O8:H9) Track 1: CFA/II$^+$ST$^-$LT$^-$;
Track 2: CFA/II$^-$ST$^-$LT$^-$.

E9035 (*E. coli* O8:H9) Track 3: CFA/II$^-$ST$^-$LT$^-$;
Track 4: CFA/II$^+$ST$^+$LT$^+$.

E219A/69 (*E. coli* O6:H16) Track 5: CFA/II$^+$ST$^+$LT$^-$;
Track 6: CFA/II$^-$ST$^+$LT$^-$.

E9187 (*E. coli* O6:H16) Track 10: CFA/II$^+$ST$^+$LT$^-$
Track 9: CFA/II$^-$ST$^-$LT$^-$ Track 8: CFA/II$^+$ST$^-$LT$^-$
Track 7: CFA/II$^-$ST$^-$LT$^-$

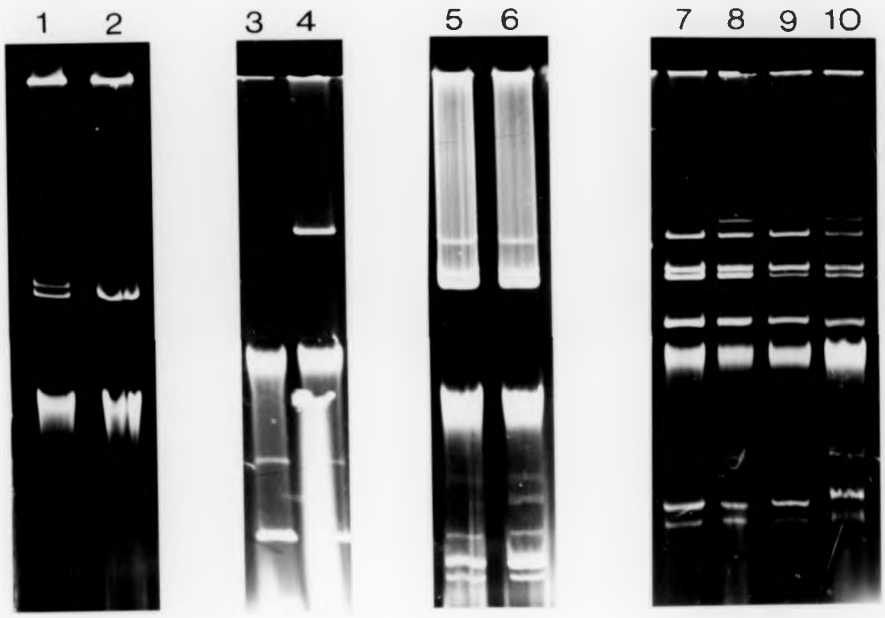


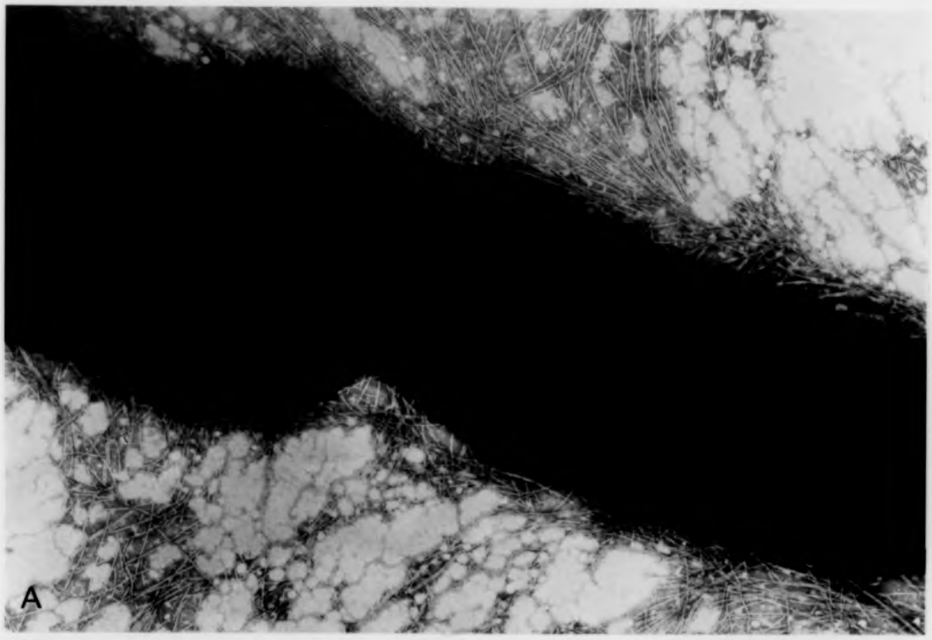
Figure 4. Agarose gel electrophoresis of partially purified plasmid DNA preparations of CFA/II$^+$ strains and their CFA/II$^-$ variants. Electrophoresis on 0.6% agarose vertical gels.

E9033 (*E. coli* 08:H9) Track 1: CFA/II$^+$ST$^-$LT$^-$;
Track 2: CFA/II$^-$ST$^-$LT$^-$.

E9035 (*E. coli* 08:H9) Track 3: CFA/II$^-$ST$^-$LT$^-$;
Track 4: CFA/II$^+$ST$^+$LT$^+$.

E219A/69 (*E. coli* 06:H16) Track 5: CFA/II$^+$ST$^+$LT$^-$;
Track 6: CFA/II$^-$ST$^+$LT$^-$.

E9187 (*E. coli* 06:H16) Track 10: CFA/II$^+$ST$^+$LT$^-$
Track 9: CFA/II$^-$ST$^-$LT$^-$ Track 8: CFA/II$^+$ST$^-$LT$^-$
Track 7: CFA/II$^-$ST$^-$LT$^-$



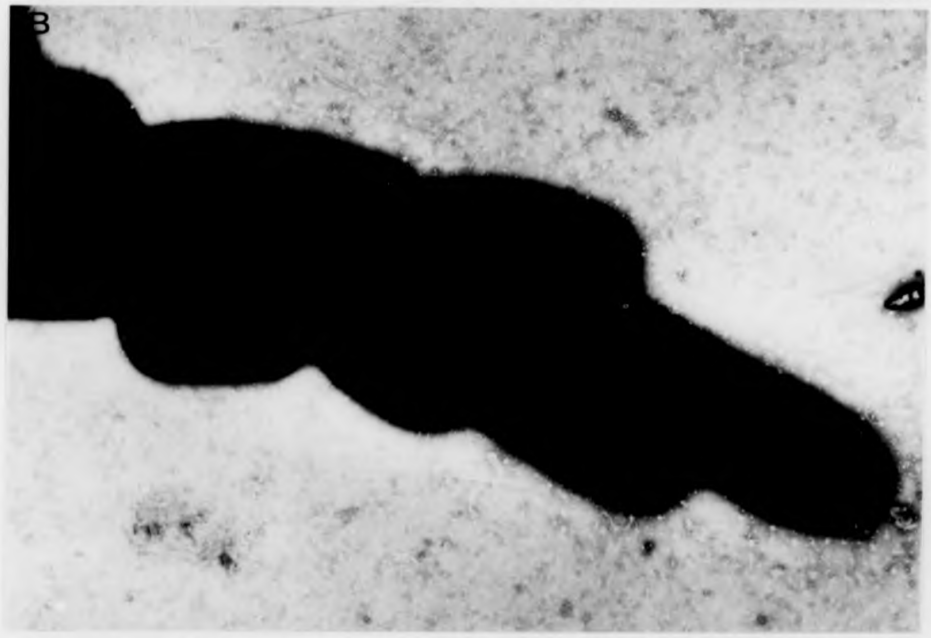


Figure 5. A) Negative-stained preparation of strain E1392 CFA/II$^+$ (*E. coli* O6:H16), magnification x 18,250. B) Similar preparation of a CFA/II$^-$ variant of E1392, magnification x 10,000.

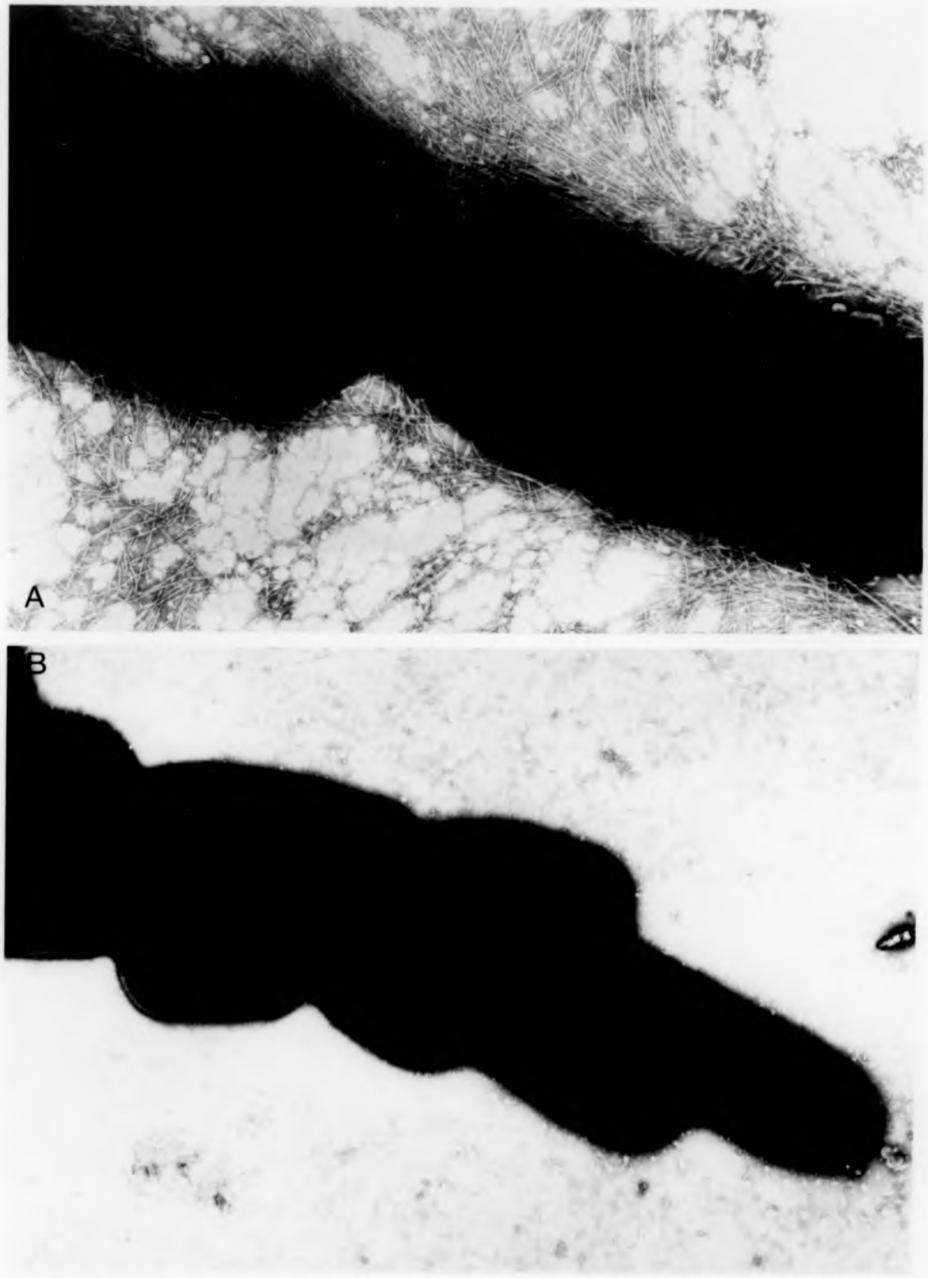


Figure 5. A) Negative-stained preparation of strain E1392 CFA/II$^+$ (*E. coli* O6:H16), magnification x 18,250. B) Similar preparation of a CFA/II$^-$ variant of E1392, magnification x 10,000.

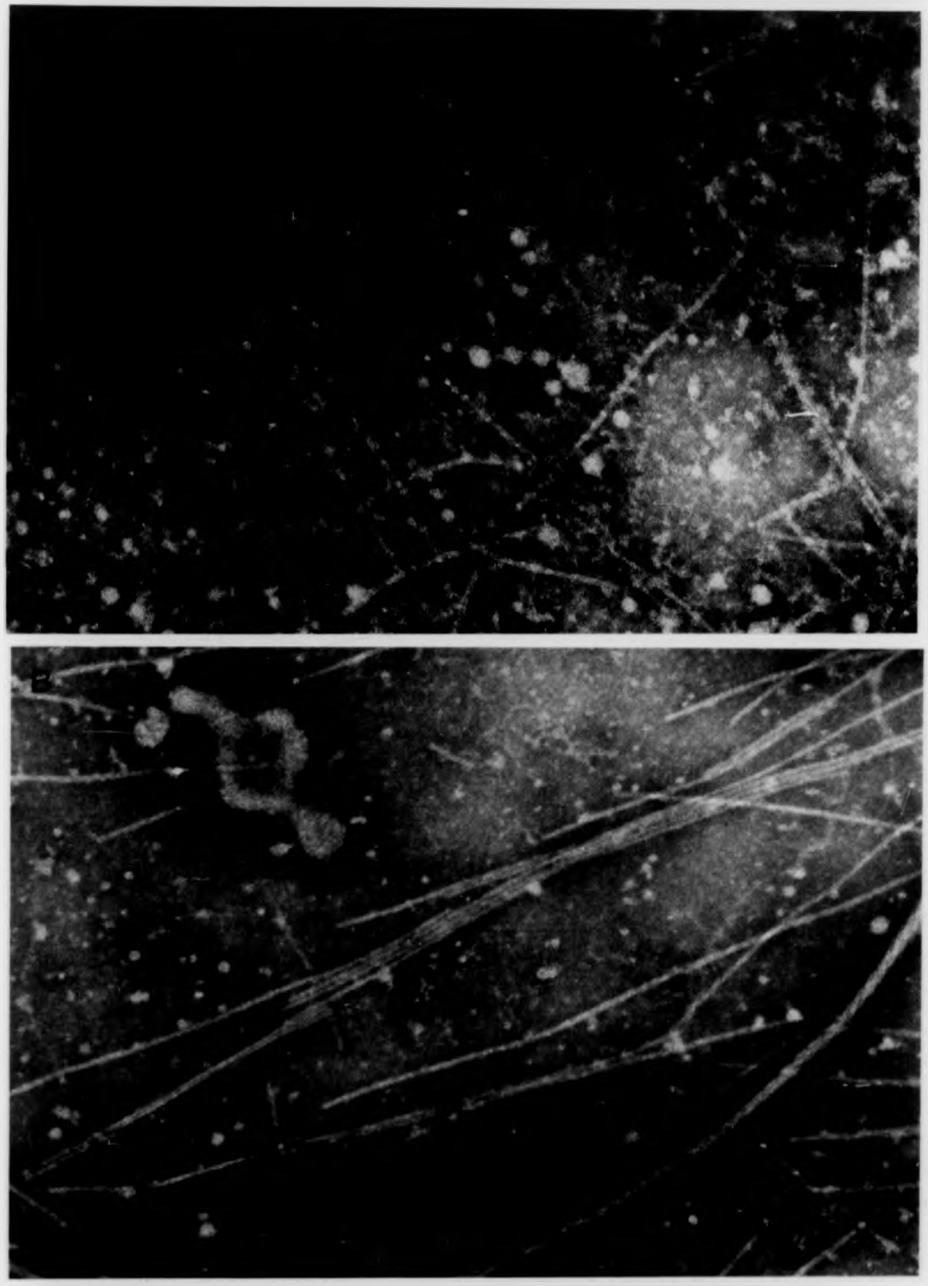
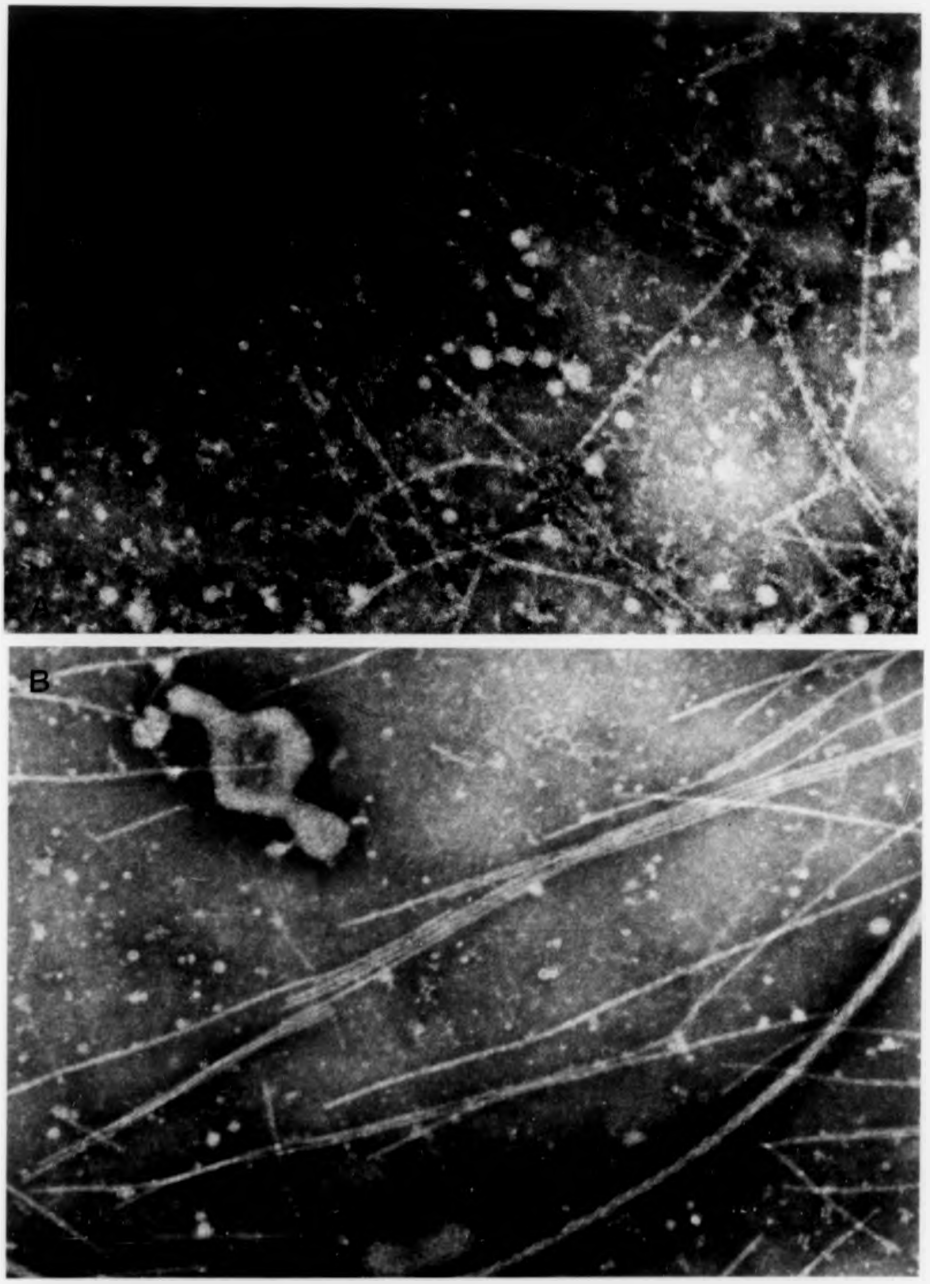


Fig. 6. Immune electron microscope studies with CFA/II antiserum. A) Strain E1392 CFA/II$^+$ (E.coli O6:H16), note fimbriae heavily coated with antiserum. B) CFA/II$^-$ variant of E1392, note fimbriae not coated with the antiserum.



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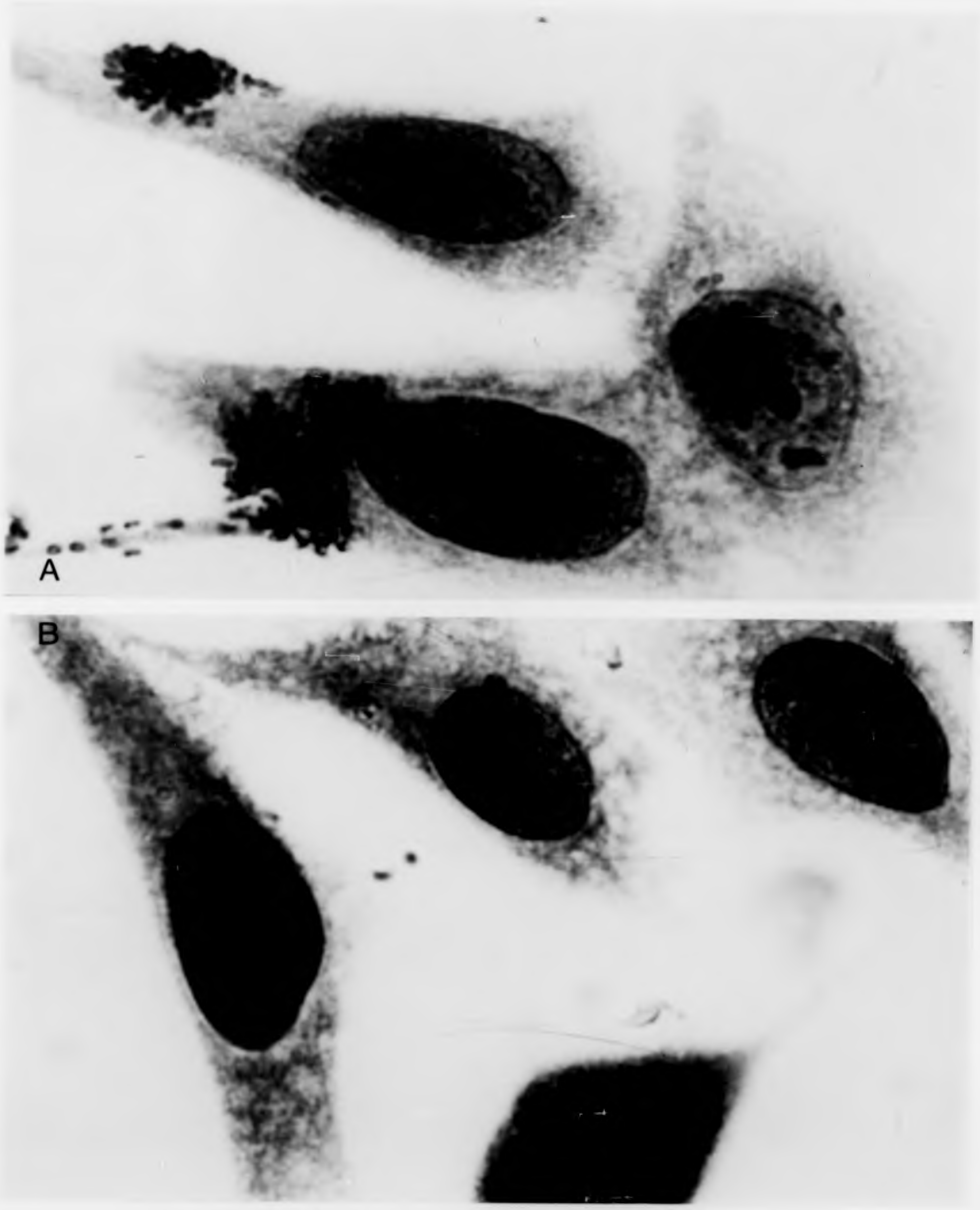
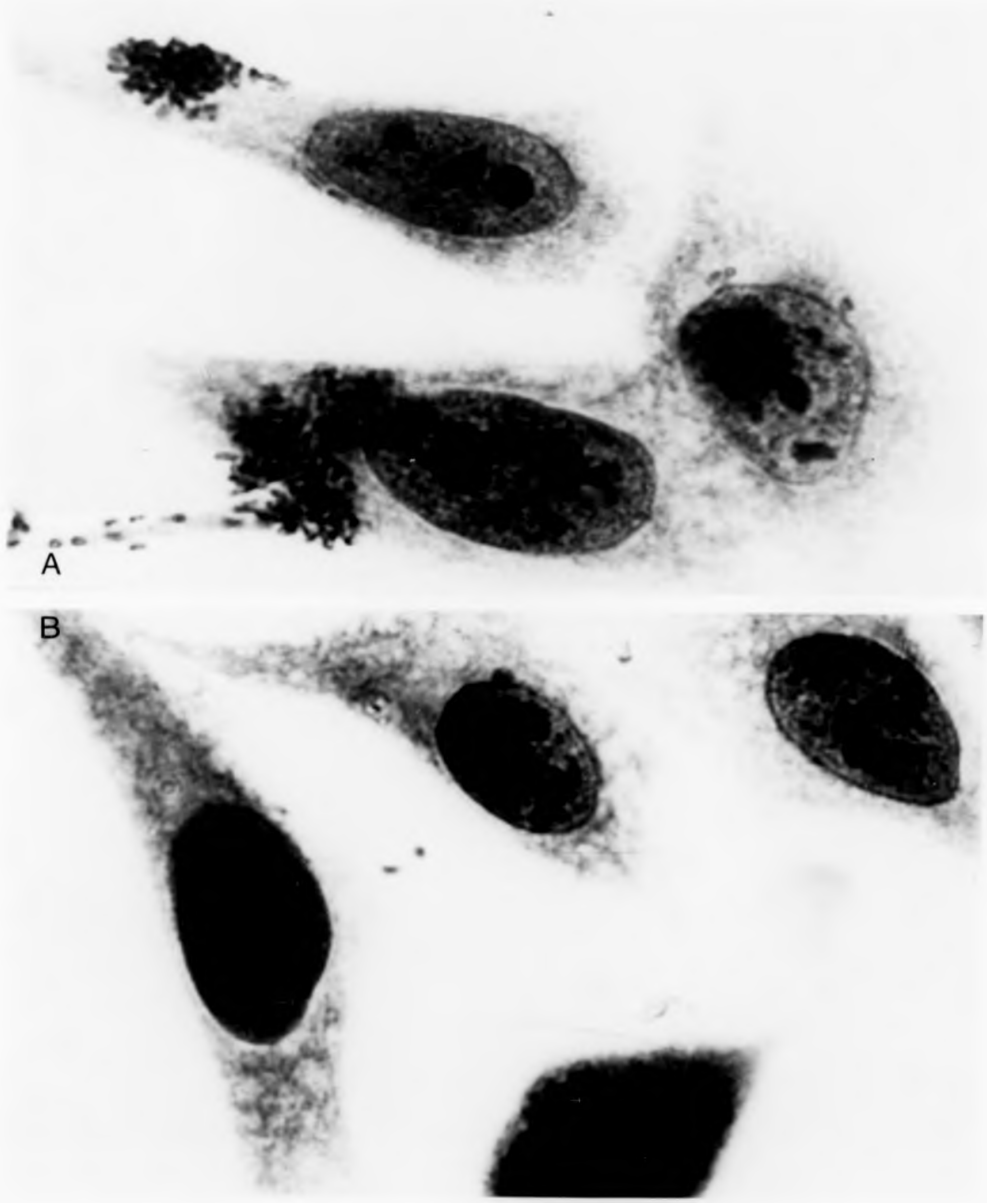


Fig. 7. HEp-2 adhesive assay of EPEC strains. A) Strain H19 (E.coli 026:H11) adhering to HEp-2 cells, magnification x 1,000. B) Strain E7542 (E.coli 026:H-) not adhering to HEp-2 cells, magnification x 1,000.



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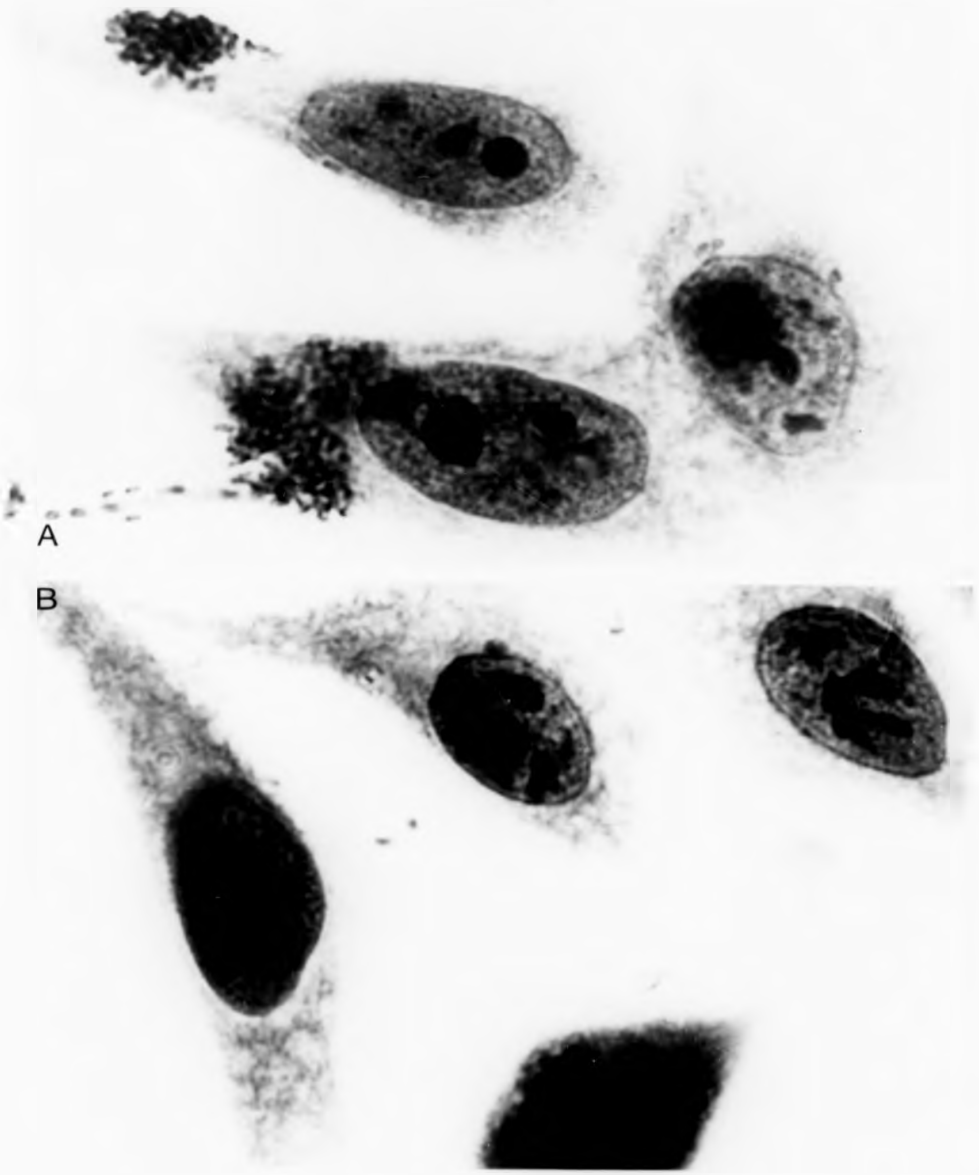
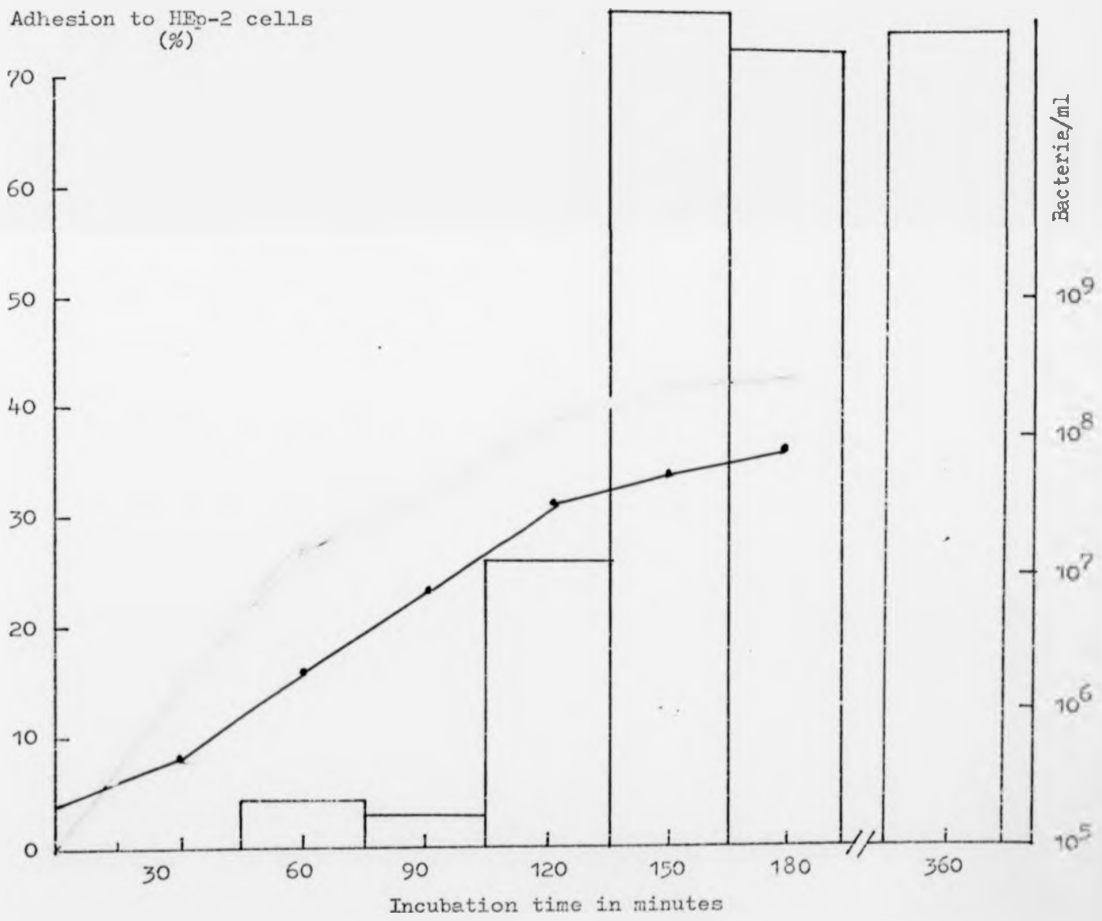


Fig. 7. HEp-2 adhesive assay of EPEC strains. A) Strain H19 (*E.coli* 026:H11) adhering to HEp-2 cells, magnification x 1,000. B) Strain E7542 (*E.coli* 026:H-) not adhering to HEp-2 cells, magnification x 1,000.

Figure 8

Effect of length of incubation on the adhesive ability of strain H19 (*E.coli* 026 K60 H11) to HEp-2 cells



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THE OCCURRENCE OF COLONISATION FACTOR (CF) IN ENTEROTOXIGENIC *ESCHERICHIA COLI*

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1. Introduction

Enterotoxin production is not in itself sufficient to enable strains of *Escherichia coli* to cause diarrhoeal disease. An ability to adhere to the intestinal epithelium is another important factor [1]. Evans and her colleagues have described a pilus-like surface antigen known as colonisation factor (CF) in an enterotoxigenic strain of *E. coli* 0.78.H11 isolated from a patient with cholera-like diarrhoea [2]. This antigen is plasmid-controlled and enables the organism to colonise the infant rabbit intestine. In a subsequent survey Ørskov and Ørskov [3] demonstrated the presence of CF in other enterotoxigenic strains of *E. coli* belonging to serogroup 078 but failed to demonstrate CF in 49 enterotoxigenic strains belonging to 5 other O groups. There was good correlation between the presence of CF and mannose-resistant ability to cause haemagglutination of human red cells.

The object of the present study was to extend the search for CF and mannose-resistant haemagglutination by the examination of enterotoxigenic *E. coli* from a wide range of serogroups.

2. Materials and Methods

2.1 Bacterial strains

89 strains of *E. coli* were examined. They had been isolated from faecal specimens of patients with diarrhoea in 14 different countries and belonged to 41 different O : H serogroups in 19 different recognised O groups. All the strains were shown to produce either heat stable enterotoxin (ST) or heat-labile enterotoxin (LT), or both, by means of the suckling mouse test for ST [4] and the Y1 [5] and CHO [6] cell tissue culture tests for LT (Table 1)

2.2. Slide agglutination test for colonisation factor

An antiserum was prepared in rabbits using as a vaccine strain H10407 (*E. coli* 078.H11) which was known to possess CF. To prepare an antiserum specific for CF this antiserum was absorbed with strain H10407-P, a variant of strain H10407 known to lack CF [2]. All 89 enterotoxigenic strains were cultured on Hartley's digest agar with the addition of 0.1% (w/v) glucose and tested for the presence of CF by slide agglutination using the specific CF antiserum.

2.3. Immunodiffusion test for colonisation factor

All the strains in which CF was demonstrated by slide agglutination were further examined by an Ouchterlony gel-immunodiffusion technique [7] using the specific CF antiserum. Antigens were prepared by simple saline extraction at 60°C for 20 min and aliquots of these were further heated at 100°C for 60 min [8]. Strains H10407 and H10407-P were used as controls.

2.4. Haemagglutination tests

All 89 enterotoxigenic *E. coli* strains were tested for mannose-resistant haemagglutination by a slide agglutination technique using washed red cells from human blood [9].

TABLE 1

Enterotoxigenic *E. coli* studied

Serotype	Number of strains			
	ST+	LT+	ST+ LT+	Total
01.H1			1	1
06.H–		1		1
06.H16		1	18	19
07.H18		1		1
08.H–		1		1
08.H9			8	8
015.H–		1		1
020.H–			1	1
020.H11	1			1
025.H42			2	2
027.H7	3			3
060.H19			1	1
063.H12	2			2
063.H30			1	1
078.H–		1		1
078.H12		1	4	5
085.H7			1	1
089.H	1			1
0114.H21	1			1
0114.H49			2	2
0115.H40			2	2
0115.H51			3	3
0128.H12	1			1
0128.H18	1			1
0128.H27	1			1
0128.H49		1		1
0148.H28	5		2	7
0153.H10	1			1
0153.H12	1			1
0159.H20	1			1
0159.H34		2	1	3
0?H		1	1	2
0?H27	2	–	–	2
0?H31		1		1
0?H33	1			1
0?H40		2		2
0 rough H–	1			1
0 rough H10		1		1
0 rough H21			1	1
0 rough H27	1			1
0 rough H55		1		1
Total	24	16	49	89

3. Results

6 of the 89 enterotoxigenic *E. coli* possessed CF as judged by slide agglutination with specific antiserum. They belonged to serogroups 063H12, 078H12 and 0153H12. All 6 caused mannose-resistant haemagglutination of human red cells. In addition three strains which lacked CF also caused mannose-resistant haemagglutination (Table 2).

TABLE 2

Strains of *E. coli* showing colonisation factor (CF) or mannose resistant haemagglutination (HA)

	Serotype	Exterotoxigenicity		CF	HA
1	078.H12	ST	LT	+	+
2	078.H12	ST	LT	+	+
3	078.H12	ST	LT	+	+
4	078.H12	ST		+	+
5	063.H12	ST		+	+
6	0153.H12	ST		+	+
7	0128.H12	ST		–	+
8	025.H42	ST	LT	–	+
9	025.H42	ST	LT	–	+

Immunodiffusion of extracts of all the CF positive strains using specific CF antiserum gave a precipitin line identical to that given by the CF positive control strain H10407 (*E. coli* 078.H11). Strain H10407-P which was known to lack CF gave no such line. However, such lines were seen only when extracts heated at 100°C for 60 min were used. Extracts prepared at 60°C and used for immunodiffusion without further heating gave no such lines.

4. Discussion

In previous studies CF has been reported only in strains belonging to serogroup 078 [3]. It is therefore of particular interest that in the present study CF was also found in strains belonging to serogroups 063 and 0153. Three additional CF-negative strains gave mannose-resistant haemagglutination using human red cells and this may indicate the presence of other antigenically distinct adhesive factors. Two of these strains belonged to serogroup 025.H42 and mannose-resistant haemagglutination has previously been observed among members of this serogroup [3].

The presence of CF could be demonstrated by immunodiffusion as well as by slide agglutination However, it is of interest that precipitin lines which correlated with the presence of CF were found only when extracts heated at 100°C for 60 min were used.

These findings contrast with those obtained in studies of the K88 antigen responsible for intestinal epithelial adhesion of many porcine enteropathogenic *E. coli*. Immunodiffusion of K88 preparations has shown that lines of precipitation are absent when the preparations are heated above 70°C [10].

80 of the 89 enterotoxigenic strains examined possessed neither CF nor the ability to cause mannose-resistant haemagglutination. All these strains were isolated from cases of human diarrhoeal disease and it seems likely that they were the causative organism in at least some of these cases. Further study is required to determine whether such strains possess other mechanisms of adhesion and intestinal colonisation.

Acknowledgements

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MANNOSE-RESISTANT HAEMAGGLUTINATION OF HUMAN ERYTHROCYTES BY STRAINS OF *ESCHERICHIA COLI* FROM EXTRAINTESTINAL SOURCES: LACK OF CORRELATION WITH COLONISATION FACTOR ANTIGEN (CFA/I)

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1. Introduction

The ability of *Escherichia coli* to agglutinate red blood cells from different animal species has been correlated with the presence of adhesive factors on the bacterial surface [1–3]. It has been shown that enterotoxigenic strains of *E. coli* (ETEC) capable of agglutinating human red cells in the presence of D-mannose possess a pilus-like colonisation factor antigen (CFA/I) that enables them to adhere to the intestinal epithelium of rabbits and humans [4]. Haemagglutination might therefore appear to be a convenient test for CFA/I. However, Minshew et al. [5] have recently shown that strains of *E. coli* from extraintestinal sources can also cause mannose-resistant haemagglutination (MRHA) of human red cells but these strains were not tested for the presence of CFA/I. In the present study a wide selection of enterotoxigenic *E. coli* strains and strains from extraintestinal sources were tested for haemagglutinating ability using human erythrocytes and for the presence of CFA/I by slide agglutination with specific antiserum.

2. Materials and Methods

2.1. Bacterial strains

563 strains of *E. coli* were examined. 122 had been isolated from specimens of cerebro-spinal fluid (CSF), 124 from urine, and 130 from blood of patients in the United Kingdom. 187 strains were ETEC isolated in many different countries from patients with diarrhoea. The ETEC strains were shown to produce either heat stable enterotoxin (ST) or heat labile enterotoxin (LT), or both, by means of the suckling mouse test for ST [6] and the Y1 [7] and CHO [8] cell tissue culture tests for LT.

Strains were identified by biochemical tests [9] and by serotyping techniques [10] using antisera for *E. coli* O groups 1–164 and H antigens 1–56. They were then maintained at room temperature on Dorset egg medium.

2.2. Haemagglutination tests

The cultures were grown overnight at 37°C on slopes of 2% agar containing 1% casamino acids (Difco) and 0.15% yeast extract (Difco) plus 0.005% $MgSO_4$, and 0.0005% $MnCl_2$. Evans et al. [2] have shown maximal production of CFA using this medium. Tests for MRHA were performed at 4°C using a slide agglutination technique with washed red cells from human group A blood [11].

2.3. Slide agglutination test for colonisation factor

An antiserum was prepared in rabbits using as a vaccine strain E6674 (*E. coli* O63.H-). This strain possesses CFA/I which we have shown by immunodiffusion techniques to be identical to that first described in strain H10407 [4]. To prepare an antiserum specific for CFA/I this crude antiserum was absorbed with strain E6674/H5, a variant of strain E6674 known to lack CFA/I. All strains that gave a positive human MRHA were tested for the presence of CFA/I by slide agglutination using the specific CFA/I antiserum.

TABLE 1

Human mannose-resistant haemagglutination (MRHA), and presence of colonisation factor antigen (CFA/I) in enterotoxigenic *E. coli* (ETEC) and *E. coli* from extraintestinal sources

	Extraintestinal *E. coli*				ETEC
	CSF[1]	Urine	Blood	Total	
Total	122	124	130	376	187
MRHA	69 (57)	47 (38)	74 (57)	190 (50)	31 (17)
CFA/I	0 (0)	0 (0)	0 (0)	0 (0)	31 (17)

[1] Figures in parentheses are percentages.

3. Results

Table 1 shows the distribution of strains according to source of isolation and the ability to give a positive human MRHA. 69 (57%) of the CSF strains, 47 (38%) of the urine strains and 74 (57%) of the blood strains gave a positive human MRHA. None of the extraintestinal strains that were positive for MRHA were agglutinated by the CFA/I specific antiserum. Of the 187 enterotoxigenic strains 31 (17%) were positive for human MRHA. All 31 of the MRHA positive enterotoxigenic strains gave good slide agglutination with the specific CFA/I antiserum.

Table 2 shows the total number of extraintestinal strains tested in each *E. coli* O-group. 39 strains did not have an identifiable O-group (O?) and 28 strains were autoagglutinable (O Rough). 276 (74%) of the strains tested belonged to only 15 O-groups. The percentage in each O-group that gave positive human MRHA varied from greater than 75% (O16, O1, O7) to less than 25% (O83,O9). On average 57% of the 276 strains in these 15 O-groups gave positive MRHA.

Table 3 shows the human MRHA results for the

TABLE 2

Extraintestinal *E. coli* strains by frequency of O-group and mannose-resistant human haemagglutination (MRHA)

O-group	Total number of strains studied	MRHA positive	(%)
O18ac	44	23	(52)
O6	39	21	(54)
O75	32	22	(69)
O7	24	18	(75)
O16	23	21	(91)
O1	21	18	(86)
O4	21	14	(67)
O2	19	9	(47)
O9	16	2	(13)
O8	10	4	(40)
O101	7	2	(29)
O26	6	2	(33)
O83	6	1	(17)
O18ab	5	0	(0)
O160	3	0	(0)
O?	39	8	(21)
O Rough	28	11	(39)
Others [a]	33	14	(42)
Total	376	190	(50)

[a] One or two isolates in 30 different O-groups.

TABLE 3

Enterotoxigenic *E. coli* studied according to serotype, mannose-resistant human haemagglutination (MRHA) and presence of colonisation factor antigen (CFA/I)

Serotype	Number of strains					
	Total	ST+	LT+	ST+ LT+	MRHA-positive	CFA-positive
O1H1	1			1	0	– [a]
O6H-	1		1		0	–
O6H16	38		3	35	0	–
O7H18	1		1		0	–
O8H-	1		1		0	–
O8H9	15		2	13	0	–
O15H-	2		2		0	–
O15H11	3		1	2	0	–
O20H11	1	1			0	–
O25H16	3		3		0	–
O25H42	2			2	2	2
O27H-	1	1			0	–

TABLE 3 (continued)

Serotype	Number of strains					
	Total	ST+	LT+	ST+ LT+	MRHA-positive	CFA-positive
O27H7	5	5			0	– [a]
O27H20	1	1			0	–
O60H19	1			1	0	–
O63H-	12		3	9	8	8
O63H12	2	2			1	1
O63H30	1			1	0	–
O75H10	1		1		0	–
O78H-	3	1	1	1	1	1
O78H11	3			3	3	3
O78H12	19	4	2	13	14	14
O78H18	1	1			0	–
O80H9	1	1			0	–
O85H7	2			2	0	–
O88H21	1		1		0	–
O89H-	1	1			0	–
O109H-	3		3		0	–
O114H?	4	4			0	–
O114H21	1	1			0	–
O114H49	2		2		0	–
O115H40	2			2	0	–
O115H51	3			3	0	–
O128H12	1	1			1	1
O128H18	1	1			0	–
O128H27	1	1			0	–
O128H49	2		2		0	–
O148H28	7	5		2	0	–
O153H10	1	1			0	–
O153H12	1	1			1	1
O159H4	5		4	1	0	–
O159H20	1	1			0	–
O159H21	3		3		0	–
O159H34	3		2	1	0	–
O?H-	3		2	1	0	–
O?H7	1		1		0	–
O?H8	1		1		0	–
O?H25	1	1			0	–
O?H27	3	3			0	–
O?H31	1		1		0	–
O?H33	1	1			0	–
O?H40	3		3		0	–
O?H49	3		3		0	–
ORH-	1	1			0	–
ORH10	1		1		0	–
ORH12	1	1			0	–
ORH21	1			1	0	–
ORH27	1	1			0	–
ORH55	1		1		0	–
Total	187	42	51	94	31	31

[a] –, not tested.

ETEC strains. The 31 MRHA positive ETEC strains belonged to eight *E. coli* O : H serotypes in five different O-groups. 27 strains produced ST and LT, and 4 produced ST only. None of the 51 ETEC strains that produced LT only were found to possess CFA/I.

4. Discussion

Our results show that approx. 50% of the extraintestinal *E. coli* isolates studied caused human MRHA. However, if only the 10 most common O groups are considered, this figure rises to almost 80%, a similar result to that of Minshew et al. [5]. Among these strains the incidence of human MRHA was approximately the same for each source of isolation. None of the extraintestinal strains that caused human MRHA gave a positive agglutination with CFA/I antiserum. Therefore the ability to cause human MRHA does not correlate with the presence of CFA/I in *E. coli* from extraintestinal sources. This conclusion is consistent with the observations of Minshew et al. [12] who have found that the ability to cause human MRHA in extraintestinal isolates is not related to the presence of a specific plasmid, while CFA/I is known to be plasmid-determined [4]. In contrast, among the enterotoxigenic *E. coli* the apparent correlation between the ability to cause human MRHA and the presence of CFA/I was confirmed.

The adhesive factor detected by human MRHA in extraintestinal *E. coli* is independent of the presence of type 1 pili, since the latter cause a mannose sensitive haemagglutination of guinea pig erythrocytes but do not cause MRHA of human erythrocytes [1, 11]. Strains of *E. coli* isolated from the urine of patients with urinary tract infection have been shown to possess a D-mannose resistant ability to adhere to human uroepithelial cells [13]. This adhesive ability was not due to the presence of type 1 pili and may be another manifestation of the adhesive factors detected by MRHA.

In a previous study using both MRHA and slide agglutination with CFA/I antiserum we reported the presence of CFA/I in strains of ETEC belonging to serotypes O78H11, O78H12, O63H12 and O153H12 [3]. We have now shown the presence of CFA/I in strains belonging to serotypes O25H42, O63H-, and

O78H-, in addition to those previously reported. However, only 17% of ETEC in our sample possessed CFA/I. It seems likely that other adhesive factors exist among ETEC and one additional factor (CFA/II) has recently been described in strains of *E. coli* O6 and O8 [14]. Further studies are required to discover additional adhesive factors of ETEC, enteropathogenic *E. coli* and *E. coli* from extraintestinal sources. Antigenic studies are needed to identify these factors and to distinguish between them.

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A PLASMID CODING FOR THE PRODUCTION OF COLONISATION FACTOR ANTIGEN I AND HEAT-STABLE ENTEROTOXIN IN STRAINS OF *ESCHERICHIA COLI* OF SERO-GROUP O78

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1. Introduction

In certain strains of *Escherichia coli*, adhesion to the intestinal mucosa is mediated by specific antigens on the bacterial cell surface. Those which have been characterised in some detail are K88 and 987P in porcine strains [1,2], K99 in bovine and ovine strains [3] and the colonisation factor antigens, CFA/I and CFA/II, identified in enterotoxigenic strains isolated from humans with diarrhoea [4–11]. These antigens are serologically distinct, but all are heat-labile fimbrial structures, which can be detected by mannose-resistant haemagglutination. Adhesion of the human enteropathogenic strain H19 to human foetal small intestine has also been demonstrated but this system is antigenically different from those described above [12,13].

The synthesis of K88 and K99 is determined by plasmids which have been transferred to *E. coli* K12 [14,15]. Adhesion in *E. coli* H19 is controlled by a plasmid which also codes for synthesis of colicin Ib and has been transferred to *E. coli* K12 [13,16]. Failure to produce CFA/I in *E. coli* strain H10407 has been correlated with the loss of a single plasmid, but transfer of this plasmid to *E. coli* K12 has not been demonstrated [4]. In some strains of *E. coli* the production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) is plasmid-mediated [17,18]. Production of CFA/I and heat-stable enterotoxin are properties which can be lost together from a number of *E. coli* strains belonging to several different serotypes [8].

We have examined a number of enterotoxigenic strains of *E. coli* serogroup O78 isolated in several different countries. The properties of these strains, including enterotoxin production, presence of CFA/I and drug resistance, have been investigated. Since there is little information on plasmid control of CFA/I synthesis we have studied the plasmid content of some of the *E. coli* strains and derivatives which have lost CFA/I and toxin production. Transfer to *E. coli* K12 of a plasmid coding for both CFA/I and ST production is described.

2. Materials and Methods

2.1. Wild-type bacterial strains

The enterotoxigenic *E. coli* strains are listed in Table 1; they were isolated in several different countries from patients with diarrhoea. The strains were identified by biochemical tests [19] and by serotyping techniques [20] using antisera for *E. coli* O groups 1–164 and H antigens 1–56. All the strains were included in a survey of enterotoxigenic *E. coli* to detect mannose-resistant haemagglutination of human erythrocytes [7]. Strains were maintained at room temperature on Dorset egg medium.

2.2. Enterotoxin tests

Heat-stable enterotoxin (ST) was detected by the suckling mouse test [21]. The production of heat-labile enterotoxin (LT) was tested with Y1 adrenal cell [22] and CHO cell [23] tissue cultures.

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A PLASMID CODING FOR THE PRODUCTION OF COLONISATION FACTOR ANTIGEN I AND HEAT-STABLE ENTEROTOXIN IN STRAINS OF *ESCHERICHIA COLI* OF SERO-GROUP O78

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1. Introduction

In certain strains of *Escherichia coli*, adhesion to the intestinal mucosa is mediated by specific antigens on the bacterial cell surface. Those which have been characterised in some detail are K88 and 987P in porcine strains [1,2], K99 in bovine and ovine strains [3] and the colonisation factor antigens, CFA/I and CFA/II, identified in enterotoxigenic strains isolated from humans with diarrhoea [4–11]. These antigens are serologically distinct, but all are heat-labile fimbrial structures, which can be detected by mannose-resistant haemagglutination. Adhesion of the human enteropathogenic strain H19 to human foetal small intestine has also been demonstrated but this system is antigenically different from those described above [12,13].

The synthesis of K88 and K99 is determined by plasmids which have been transferred to *E. coli* K12 [14,15]. Adhesion in *E. coli* H19 is controlled by a plasmid which also codes for synthesis of colicin Ib and has been transferred to *E. coli* K12 [13,16]. Failure to produce CFA/I in *E. coli* strain H10407 has been correlated with the loss of a single plasmid, but transfer of this plasmid to *E. coli* K12 has not been demonstrated [4]. In some strains of *E. coli* the production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) is plasmid-mediated [17,18]. Production of CFA/I and heat-stable enterotoxin are properties which can be lost together from a number of *E. coli* strains belonging to several different serotypes [8].

We have examined a number of enterotoxigenic strains of *E. coli* serogroup O78 isolated in several different countries. The properties of these strains, including enterotoxin production, presence of CFA/I and drug resistance, have been investigated. Since there is little information on plasmid control of CFA/I synthesis we have studied the plasmid content of some of the *E. coli* strains and derivatives which have lost CFA/I and toxin production. Transfer to *E. coli* K12 of a plasmid coding for both CFA/I and ST production is described.

2. Materials and Methods

2.1. Wild-type bacterial strains

The enterotoxigenic *E. coli* strains are listed in Table 1; they were isolated in several different countries from patients with diarrhoea. The strains were identified by biochemical tests [19] and by serotyping techniques [20] using antisera for *E. coli* O groups 1–164 and H antigens 1–56. All the strains were included in a survey of enterotoxigenic *E. coli* to detect mannose-resistant haemagglutination of human erythrocytes [7]. Strains were maintained at room temperature on Dorset egg medium.

2.2. Enterotoxin tests

Heat-stable enterotoxin (ST) was detected by the suckling mouse test [21]. The production of heat-labile enterotoxin (LT) was tested with Y1 adrenal cell [22] and CHO cell [23] tissue cultures.

TABLE 1

Enterotoxigenic strains of *Escherichia coli* of serogroup O78

Strain No. [a]	H antigen	Origin	Enterotoxin production		Colonisation factor antigen CFA/I	Biotype pattern	Drug resistance [b]
			ST	LT			
H10407	11	Bangladesh c. 1971	+	+	+	1	–
E9642	11	Bangladesh	+	+	+	1	–
E9643	11	Bangladesh	+	+	+	2	SmTc
E9505	12	Bangladesh 1977	+	–	+	7	–
E9506	12	Bangladesh 1977	+	+	+	9	–
E9507	12	Bangladesh 1977	+	+	+	5	–
E9508	12	Bangladesh 1977	+	+	+	9	–
E9640	12	Bangladesh	+	+	+	5	–
E9641	12	Bangladesh	+	–	+	5	–
E5258	12	India ca. 1971	+	+	–	3	–
E6085	12	India 1976	+	+	+	6	SmSu
E9426	12	Thailand 1975	+	+	+	5	–
E9434	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9435	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9436	12	Thailand 1975	+	+	+	5	CmSmSuTc
E9443	12	Thailand 1977	+	–	–	3	ApCmTc
E9446	12	Thailand 1977	–	+	–	5	CmSmSuTc
E7464	12	South Africa 1977	+	+	+	4	–
E7473	12	South Africa 1977	+	+	+	3	SmSu
E7479	12	South Africa 1977	+	–	+	7	ApSmSuTc
E11936	12	South Africa 1978	+	+	+	4	–
E9570	12	Ethiopia 1977	+	–	+	4	–
E9060	18	Costa Rica 1977	+	–	–	5	–
E5541	–	South Africa 1976	–	+	–	8	–
E9442	–	Thailand 1977	+	–	–	3	Cm
E9562	–	Ethiopia 1977	+	+	+	4	–

[a] Strains were received from the following people: H10407, E9640-9643 (Dr. D. G. Evans), E9505-9508 (Dr. R. E. Black), E6085 (Dr. A. Varghese), E9426, 9434-9436, 9442, 9443, 9446 (Dr. M. L. Ratanasuda Phan-Urai), E9562, E9570 (Dr. T. Wadstrom), E7464, 7473, 7479, 11936 (Dr. H. J. Koornhof), E9060 (Dr. L. J. Mata), E5541 (Dr. A. S. Greeff).

[b] Symbols for drug resistances: Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulphathiazole; Tc, tetracycline.

2.3. Haemagglutination, slide agglutination and immunodiffusion tests

Detection of colonisation factor antigen CFA/I by haemagglutination of human erythrocytes, slide agglutination using specific CFA/I antiserum and immunodiffusion was as described previously [6,7].

2.4. Biotyping

Bacterial strains were tested for their ability to decarboxylate arginine, lysine or ornithine using the Moeller method and for their ability to utilise mucate and to ferment adonitol, arabinose, cellobiose, dulcitol, glucose, glycerol, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, trehalose and xylose [24]. Results of decarboxylation tests were recorded for 4 days; results of other tests were recorded for 14 days.

2.5. Tests for resistance to antibacterial drugs and heavy metals

Bacterial strains were tested for resistance to ampicillin, chloramphenicol, furazolidone, gentami-

cin, kanamycin, nalidixic acid, streptomycin, sulphatiazole, tetracycline and trimethoprim as described previously [25]. Resistance to mercuric chloride, potassium tellurite and silver nitrate was determined by the strip diffusion technique [26].

2.6. Tests for colicin and haemolysin production

Colicin production was examined by the agar overlay method of Fredericq [27] and haemolysin production was tested by plating the strains on newly poured nutrient agar containing 2.5% horse blood.

2.7. Transfer of a plasmid coding for CFA/I and ST production (CFA/I-ST)

For bacterial crosses nutrient broth cultures of the donor and recipient strains (ca. $2 \cdot 10^8$ cells/ml) were mixed in equal volumes and incubated overnight at 37°C. The R factor R1-19K$^-$ (ApCmSmSu) [28], which was used to mobilise the CFA/I-ST plasmid, was introduced from *E. coli* K12 into the wild-type enterotoxigenic strain. Transconjugants were selected on MacConkey agar containing chloramphenicol (20 μg/ml) and the *E. coli* K12 donor was eliminated with colicin E2 [25]. A transconjugant carrying R1-19K$^-$ which was checked for CFA/I and enterotoxin production was used in a cross with *E. coli* K12 *lac*$^-$ Nal^r. Transconjugants were selected on MacConkey agar containing chloramphenicol and nalidixic acid (30 μg/ml). Thirty colonies were examined for drug resistance and for lysis by the F-specific phage μ2 in surface spot tests [25]. Strains containing R1-19K$^-$ alone normally gave visible lysis when spotted with μ2. Those strains that were not lysed by μ2 were tested for CFA/I, ST and LT production.

2.8. Preparation of partially purified plasmid DNA

Wild-type strains of *E. coli* were grown in 50 ml volumes of nutrient broth. The cells were lysed with a mixture of Brij 58 and sodium deoxycholate [29]. Plasmid DNA was partially purified by phenol extraction of cleared lysates [30].

2.9. Agarose gel electrophoresis

Plasmid DNA preparations were analysed by electrophoresis on 0.75% agarose (B.D.H.) vertical slab gels as described previously [31]. The molecular sizes of the plasmids present in the wild-type strains were determined by reference to the migration of plasmids of known molecular weight subjected to electrophoresis on the same gel [30]. The standard plasmids ranged in size from 3.7 to $78 \cdot 10^6$.

3. Results

3.1. Properties of enterotoxigenic E. coli *strains of serogroup O78*

19 of the 26 strains of serogroup O78 were of flagellar type H12 and the others were H11, H18 and H$^-$. 20 of the strains possessed CFA/I which was detected by haemagglutination, slide agglutination and immunodiffusion tests. All these 20 CFA/I$^+$ strains produced ST and 16 of them also produced LT. Of the 6 strains which lacked CFA/I, 1 was ST$^+$ LT$^+$, 3 were ST$^+$ and 2 were LT$^+$. 10 of the strains were drug-resistant, including 6 isolated in Thailand, but none of the strains studied was resistant to heavy metals and they did not produce haemolysin or colicin.

All strains of *E. coli* O78 fermented arabinose, glycose, glycerol, lactose, maltose, mannitol, rhamnose, sorbitol and trehalose; they all failed to ferment cellobiose, inositol and inulin and did not decarboxylate arginine. There were differences in the reactions in the remaining tests and nine biotypes could be recognised (Table 2). The biotype of each strain is given in Table 1. Derivatives of strains H10407, E6085, E7464, E7473, E9426, E9434 and E9436 which had lost CFA/I and ST production (see section 3.2) were also biotyped; none of the derivatives differed in any reaction from the original CFA/I$^+$ ST$^+$ strain.

3.2. Loss of CFA/I and heat-stable enterotoxin production

Strains were investigated for loss of CFA/I by examining single colonies of cultures stored on Dorset egg medium by slide agglutination using specific CFA/I antiserum and by haemagglutination. The presence or absence of CFA/I was confirmed by immunodiffusion tests. Derivatives which lacked

TABLE 2

Biotyping reactions

Pattern	Decarboxylation of		Mucate utilisation	Fermentation of						
	Lysine	Ornithine		Adonitol	Dulcitol	Raffinose	Salicin	Sorbose	Sucrose	Xylose
1	+	–	–	+	–	–	(+)	–	–	–
2	+	–	+	+	+	+	(+)	–	+	+
3	+	+	+	–	+	+	(+)	+	(+)	+
4	+	+	–	–	+	+	(+)	+	+	+
5	+	+	+	–	(+)	+	(+)	–	+	+
6	+	+	+	–	–	+	–	–	+	+
7	+	+	+	–	(+)	+	–	–	+	+
8	+	+	+	–	–	+	(+)	+	+	+
9	–	+	+	–	(+)	+	–	–	+	+

+, positive on day 1 or 2.
(+), delayed positive reaction.
–, negative.

CFA/I were then tested for enterotoxin production. Loss of CFA/I was detected in 10 strains and in all cases this was accompanied by loss of ST production. 8 of these strains were also LT$^+$ but this property was not lost with CFA/I and ST production in any of the strains.

3.3. Examination of the plasmid content of the E. coli *strains*

The plasmid content of several of the enterotoxigenic strains was examined by agarose gel electrophoresis. Derivatives which had lost CFA/I and ST

TABLE 3

Plasmid content of enterotoxigenic *E. coli* strains

Strain No.	Colonisation factor antigen (CFA/I)	Enterotoxin production		Molecular weights of the plasmide ($\times 10^6$)			
		ST	LT				
H10407	+	+	+	56	42	3.8	
H10407-P	–	–	+		42	3.9	
E9505	+	+	–	57	42	4.1	
	–	–	–		41	4.0	
E7464	+	+	+	60	55	34	
	–	–	+		54	34	
E7473	+	+	+	61	54	42	4.4
	–	–	+		54	42	4.3
E9570	+	+	–	60	44		
	–	–	–		43		
E6085	+	+	+	58	4.6	4.1	
	–	–	+	59	4.5	4.0	
E9434	+	+	+	76	58	<3	
	–	–	+	78	57	<3	

production as described above were also studied and the number and sizes of plasmids present were compared with those in the original strains. The properties and plasmid content of seven pairs of strains are given in Table 3, and the results of gel electrophoresis of plasmid preparations from four pairs of strains are shown in Fig. 1.

A plasmid of molecular weight $56 \cdot 10^6$ was present in H10407 but absent in the CFA/I derivative H10407-P; this was observed by Evans et al. [4] who concluded that the plasmid coded for synthesis of CFA/I. Our observations indicated that this plasmid coded for production of heat-stable enterotoxin as well as CFA/I. With strains E9505, E7464, E7473 and E9570 there was also a single plasmid difference between the CFA/I$^+$ ST$^+$ and CFA/I$^-$ ST$^-$ pairs. The CFA/I$^+$ ST$^+$ strains carried, respectively, plasmids of $57 \cdot 10^6$, $60 \cdot 10^6$, $61 \cdot 10^6$ and $60 \cdot 10^6$ not detected in CFA/I$^-$ ST$^-$ lines. Two other strains (E6085 and E9434) from which CFA/I$^-$ ST$^-$ derivatives were obtained showed no clear difference between pairs in the number and size of the plasmids. However, in these cases the CFA/I$^-$ ST$^-$ derivatives that produced LT only carried a plasmid of approx. $57–59 \cdot 10^6$ and this may obscure the detection of another plasmid of very similar size in the CFA/I$^+$ ST$^+$ LT$^+$ strains. It is also possible that the isolation procedure did not recover detectable amounts of another plasmid in these strains.

7 other strains which were CFA/I$^+$ ST$^+$ LT$^+$ were also examined for their plasmid content; each of them carried a plasmid of molecular weight $57–60 \cdot 10^6$ as well as plasmids of other sizes. We have not yet isolated CFA/I$^-$ ST$^-$ derivatives from these strains.

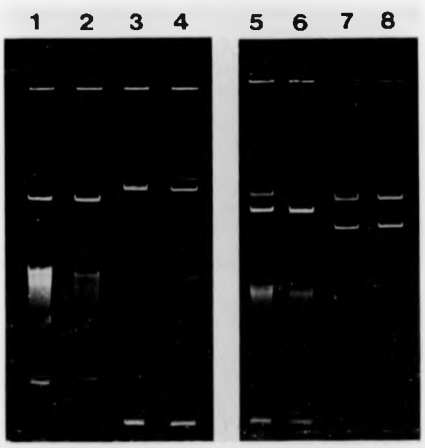

Fig. 1. Agarose gel electrophoresis of plasmid DNA from CFA/I$^+$ ST$^+$ strains and from derivatives which had lost CFA/I and ST production. Partially purified plasmid DNA was subjected to electrophoresis on 0.75% agarose gels [31]. Track 1: H10407 CFA/I$^+$ ST$^+$ LT$^+$; Track 2: H10407-P CFA/I$^-$ ST$^-$ LT$^+$; Track 3: E9434 CFA/I$^+$ ST$^+$ LT$^+$; Track 4: E9434 CFA/I$^-$ ST$^-$ LT$^+$; Track 5: E9505 CFA/I$^+$ ST$^+$; Track 6: E9505 CFA/I$^-$ ST$^-$; Track 7: E7464 CFA/I$^+$ ST$^+$ LT$^+$; Track 8: E7464 CFA/I$^-$ ST$^-$ LT$^+$.

3.4. Transfer of a CFA/I-ST plasmid to E. coli *K12*

R1-19K$^-$ was transferred into the wild-type *E. coli* strain E7473 and an R$^+$ colony was used as donor in a cross with *E. coli* K12. Five out of 30 ApCmSmSu transconjugants were not lysed by phage $\mu 2$ and were CFA/I$^+$ ST$^+$ and LT$^-$. These results suggested that a CFA/I-ST plasmid, which caused fertility inhibition, was transferred with R1-19K$^-$. After loss of the R factor from one transconjugant it was shown by gel electrophoresis that a single plasmid of molecular weight $60 \cdot 10^6$ was present. The properties of this plasmid which coded for CFA/I and ST production will be described later (M.M. McConnell et al., unpublished).

4. Discussion

In a survey of human enterotoxigenic *E. coli* belonging to 23 serogroups, 31 of 187 strains possessed CFA/I [7]. 20 of these positive strains were in serogroup O78, and are described in this paper. All CFA/I$^+$ strains produced heat-stable enterotoxin but none of 51 LT$^+$ only strains had CFA/I. In the present study of O78 strains, loss of CFA/I was always accompanied by loss of ST production; this was observed previously in strains of serogroups O15, O25, O63 and O78 [8]. Plasmid analysis of some pairs of CFA/I$^+$ ST$^+$ and CFA/I$^-$ ST$^-$ strains showed a difference of a single plasmid of molecular weight $56–61 \cdot 10^6$. Transfer to *E. coli* K12 of a plasmid

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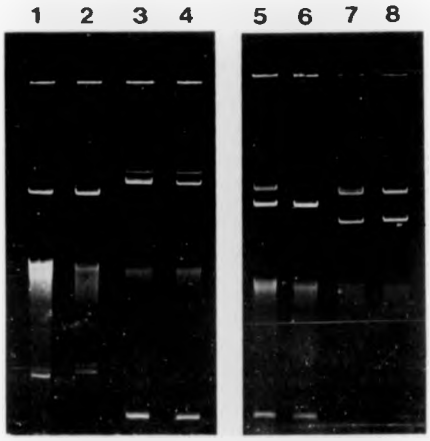

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which coded for both CFA/I and ST production confirmed that these two properties were determined by a single plasmid. Some of the CFA/I$^-$ ST$^-$ derivatives still produced LT and investigation of such strains showed that this property was also plasmid-mediated. LT plasmids have been transferred to *E. coli* K12 from most of the O78 strains described in this paper; a detailed analysis of these plasmids will be reported (M.M. McConnell et al., unpublished).

Recent studies of an ST plasmid derived from a bovine *E. coli* strain showed that the genes encoding ST were on a transposable element [32]. It was suggested in this paper that transposition may result in the ST genes being acquired by a plasmid which determined adherence to the intestinal mucosa and it was stated that one such a plasmid had been identified. There has been a report that all K99$^+$ *E. coli* strains from calves also produced ST [33]; however, it was not shown whether a single plasmid determined these properties. Other combinations of plasmid-coded genes have been described in wild-type *E. coli* strains. For example, a plasmid which coded for both enterotoxin production and drug resistance was identified [34] and recent work in this laboratory demonstrated that an enterotoxin-drug resistance plasmid was formed by transposition of ampicillin resistance in an enterotoxigenic *E. coli* strain [35]. Further studies will probably reveal the presence of other recombinant plasmids which affect the pathogenicity of *E. coli* strains.

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An Adhesive Factor Found in Strains of *Escherichia coli* Belonging to the Traditional Infantile Enteropathogenic Serotypes

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Abstract. *Escherichia coli* strains isolated from outbreaks of diarrheal disease were tested for the presence of adhesive factors. Fifty-one of these strains belonged to traditional infantile enteropathogenic serotypes (EPEC) and 17 belonged to other serotypes. None of these strains were enterotoxigenic and none possessed colonization factors CFA/I or CFA/II, which have been described among strains of enterotoxigenic *E. coli* (ETEC). Enterotoxigenic *E. coli* strains from patients with diarrhea and strains which were neither EPEC nor ETEC from subjects without diarrhea were also examined. By means of a tissue culture technique using HEp-2 cells, a new adhesive factor was found to occur with greater frequency in EPEC strains. The adhesive factor was found less frequently in the other groups of *E. coli* studied. It was distinct from type 1 pili and was not inhibited by the presence of D-mannose.

The ability of certain bacteria to adhere to epithelial cells contributes to their pathogenicity [21]. Some enterotoxigenic strains of *Escherichia coli* (ETEC) capable of causing diarrheal disease of man and animals have been shown to possess pilus-like surface factors which enable them to adhere to intestinal epithelial cells and to colonize the small bowel [22]. These factors are host specific and are protein in nature. They are antigenic and can be identified by mannose-resistant hemagglutination (MRHA) of red blood cells and by using specific antisera. So far, two antigenically distinct colonization factors (CFA/I and CFA/II) have been recognized among ETEC strains isolated from humans [7,9]. CFA/I causes MRHA of human and bovine red cells, while CFA/II causes MRHA of bovine red cells only. They have been shown by serological and immunological tests to be distinct from type 1 pili.

Strains of *E. coli* belonging to the traditional infantile enteropathogenic serotypes (EPEC) have been the cause of numerous outbreaks of infantile enteritis in many countries [23]. Most strains do not produce enterotoxins detected by the tests widely used for the identification of ETEC [10]. Nevertheless, a number have been shown to elaborate toxic substances which cause fluid secretion in a rat gut perfusion model [12], and their ability to cause diarrhea has been confirmed by feeding experiments with adult volunteers [14]. The occurrence of colonization factors, as distinct from type 1 pili, has not been reported among nonenterotoxigenic EPEC strains. In the present study, EPEC strains isolated during outbreaks of enteritis were tested for the presence of adhesive factors by means of hemagglutination and serological tests and by tests for the ability to adhere to HEp-2 cells in tissue culture. ETEC strains and strains from the feces of subjects without diarrhea were also examined.

Materials and Methods

Organisms. The *Escherichia coli* strains examined were from the collection of the Division of Enteric Pathogens, Colindale, where they had been maintained on Dorset egg medium at room temperature. They were identified as *E. coli* by biochemical tests [2] and were serotyped using antisera for *E. coli* O groups 1 to 164 and for flagella antigens H1 to H56 [17].

All the strains were tested for production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) by means of the infant mouse test for ST [4] and the Y1 [5] and CHO [11] cell tissue culture tests for LT.

The strains in the study belonged to four main groups. Group A: *E. coli* belonging to traditional infantile enteropathogenic serotypes (EPEC). Group B: *E. coli* strains that produced heat-stable and/or heat-labile enterotoxins (ETEC). Group C: *E. coli* strains that did not belong to traditional infantile EPEC serotypes and

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Table 1. Adhesive factors among *Escherichia coli* from various sources.

Group	Source	No. strains tested	No. human MRHA (%)[a]	No. CFA/I positive (%)	No. bovine MRHA (%)[a]	No. CFA/II positive (%)	No. type 1 pili (%)[c]	No. HEp-2 adhesion positive (%)[d]
A	Traditional EPEC	51	0 (0)	0 (0)	0 (0)	0 (0)	38 (75)	41 (80)
B	ETEC	22	16 (73)	16 (73)	18 (82)	2 (9)	17 (77)	3 (14)
C	Non-EPEC, non-ETEC *E. coli*	17	2 (12)	0 (0)	0 (0)	0 (0)	13 (76)	5 (29)
D	*E. coli* from subjects without diarrhea	23	4 (17)	0 (0)	2 (9)	0 (0)	12 (52)	4 (17)
Total		113	22 (19)	16 (14)	20 (18)	2 (2)	80 (71)	53 (47)

[a] MRHA, mannose-resistant hemagglutination.
[b] CFA, colonization factor antigen.
[c] χ^2 1.39 Df3 $p > 0.5$ for groups A to D.
[d] χ^2 24.03 Df3 $p < 0.001$ for groups A to D.

Table 2. Adhesive factors among enteropathogenic *Escherichia coli* (Group A).

Source	Serotype	Patients affected	No. cultures tested	No. human MRHA (%)[a]	No. CFA/I positive (%)[b]	No. bovine MRHA (%)	No. CFA/II positive (%)	No. type 1 pili (%)	No. HEp-2 adhesion (%)
Teeside 1967	O128: H2	Infants	4	0	0	0	0	2	2
Manchester 1968–69	O114: H2	Infants	4	0	0	0	0	4	3
Taunton 1969	O127: H6	Infants	4	0	0	0	0	4	2
Wakefield 1969	O119: H6	Infants	4	0	0	0	0	4	4
Glasgow 1969–71	O142: H6	Infants	4	0	0	0	0	3	2
Australia 1972	O111: H-	Adults	4	0	0	0	0	2	4
Dublin 1972	O126: H2	Infants	4	0	0	0	0	1	2
London 1972	O119: H-	Infants	4	0	0	0	0	4	4
Norwich 1973	O127: H4	Adults	4	0	0	0	0	0	3
London 1976	O125: H21	Infants	4	0	0	0	0	4	4
Sheffield 1976	O111: H12	Infants	4	0	0	0	0	3	4
Birmingham 1978	O125: H*	Infants	7	0	0	0	0	7	7
Total			51	0(0)	0(0)	0(0)	0(0)	38(75)	41(80)

[a] MRHA, mannose-resistant hemagglutination.
[b] CFA, colonization factor antigen.

did not produce ST or LT, but were isolated from outbreaks of diarrhea. Group D. *E. coli* strains isolated from children without diarrhea (Table 1).

Group A consisted of 51 EPEC strains which were isolated from the feces of patients involved in outbreaks of diarrheal disease in the United Kingdom, Australia, and Ireland (Table 2). Twenty of these were isolated during five well-documented outbreaks of infantile enteritis, which have previously been described [10].

The 22 ETEC strains in Group B were isolated in several countries from the feces of patients with diarrhea (Table 3). All 22 produced ST and 19 also produced LT. They were part of a collection of strains tested for colonization factor antigen (CFA) in a previous study [3], and all strains possessed either CFA/I or CFA/II.

Group C consisted of 17 *E. coli* strains which were isolated from outbreaks of diarrheal disease in the United Kingdom (Table 4). The evidence from serotyping and epidemiology suggested that these serotypes were the epidemic agents, although they did not belong to EPEC serotypes and were not enterotoxigenic.

The 23 strains in Group D were isolated from the feces of children and adults without diarrhea in the United Kingdom (Table 1). The strains were nonenterotoxigenic and did not belong to EPEC serotypes.

Hemagglutination tests. Test strains were cultured on CFA agar slopes [8] consisting of 2% agar containing 1% Casamino Acids (Difco Laboratories, Detroit, Michigan) and 0.15% yeast extract (Difco) plus 0.005% $MgSO_4$ and 0.0005% $MnCl_2$.

All the strains were tested for mannose-sensitive (MSHA) and mannose-resistant (MRHA) hemagglutination at 4°C by a slide agglutination technique [6], using washed red cells from human group A, bovine, and guinea pig blood.

Immunodiffusion test for colonization factor antigens I and II. Antisera were prepared in rabbits; E6674 (*E. coli* O63:H) and E1392/75 (*E. coli* O6:H16) were used as vaccine strains. These strains possess CFA/I and CFA/II, respectively, and these antigens were shown by immunodiffusion techniques to be identical to those first described in strains H10407 and PB-176, respectively, by Evans et al. [7,9]. Specific antisera were prepared by absorption with laboratory-derived variants lacking CFA/I and CFA/II.

All 113 strains grown on CFA agar were tested for the presence of CFA/I and CFA/II by slide agglutination and by the Ouchterlony gel-immunodiffusion technique, using the specific antisera [19]. Antigens for immunodiffusion were prepared by simple saline extraction at 60°C for 20 min and aliquots of these were further heated at 100°C for 60 min [18].

HEp-2 adhesive assay. The tissue culture method was adapted from that described by Labrec et al. [13], which used HeLa cells for the study of bacterial invasion.

Bacterial strains were grown overnight at 37°C in 1% tryptone water with 1% D-mannose added to inhibit adhesion due to type 1 pili [20]. A total of 0.1 ml of the bacterial culture was mixed with 4.9 ml of 1× basal medium Eagle (1×BME) (Flow Laboratories) with Hanks salts, containing 0.35 g/liter sodium bicarbonate, 0.5 ml/100 ml glutamine (Flow), and 15% fetal bovine serum (Flow). This mixture gave a concentration of 10^7 – 10^8 bacteria/ml. HEp-2 cells (Flow) were grown in the same medium with the addition of 100 μg streptomycin/ml, 100 μg penicillin/ml, and 100 μg amphotericin B/ml.

Two milliliters of a suspension containing HEp-2 cells at a concentration of 2×10^5 cells/ml was poured into 30-×-10-mm sterile plastic tissue culture Petri dishes (Nunc) into which four sterile rectangular cover slips had been placed. After overnight incubation at 37°C, the cover slips were washed three times with

Table 3. Adhesive factors among enterotoxigenic *Escherichia coli* (Group B).

Serotype	Enterotoxin(s) produced[a]	No. tested	Human MRHA (%)[b]	CFA/I positive (%)[c]	Bovine MRHA (%)	CFA/II positive (%)	Type 1 pili (%)	HEp-2 adhesion positive (%)
O6:H16	ST LT	2	0	0	2	2	0	0
O25:H42	ST LT	2	2	2	2	0	1	0
O63:H⁻	ST LT	2	2	2	2	0	2	0
O63:H12	ST	1	1	1	1	0	1	0
O78:H11	ST LT	2	1	1	1	0	2	0
O78:H12	ST LT	11	8	8	8	0	8	2
O128:H12	ST	1	1	1	1	0	2	1
O153:H12	ST	1	1	1	1	0	1	0
Total		22	16 (73)	16 (73)	18 (82)	2 (9)	17 (77)	3 (14)

[a] ST, heat stable; LT, heat labile.
[b] MRHA, mannose-resistant hemagglutination.
[c] CFA, colonization factor antigen.

Table 4. Adhesive factors among non-EPEC, non-ETEC strains of *Escherichia coli*.

Source	Serotype	Patient affected	No. cultures tested	No. human MRHA (%)[a]	No. CFA/I positive (%)[b]	No. bovine MRHA (%)	No. CFA/II positive (%)	No. type 1 pili (%)	No. HEp-2 adhesion positive (%)
Winchester 1967	O91:H7	Infants	4	0	0	0	0	4	1
Staines 1972	O83:H4	Adults	2	0	0	0	0	2	2
Ashford 1972	O132:H12	Adults	3	0	0	0	0	3	0
Huddersfield 1974	O6:H1	Adults	4	0	0	0	0	2	2
London 1976	O149:H⁻	Infants	4	2	0	0	0	2	0
Total			17	2 (12)	0 (0)	0 (0)	0 (0)	13 (76)	5 (29)

[a] MRHA, mannose-resistant hemagglutination.
[b] CFA, colonization factor antigen.

Hanks balanced salt solution (HBSS) and 1 ml of the bacterial culture in 1×BME was added. After 3 h of incubation, two cover slips were removed with sterile forceps and immersed in 2 ml of phosphate-buffered saline (PBS) (3-h test). The remaining cover slips were washed three times with HBSS and 2 ml of fresh 1×BME without antibiotics was added. After a further 3-h incubation, the cover slips were immersed in PBS (6-h test). Immediately following the appropriate incubation period, the cover slips were washed three times with PBS, fixed with 70% methanol, and stained with 10% Giemsa. The stained cover slips were mounted on glass slides and examined under oil immersion with a light microscope. Since the results of the 3- and 6-h tests were invariably identical, only the results of the latter will be considered.

Results

In the HEp-2 assay, adhesive and nonadhesive strains could easily be differentiated. Strains which were considered as positive adhered to at least 40% of the HEp-2 cells, while nonadhering strains attached to less than 10%. Intermediate results were not found.

The occurrence of adhesive factors among the different groups of *Escherichia coli* strains is summarized in Table 1. The incidence of HEp-2 adhesion was significantly higher among EPEC strains than among the other three groups studied ($p < 0.001$ by χ^2 test).

Of the 51 strains belonging to traditional infantile EPEC serotypes, none gave a positive human or bovine MRHA and none possessed CFA/I or CFA/II as judged by slide agglutination or immunodiffusion with specific antisera. Forty-one strains (80%) adhered to HEp-2 cells and 21 of these also possessed Type 1 pili. A further 17 strains which did not adhere to HEp-2 cells nevertheless possessed Type 1 pili. In all the EPEC outbreaks, some or all of the strains adhered to HEp-2 cells (Table 2).

Of the 22 ETEC strains, 16 (73%) gave a positive human MRHA. The presence of CFA/I was confirmed in all 16 strains by slide agglutination and immunodiffusion using a specific antiserum (Table 3). Eighteen (82%) of the ETEC gave a positive bovine MRHA. However only 2 strains, both *E. coli* O6:H16, were positive for CFA/II by slide agglutination and immunodiffusion. Type 1 pili were found in 17 (77%) of the ETEC studied. In spite of the high incidence of CFA/I and II in these ETEC strains, only 3 gave a positive result in the HEp-2 assay (Table 3).

Among the 17 strains which were neither EPEC nor ETEC but were the possible causative strains in outbreaks of diarrheal disease, only 2 strains, both belonging to serogroup O149, gave a positive human

MRHA. All 17 strains were negative for bovine MRHA (Table 4) and for CFA/I and CFA/II by slide agglutination and immunodiffusion with specific antisera. Type 1 pili were found in 13 strains (76%), and 5 (29%) gave a positive HEp-2 adhesion assay. Strains which adhered to HEp-2 cells were found in only three of the five outbreaks studied (Table 4).

Of the 23 *E. coli* strains from subjects without diarrhea, 4 (17%) gave a positive human MRHA, and 2 (9%) gave a positive bovine MRHA. None of the 23 strains were positive for CFA/I or CFA/II by slide agglutination or immunodiffusion using specific antisera. Twelve strains (52%) had type 1 pili and 4 (17%) were positive in the HEp-2 adhesion assay (Table 1).

Discussion

By means of a tissue culture test, the presence of an adhesive factor was demonstrated in a high percentage of epidemic strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic (EPEC) serotypes. The bacterial adhesion demonstrated by this method was not due to the presence of CFA/I or CFA/II, which are known to occur among ETEC strains, nor was it dependent on the presence of type 1 pili. Nevertheless, three ETEC strains that possessed CFA/I also adhered to the HEp-2 cells. It therefore appears that some ETEC strains may possess both of these adhesive characters. Lack of D-mannose in the culture medium caused an indiscriminate attachment of the bacteria to the glass slides in strains with type 1 pili whether they were positive or negative in the HEp-2 assay. The ability to adhere to HEp-2 cells was not inhibited by D-mannose at the concentrations used in this study, which suggests that the HEp-2 adhesive factor is distinct from that studied by Ofek and Beachey [15] using mannan-binding yeast cells and from that demonstrated by other authors using buccal epithelial cells [1,16].

The HEp-2 adhesive strains were found in all the EPEC outbreaks studied. In the case of strains isolated from outbreaks which occurred after 1972, almost all strains were adhesive, whereas non-adhesive strains were found more frequently in earlier outbreaks. This result may be due to loss of the adhesive factor during the storage of these strains, and therefore suggests the possibility that the HEp-2 adhesive factor may be plasmid mediated.

In a recent study [24], adhesion of the human EPEC strain H19 (*E. coli* O26:K60:H11) to human fetal small intestine cells was shown to be plasmid mediated. It was reported that the plasmid coding for adhesion also controlled the production of colicin Ib. In preliminary studies we have shown that strain H19 adheres to HEp-2 cells. However, following the conjugal transfer of the ColIb plasmid from strain H19, the *E. coli* K12 transconjugants that acquired ColIb were not HEp-2 adhesive. Furthermore, many of the HEp-2 adhesive EPEC strains in the present study were noncolicinogenic. Studies are in progress to examine the genetic control of the HEp-2 adhesion factor and its possible relationship to the adhesive factor reported in strain H19.

The incidence of HEp-2 adhesive strains among ETEC and among non-EPEC, non-ETEC (Group C) strains from outbreaks of diarrhea is similar to that found among the control strains obtained from individuals with diarrhea. On the other hand, the EPEC strains seem to have a greater affinity for receptors in the HEp-2 cells. The assay can be used for the selection and study of *E. coli* strains possessing an adhesive factor different from those already described, and preferentially found in EPEC serotypes. Antigenic studies are in progress to determine whether all HEp-2 adhesion is due to a single factor or whether a number of antigenically distinguishable factors exist.

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