The Possession of Three Novel Coli Surface Antigens by Enterotoxigenic
Escherichia coli Strains Positive for the Putative Colonization Factor
PCF8775

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A possible colonization factor, E8775, has previously been described for enterotoxigenic
Escherichia coli strains of serogroups O25, O115 and O167. Re-examination of these strains by
immunodiffusion has revealed that the antigenic nature of this factor, renamed putative
colonization factor (PCF) 8775, is more complex than was first thought. All the strains of
serogroup O25 tested possessed two antigenic components, termed CS4 and CS6, and gave
mannose-resistant haemagglutination (MRHA) of human and bovine erythrocytes. Spon-
taneous variants possessing CS6 only did not give MRHA. Strains of serogroups O115 and O167
had the antigenic components CS5 and CS6, and gave MRHA of human, bovine and guineapig
erythrocytes. Using immune electron microscopy, the components CS4 and CS5 were
identified as fimbriae. No fimbriae were associated with CS6.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) cause diarrhoea in humans and animals by
colonizing the small intestine and producing enterotoxins. In many ETEC strains the adhesion
to the intestinal mucosa is mediated by fimbriae which are specific antigens. The first adhesive
factor found on ETEC strains isolated from humans was the colonization factor antigen (CFA) I
(Evans et al., 1975). CFA/I is a fimbrial antigen which causes mannose-resistant haemagglutina-
tion (MRHA) of human and bovine erythrocytes (Evans et al., 1977). The second adhesive
factor reported, CFA/II, was originally described as a single fimbrial antigen which caused
MRHA of bovine erythrocytes (Evans & Evans, 1978). However it is now realized that CFA/II
is composed of more than one antigenic component. These components are called E. coli surface-
associated antigens CS1, CS2 and CS3 (Cravioto et al., 1982; Smyth, 1982). Nearly all CFA/II-
positive E. coli possess CS3, which probably corresponds to the original CFA/II as defined by
Evans & Evans (1978). In addition to CS3, strains of serogroup O6 can produce either CS1 or
CS2. Production of CS1 or CS2 correlates with the biotype of the host strain (Cravioto et al.,
1982). Components CS1 and CS2 are fimbriae (Smyth, 1982; Mullany et al., 1983) and it was
recently shown that CS3 consists of flexible fibrils (Knutton et al., 1984; Levine et al., 1984).

A new fimbrial antigen, designated E8775, was shown on strain E8775 (O25.H42) which gave
MRHA of human and bovine erythrocytes (Thomas et al., 1982). An antiserum was prepared
against strain E8775 and absorbed with a variant strain, E8775B, which did not have fimbriae
and which was MRHA-negative. When tested by immunodiffusion with this antiserum, strain
E8775 gave a single precipitin line and strain E8775B did not show any line. In a survey of
ETEC strains isolated in Thailand and Bangladesh and from travellers returning to Japan from

Abbreviations: CFA, colonization factor antigen; CS, coli surface antigen; ETEC, enterotoxigenic Escherichia
coli; LT, heat-labile enterotoxin; MRHA, mannose-resistant haemagglutination; PCF, putative colonization
factor; ST, heat-stable enterotoxin.
abroad, 5% of the strains possessed the E8775 antigen when assessed by immunodiffusion with the absorbed antiserum (Thomas & Rowe, 1982). These strains belonged to O groups O25, O115 and O167. None of the strains isolated in Thailand were positive with this antiserum.

Certain strains of serogroup O25 have now been shown to give more than one precipitin line on immunodiffusion with the absorbed antiserum to strain E8775 and this was investigated further in the present study. To distinguish between the strain E8775 and the putative colonization factor E8775, the latter will be designated PCF8775.

**METHODS**

*Bacterial strains.* A total of 54 ETEC strains were examined: 48 strains were isolated from patients with diarrhoea in Bangladesh and 6 strains were isolated from travellers returning to Japan from abroad. In a previous study these strains gave a precipitin line when tested by immunodiffusion with the absorbed antiserum of strain E8775 (Thomas & Rowe, 1982). The strains did not react with the antisera for CFA/I or CFA/II.

*Tests for enterotoxin production.* The production of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) was detected as described previously (Thomas et al., 1982).

*Mannose-resistant haemagglutination.* The strains were grown overnight at 37 °C on colonization factor antigen (CFA) agar, and then tested for MRHA of human, bovine and guinea-pig erythrocytes (Evans & Evans, 1978; Duguid & Gillies, 1957).

*Immunodiffusion tests.* An antiserum was prepared in rabbits against strain E8775 (O25. H42) and absorbed with the MRHA-negative variant E8775B (Thomas et al., 1982). Antisera were also prepared against ETEC strains E17374 (O25. H42) and E17018 (O167. H5) which gave an MRHA of human and bovine erythrocytes and reacted with absorbed E8775 antiserum (Thomas & Rowe, 1982). These two antisera were absorbed with strains E17374B and E17018B respectively, which were spontaneous variants which failed to give MRHA and which did not react with the absorbed E8775 antiserum. The absorbed antisera of strains E17374 and E17018 were tested by immunodiffusion against the absorbed antisera of strains E8775, E17374 and E17018, using the gel immunodiffusion technique described by Ouchterlony (1949).

*Isolation of MRHA-negative variants.* Strains of serogroup O25 were screened for loss of MRHA by testing individual colonies for the ability to give MRHA of human erythrocytes. Strains of serogroups O115 and O167 were screened for loss of MRHA by testing individual colonies for MRHA of guinea-pig erythrocytes. MRHA-negative variants were then checked for the additional loss of ability to give MRHA of bovine, or human and bovine erythrocytes, and tested by immunodiffusion for the presence of the PCF8775 antigenic components. They were also tested for enterotoxin production. Some of the MRHA-negative variants were tested by ELISA.

*Enzyme-linked immunosorbent assays (ELISAs).* Antiserum to CS4 was prepared using E17374 (CS4+ CS6+) as the vaccine strain. The resulting serum was absorbed first with a MRHA-negative variant of E17374 which lacked CS4 and CS6 and then with E17018 (CS5+ CS6+). Antiserum to CS6 was prepared using E11881/16, a MRHA-negative variant of E11881 which produced only CS6 (Table 2), as the vaccine strain and absorbing the resulting antiserum with an MRHA-negative variant of E11881 which lacked CS4 and CS6 (E11881/2; Table 2). IgGs were prepared from the absorbed antiserum and conjugates made as previously described (Mullany et al., 1983). Strains to be tested were grown on CFA agar plates overnight, harvested into 1 ml saline and heated at 60 °C for 30 min. The extracts were tested in the ELISA as previously described (Mullany et al., 1983).

*Electron microscopy.* Bacteria were grown on CFA agar (Evans & Evans, 1978) for two successive 24 h periods and studied by electron microscopy and immune electron microscopy as previously described (Mullany et al., 1983).

**RESULTS**

*Haemagglutination*

All the strains of serogroup O25 gave a good MRHA reaction with human and bovine erythrocytes, but did not react with guinea-pig erythrocytes. All the strains of serogroups O115 and O167 gave a weaker MRHA of human and bovine erythrocytes and reacted faster and more completely with guinea-pig erythrocytes.

*Serological studies*

All the strains gave an identical precipitin line halfway between the antigen and antiserum wells when tested by immunodiffusion against the absorbed antisera of strains E8775 (O25. H42), E17374 (O25. H42) and E17018 (O167. H5) (Fig. 1). Apart from strain E8775, all
Surface antigens of enterotoxigenic E. coli

![Image](https://www.microbiologyresearch.org)

Fig. 1. Immunodiffusion of crude antigen preparations of strains of E. coli. A, strain E8775; B, strain E17374; C, strain E17018. Central wells contain absorbed antiserum for (S1) E17374 (CS4, CS6) and (S2) E17018 (CS5, CS6).

Table 1. Enterotoxin production and number of ETEC strains positive for MRHA pattern and antigenic components

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of strains tested</th>
<th>Enterotoxin</th>
<th>MRHA pattern</th>
<th>Production of PCF8775 antigenic components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ST</td>
<td>LT</td>
<td>Human and bovine</td>
</tr>
<tr>
<td>O25. H−</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>O25. H42</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>−</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>3</td>
</tr>
<tr>
<td>O115. H40</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>O167. H5</td>
<td>21</td>
<td>+</td>
<td>−</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the strains of serogroup O25 showed an additional slower precipitin line close to the antigen well when tested by immunodiffusion against the absorbed antisera of strains E8775 and E17374. When tested with these antisera, strains of serogroups O115 and O167 did not show this line. All the strains of serogroups O115 and O167 showed an additional precipitin line close to the antigen well when tested by immunodiffusion against the absorbed antiserum of strain E17018. Strains of serogroup O25 did not show this line (Fig. 1).

To differentiate between the three antigenic components of PCF8775 the term CS (coli surface associated antigen) is used. This term was used by Smyth (1982) to describe the three antigenic components of CFA/II: CS1, CS2 and CS3. In the present study the common antigen found on all the strains was designated CS6. The slow component found on strains of serogroup O25 was called CS4 and that on strains of serogroups O115 and O167 was called CS5. Table 1 summarizes the MRHA pattern, the enterotoxin production and the antigenic components of the strains. Possession of CS4 and CS6 correlated with the MRHA pattern of human and bovine erythrocytes. Possession of CS5 and CS6 correlated with the MRHA pattern of human, bovine and guinea-pig erythrocytes. Four CS4+CS6+ strains had lost the ability to produce an enterotoxin when retested.

Five MRHA-positive O25. H42 strains including E8775 were tested in an ELISA for CS4 and CS6 (Table 2). All were positive. E8775 has been included in Table 1 as CS4+ in spite of giving a negative result in the immunodiffusion test because antibodies to CS4 were produced when it was used as a vaccine strain and it was CS4+ in the ELISA.
Table 2. Expression of CS4 and CS6 antigens by E. coli O25, H42 strains and their MRHA-negative derivatives measured by ELISA

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MRHA (bovine)</th>
<th>Enterotoxin production</th>
<th>Absorbance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ST</td>
<td>LT</td>
</tr>
<tr>
<td>E8775</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E8775B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E17374</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E17374B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E11881</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E11881/16</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E11881/2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E14341</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E14341/4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E14884</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E14884/7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Absorbance was read at 490 nm using specific IgG conjugates against CS4 and CS6. The test strains were measured against blanks prepared in the same way except that the antigen was replaced by saline. Each strain was tested in duplicate. Results of a typical experiment are shown. All observations were repeated two or more times and similar data were obtained each time.

MRHA-negative variants

MRHA-negative variants of serogroup O25 were relatively easy to obtain. In some cases up to 80% of the colonies examined were MRHA-negative. Strain E8775 was particularly unstable for MRHA. In general, MRHA-negative variants derived from CS4+ CS6+ strains were negative by immunodiffusion for CS4 and CS6. However, MRHA-negative variants (E11881/16, E14341/4 and E14884), isolated from three CS4+ CS6- wild-type strains of serogroup O25 (strains E11881, E14341, E14884), were positive for CS6 only. Strain E11881/16 still produced ST and LT, strain E14341/4 was derived from a non-toxigenic parent strain and strain E14884/7 had lost the ability to produce ST and LT. An MRHA-negative variant (E11881/2) was also isolated from strain E11881, which was negative for all the PCF8775 components but positive for ST and LT. The immunodiffusion results were confirmed by ELISA (Table 2).

MRHA-negative variants of serogroup O115 were not isolated after 100 colonies were tested from each strain. MRHA-negative variants were derived from four CS5+ CS6- strains of serogroup O167 and were negative for CS5 and CS6.

Electron microscopy

Examination of strain E17374 (CS4+ CS6+) and strain E17018 (CS5+ CS6+) revealed that both strains had a moderate number of fimbriae on their surfaces. These fimbriae were morphologically indistinguishable and measured approximately 7 nm in width (Figs 2a and 2c). The antigenic nature of the fimbriae was investigated using the absorbed antisera of strains E17374 and E17018. The absorbed antiserum to strain E17374, with antibodies for CS4 and CS6, coated all the fimbriae of strain E17374 (Fig. 2b). The absorbed antiserum to strain E17018, with antibodies for CS5 and CS6, did not attach to the fimbriae of strain E17374. The absorbed antiserum of strain E17018, with antibodies to CS5 and CS6, coated all the fimbriae of strain E17018 (Fig. 2d). The absorbed antiserum of strain E17374, with antibodies to CS4 and CS6 did not coat the fimbriae of strain E17018 (Fig. 2e).

The three variants of the wild-type strains which produced CS6 only were also examined by electron microscopy. Strain E14341/4 did not show any surface structures. Up to one-third of the bacteria in cultures of strains E11881/16 and E14884/7 had curly, wiry strands, approximately 3 nm wide, on their surfaces (Figs 3a and 3b). The absorbed antisera of strains E17374 and E17018 did not coat these structures. The structures were also seen on strain E11881/2, the variant strain which had lost the ability to produce CS4 and CS6. None of the three CS6- variants showed any other structures which could be identified as CS6.
DISCUSSION

Further examination of strains positive for PCF8775 has revealed that the serology of this putative colonization factor was more complicated than was first realized. Strains of serogroup O25 produced a fimbrial antigen (CS4) and gave MRHA of human and bovine erythrocytes. Strains of serogroups O115 and O167 produced serologically distinct fimbriae (CS5) and gave MRHA of human, bovine and guinea-pig erythrocytes. In addition all the strains possessed another antigenic component (CS6). Strain E8775 showed only CS6 on immunodiffusion. However the strain was MRHA-positive and produced fimbriae (Thomas et al., 1982). When E8775 was used as a vaccine strain antibodies to CS4 were produced. The production of CS4 by E8775 was shown by ELISA. Variant strains of serogroup O25 which produced CS6 only did not give MRHA. As CS4 and CS5 appeared very close to the antigen well and it was sometimes difficult to discern the precipitin lines for these antigens, the ELISA was used to confirm that CS4 was not produced by the CS6-only variant strains. CS6-only variants were not isolated from CS5\(^+\) CS6\(^+\) strains of serogroups O115 and O167.
The results of this study are comparable to those obtained with CFA/II in that the strains possess two possible adhesive antigens, one antigen being associated with fimbriae which give pronounced MRHA and another antigen being present on all the strains. The antigen found on all CFA/II-positive strains (CS3) is now known to be associated with flexible fibrils. The structure of the antigen found on all PCF8775-positive strains (CS6) has not been determined and different staining techniques may reveal its structure. The curly, wiry strands found on some strains in this study were not thought to be CS6; their presence did not correlate with that of CS6 and they did not react antigenically as CS6 when tested by immune electron microscopy. The CFA/II components CS2 and CS3 were found in strains of the same serotype but with different biotypes (Cravioto et al., 1982). The PCF8775 components CS4 and CS5 were found in different serotypes. The production of CFA/I and CFA/II components has been shown to be plasmid-mediated and linked to the production of ST and LT (Mullany et al., 1983; Peiharanda et al., 1980; Smith et al., 1979). A plasmid which codes for CS5, CS6 and ST has been transferred from an O167 strain into E. coli K12 (L. V. Thomas, unpublished work). It is probable that CS4 and CS6 are plasmid-encoded, but it is not yet clear whether genes coding for CS4, CS6, ST and LT are present on the same plasmid. Although non-enterotoxigenic CS4+ CS6+ variants and CS6− only enterotoxigenic variants were isolated, they could have arisen as a consequence of deletion mutations.

The possession of two or more adhesive antigens has been shown previously with animal strains of ETEC which produce two fimbriae, K99 and F41 (Morris et al., 1980). A similar
multiplicity of fimbriae has also been demonstrated on uropathogenic \textit{E. coli} (Ørskov et al., 1980; Väisänen et al., 1981).

Adhesive fimbriae which are found on pathogenic \textit{E. coli} have been designated as F antigens by Ørskov & Ørskov (1984). CFA/I was renamed F2 and CFA/II was called F3. However CFA/II has now been shown to consist of three fimbrial antigens (Cravioto et al., 1982; Smyth, 1982) so that reallocation of F numbers or subdivision of F3 is now required. Smyth (1982) originally used the term CS to describe the antigens of the CFA/II complex. We felt that this terminology was useful where the morphology of the antigen was not established and we have therefore provisionally called the three antigens of the PCF8775 complex CS antigens. While the two fimbrial antigens CS4 and CS5 could not now be given an F number in the Ørskov scheme, CS6 could not since its structure has not yet been established nor indeed has its adhesive properties.

Although the CS6 antigen appeared to be non-fimbrial and non-haemagglutinating this does not exclude the possibility that it has adhesive properties \textit{in vivo}. The animal adhesive fimbrial antigen 987P, which is found on certain porcine ETEC strains, lacks haemagglutinating ability (Isaacson & Richter, 1981). Fimbriae appear to play no part in the adhesion of ETEC to HEp-2 tissue culture cells (Scotland et al., 1983) and similarly a number of \textit{E. coli} strains in which fimbriae could not be demonstrated by electron microscopy were shown to adhere to human uroepithelial cells (Van den Bosch et al., 1980). Human volunteer feeding experiments would evaluate the possible role of the different antigenic components of PCF8775 in intestinal colonization by ETEC strains.

\textbf{REFERENCES}


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