INFECTION AND IMMUNITY, Aug. 1978, p. 638-647
0019-9567/78/0021-0638$02.00/0

Vol. 21, No. 2
Printed in U.S.A.

New Surface-Associated Heat-Labile Colonization Factor Antigen (CFA/II) Produced by Enterotoxigenic *Escherichia coli* of Serogroups O6 and O8

DOLORES G. EVANS* AND DOYLE J. EVANS, JR.

Program in Infectious Diseases and Clinical Microbiology, The University of Texas Medical School at Houston, Houston, Texas 77030

Received for publication 13 March 1978

Enterotoxigenic *Escherichia coli* (ETEC) belonging to serogroups O6 and O8 do not possess the H-10407-type colonization factor antigen (CFA/I). However, these frequently isolated ETEC were found to possess a second and distinct heat-labile surface-associated colonization factor antigen, termed CFA/II. Whereas CFA/I mediates mannose-resistant hemagglutination of human group A erythrocytes, CFA/II does not. CFA/II mediates mannose-resistant hemagglutination of bovine erythrocytes, and mannose-resistant hemagglutination is rapid only at reduced temperature (4°C). Because CFA/II, like CFA/I, is spontaneously lost by many ETEC isolates in the laboratory, it was possible to produce specific anti-CFA/II serum by preparing antiserum against living cells of a prototype strain (PB-176) and adsorbing this serum with living and heat-treated cells of its CFA/II-negative derivative strain PB-176-P. This serum, which neutralized the colonization factor activity of CFA/II-positive strains in infant rabbits, was employed to confirm the presence of CFA/II on ETEC which exhibited mannose-resistant hemagglutination of bovine but not human erythrocytes. CFA/II, like CFA/I, mediates adherence of the bacteria to the mucosal surface of the small intestine, as demonstrated by indirect immunofluorescence. CFA/II appears to be an important virulence factor for humans since CFA/II-positive ETEC are frequently isolated from diarrhea cases, particularly travelers' diarrhea, in Mexico; these ETEC were not uncommon in a collection of isolates from Bangladesh. The O6:H16 strain of ETEC responsible for an outbreak of diarrhea in the United States was also shown to be CFA/II positive. CFA/I and CFA/II were never found on the same serotypes of ETEC, but 98% of the heat-stable and heat-labile enterotoxin-producing ETEC belonging to the frequently isolated serogroups O6, O8, O15, O25, O63, and O78 were positive for either CFA/I or CFA/II.

Enterotoxigenic *Escherichia coli* (ETEC) cause an acute cholera-like diarrhea in both humans and animals. The disease process involves colonization of the mucosal surfaces of the small intestine, without tissue invasion, followed by elaboration of a choleragen-like heat-labile enterotoxin (LT) and/or a heat-stable enterotoxin (ST) (9, 12). Intestinal colonization by ETEC is mediated by specific surface-associated fimbriate antigens by which the bacteria adhere to epithelial cell surfaces. To date, three different colonization-related antigens have been characterized. These are the K88 antigen of swine-specific ETEC (14), the K99 antigen of calf- and sheep-specific ETEC (20), and the colonization factor antigen, or CFA, of human-associated ETEC (9). K88, K99, and CFA are antigenically unrelated to the type 1 pili generally associated with *E. coli*, and each is demonstrable by mannose-resistant hemagglutination (MRHA) activity, whereas the type 1 pili mediate only mannose-sensitive hemagglutination (1, 6, 14, 19).

We have characterized the CFA of ETEC isolated from humans, using the infant rabbit model, and recently reported the results of volunteer studies which confirmed the hypothesis that CFA is important as a virulence factor for humans (7, 9). One interesting observation was that CFA could be detected on ETEC of the frequently isolated serogroups O15, O25, O63, and O78 but not on ETEC of serogroups O6 and O8, which are also frequently associated with acute diarrhea in humans (7, 12, 17). We document here evidence that ETEC of serogroups O6 and O8 possess a fimbriate CFA (termed CFA/II) distinct from the previously described CFA (now termed CFA/I).

MATERIALS AND METHODS

Bacterial cultures. A total of 60 ETEC isolates, as well as 29 laboratory derivatives of these obtained by isolating clones which were either ST only, LT only, nontoxigenic, or CFA negative, were used in these studies. These laboratory-passed derivatives, or P-strains, have been described previously (6, 7, 12). Strains 36025/5, 36025/10, 36035/6, 36027/10, and 36068/9, originating in Bangladesh, were gifts of S. H. Richardson. Other strains from Bangladesh are those with unlettered numbers and numbers with the prefix "H" (10, 13). Strains with the prefix "PB" were obtained during studies on travelers' diarrhea in Mexico (4), as were those with the prefix "TD" (16). TD cultures and strain CL-9620 were obtained from the Center for Disease Control, Atlanta, Ga. Strain CL-9620 is the epidemic strain isolated during an outbreak of acute diarrhea involving adults at Crater Lake (21). Strain NIH-074 was isolated from an individual adult case of diarrhea in Houston; strain TX-CH is the epidemic strain associated with a nosocomial outbreak of severe diarrhea in a hospital in Houston (22).

Serotyping was carried out by F. Ørskov and I. Ørskov of the WHO International Escherichia Centre, Copenhagen, Denmark, and B. Rowe of the Central Public Health Laboratory, London, England. Cultures which were gifted were usually serotyped by the source. It should be noted that the cultures designated here as serogroup O63 were previously reported as belonging to serogroup O20 (6, 7).

Culture media. Stock cultures were kept at room temperature on slants composed of 2% peptone–0.5% NaCl–2% agar. Long-term storage was accomplished by storing cultures at −65°C; for this purpose the cells were suspended in CYE medium (13) with 15% glycerol added. Highly unstable ETEC cultures were maintained by lyophilization. A special medium, CFA agar (6), was employed to grow cultures for the assay of CFA/I and CFA/II. CFA agar consists of 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.15% yeast extract (Difco), and salts with 2% agar, pH 7.4. CYE broth (13), also a Casamino Acids-yeast extract-salts medium, was used to grow cultures for the assay of ST and LT. Culture supernatant fluids were assayed for ST by the method of Dean et al. (2) and for LT by the in vitro passive immune hemolysis technique (11) and by the Y-1 cultured adrenal cell assay of Donta et al. (3).

Hemagglutination tests. Type A human blood was drawn from volunteer donors and placed into a tube containing 1.0 ml of 3.8% citric acid, in distilled water, per 9.0 ml of blood. Blood was diluted 1:4 with phosphate-buffered saline (PBS), pH 7.2, to test for hemagglutination and 1:4 with 1% mannose in PBS to test for MRHA. The same procedure was used for bovine blood obtained from Flow Laboratories, Inc., Rockville, Md. The slide agglutination test was performed as follows. Bacterial growth obtained from 18-h CFA agar cultures, either isolated colonies or confluent growth as required, was picked up with a sterile wooden toothpick and mixed with a drop of blood (0.025 ml) on a microscope slide at room temperature. After approximately 1 min, slides with less than maximum agglutination were placed on the surface of ice and cooled. Results were recorded as follows. A 4+ reaction was instantaneous and complete, involving all of the erythrocytes; lesser degrees of reaction were graded 3+, 2+, or 1+ (6). A 4+ reaction in the case of CFA/II-positive *E. coli* required cooling; however, cooling did not increase the degree of agglutination of CFA/I-positive *E. coli* with bovine erythrocytes.

Preparation of anti-CFA sera. The preparation of anti-CFA/I serum, using purified CFA/I, has been described (7). The bacterial cell adsorption method for the preparation of anti-CFA/I-specific serum has also been described (6, 9). In this method, hyperimmune serum from rabbits immunized with the CFA-positive strain H-10407 is repeatedly adsorbed with heat-killed and living cells of the CFA-negative strain H-10407-P (9). Anti-CFA/II serum was prepared by the same method, using the CFA/II-positive prototype strain PB-176 to prepare the immune serum and the CFA/II-negative derivative strain PB-176-P to adsorb the serum; the resultant serum agglutinates at high titer only living PB-176 cells or other living bacteria possessing the CFA/II antigen (see below).

Bacterial agglutination with anti-CFA serum. The microtiter technique was used to test *E. coli* cultures for CFA/I and for CFA/II with specific serum prepared as described above. Bacterial cells were harvested with PBS, pH 7.2, from 18-h CFA agar cultures grown at 37°C. The cell suspensions were adjusted to an optical density of approximately 2.0 at 640 nm with PBS. Serial twofold dilutions of the specific anti-CFA serum were prepared in U-well microtiter plates (Linbro Scientific Co., Hamden, Conn.) with PBS plus 0.02% bovine serum albumin as diluent. An equal volume (0.025 ml) of cell suspension was then added to the appropriate wells, and the plate was sealed and incubated for 60 min at 37°C. Agglutination was determined visually, using cells plus diluent alone as controls. The agglutination titer was the reciprocal of the highest dilution of anti-CFA serum producing agglutination. Cells that had been heated at 100°C for 2 h also served as controls because both CFA/I and CFA/II are heat labile.

Preparation of homogenates for immunodiffusion tests. Immunodiffusion tests for CFA/I and CFA/II were performed with specific antiserum and homogenate antigen preparations prepared as follows. For each culture to be tested, a large Roux bottle containing CFA agar was inoculated for confluent growth and incubated at 37°C for 48 h. The cells were harvested with PBS containing 0.02% sodium azide in a minimum volume, and the thick suspension was homogenized with a Waring blender for a total of 6 min, maintaining a temperature of 4°C. The cells were then removed by centrifugation at 12,000 × *g* for 45 min and the supernatant fluid was ultrafiltered (Millipore Corp., Bedford, Mass.; 0.8 μm) with a syringe filter.

Assays for colonization factor activity in infant rabbits. Three-day-old rabbits were obtained and fasted for 24 h before administration of bacteria. Challenge strains were prepared as 4-h cultures in brain heart infusion broth (Difco), and 2.0 ml of each inoculum was injected directly into the lumen of the intestine as previously described (7, 9). The inoculum size was standardized in all cases at 10^7 bacteria per

rabbit. After 18 h, the rabbits were sacrificed, and the intestines were removed. Small sections of each intestine were frozen in liquid nitrogen for later processing, and fluid accumulation was determined as milliliters per milligram of intestinal tissue (7). The intestines were weighed before and after removal of contents. After the first weighing, sections approximately 1 cm in length were placed on absorbent paper, and the contents were expressed with a flat instrument. The resultant values for uninoculated control rabbits were approximately 2.0 ml of fluid per mg of tissue due primarily to the fragility of the mucosal tissue. Data shown in Tables 2 and 5 are uncorrected values. The frozen segments were used to prepare thin (5 to 8 μm) cross sections, using an IEC cryostat microtome (International Equipment Co., Div. of Damon Corp., Needham Heights, Mass.). These sections were submitted to analysis by indirect immunofluorescence for the test strains as follows. The sections were fixed in acetone, air dried, washed in PBS, and exposed first to antibody against the appropriate *E. coli* somatic antigen and then after another washing to goat anti-rabbit immunoglobulin (globulin fraction; Cappel Laboratories, Inc., Dowington, Pa.) conjugated with fluorescein isothiocyanate. The goat antiserum was adsorbed first with an acetone-dried powder of rabbit muscle to reduce nonspecific fluorescence. Antibody-treated sections were mounted in glycerol-PBS (3:1) and observed by fluorescence microscopy.

Electron microscopy. Bacteria were observed with a JOEL JEM 100 transmission electron microscope. Test materials were treated essentially as previously described (7). Samples were placed on carbon-coated collodion grids, air dried, and stained with 1% phosphotungstic acid, pH 6.8.

RESULTS

MRHA by ETEC of serogroups O6 and O8. ETEC possessing CFA/I cause MRHA of human type A erythrocytes (6). This reaction is rapid and complete at room temperature, or 25°C. CFA/I-positive ETEC, such as the prototype strain H-10407, also cause rapid MRHA of bovine erythrocytes at 25°C, but to a lesser degree. CFA/I-negative laboratory derivatives of these strains, such as H-10407-P, do not cause MRHA of either human or bovine erythrocytes. In the process of screening numerous ETEC isolates, we observed that many of those ETEC belonging to serogroups O6 and O8 caused MRHA with bovine, but not human, erythrocytes and that these were 4+ positive only when the tests were carried out on ice. These preliminary results (Table 1) were significant because bovine-only MRHA was associated with ETEC serotype and also because this property was frequently lost during subculture in the laboratory. These data (Table 1) indicated that ETEC of serogroups O6 and O8 might possess a fimbriate colonization antigen other than CFA/I.

Intestinal colonization by ETEC of serogroups O6 and O8. The results presented above served as a guide for selecting specific ioslates of CFA/I-negative ETEC to test for colonization factor activity in the infant rabbit model. Strain PB-176 (serotype O6:H16, bovine MRHA-positive ETEC) was selected as the prototype strain particularly since an MRHA-negative laboratory derivative (PB-176-P) was available for comparison. Fluid accumulation was employed as a measure of virulence, and the indirect immunofluorescence technique was used to detect adherence of the bacteria in the intestine. Table 2 shows that of the two test strains only PB-176 produced a diarrheal response in rabbits and that this strain adhered to and colonized the infant rabbit intestine, whereas strain PB-176-P did not. Colonization factor activity was subsequently demonstrated with other ETEC isolates of serogroup O6, ETEC belonging to serogroup O8 and an O85:H7 isolate, all of which are CFA/I negative but bovine MRHA positive (Table 3).

Demonstration of a specific heat-labile surface-associated antigen (CFA/II) on ETEC of serogroups O6, O8, and O85. The correlation observed between bovine MRHA activity, colonization factor activity in the infant rabbit model, and the presence of fimbrial structures on the surface of CFA agar-grown strain PB-176 (which does not produce type 1 pili when grown on CFA agar [manuscript in preparation]) (Fig. 1) led us to test for a CFA on this strain. Antiserum specific for heat-labile surface antigens of strain PB-176 was prepared by the bacterial adsorption method previously used to prepare anti-CFA/I antiserum (9). Figures 2 and 3 show the results of immunodiffusion tests dem-

TABLE 1. *Summary of preliminary observations on MRHA of bovine erythrocytes by ETEC possessing the H-10407-type CFA (CFA/I) and by CFA/I-negative ETEC belonging to serotypes O6:H16 and O8:H9*

Test strains[a]	MRHA with:	
	Human type A erythrocytes	Bovine erythrocytes
CFA/I$^+$, ST$^+$LT$^+$ ETEC of serotypes O25:H42, O78:H11	4$^+$ at 25°C	3$^+$ at 25°C
CFA/I$^-$, P-strains of serotypes O25:H42, O78:H11	Negative	Negative
CFA/I$^-$, ST$^+$LT$^+$ ETEC of serotypes O6:H16, O8:H9	Negative	4$^+$ at 4°C only
CFA/I$^-$, P-strains of serotypes O6:H16, O8:H9	Negative	Negative

[a] P-strains are spontaneous derivatives of ST$^+$LT$^+$ ETEC which no longer produce one of the enterotoxins; P-strains of CFA/I-positive ETEC usually, but not always, fail to produce CFA/I (see Table 5).

onstrating that the bovine MRHA-positive ETEC possess an antigen which is distinct from CFA/I; this antigen, shared by ETEC of serogroups O6, O8, and O85, is referred to as CFA/II. CFA/II is also demonstrable by the bacterial agglutination technique. Note in Table 4 that CFA/II was destroyed by boiling. Also, many ETEC of serogroups O6 and O8 produced 16-fold-lower titers with the anti-CFA/II serum than did the prototype strain PB-176, but these ETEC did produce equally strong precipitin lines in the immunodiffusion tests. Note also that none of the CFA/II-positive strains reacted with anti-CFA/I antiserum.

Neutralization of CFA/II activity in infant rabbits by specific antiserum. The following experiments were performed to test for antibody neutralization of the colonizing activity of strain PB-176. One group of rabbits was challenged with strain PB-176 premixed with a 1:50 dilution of normal rabbit serum, and another group was challenged with the same dose of PB-176 which had been premixed with a 1:50 dilution of the adsorbed anti-PB-176-specific antiserum. Table 5 shows that both adherence and fluid accumulation were prevented by the PB-176-specific adsorbed serum, confirming that this strain does possess a surface-associated CFA.

TABLE 2. *Demonstration of colonization factor activity by ETEC strain PB-176, using the infant rabbit model, and failure of strain PB-176-P to colonize: correlation with bovine-only MRHA activity*

E. coli strain[a]	Adherence, assayed by indirect immunofluorescence	Fluid accumulation (ml/mg of intestine)[b]
PB-176 (MRHA$^+$)	Positive	4.99 ± 0.582 SD[c]
PB-176-P (MRHA$^-$)	Negative	2.025 ± 0.341 SD

[a] Each group consisted of 10 animals; inoculum, 10^7 bacteria, intraluminal; sacrifice time, 18 h.
[b] Uncorrected values; see text.
[c] SD, Standard deviation.

Relationships between production of CFAs, serotype, and enterotoxigenicity. ETEC may produce either ST or LT or both, and all three varieties of ETEC have been isolated from cases of acute diarrhea. Also, ETEC which produce both toxins when isolated may lose the ability to produce either or both toxins (12). Against this background, it was of interest to consider why CFA/I-positive ETEC isolates are almost invariably positive for both ST and LT and whether this relationship also applies to ETEC with the CFA/II antigen. This was investigated by testing freshly cloned, i.e., homogeneous, cultures of CFA/I- and CFA/II-positive ETEC, respectively, and CFA-negative derivatives and isolates for ST and LT production. Several ST-only and LT-only isolates were also submitted to analysis; these are included in Tables 6 and 7 according to serotype. Table 6 lists 31 isolates of ST/LT ETEC possessing CFA/I and 25 related isolates and P-strains. There are several points of interest. First, there are few different serogroups on the list. Of 31 serotyped isolates studied, 30 belong to serogroup O15, O25, O63, or O78; serogroup O128 is represented by one isolate. Five O78 isolates gave rise to derivatives which were negative for both CFA/I and ST but remained stable for LT production. Also, different serotypes apparently exhibit different patterns of instability. ETEC of serotype O25:H42 produced a variety of CFA/I-negative

TABLE 3. *Correlation between MRHA of bovine erythrocytes and colonization factor activity in the infant rabbit model*

E. coli strain	Serotype	MRHA		Colonization of rabbit (immunofluorescence)
		Human A	Bovine	
PB-176	O6:H16	−	4$^+$	Positive
PB-176-P		−	−	Negative
PB-407	O6:H16	−	4$^+$	Positive
PB-407-P		−	−	Negative
CL-9620	O6:H16	−	4$^+$	Positive
CL-9620-P		−	−	Negative
H-10400	O8:H$^-$	−	4$^+$	Positive
H-10400-P		−	−	Negative
H-15697-3	O8:H?	−	4$^+$	Positive
H-15697-3-P		−	−	Negative
19935	O85:H7	−	4$^+$	Positive
NIH-074	O6:H16	−	4$^+$	Positive
PB-67	O8:H9	−	−	Negative
H-10407 (CFA/I$^+$)	O78:H11	4$^+$	3$^+$	Positive
H-10407-P (CFA/I$^-$)		−	−	Negative

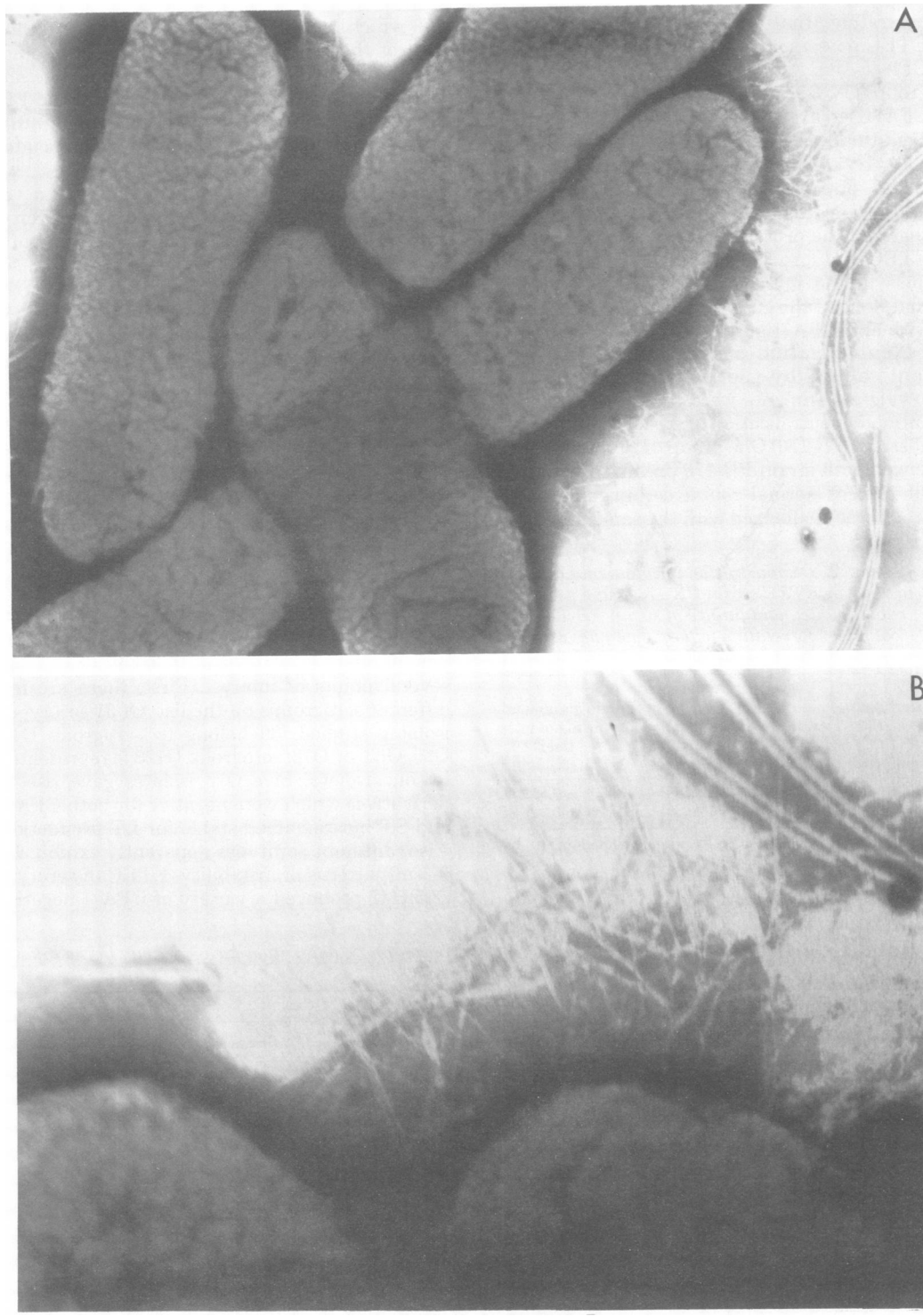

FIG. 1. *Electron micrographs of negatively stained preparation of E. coli PB-176. (A) × 35,000; (B) × 90,000. Note the presence of very thin fimbriae; large filaments are flagella.*

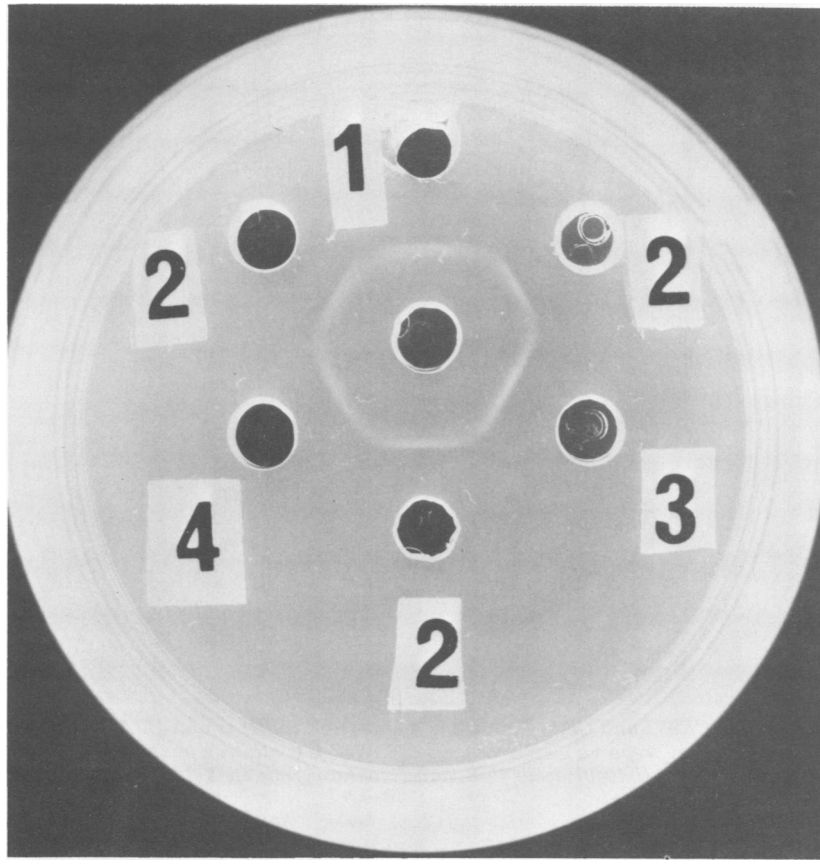

FIG. 2. *Immunodiffusion plate with anti-CFA/II serum in the center well. Other wells contain crude antigen preparations derived from the homologous strain PB-176 (no. 2), strain 19935 (O85:H7) (no. 1), strain CL-9620 (O6:H16) (no. 3), and strain H-10400 (O8:H⁻) (no. 4).*

derivatives with respect to ST and LT. The O15:H11 isolates were characteristically very unstable for CFA/I production and rapidly lost this property.

Table 7 lists 17 isolates of ST/LT ETEC possessing CFA/II and 12 related isolates and P-strains negative for ST, LT, or CFA/II. Note that of the 21 serotyped isolates 10 belong to serogroup O6, mostly O6:H16, and 9 belong to serogroup O8, mostly O8:H9; serogroups O80 and O85 are represented by single isolates. As with CFA/I, the loss of CFA/II was usually accompanied by loss of either ST or LT production.

CFA/II was never detected on ETEC of serogroup O15, O25, O63, or O78, and CFA/I was never detected on isolates belonging to serogroups O6 and O8. Also, although some infrequently isolated ETEC serogroups such as O128 and O85 did possess either CFA/I or CFA/II, others did not. One ST/LT isolate each of serotypes O103:H21, O88:H⁻, and O9:H⁻ (not included in the tables) were found to be negative for the known CFAs.

DISCUSSION

ETEC belonging to serogroups O6 and O8 share the ability to produce a surface-associated heat-labile antigen, CFA/II, and preliminary evidence indicates that this antigen is a distinct form of fimbriae. CFA/II-positive ETEC such as the prototype strain PB-176 possess fimbriae when grown on CFA agar, whereas CFA/II-negative derivatives such as PB-176-P do not. Also, CFA/II-positive ETEC hemagglutinate bovine erythrocytes in the presence of mannose, whereas CFA/II-negative laboratory derivatives such as PB-176 do not. Distinct MRHA patterns are characteristic of the other fimbrial ETEC colonization factors K88, K99, and CFA/I (1, 6, 14, 19). CFA/II, like CFA/I, confers upon ETEC the ability to adhere to and colonize the mucosal surfaces of the small intestine of infant rabbits, and this colonization factor activity is neutral-

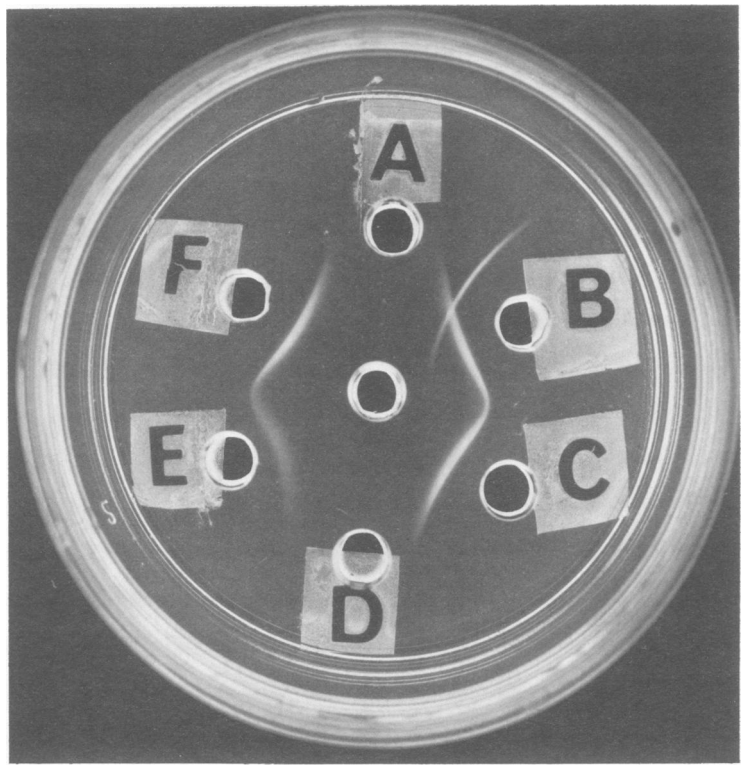

FIG. 3. *Nonidentity of CFA/I and CFA/II antigens. Center well contains anti-CFA/I serum; wells A and D contain anti-CFA/II serum prepared with strain PB-176. Well B contains a mixture of CFA/II from strain PB-176 and CFA/I from strain H-10407; wells C, E, and F contain crude CFA/I preparations from strains H-9195, PB-40, and PB-200, respectively.*

TABLE 4. *Demonstration of the CFA/II antigen on ETEC of serogroups O6 and O8 and the absence of CFA/II on P-strains*

E. coli strain	Serotype	Bovine-specific MRHA	Titer with anti-CFA/I[a]	Titer with anti-CFA/II	CFA/II by immunodiffusion
PB-176 (living)	O6:H16	+	<1:2	1:512	+
PB-176 (boiled)		NA[b]	<1:2	<1:2	NA
PB-176-P (living)		−	<1:2	<1:2	−
PB-407 (living)	O6:H16	+	<1:2	1512	+
PB-407 (boiled)		NA	<1:2	<1:2	NA
PB-407-P (living)		−	<1:2	<1:2	−
CL-9620 (living)	O6:H16	+	<1:2	1:32	+
CL-9620 (boiled)		NA	<1:2	<1:2	NA
CL-9620-P (living)		−	<1:2	<1:2	−
H-10400 (living)	O8:H$^-$	+	<1:2	1:32	+
H-10400 (boiled)		NA	<1:2	<1:2	NA
H-10400-P (living)		−	<1:2	<1:2	−
H-15697 (living)	O8:H?	+	<1:2	1:32	+
H-15697 (boiled)		NA	<1:2	<1:2	NA
H-15697-P (living)		−	<1:2	<1:2	−
H-10407 (living)	O78:H11	−	1:512	<1:2	−
H-10407 (boiled)		−	<1:2	<1:2	NA
H-10407-P (living)		−	<1:2	<1:2	−

[a] Bacterial agglutination titers; the same anti-CFA/II serum was used for bacterial agglutination tests and for immunodiffusion.

[b] NA, Not applicable.

ized by antibody prepared against the surface antigen. Neutralization of CFA/II also prevents diarrhea in the animal model.

TABLE 5. *Neutralization of adherence and diarrhea production in infant rabbits of strain PB-176 by PB-176-specific (adsorbed) immune serum*

E. coli strain[a]	Adherence, assayed by indirect immunofluorescence	Fluid accumulation (ml/mg of intestine)[b]
PB-176 premixed with NRS	Positive	5.91 ± 1.259 SD[c]
PB-176 premixed with immune serum	Negative	2.939 ± 0.347 SD

[a] Animals, 8 with immune serum and 10 with normal rabbit serum (NRS); inoculum, 10^7 bacteria, intraluminal; sacrifice time, 18 h. Both NRS and immune serum were used at a final dilution of 1:50.

[b] Uncorrected values; see text.

[c] SD, Standard deviation.

An important question naturally arises; i.e., is there evidence that CFA/II is a virulence factor for humans? Indirect evidence is as follows. CFA/II has the same general properties as CFA/I (i.e., heat lability, surface association, MRHA activity, colonization factor activity in the animal model, antibody neutralization of function, spontaneous loss, relationship with a well-defined group of serotypes, relationship with enterotoxigenicity, association with naturally acquired diarrhea), and CFA/I has been confirmed as a virulence factor for humans by volunteer studies (8). More direct evidence, although not conclusive, is the fact that CFA/II is associated with two of the more frequently isolated ETEC serogroups, O6 and O8, which do not produce CFA/I. Specifically, CFA/II-positive ETEC were isolated from individual diarrhea cases in Bangladesh and from two different sites in Mexico in association with travelers'

TABLE 6. *Production of CFA/I in relation to enterotoxigenicity and serotype of E. coli isolates and P-strains*

Phenotype	Serotype	No. of isolates	Identity[a]
ST^+LT^+CFA/I^+	O78:K80:H11	6	H-9190, H-9192, H-10401, H-10407, H-10409, 36027/10, 36035/6
	O78:K80:H12	2	36025/5
	O63:H$^-$	5	PB-40, PB-426, PB-312, PB-200, PB-66
	O25:H42	8	PB-434, PB-379, PB-11, PB-182, PB-37, PB-413, H-9195, 16173-1
	O25:H$^-$	2	PB-334, PB-302
	O25:H?	1	PB-143
	O15:H11	2	H-10408, 16095
	O15:H$^-$	1	PB-455
	Not typed	4	23505, PB-202, PB-60, PB-418
ST^+LT^-CFA/I^+	O78:K80:H12	2	TX-CH (multiple isolates), 36025/10
	O128:K67:H12	1	16098-1
	O63:H$^-$	1	19123
ST^-LT^+CFA/I^-	O78:K80:H11	5	H-9190-P, H-9192-P, H-10401-P, H-10407-P, H-10409-P
	O63:H$^-$	2	PB-40-P, PB-200-P
	O25:H42	1	PB-434-P
	O25:H$^-$	1	PB-302-P
	O15:H11	2	PB-455-P, 13634
	O15:H$^-$	1	PB-258-P
	Not typed	1	PB-202-P
ST^+LT^+CFA/I^-	O25:H42	1	H-9195-P
	O15:H11	2	H-10408-P, 16095-P
ST^-LT^-CFA/I^+		0	
ST^-LT^+CFA/I^+		0	
ST^+LT^-CFA/I^-	O25:H42	2	PB-37-P, PB-182-P
	O25:H?	1	PB-143-P
ST^-LT^-CFA/I^-	O25:H42	1	PB-11-P
	O63:H$^-$	1	PB-66-P

[a] See text for description of sources.

TABLE 7. *Production of CFA/II in relation to enterotoxigenicity and serotype of E. coli isolates and P-strains*

Phenotype	Serotype	No. of isolates	Identity[a]
ST^+LT^+CFA/II^+	O6:K15:H16	2	CL-9620 (multiple isolates), 36068/9
	O6:K?:H16	6	TD-260, TD-415, TD-219, PB-407, PB-176, NIH-074
	O6:K?:H^-	1	PB-166
	O8:K40:H9	3	15862-2, 16060-2, 19942
	O8:K40:H^-	2	15697-3, H-10400
	O8:K40:H?	1	18646
	O85:K?:H7	1	19935
	O80:H9	1	PB-122
ST^+LT^-CFA/II^+	O80:K?:H9	1	PB-122-P
	O6:H16	1	PB-166-P
ST^-LT^+CFA/II^-	O6:H16	1	PB-176-P
ST^+LT^+CFA/II^-	O8:K?:H9	2	PB-67, 16932
ST^-LT^-CFA/II^+		0	
ST^-LT^+CFA/II^+	O6:K15:H16	1	22743
	O8:K?:H9	1	19796
ST^+LT^-CFA/II^-		0	
ST^-LT^-CFA/II^-	O6:K15:H16	1	CL-9620-P
	O6:H?:H16	1	PB-407-P
	O8:K40:H^-	2	H-10400-P, 15697-3-P
	O8:H9	1	PB-67-P

[a] See text for description of sources.

diarrhea (4, 16). We have also isolated CFA/II-positive ETEC from diarrhea cases in Houston; for example, strain NIH-O74. A rather striking example of ETEC virulence for adults is the Crater Lake outbreak reported by Rosenberg et al. (21); the CFA/II-positive strain CL-9620 reported here is the epidemic strain isolated from that source. In fact, 49 of 50 (98%) of the ST/LT isolates belonging to the frequently isolated ETEC serogroups were found to possess either CFA/I or CFA/II, but never both. This suggests that these two antigens constitute the predominant colonization factors of ETEC associated with diarrhea in humans. The only reservation to this conclusion is that so few ST/LT ETEC isolates from infantile diarrhea cases have been studied that the failure to isolate such ETEC from young children and infants in the United States is itself conspicuous. ST-only and LT-only ETEC are available from this age group (5), but as the data presented here show, the CFAs are only infrequently associated with such ETEC.

Further evidence for the importance of CFA/II as a virulence factor for humans is accumulating from studies concerned with the humoral antibody response, or seroconversion, with respect to CFA/I and CFA/II in the course of naturally acquired diarrhea. We report elsewhere (manuscripts in preparations; D. J. Evans, Jr., A. Willett, H. L. DuPont, and S. M. Hamilton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C11, p. 79; T. R. Deetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C12, p. 279) the results of two studies in which antibody responses to CFA/I and CFA/II, respectively, are documented.

CFAs, unlike the enterotoxins, present a serious problem of detection since the infant rabbit model is not suitable for rapid screening of isolates. MRHA of bovine erythrocytes, performed by a simple slide test, is of particular value as a screening method, but results are not conclusive since the presence of CFA/II must be confirmed by the use of specific antiserum. We report elsewhere the results of work designed to determine the reliability of MRHA tests for CFA/I- and CFA/II-positive ETEC and describe an improved approach for employing this screening method (manuscript in preparation). Bacterial agglutination is not always reliable since clinical isolates of CFA/II-positive ETEC may autoagglutinate or possess other surface features which interfere with specific agglutination. On the other hand, anti-CFA/II serum prepared by the bacterial adsorption method, using the proto-

type strains PB-176 and PB-176-P, proved to be very reliable as a reagent when used in immunodiffusion tests using crude antigen preparations of a large number of strains. Also, we have found the staphylococcus co-agglutination test (15) adapted for CFA/I-positive ETEC (18) valuable for screening isolates and have also applied this technique for detecting CFA/II-positive ETEC (D. G. Evans, D. J. Evans, Jr., and T. R. Deetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B8, p. 15).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-13385 from the National Institute of Allergy and Infectious Diseases.

We acknowledge N. Ford, S. M. Hamilton, and J. A. Pauley for technical assistance and Irma Bujnoch and Sandra Cobb for typing this manuscript.

LITERATURE CITED

1. **Burrows, M. R., R. Sellwood, and R. A. Gibbons.** 1976. Hemagglutinating and adhesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. J. Gen. Microbiol. **96**:269–275.
2. **Dean, A. G., C. Yi-Chuan, G. Williams, and B. Barden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
3. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183**:334–336.
4. **DuPont, H. L., J. Olarte, D. G. Evans, L. K. Pickering, E. Galindo, and D. J. Evans, Jr.** 1976. Comparative susceptibility of Latin American and United States students to enteric pathogens. N. Engl. J. Med. **295**:1520–1521.
5. **Evans, D. G., D. J. Evans, Jr., and H. L. DuPont.** 1977. Virulence factors of enterotoxigenic *Escherichia coli*. J. Infect. Dis. **136**:S118–S123.
6. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
7. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of the colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
8. **Evans, D. G., T. K. Satterwhite, D. J. Evans, Jr., and H. L. DuPont.** 1978. Differences in serological responses and excretion patterns of volunteers challenged with enterotoxigenic *Escherichia coli* with and without the colonization factor antigen. Infect. Immun. **19**:883–888.
9. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
10. **Evans, D. J., Jr., and D. G. Evans.** 1973. Three characteristics associated with enterotoxigenic *Escherichia coli* isolated from man. Infect. Immun. **8**:322–328.
11. **Evans, D. J., Jr., and D. G. Evans.** 1977. Direct serological assay for the heat-labile enterotoxin of *Escherichia coli*, using passive immune hemolysis. Infect. Immun. **16**:604–609.
12. **Evans, D. J., Jr., D. G. Evans, H. L. DuPont, F. Ørskov, and I. Ørskov.** 1977. Patterns of loss of enterotoxigenicity by *Escherichia coli* isolated from adults with diarrhea: suggestive evidence for an interrelationship with serotype. Infect. Immun. **17**:105–111.
13. **Evans, D. J., Jr., D. G. Evans, and S. L. Gorbach.** 1973. Production of vascular permeability factor by enterotoxigenic *Escherichia coli* isolated from man. Infect. Immun. **8**:725–730.
14. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 with haemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**:135–144.
15. **Kronvall, G.** 1972. A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A containing staphylococci. J. Med. Microbiol. **6**:187–190.
16. **Merson, M. H., G. K. Morris, D. A. Sack, J. G. Wells, J. C. Feeley, R. B. Sack, W. B. Creech, A. Z. Kapikian, and E. J. Gangarosa.** 1976. Travelers' diarrhea in Mexico. A prospective study of physicians and family members attending a congress. N. Engl. J. Med. **294**:1299–1305.
17. **Ørskov, F., I. Ørskov, D. J. Evans, Jr., R. B. Sack, D. A. Sack, and T. Wadstrom.** 1976. Special *Escherichia coli* serotypes among enterotoxigenic strains from diarrhoea in adults and children. Med. Microbiol. Immunol. **162**:73–80.
18. **Ørskov, I., and F. Ørskov.** 1977. Special O:K:H:, serotypes among enterotoxigenic *E. coli* strains from diarrhea in adults and children. Occurrence of the CF (colonization factor) antigen and of hemagglutinating abilities. Med. Microbiol. Immunol. **163**:99–110.
19. **Ørskov, I., F. Ørskov, B. Jann, and K. Jann.** 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. **41**:667–710.
20. **Ørskov, I., F. Ørskov, H. W. Smith, and W. J. Sojka.** 1975. The establishment of K99, a thermolabile, transmissible *Escherichia coli* K antigen, previously called "Kco," possessed by calf and lamb enteropathogenic strains, Acta Pathol. Microbiol. Scand. **83**:31–36.
21. **Rosenberg, M. L., J. P. Koplan, I. K. Wachsmuth, J. G. Wells, E. J. Gangarosa, R. L. Guerrant, and D. A. Sack.** 1977. Epidemic diarrhea at Crater Lake from enterotoxigenic *Escherichia coli*. A large waterbourne outbreak. Ann. Intern. Med. **86**:714–718.
22. **Ryder, R. W., I. K. Wachsmuth, A. E. Buxton, D. G. Evans, H. L. DuPont, E. Mason, and F. F. Barrett.** 1976. Infantile diarrhea produced by heat-stable enterotoxigenic *Escherichia coli*. N. Engl. J. Med. **295**:849–853.