INFECTION AND IMMUNITY, Jan. 1986, p. 10–15
0019-9567/86/010010-06$02.00/0

Vol. 51, No. 1

New Fimbrial Antigen F165 from *Escherichia coli* Serogroup O115 Strains Isolated from Piglets with Diarrhea

JOHN M. FAIRBROTHER,* SERGE LARIVIÈRE, AND RÉAL LALLIER

Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, C.P. 5000, Saint Hyacinthe, Province of Quebec, Canada J2S 7C6

Received 2 July 1985/Accepted 17 September 1985

Sixteen strains of *Escherichia coli* serogroup O115 isolated from piglets with diarrhea were examined for mannose-sensitive or mannose-resistant hemagglutination (MSHA or MRHA, respectively) for the presence of fimbriae by electron microscopy and for enterotoxigenicity by the ligated gut loop technique in 10-day-old piglets. Four strains demonstrated MRHA of sheep, goat, pig, dog, cat, chicken, and human erythrocytes but no MRHA of calf, horse, guinea pig, and rabbit erythrocytes. They were divided into pattern I (MSHA negative) and pattern II (MSHA positive). The remaining 12 strains were classified as pattern III (MRHA negative, MSHA positive) and pattern IV (hemagglutination negative). An antiserum produced against the MRHA-positive, MSHA-negative strain 4787 and absorbed by the same strain grown at 15°C agglutinated all of the MRHA-positive strains but none of the MRHA-negative strains and completely inhibited the MRHA of these strains. The surface antigen against which this absorbed antiserum was directed was designated "F165." Fimbriae (pili) purified from strain 4787 hemagglutinated erythrocytes in the same mannose-resistant pattern as the strain itself and reacted with the anti-F165 antiserum in an enzyme-linked immunosorbent assay, thus demonstrating the fimbrial nature of the hemagglutinating F165 antigen. The F165 antigen showed no serological relationship with the fimbrial antigens F4, F5, F6, and "F41." A positive correlation between the presence of F165 and the lack of enterotoxigenicity was demonstrated. Thus, we found a new mannose-resistant, hemagglutinating fimbrial antigen, F165, which is produced only by nonenterotoxigenic strains of *E. coli* serogroup O115. The possible role of F165 as a virulence attribute of *E. coli* strains causing extraintestinal disease is discussed.

Strains of *Escherichia coli* have been associated with a wide variety of syndromes in animals and humans. These include the enterotoxic, enterotoxemic, enteroinvasive, and enteropathogenic enteropathies, urinary infection, meningitis, and septicemia. Fimbriae (pili) play an important role in the pathogenesis of enterotoxigenic *E. coli* diarrhea. They facilitate the attachment to and colonization of the small intestine by enterotoxigenic *E. coli*. A limited number of antigenically different fimbrial adhesins have been shown to be associated with diarrhea (13). Each adhesin results in a specific pattern of mannose-resistant (MR) hemagglutination (MRHA) against erythrocytes of different animal species (6). In newborn piglets and calves, the adhesins F4, F5, and F6 (K88, K99, and 987P) and "F41" have been described to date (13, 27). Of these adhesins, only F6 has not demonstrated MRHA (13). MR hemagglutinating fimbriae also have been found on *E. coli* associated with urinary tract infections in humans (27). In addition, a high proportion of *E. coli* strains isolated from such extraintestinal sources as cerebrospinal fluid, urine, and blood carry MR hemagglutinins (4, 11, 25). The majority of pathogenic and nonpathogenic *E. coli* strains also express the F1 (type 1) common fimbrial adhesin which causes a mannose-sensitive (MS) hemagglutination (MSHA) of erythrocytes from various animal species (6, 27).

Strains of *E. coli* belonging to serogroup O115 have been associated with severe catarrhal to hemorrhagic enteritis and septicemia in calves and pigs (14, 15, 26). Results of our preliminary work (unpublished data) has suggested that certain strains of this serogroup colonize the ileum of naturally infected piglets but do not produce any of the known fimbrial adhesins F4, F5, F6, or F41.

Thus, the purpose of this study was to investigate the possibility that a previously undetected fimbrial adhesin is produced by strains of this serogroup. We found that nonenterotoxigenic strains of this serogroup produce an MR, hemagglutinating fimbrial antigen which we designated "F165."

MATERIALS AND METHODS

Bacterial strains. A total of 16 encapsulated, nonhemolytic strains belonging to the serogroup O115:K"V165" were examined. These strains were isolated at the Faculty of Veterinary Medicine, St-Hyacinthe, from the intestinal contents of diarrheic piglets (age, 1 to 3 weeks) and were stored at −70°C until this investigation was carried out. Each strain originated from a different farm. The strains were serotyped with antisera for the *E. coli* OK serogroups as described previously (24). In addition, the *E. coli* K-12 strains carrying the F4 or F5 plasmid, *E. coli* B41MC (O101:F41:F5$^-$) obtained from J. A. Morris (Central Veterinary Laboratory, Weybridge, Surrey, England), and the *E. coli* field strain 603A (O9:F6) were used to produce reference antisera.

E. coli 2347 (O9:K"2347":F4), obtained from P. A. M. Guinée (National Institute for Public Health, Bilthoven, The Netherlands); B44 (O9:K30:F5), obtained from W. J. Sojka (Central Veterinary Laboratory); and 1592 (O9:K103:F6), obtained from H. W. Moon (National Animal Disease Center, Ames, Iowa), were also used as control strains producing the fimbrial antigens F4, F5, and F6, respectively.

* Corresponding author.

Culture media. Bacteria were grown on Minca agar-IsoVitaleX (Minca-Is) (17) at 37°C for 20 to 24 h to test for fimbrial antigens and hemagglutination. Bacteria were cultivated statically in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 48 h for examination of hemagglutination.

Production of antisera. Antisera against the fimbrial antigens F4, F5, F6, and F41 were prepared in rabbits. Immunization was carried out by method 1 for the production of *E. coli* OK antisera (7) with strains K-12:F4, K-12:F5, 603A, and B41MC. The K-12:F4 and K-12:F5 antisera were absorbed with the parent nonfimbriated K-12 strain. The 603A and B41MC antisera were absorbed with the homologous nonfimbriated strains grown at 15°C. These antisera were considered to be specific for the respective fimbrial antigens and were used in an indirect immunofluorescence test at dilutions of 1:40 (F41, F4) or 1:50 (F5, F6).

Antiserum was produced similarly against field strain 4787 (O115:KV165:H?). This strain was grown on Minca-Is before inoculation into broth for antigen production. To remove O and K antibodies, the antiserum was absorbed with strain 4787 grown at 15°C. The absorbed antiserum agglutinated strain 4787 grown at 37°C but did not agglutinate the same strain grown at 15°C. The antigen against which antibody in the absorbed antiserum was directed was designated F165. Absorption and slide agglutination procedures were performed in a standard manner as described by Edwards and Ewing (7).

Immunofluorescence. An indirect immunofluorescence technique was used for detection of fimbrial antigens. The first antibody was the absorbed antiserum, and the second antibody was a fluorescein-labeled goat anti-rabbit globulin conjugate (GIBCO Laboratories, Grand Island, N.Y.). Preparations were examined with Laborlux 12 fluorescent microscope (Leitz, Wetzlar, Federal Republic of Germany) with epi-illumination.

Hemagglutination. Hemagglutination tests were performed as described by Duguid et al. (6). Guinea pig erythrocytes were used to demonstrate MSHA. Cultures were grown serially in TSB incubated statically at 37°C for 48 h, with up to four subcultures of each series being tested. Tests were performed in the presence and absence of 1.5% (wt/vol) D-mannose. The rocked-tile method was used to demonstrate MRHA. Cultures were grown at 37°C for 18 h on Minca-Is. One drop each of a dense suspension of the bacteria, 1.5% D-mannose, and 3% erythrocytes was mixed on a glass tile at 20°C. The tile was chilled to 0°C, and the test result was read within 30 s after the tile was returned to 20°C. Titration for MRHA was carried out in the following manner. Bacteria grown on Minca-Is were suspended in saline to a concentration of 5×10^{10} organisms per ml and diluted in twofold steps. One drop of each dilution was transferred to a glass tile for determination of MRHA. The titer was the highest dilution showing MRHA. Tests for hemagglutination of purified fimbrial preparations were performed in microtiter plates as described by de Graaf and Roorda (5).

Hemagglutination inhibition. A volume of 0.05 ml of each twofold serial dilution of the absorbed F165 antiserum was mixed on a glass tile with an equal volume of bacterial suspension at a concentration of 5×10^9 organisms per ml. After incubation at 37°C for 20 min, the agglutination was read, and 0.05 ml of a 3% suspension of erythrocytes was added. The MRHA was read after the tile was chilled to 0°C. As a control, bacteria were incubated with antiserum to obtain a F165-negative O115:KV165 strain.

Purification of fimbriae. The flagellated strain 4787 was grown on Minca-Is at 37°C for 24 h and suspended in phosphate-buffered saline at pH 7.3. The suspension was agitated at high speed for 10 min in a Virtis-45 homogenizer (Virtis Inc., Gardiner, N.Y.) and centrifuged at 12,000 × *g* for 20 min. The pellet was discarded, and the fimbriae were purified from the supernatant by the technique of Paranchych et al. (30). Briefly, the sodium chloride concentration of the supernatant solution was adjusted to 0.5 M. The fimbriae were precipitated overnight at 4°C by adding 1% (wt/vol) polyethylene glycol 6000. After centrifugation at 12,000 × *g* for 20 min, the pellet was suspended in 10% (wt/vol) ammonium sulfate (pH 4.0) and allowed to stand at 4°C for 2 h. After centrifugation at 12,000 × *g* for 15 min, the pellet was suspended in 0.85% saline (pH 7.4) and dialyzed exhaustively against saline. The fimbriae were further purified by ultracentrifugation on a 20 to 60% discontinuous sucrose gradient. After 3 h of centrifugation at 30,000 rpm in an SW27 rotor with a Beckman L5-65 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.), the fimbrial band was recovered and dialyzed against saline. Examination of this preparation on sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis revealed only one protein band.

To produce antisera against the purified fimbriae, individual rabbits were immunized intradermally with 40 μg of purified fimbriae emulsified in complete Freund adjuvant divided among 20 to 30 sites. Immunization was repeated in alternate sites after 14 days with incomplete Freund adjuvant. Two additional injections of 20 μg of the purified fimbriae were administered intravenously at weekly intervals. The rabbits were bled 2 weeks after the final injection. To remove any possible O and K antibodies, the antiserum was absorbed with strain 4787 grown at 15°C.

Protein estimation. Protein was estimated by the method of Bradford (3) with bovine serum albumin used as the standard.

Electron microscopy. Dense bacterial suspensions of strains grown in TSB and on Minca-Is were prepared as described above for hemagglutination. Strain 4787 was also grown on Minca-Is at 15°C. A drop of each suspension was placed on a 200-mesh Formvar-coated carbon electron microscope grid and air dried. A drop of 2% potassium phosphotungstate (pH 7.0) was then applied to the grid. Grids were examined on a Philips 201 electron microscope operating at 80-kV, 10-nA emission.

Detection of enterotoxin. Strains were tested for enterotoxigenicity (both heat-labile and heat-stable enterotoxin) by the ligated gut loop technique in 10-day-old piglets (23). For each strain, 1 ml of an overnight broth culture was injected into a ligated intestinal segment, and at least three tests in different animals were performed.

Enzyme-linked immunosorbent assay. An enzyme-linked immunosorbent assay (ELISA) was performed as described previously (12), except that a single dilution of antiserum was used, and results were read on a Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.). The antigens used were the purified fimbrial preparation from strain 4787, O115 lipopolysaccharide (LPS) from an O115:KV165 strain (33), and KV165 capsular polysaccharide isolated from an O115:KV165 strain (21). The antigens were coated onto the wells of polystyrene microtiter plates in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. The antisera were diluted 1:400, 1:50, and 1:40 for the fimbrial, LPS, and capsular antigens, respectively. The optical density was measured at 414 nm 10 min after addition of the 2,2-azino-di(3-ethylbenzthiazoline sulfonic acid).

RESULTS

Hemagglutination patterns of O115 strains. Sixteen strains belonging to the serogroup O115:KV165 were examined for MSHA with guinea pig erythrocytes and for MRHA with sheep, goat, pig, chicken, human type O, dog, cat, guinea pig, rabbit, calf, and horse erythrocytes. Four different patterns of hemagglutination (I, II, III, and IV) were found (Table 1). Four strains produced MRHA of sheep, goat, pig, chicken, human type O, dog, and cat erythrocytes. These were further divided into MSHA-negative (pattern I) and MSHA-positive (pattern II) strains. The remaining 12 strains were MRHA-negative with all tested erythrocytes but most demonstrated MSHA (pattern III). By using sheep erythrocytes, the MRHA titer of pattern I and II strains was found to be between 32 and 128.

Relationship of MRHA to F165 antigen. A rabbit antiserum against pattern I MRHA-positive, MSHA-negative, flagellated strain 4787 was absorbed with the homologous strain grown at 15°C. The agglutinins remaining in the absorbed antiserum were considered to be directed against a surface antigen that we designated F165. Among the O115 strains, only the strains demonstrating MRHA were agglutinated by the F165 antiserum (with titers of 320 or 640) and were positive on examination by immunofluorescence with the F165 antiserum. The MRHA of erythrocytes from all animal species testing positive was completely inhibited by the F165 antiserum and by the antiserum against the purified fimbriae from strain 4787 diluted 1:10. The MRHA of sheep erythrocytes was inhibited by the F165 antiserum to titers of between 80 and 320. In control tests, MRHA was not inhibited by an antiserum for an O115:KV165, F165-negative strain nor was the MSHA of guinea pig erythrocytes inhibited by the F165 or the antifimbrial antiserum.

Relationship of F165 antigen to other fimbrial antigens. Control strains 2347, B44, 1592, and B41MC producing the fimbrial antigens F4, F5, F6 or F41, respectively, were negative on examination by agglutination and immunofluorescence with the F165 antiserum. Similarly, 4787 and the other three F165-positive strains were negative on examination by agglutination and immunofluorescence with the absorbed F4, F5, F6, and F41 antisera. In addition, the absorbed F4, F5, F6, and F41 antisera were negative when tested in an ELISA with the purified fimbrial antigen.

Detection of enterotoxin. The four F165-positive strains of hemagglutination patterns I and II were enterotoxin negative when tested as live cultures by the ligated gut loop test in piglets. On the other hand, 10 of 12 of the F165-negative strains, belonging to hemagglutination patterns III and IV, were enterotoxin positive.

TABLE 1. Hemagglutination patterns of 16 *E. coli* strains of serogroup O115

Hemagglutination pattern	No. of strains	MRHA of erythrocytes from[a]:				MSHA[b]
		Sheep, goat	Pig, chicken, human	Dog	Cat	
I	2	4	3	2	1	0
II	2	4	3	2	1	4
III	11	0	0	0	0	4
IV	1	0	0	0	0	0

[a] Cultures grown on Minca-Is agar; 1 to 4, weak to strong hemagglutination in the presence of 1.5% D-mannose; 0, no hemagglutination.

[b] Cultures were grown in TSB for 48 h and tested with guinea pig erythrocytes; 4, strong hemagglutination inhibited by 1.5% D-mannose; 0, no hemagglutination.

TABLE 2. Relationship between hemagglutination pattern and the presence of fimbriae on *E. coli* strains of serogroup O115, as demonstrated by electron microscopy

Hemagglutination pattern	No. of strains:		
	Tested	Fimbria positive after growth on:	
		TSB	Minca-Is agar
I	2	2	2
II	2	2	2
III	11	10	2[a]
IV	1	0	1[a]

[a] Fewer than 10% of cells were fimbria positive. For all other strains, fimbriae were observed on most cells.

Demonstration of fimbriae by electron microscopy. The four strains of patterns I and II, which were F165-positive and MRHA positive for sheep erythrocytes, were flagellated and highly fimbriated when grown either on Minca-Is or in TSB (Table 2). On the other hand, the strains of patterns III and IV, which were F165 negative and MRHA negative and mostly MSHA positive, were usually fimbriated when grown in TSB but were nonfimbriated or poorly fimbriated when grown on Minca-Is. Eight of these strains were flagellated. The fimbriae on the MRHA-positive bacteria grown on Minca-Is were long and straight and had a diameter of approximately 6 nm (Fig. 1). When grown on Minca-Is at 15°C, strains of hemagglutination patterns I and II were MRHA negative and F165 negative as demonstrated by agglutination and immunofluorescence, flagellated, and nonfimbriate.

Characterization of the fimbriae produced by strain 4787. The purified fimbriae from the F165-positive, MRHA-positive strain 4787 resembled those observed on the bacterial cells of the same strain (Fig. 2). Their width varied from 5 to 8 nm. The purified fimbrial preparation demonstrated the same hemagglutination pattern as strain 4787. Antibody for the purified fimbriae from strain 4787 was detected by ELISA in the absorbed F165 antiserum at a lower level than in the antiserum for the purified fimbrial preparation (Table 3). Antibody for the LPS and capsule was not observed in either antiserum.

DISCUSSION

Examination of *E. coli* serogroup O115 strains revealed four patterns of hemagglutination which indicate the presence of at least two distinct hemagglutinins. The first type of hemagglutinin, found on strains of patterns II and III, was MS and associated with a fimbria which was produced in broth but not on agar; these properties are strongly suggestive of the F1 or type I fimbria (6, 27). The second type of hemagglutinin induced MRHA of erythrocytes from a wide variety of animal species and was found on strains of pattern I or together with F1 on strains of pattern II. The coexistence of MR and MS hemagglutinins in the same culture of an *E. coli* strain has been reported previously (6, 11). An absorbed antiserum against MRHA-positive F1-negative strain 4787 revealed the presence of a temperature-dependent surface antigen, F165, which was found on all the MRHA-positive strains. Inhibition of the MRHA of erythrocytes from all positive animal species by the absorbed F165

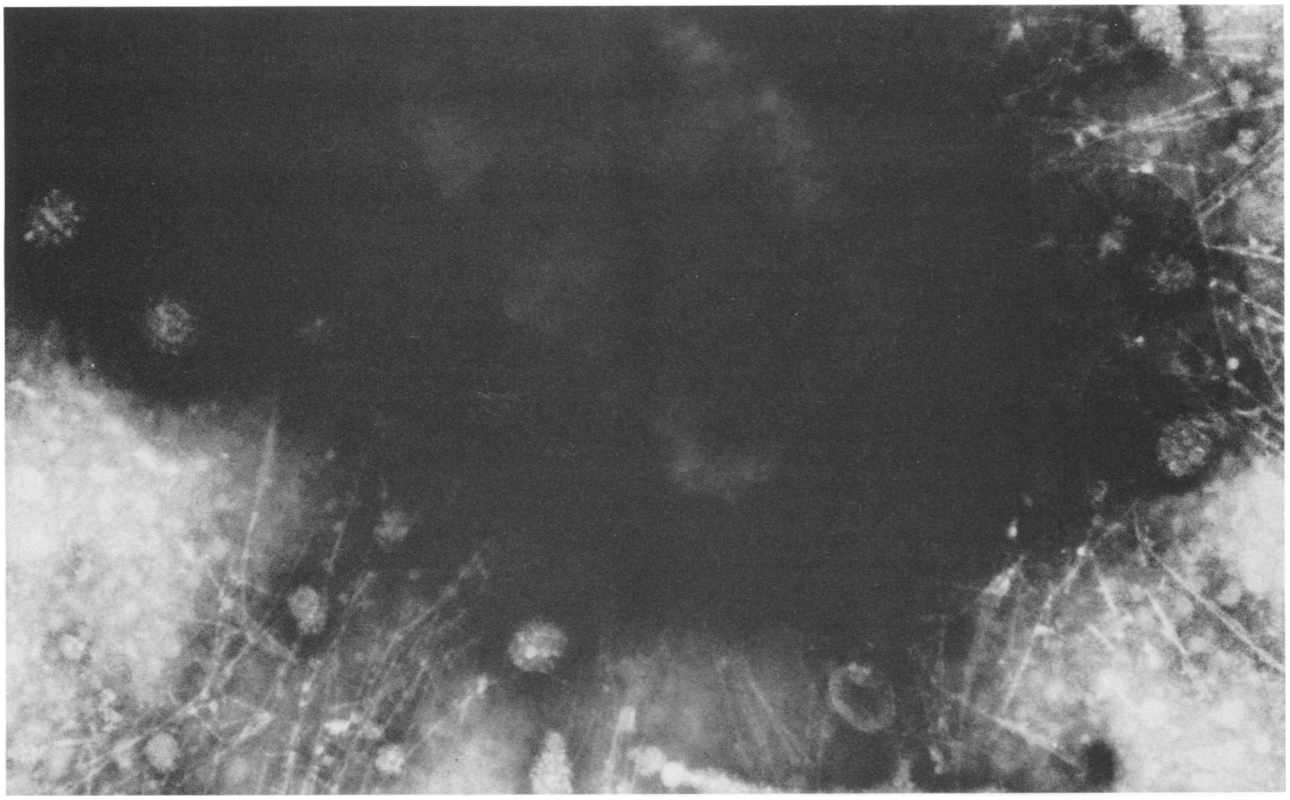

FIG. 1. Electron micrograph of a negatively stained cell of *E. coli* 4787 grown on Minca-Is agar showing many long, thin fimbriae attached to the cell. Negatively stained with 2% potassium phosphotungstate; ×90,000.

antiserum demonstrates that the F165 and MR hemagglutinin are identical. The observation by electron microscopy that all of the F165-positive strains are highly fimbriated when cultivated on Minca-Is agar at 37°C but not at 15°C suggests the fimbrial nature of the F165 antigen. The purified fimbriae isolated from strain 4787 demonstrated the same hemagglutination pattern as the parent strain. In addition, this fimbrial preparation showed a positive reaction with the absorbed F165 antiserum by ELISA. These findings confirm the fimbrial nature of the hemagglutinating F165 antigen.

The pattern of hemagglutination induced by the F165-positive strains was similar to those of patterns 1 and 2 described by Duguid et al. (6). However, these patterns differed from that of F165 in the following way. Pattern I was MRHA negative for chicken erythrocytes and MRHA positive for rabbit erythrocytes, whereas pattern 2 was MRHA negative for both chicken and human erythrocytes. The F165 pattern also resembles the hemagglutination type VI-A and VI-B patterns described by Evans et al. (11). In the latter study, in which only human, bovine, chicken, monkey, and guinea pig erythrocytes were tested, patterns VI-A and VI-B were MRHA positive for human, chicken, and monkey erythrocytes. However, no resemblance was noted between the hemagglutination pattern of F165-positive strains and those reported for either the enterotoxigenic strains of human origin positive for F2 or F3 (CFA/I or CFA/II) (10, 11) or the bovine or porcine strains positive for F4 (20), F5 (29, 32), F6 (19), or F41 (5). In addition, no similarity was observed between the F165 hemagglutination pattern and those of the F4, F5, F6, and F41-positive strains tested in our laboratory (data not shown). We also showed by agglutination, immunofluorescence, and ELISA that there is no serological relationship between the F165 antigen and the fimbrial adhesins F4, F5, F6, and F41 that have already been described in strains of porcine origin.

An important finding of our study was the relationship between the presence of F165 and the absence of production of enterotoxin among O115 serogroup strains. In the noninvasive enterotoxigenic *E. coli* strains, there is a strong positive correlation between the production of enterotoxin and the presence of the fimbrial adhesins F2 (9); F3 (8); and F4, F5, or F6 (16). Although all of the strains examined in this study were isolated from the intestinal contents of young piglets with diarrhea, strains of this serogroup have been associated previously with septicemia in calves and piglets (14, 15). Thus, although the F165 antigen is not associated with enterotoxigenic *E. coli* strains, it could contribute to the pathogenicity of those strains which translocate extraintestinally and induce septicemia. Evans et al. (11) have found an association between the presence of their type VI pattern of hemagglutination and bacteremia-related strains of facultatively enteropathogenic *E. coli* serogroups. The presence of fimbriae or MR hemagglutinins has been associated with the ability of *E. coli* strains to adhere to urinary

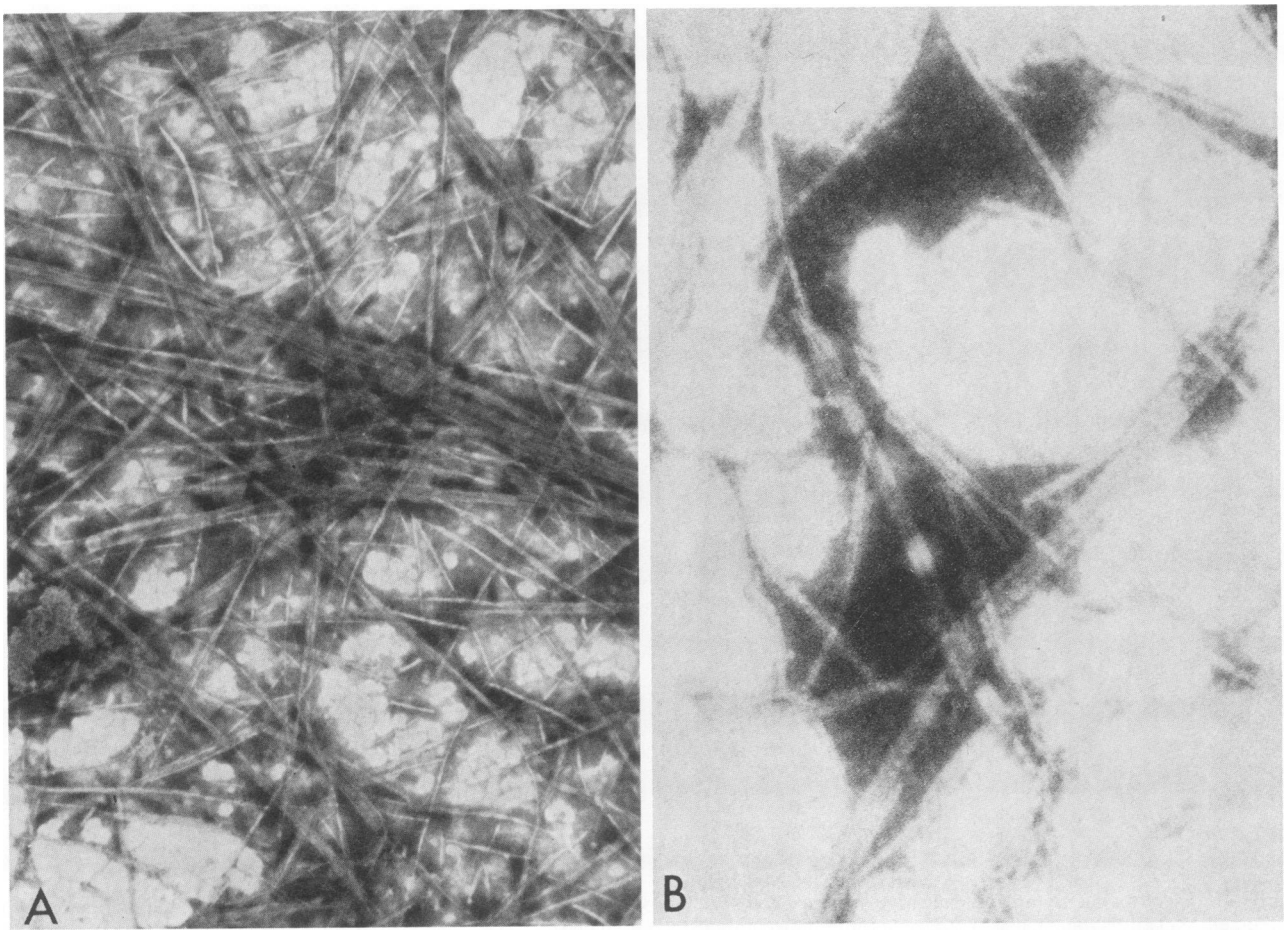

FIG. 2. Fimbriae isolated from *E. coli* 4787. Negatively stained with 2% potassium phosphotungstate. (A) ×40,000; (B) ×190,000.

tract epithelial cells in vitro (18, 22, 31). The presence of certain MR fimbriae may also help strains of *E. coli* to resist phagocytosis either by protecting the bacteria from polymorphonuclear leukocyte killing (1) or because of a lack of receptors for the MR fimbriae, as opposed to MS fimbriae, on the phagocyte (2). We found that only the F165-positive strains in the O115 serogroup are enteropathogenic and induce septicemia in newborn piglets. Thus, we are investigating at present the role of F165 in the development of this disease.

TABLE 3. Antibody levels in F165 and purified *E. coli* 4787 fimbriae antisera produced in rabbits, by ELISA

ELISA antigen	Absorbance at OD_{414} for:			
	Preimmune serum	Antiserum[a] for:		
		Whole cells[b]	F165[c]	Purified fimbriae
Purified fimbriae	0.08	0.43	0.31	0.68
LPS	0.12	0.32	0.12	0.15
Capsule	0.10	0.14	0.08	0.10

[a] Antisera were diluted 1:400, 1:50, and 1:40 for the fimbrial, LPS, and capsular antigens, respectively.
[b] Antiserum for whole cells of strain 4787.
[c] Strain 4787 antiserum, absorbed as described in the text.

ACKNOWLEDGMENTS

This work was supported by grant EQ 2558 (Fonds pour la Formation de Chercheurs et l'Aide à la Recherche) from the Government of Quebec and by a grant A2853 from the Natural Sciences and Engineering Research Council of Canada.

We thank Claudette Cormier and Clarisse Moreau for invaluable technical assistance.

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