JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1996, p. 2921–2928 Vol. 34, No. 12
0095-1137/96/$04.00+0

Rapid and Specific Detection of F17-Related Pilin and Adhesin Genes in Diarrheic and Septicemic *Escherichia coli* Strains by Multiplex PCR

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Received 22 March 1996/Returned for modification 7 June 1996/Accepted 22 August 1996

The F17-related adhesins are prevalent in *Escherichia coli* strains isolated from calves with diarrhea or septicemia and from lambs with nephropathy. The F17 family includes the F17a, F17b, F17c, and F111 fimbriae produced by bovine *E. coli* strains and the G agglutinin produced by human uropathogenic *E. coli* strains. An easy and inexpensive multiplex PCR method was developed to detect all the F17-related fimbriae and to identify four subtypes of structural subunit genes and two distinct subfamilies of adhesin genes by only two runs of amplification. A strict correlation was observed between the phenotypic assays and the multiplex PCR method when 166 pathogenic *E. coli* strains isolated from intestinal content of calves or lambs were tested. Genes encoding the F17c structural subunit and the subfamily II adhesins were prominent among bovine and ovine isolates, and the capsule-like CS31A antigen was strictly associated with the F17c fimbriae. The F17b subtype fimbriae were prominent among bovine isolates producing the CNF2 toxin, whereas the F17a subtype fimbriae were associated with the bovine isolates producing neither the CS31A antigen nor the CNF2 toxin. Five bacterial strains possessed two distinct and complete F17-related fimbrial gene clusters, and two of them produced two F17-related fimbriae at the bacterial cell surface. The related fimbrial gene clusters are probably organized in mosaic operons consisting of F17-related pilin and adhesin genes, and horizontal gene transfer may occur among *E. coli* strains isolated from different animal species.

The colonization of host intestinal cells is an important initial step in the development of intestinal and extraintestinal infections (15, 16). The binding of pathogenic bacteria to the host receptors is mediated by fimbriae expressed on the bacterial cell surface (15, 16). Hence, an essential element of vaccine development is the detection of fimbrial antigens common to most of the pathogenic isolates able to induce antibodies that block bacterial adhesion (17). The bovine fimbrial antigens incorporated in vaccines to protect newborn calves from diarrhea are the K99 (14), F41 (7), and F17 (19) (formerly called FY [13]) fimbriae (6). Experimental infections revealed that the best protection against bovine enterotoxigenic *Escherichia coli* (ETEC) strains was obtained only when antibodies against K99, F41, and F17 were associated in the colostrum of vaccinated cows (6).

Recently, a new F17-related fimbrial adhesin called 20K has been described on *E. coli* strains associated with bovine diarrhea or septicemia and with lambs showing a severe tubular disease (nephrosis) (3). Comparison of amino acid sequences revealed that the structural subunit and the adhesin of the 20K fimbriae were identical to those of the G fimbriae produced by a human uropathogenic *E. coli* (UPEC) strain responsible for glucosamine-sensitive hemagglutination of endo-β-galactosidase-treated human erythrocytes (22). In view of its homology with the structural subunit and adhesin of F17a (18, 20) and F17b (10) gene clusters, the 20K fimbria was termed F17c (22). The family of F17-related fimbriae includes (i) F17a fimbriae expressed by bovine ETEC (19), (ii) F17b fimbriae expressed by *E. coli* isolated from septicemic and diarrheic lambs and calves (10, 25), (iii) F17c fimbriae isolated from septicemic *E. coli* strains (3) and the identical G fimbriae isolated from human UPEC (31), and (iv) F111 fimbriae previously described on bovine ETEC (2). It is of interest that pathogenic *E. coli* strains producing F17-related fimbriae represent a significant part of isolates from diarrheic calves in France and Belgium (43%) (3) and from lambs with nephrosis in Scotland (29%) (3).

These fimbriae promote inhibitable *N*-acetyl-D-glucosamine (GlcNac) hemagglutination of bovine erythrocytes and in vitro adhesion to the brush border of intestinal calf villi and/or to the human Caco-2 cell line (3). However, the F17c and F17b fimbriae bind to terminal residues with higher affinity than the F17a, G, or F111 fimbriae, and each F17-related adhesin probably recognizes different oligosaccharide sequences or receptors on host tissues (3).

Most of the F17-related positive *E. coli* strains originating from Europe also express the nonfimbrial adhesin CS31A (3, 12). In contrast, none of the F17-related positive *E. coli* strains isolated from lambs with nephrosis is CS31A positive (3). The F17b fimbriae are associated with the cytotoxic necrotizing factor type 2 (CNF2) on the Vir plasmid (10, 24).

In the present report, we describe the use of a multiplex PCR protocol to detect and identify the members of the F17 family. The PCR protocol was used as an alternative to the phenotypic identification of the fimbrial adhesins based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), adhesion, and immunological studies. We established a quick and convenient PCR protocol to detect and identify four subtypes of structural subunit genes and two subfamilies of adhesin genes by only two runs of amplification. To validate the protocol, PCR and phenotypic detections were performed on different F17 reference strains. In addition, the subtype of 129 pathogenic F17-producing *E. coli* strains was

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TABLE 1. Oligonucleotide primers used in this study[a]

Gene(s)	Primer	Oligonucleotide sequence (5′-3′)	Location	Size of the amplified product (bp)
F17 family (F17a-A, F17b-A, F17c-A/*gafA*, F111-A)	P1	GCAGAAAATTCAATTTATCCTTGG	3–26	537[b]
F17 family (F17a-A, F17b-A, F17c-A/*gafA*, F111-A)	P2	CTGATAAGCGATGGTGTAATTAAC	517–540	
F17a-A	P3	GCTGGAAGGGTGCAATACGCCTG	219–241	321[b]
F17b-A	P4	CAACTAACGGGATGTACAGTTTC	217–239	323[b]
F17c-A/gafA	P5	GCAGGAACCGCTCCCTTGGC	124–143	416[b]
F111-A	P6	GATAGTCATAACCTTAATATTGCA	301–324	239[b]
Subfamily I adhesins (F17a-G, F111)	P7	CGGAGCTAATACTGCATCAACC	399–420	615[c]
Subfamily II adhesins (F17b-G, F17c-G/*gafD*)	P8	CGTGGGAAATTATCTATCAACG	396–417	615[c]
Subfamilies I and II adhesin genes	P9	TGTTGATATTCCGTTAACCGTAC	992–1014	

[a] The MXP1 primer set contained the P1, P2, P3, P6, P7, and P9 primers. The MXP2 primer set contained the P2, P4, P5, P8, and P9 primers.
[b] Size of the PCR product obtained when P1, P3, P4, P5, or P6 primer was used in association with P2 primer.
[c] Size of the PCR product obtained when P7 or P8 primer was used in association with P9 primer.

determined and the association with CS31A antigen and CNF2 toxin was analyzed.

MATERIALS AND METHODS

Reference bacterial strains and growth conditions. *E. coli* 25KH9, S5, 31A, HB101(pRR5), and 111KH86 were used as positive controls for the detection of F17a, F17b, F17c, G, and F111 fimbrial systems, respectively, and have been described elsewhere (2, 12, 19, 25, 31). *E. coli* HB101 was used as a negative control.

Bacteria tested by PCR were grown in Luria-Bertani broth medium containing (per liter) 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.0) for 16 h at 37°C. Immunoblotting assays (Western blot and dot blot) were performed on fimbrial extracts obtained from an overnight Minca agar culture (14).

PCR primers. The nucleotide sequences of the F17-related structural or adhesin subunit genes were aligned by using the FASTA program (28) from the Wisconsin Genetics Computer Group (GCG) software package. The primers were selected on the basis of annealing temperature, degree of homology, and size of the amplified products. Oligonucleotide primers were purchased from Eurogentec (Seraing, Belgium). Following synthesis, the primers were purified by PAGE and filtered through a G25-Sephadex spin column to remove salts.

Amplification procedure. DNA to be amplified was released from whole organisms by boiling. Bacteria were harvested from 1 ml of an overnight broth culture, suspended in 1 ml of sterile water, and incubated at 100°C for 15 min. PCR was carried out in a total volume of 50 μl containing 5 μl of the template, 1.5 μl of each oligonucleotide primer at 20 μM, 200 μM (each) of the four deoxynucleoside triphosphates, 5 μl of enzyme buffer (Appligene-Oncor, Illkirch, France), and 0.2 U of Ampli*Taq* DNA polymerase (Appligene). The samples were overlaid with 50 μl of mineral oil, denatured for 5 min at 94°C, and subjected to 25 cycles of amplification on a thermal cycler (Techne). The parameters were denaturation for 2 min at 94°C, annealing of primers for 1 min at 55 or 59°C, and primer extension for 1 min at 72°C. Amplification of DNA fragments larger than 4 kb was performed by the same procedure with the following modifications: 10 μl of the template was used and samples were subjected to 35 cycles of amplification by the *Taq*$^+$ DNA polymerase (Stratagene) with the low salt buffer recommended by the manufacturer. The parameters were 2 min at 94°C, 1 min at 55°C, and 4 min at 72°C. Five or ten microliters of the reaction was then mixed with loading buffer and separated by electrophoresis on a 1.4 or 0.5% (for amplified fragment larger than 4 kb) agarose gel. The DNA fragments were visualized by ethidium bromide staining.

DNA from the reference strains producing F17-related fimbriae were included in every PCR procedure. *E. coli* HB101 was included in every PCR procedure to prevent false-positive results. The sizes of the amplified products were compared with those of positive-control amplified products and the DNA mass marker VIII (Boehringer Mannheim).

Immunological assays and CNF2 toxin detection. Dot blotting and Western blotting assays were performed as previously described (3). Antibodies raised against F17c fimbriae (anti-F17 antibodies) cross-reacted with all the denatured F17-related fimbrial subunits (3, 23) when used in Western blotting assays. The anti-F17a- and -F111 antibodies were supplied by Henri de Greve (Laboratory of Genetic Virology, Brussels, Belgium). The specific anti-F17b and -CS31A antibodies were described elsewhere (12, 23). The specific anti-F17a, -F17b, -F17c, -F111, and -CS31A antisera were used in dot blotting assays. Genotypic and phenotypic detection of CNF2 were performed as described elsewhere (26).

Collection of pathogenic *E. coli* strains tested by the multiplex PCR protocol. Pathogenic *E. coli* strains isolated from intestinal contents of diseased calves and lambs were used to standardize the multiplex PCR approach. A total of 129 *E. coli* strains reacting with the anti-F17 antibodies and adhering in vitro to calf intestinal villi were tested. In addition, 37 bacterial strains producing no F17-related fimbriae and isolated from calves with diarrhea or septicemia (5, 30) were used as negative controls.

The strains included in groups I and II were isolated in France and Belgium in the course of a study on bovine septicemic *E. coli* strains. The 38 *E. coli* strains included in group I produced both F17-related fimbriae and CS31A antigens (5, 29). CS31A is a plasmid-encoded major surface antigen (12) associated with multiple antibiotic resistance and siderophore aerobactin synthesis (8). The 37 *E. coli* strains included in group II produced only F17-related fimbriae (11).

Group III comprised 23 *E. coli* strains isolated from feces or organs of septicemic calves (25). All these strains produce the plasmid-encoded CNF2 (25). CNF2 is a dermonecrotic toxin responsible for a specific thermolabile cytopathic effect of multinucleation in an HeLa cell assay and is lethal to rabbits, mice, and chicken (24, 27). In addition, these strains hybridize with a probe subcloned from the pPLHD2 plasmid encoding the F17a fimbria (25).

Group IV comprised 31 *E. coli* strains (4) isolated in Scotland from intestinal contents of lambs with nephrosis (1). A clinicopathological survey of lambs with nephropathy showed that about half of the investigated lambs showed clinical evidence of diarrhea (1). Fecal samples were examined routinely for possible enteropathogens; 29% of the bacterial strains studied in our laboratory expressed F17-related fimbriae whereas the CS31A antigen was not detected (3).

RESULTS

Selection of oligonucleotide primers. In order to identify variable and conserved regions the nucleotide sequences of the F17a-A, F17b-A, F17c-A, and F111-A genes encoding the F17a, F17b, F17c, and F111 structural subunits, respectively (10, 20, 22), were aligned for maximum homology. The F17a-G, F17b-G, and F17c-G adhesin gene sequences (10, 18, 22) were also compared (the nucleotide sequence of the F111 adhesin gene was not available). The structural subunit and adhesin genes of F17c fimbriae (F17c-A and F17c-G) are identical to those of the G fimbriae (*GafA* and *GafD*) (22). The oligonucleotide sequences and location of primers are given in Table 1.

The P1/P2 primer pair (Table 1) located at the highly conserved 3′ and 5′ ends of the F17a-A, F17b-A, F17c-A, and F111-A genes was used to detect the genes encoding the structural subunit of all the members of the F17 family. No PCR product was obtained with the HB101 strain. Nucleotide sequences specific for one particular structural subunit gene and sufficiently different from all the others to avoid cross-hybridization were identified (Table 1). To evaluate the specificity of the primer sets, individual primers pairs were used to detect the structural subunit genes among the F17 reference strains. The distribution and size of the amplified products were as predicted (data not shown).

When the P7 primer designated as specific to F17a-G adhesin was used with the P9 primer, an amplified product of 615 bp was obtained from the F17a and F111 reference strains. The nucleotide sequence of the F111 adhesin was not available but the results suggest that the P7 and P9 primers should be located on conserved nucleotide sequences between the F17a-G

and F111 adhesin genes. The DNAs extracted from the strains producing F17b or F17c fimbriae failed to produce an amplified product with the P7/P9 primer pair. Because of the high degree of homology among nucleotide sequences the PCR approach was unable to discriminate between F17b-G and F17c-G genes. Therefore, when the P8/P9 primer pair was used, an amplified product of 615 bp was obtained with both F17b and F17c reference strains (results not shown). No DNA fragment was amplified from the F17a and F111 reference strains when the P8/P9 primer pair was used. In conclusion, the P7/P9 and the P8/P9 primer pairs were designated to detect the subfamily I (F17a-G and F111) and subfamily II (F17b-G and F17c-G) adhesin genes, respectively.

Specificity of the primers and validation of the multiplex PCR primer sets. Because of the similarity in the size of the DNA fragments amplified from F17a-A and F17b-A (321 bp and 323 bp) and from adhesin genes of the two subfamilies (615 bp) (Table 1), the primers could not all be used together in a single assay. Therefore, two multiplex PCR primer sets were designated and used as follows: (i) MXP1, containing the P1, P2, P3, P6, P7, and P9 primers, was used to detect F17a-A, F111-A, subfamily I adhesin genes, and structural subunit genes of all F17-related fimbriae; and (ii) MXP2, containing the P2, P4, P5, P8, and P9 primers, was used to detect F17b-A, F17c-A/*gafA*, and subfamily II adhesin genes (Table 1). The annealing temperatures used during PCR were 55 and 59°C for MXP1 and MXP2, respectively. The detection of the corresponding fimbrial genes among the reference strains demonstrated the specificity of the MXP1 and MXP2 cocktails (Fig. 1). To validate the PCR protocol, immunological detection was performed on F17 reference strains. Results obtained by Western blotting assays (Fig. 2) and dot blotting assays (results not shown) were as predicted.

In conclusion, MXP1 and MXP2 were used for (i) detection of the structural subunit gene of all the F17-related fimbriae, (ii) identification of the structural subunit gene of the four members of the F17 family, and (iii) detection of two subfamilies of F17-related adhesin genes.

Detection of F17-related fimbriae among bovine and ovine isolates. To assess and standardize the multiplex PCR protocol, 129 F17-positive strains (groups I to IV) (Table 2) and 37 F17-negative strains were tested. When the F17-positive strains were tested an expected 537-bp PCR product was generated with MXP1 (containing the P1/P2 primer pair). In contrast, none of the F17-negative *E. coli* strains were positive by PCR.

Group I. Group I included 38 *E. coli* strains isolated from septicemic calves and producing both F17-related fimbriae and CS31A antigen (5, 29). An amplified product specific to the F17c-A/*gafA* structural subunit gene was obtained from the 38 (100%) strains. In agreement with these results, analysis of dot blotting assays revealed an immunological reactivity with anti-F17c antiserum but not with antibodies specific to F17a, F17b, or F111 subtype fimbriae (data not shown). Western blotting assays using anti-F17-antibodies revealed a 20,000-Da polypeptide band (data not shown). In addition, a PCR product specific to subfamily II adhesin genes (F17b-G and F17c-G/*gafD*) was generated from the DNAs extracted from the 38 *E. coli* strains tested, confirming the association between the F17c-A structural subunit gene and subfamily II adhesin genes observed on the 31A reference strain.

Group II. The 37 bovine *E. coli* strains included in group II produced F17-related fimbriae but no CS31A antigen (11). PCR analysis showed that the F17a-A and the F111-A structural subunit genes were detected on 32 (87%) and 4 (11%) strains, respectively. The F17b-A gene was not detected, and

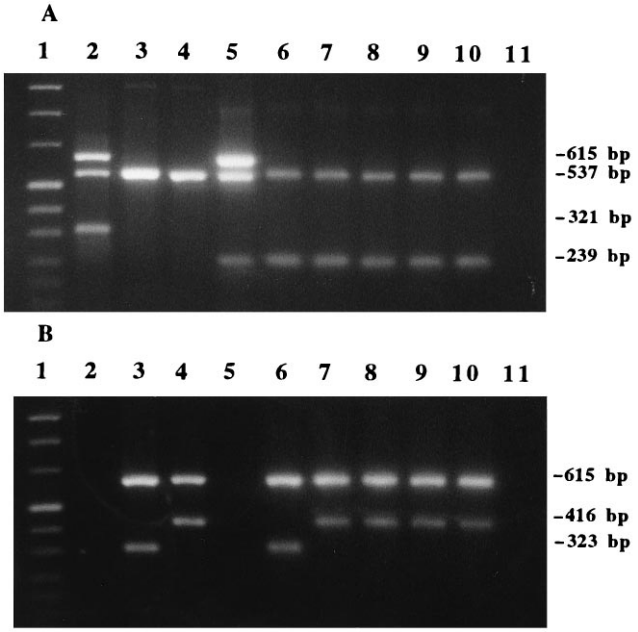

FIG. 1. Amplification of DNAs extracted from F17-positive strains by using MXP1 (A) and MXP2 (B) primer cocktails. Lanes 1, molecular size marker (DNA mass marker VIII; Boehringer Mannheim). DNAs were amplified from the following F17 reference strains: 25KH9 (F17a) (lanes 2), S5 (F17b) (lanes 3), 31A (F17c) (lanes 4), and 111KH86 (F111) (lanes 5). DNAs were amplified from the following strains genetically associated with two distinct F17-related structural subunits: JL21 (lanes 6), BM2-10 (lanes 7), B20a (lanes 8), 88-612-1 (lanes 9) and GB207 (lanes 10). Lanes 11, HB101 as negative control. The PCR products (10 μl) were separated on a 1.4% agarose gel and visualized by ethidium bromide staining.

only one *E. coli* strain was F17c-A positive. A strict correlation between genotypic and phenotypic assays was observed. In agreement with PCR results obtained with the F17a and F111 reference strains, 34 (95%) *E. coli* strains containing F17a-A or F111-A structural subunit genes were PCR positive for the subfamily I adhesin genes (F17a-G and F111 adhesins). Although *E. coli* 364 was F17a-A positive by PCR, a DNA fragment of 615 bp was amplified with the primers specific to the subfamily II adhesin genes included in the MXP2 cocktail

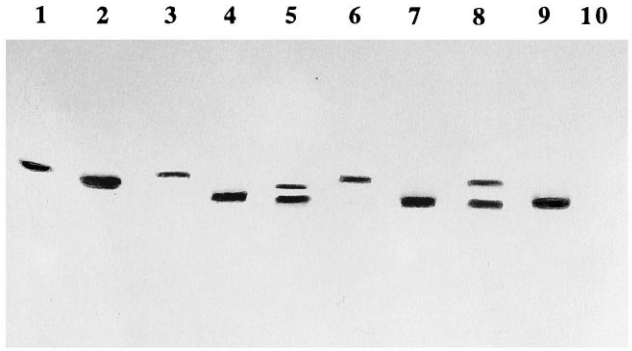

FIG. 2. Western blotting analysis of F17-positive fimbrial extracts with anti-F17-antibodies. Fimbrial extracts of the F17 reference strains were as follows: lane 1, 25KH9 (F17a); lane 2, S5 (F17b); lane 3, 31A (F17c); and lane 4, 111KH86 (F111). Fimbrial extracts of the strains genetically associated with two distinct F17-related structural subunits: lane 5, JL21; lane 6, BM2-10; lane 7, B20a; lane 8, 88-612-1; and lane 9, GB207. Lane 10, HB101 as negative-control. Western blotting assays were performed with anti-F17-antibodies (3) (diluted 1:100) after transfer from SDS-PAGE to nitrocellulose.

TABLE 2. Detection and identification of F-17 related fimbrial genes by using the multiplex PCR protocol

Group and strain	PCR amplification[a]						Size of structural subunit (kDa)[b]	CS31A[c]	CNF2[d]
	Structural subunit gene				Adhesin gene				
	F17a-A	F17b-A	F17c-A/*gafA*	F111-A	Subfamily I	Subfamily II			
Reference									
25KH9	+	−	−	−	+	−	20.0	−	−
S5	−	+	−	−	−	+	19.5	−	+
31A	−	−	+	−	−	+	20.0	+	−
HB101(pRR5)	−	−	+	−	−	+	20.0	−	−
111KH86	−	−	−	+	+	−	18.5	−	−
HB101	−	−	−	−	−	−		−	−
I									
78b	−	−	+	−	−	+	20.0	+	−
154	−	−	+	−	−	+	20.0	+	−
110c	−	−	+	−	−	+	20.0	+	−
A55	−	−	+	−	−	+	20.0	+	−
39a	−	−	+	−	−	+	20.0	+	−
12	−	−	+	−	−	+	20.0	+	−
21a	−	−	+	−	−	+	20.0	+	−
94I	−	−	+	−	−	+	20.0	+	−
75	−	−	+	−	−	+	20.0	+	−
55a	−	−	+	−	−	+	20.0	+	−
A54	−	−	+	−	−	+	20.0	+	−
18C	−	−	+	−	−	+	20.0	+	−
G2820	−	−	+	−	−	+	20.0	+	−
48I	−	−	+	−	−	+	20.0	+	−
4I	−	−	+	−	−	+	20.0	+	−
151	−	−	+	−	−	+	20.0	+	−
84I	−	−	+	−	−	+	20.0	+	−
A57	−	−	+	−	−	+	20.0	+	−
1153	−	−	+	−	−	+	20.0	+	−
107	−	−	+	−	−	+	20.0	+	−
116KH87	−	−	+	−	−	+	20.0	+	−
203KH87	−	−	+	−	−	+	20.0	+	−
23KH87	−	−	+	−	−	+	20.0	+	−
192KH88	−	−	+	−	−	+	20.0	+	−
280KH88	−	−	+	−	−	+	20.0	+	−
47KH89	−	−	+	−	−	+	20.0	+	−
121KH90	−	−	+	−	−	+	20.0	+	−
609KH91	−	−	+	−	−	+	20.0	+	−
24KH91	−	−	+	−	−	+	20.0	+	−
190KH88	−	−	+	−	−	+	20.0	+	−
129KH90	−	−	+	−	−	+	20.0	+	−
490KH91	−	−	+	−	−	+	20.0	+	−
130KH90	−	−	+	−	−	+	20.0	+	−
240KH88	−	−	+	−	−	+	20.0	+	−
381KH90	−	−	+	−	−	+	20.0	+	−
380KH90	−	−	+	−	−	+	20.0	+	−
382KH90	−	−	+	−	−	+	20.0	+	−
369KH91	−	−	+	−	−	+	20.0	+	−
II									
262-15	+	−	−	−	+	−	20.0	−	−
AY4	+	−	−	−	+	−	20.0	−	−
orne 5	−	−	+	−	−	+	20.0	−	−
80198	−	−	−	+	−	−	18.5	−	−
AY2	+	−	−	−	+	−	20.0	−	−
167	+	−	−	−	+	−	20.0	−	−
A72	+	−	−	−	+	−	20.0	−	−
28a	−	−	−	+	+	−	18.5	−	−
AY3	+	−	−	−	+	−	20.0	−	−
5007	+	−	−	−	+	−	20.0	−	−
120a	−	−	−	+	+	−	18.5	−	−
364	+	−	−	−	−	+	20.0	−	−
CKH9	+	−	−	−	+	−	20.0	−	−
3772	+	−	−	−	+	−	20.0	−	−
64I	+	−	−	−	+	−	20.0	−	−
1503b	−	−	−	+	+	−	18.5	−	−

Continued on following page

TABLE 2—*Continued*

Group and strain	PCR amplification[a]						Size of structural subunit (kDa)[b]	CS31A[c]	CNF2[d]
	Structural subunit gene				Adhesin gene				
	F17a-A	F17b-A	F17c-A/*gafA*	F111-A	Subfamily I	Subfamily II			
X Loire	+	−	−	−	+	−	20.0	−	−
753	+	−	−	−	+	−	20.0	−	−
Y Decize	+	−	−	−	+	−	20.0	−	−
182KH9	+	−	−	−	+	−	20.0	−	−
5008	+	−	−	−	+	−	20.0	−	−
11a	+	−	−	−	+	−	20.0	−	−
1106	+	−	−	−	+	−	20.0	−	−
880M	+	−	−	−	+	−	20.0	−	−
64a	+	−	−	−	+	−	20.0	−	−
AY5	+	−	−	−	+	−	20.0	−	−
262-6	+	−	−	−	+	−	20.0	−	−
7KH9	+	−	−	−	+	−	20.0	−	−
240	+	−	−	−	+	−	20.0	−	−
EKH9	+	−	−	−	+	−	20.0	−	−
876	+	−	−	−	+	−	20.0	−	−
83380	+	−	−	−	+	−	20.0	−	−
834M	+	−	−	−	+	−	20.0	−	−
263	+	−	−	−	+	−	20.0	−	−
165	+	−	−	−	+	−	20.0	−	−
733	+	−	−	−	+	−	20.0	−	−
875M	+	−	−	−	+	−	20.0	−	−
III									
B177	−	+	−	−	−	+	19.5	−	+
1404	−	+	−	−	−	+	19.5	−	+
JL21	−	+	−	+	−	+	18.5/19.5	−	+
JL10	−	+	−	−	−	+	19.5	−	+
JL9	−	−	+	−	−	+	20.0	−	+
GB240	−	+	−	−	−	+	19.5	−	+
BM2-10	−	−	+	+	−	+	20.0	−	+
B20a	−	−	+	+	−	+	18.5	−	+
B28b	−	+	−	−	−	+	19.5	−	+
711pVir	−	+	−	−	−	+	19.5	−	+
88-612-1	−	−	+	+	−	+	18.5/20.0	−	+
GB212	−	+	−	−	−	+	19.5	−	+
MV291-1	−	+	−	−	−	+	19.5	−	+
GB207	−	−	+	+	−	+	18.5	−	+
GB208	−	+	−	−	−	+	19.5	−	+
GB229	−	−	−	−	−	−	19.5	−	+
B9S2	−	−	+	−	−	+	20.0	−	+
GB236	−	+	−	−	−	+	19.5	−	+
GB211	−	+	−	−	−	+	19.5	−	+
GB230	−	+	−	−	−	+	19.5	−	+
224KH88	−	−	−	−	−	+	18.5	−	+
88-388-2	−	−	−	+	+	−	18.5	−	+
GB228	−	−	−	−	−	+	18.5	−	+
IV									
A50	−	+	−	−	−	+	19.5	−	+
A54	−	+	−	−	−	+	19.5	−	+
A66	−	−	+	−	−	+	20.0	−	−
A85	−	−	−	+	+	−	18.5	−	−
A99	+	−	−	−	−	+	20.0	−	−
A112	−	−	−	+	+	−	18.5	−	−
A128	−	−	−	+	+	−	18.5	−	−
A135	−	−	−	−	−	+	20.0	−	−
A166	−	−	+	−	−	+	20.0	−	−
A216	−	−	+	−	−	+	20.0	−	−
A218	−	−	+	−	−	+	20.0	−	−
A225	−	−	+	−	+	+	20.0	−	−
A268	−	−	−	+	+	−	18.5	−	−
A283	+	−	−	−	−	−	20.0	−	−
A284	−	−	+	−	−	+	20.0	−	−
A313	−	−	+	−	−	+	20.0	−	−
A321	−	−	+	−	−	+	20.0	−	−

Continued on following page

TABLE 2—*Continued*

Group and strain	PCR amplification[a]						Size of structural subunit (kDa)[b]	CS31A[c]	CNF2[d]
	Structural subunit gene				Adhesin gene				
	F17a-A	F17b-A	F17c-A/*gafA*	F111-A	Subfamily I	Subfamily II			
A333	−	−	+	−	−	+	20.0	−	−
A338	−	−	+	−	−	+	20.0	−	−
A339	+	−	−	−	−	+	20.0	−	−
A348	+	−	−	−	−	+	20.0	−	−
A356	+	−	−	−	−	+	20.0	−	−
A366	−	−	+	−	−	+	20.0	−	−
A368	−	−	+	−	−	+	20.0	−	−
A397	−	−	+	−	−	+	20.0	−	−
A407	−	−	+	−	−	+	20.0	−	−
A409	−	−	−	+	+	−	18.5	−	−
A417	+	−	−	−	−	+	20.0	−	−
A532	−	−	+	−	−	+	20.0	−	−
A592	−	−	−	−	−	+	20.0	−	−
A632	−	−	+	−	−	+	20.0	−	−

[a] PCR products were obtained by using the MXP1 and MXP2 oligonucleotide primer cocktails. Subfamily I comprised F17a-G and F111 adhesin genes. Subfamily II comprised F17b-G and F17c-G/*gafD* adhesin genes.
[b] Size of the structural subunits was determined by Western blotting assays using anti-F17 antibodies.
[c] CS31A antigen was detected by dot blotting assays using specific antibodies.
[d] Phenotypic and genotypic detection of the CNF2 toxin was performed as described elsewhere (27).

(F17b-G and F17c-G/*gafD*). In addition, the 88198 strain was PCR positive for the F111-A structural subunit gene but failed to produce a DNA fragment specific to the subfamily I or II adhesin genes.

Group III. The bacterial strains of group III were isolated from feces or organs of septicemic calves and produced the CNF2 toxin (25). Among the 23 strains tested, 13 (56%) were genetically associated with both the F17b-A structural subunit and the subfamily II adhesin (F17b-G and F17c-G/gafD). In agreement with these results, these 13 F17b-A-positive strains showed a 19,500-Da polypeptide band by Western blotting (data not shown). The F17a-A structural subunit gene was not detected by PCR among the 23 CNF2-producing strains.

DNAs extracted from 4 *E. coli* strains (BM2-10, B20a, 88-612-1, and GB207) generated two PCR fragments specific to F17c-A and F111-A structural subunit genes (Fig. 1). In addition, the JL21 strain was genetically associated with F17b-A and F111-A structural subunits (Fig. 1). When the F17 family antibodies were used, two polypeptide bands with apparent molecular masses of 18,500 and 20,000 Da and 18,500 and 19,500 Da were detected by Western blotting for the 88-612-1 and JL21 bacterial strains, respectively (Fig. 2). In contrast, only one polypeptide band was detected for the B20a, BM2-10, and GB207 *E. coli* strains (Fig. 2).

Three bacterial strains (GB229, 224KH88, and GB228) produced a F17-related structural subunit at the cell surface and possessed an F17-related structural subunit gene but failed to produce an amplified fragment specific to one of the four members of the F17 family. However, two of them were genetically associated with a subfamily II adhesin. These results suggest that these *E. coli* strains probably produced new F17 subtype fimbriae.

Group IV. Group IV comprised 31 *E. coli* strains (4) isolated from the intestinal contents of lambs with a severe renal tubular disease (1). Analysis of the amplified products revealed that 16 (50%) of the 32 ovine *E. coli* strains possessed the F17c-A structural subunit gene, whereas 7 (22%), 6 (19%), and 2 (6%) were PCR positive for the F17a-A, F111-A, and F17b-A structural subunit genes, respectively. A strict correlation was observed between genotypic and phenotypic assays. In agreement with the PCR results obtained with the F17 reference strains, the F17c-A- or F17b-A-positive strains were genetically associated with subfamily II adhesin genes, and the F111-A-positive strains were genetically associated with subfamily I adhesin. In contrast to the results obtained with the F17a reference strain, 6 of the 7 F17a-A-positive strains yielded a PCR product specific to subfamily II adhesin genes.

Size of entire operons. Among the 166 strains tested in this study, we have demonstrated that 5 possess two different genes encoding for two distinct F17-related structural subunits: F17c-A and F111-A for the BM2-10, B20a, 88-612-1, and GB207 strains, and F17b-A and F111-A for the JL21 strain. In these five strains, only the subfamily II adhesin genes were detected, although the subfamily I was associated with F111-A in all the other strains in this collection. To investigate whether both structural subunit genes are located on the same operon together with the adhesin genes, or whether two operons, each carrying a complete cluster of genes, are present simultaneously, further PCR assays were performed. Use of the pair of primers P6 (specific for the F111-A gene) (Table 1) and P10 (AACGCTGTTATCCAGCTTCAGAAAC) (located downstream of the adhesin gene and common to all the F17-related gene clusters studied so far) (Fig. 3) showed that each strain carried a gene cluster of approximately 4.7 kb (Fig. 3). By analogy with the F17a gene cluster, for which the entire nucleotide sequence is known (18), this is the expected size of an entire gene cluster, with no more than one pilin gene. Amplification of DNA from the JL21 strain with the pair of primers P4 (specific for the F17b-A gene) (Table 1) and P10 (Fig. 3) and of DNAs from BM2-10, B20a, 88-612-1, and GB207 strains with P5 (specific for the F17c-A gene) (Table 1) and P10 (Fig. 3) gave similar results (DNA fragments of approximately 4.8 kb and 4.9 kb, respectively [Fig. 3]). Thus, these strains possess two complete gene clusters, one encoding the F111 fimbrial system and the other the F17b or F17c fimbrial system, depending on the strains. Amplification with the P11 primer (CGTTGATAGATAATTTCCCACG), a subfamily II adhesin specific primer (Fig. 3), and either P6 (for F111-A), P4 (for F17b-A), or P5 (for F17c-A) (Table 1) showed that each gene cluster encodes a subfamily II adhesin, since a DNA fragment

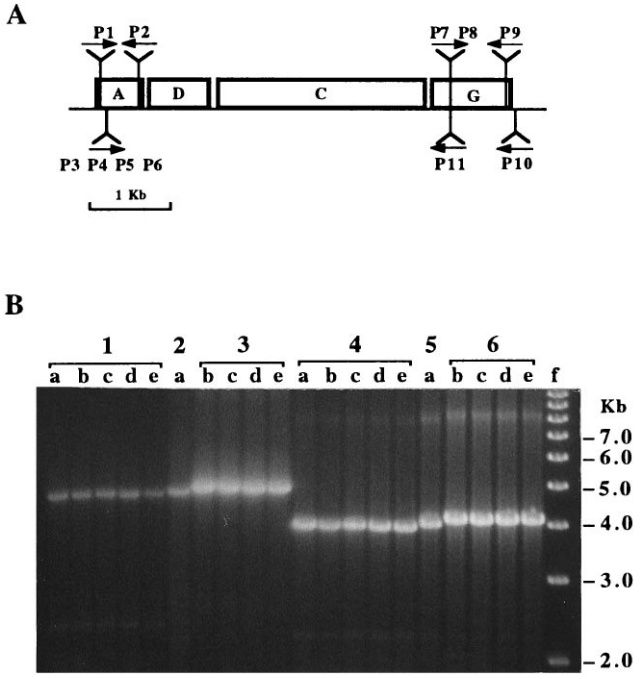

FIG. 3. Location of the primers used and amplification of DNA extracted from the *E. coli* strains genetically associated with two distinct F17-related structural subunits. (A) The *F17a* gene cluster is described elsewhere (19). The F17a-A, F17a-D, F17a-C, and F17a-G genes encode the structural subunit, chaperone, usher, and adhesin, respectively. Primers are identified by arrows. (B) DNAs were amplified from the following strains: JL21 (lanes a), BM2-10 (lanes b), B20a (lanes c), 88-612-1 (lanes d), and GB207 (lanes e). The primer pairs used for the amplification procedure were as follows: 1, P6 (specific of F111-A) and P10; 2, P4 (specific of F17b-A) and P10; 3, P5 (specific of F17c-A) and P10; 4, P6 (specific of F111A) and P11; 5, P4 (specific of F17b-A) and P11; and 6, P5 (specific of F17c-A) and P11. Lane 7, molecular mass marker (1 kb) (Life Technologies). The PCR products (5 μl) were separated on a 0.5% agarose gel and visualized by ethidium bromide staining.

with the expected size (approximately 4.0, 4.1, and 4.2 kb, respectively) was amplified in each case (Fig. 3). Thus, in these strains, the subfamily II adhesin genes were associated with the F111-A gene in contrast with strains harboring only the F111 gene cluster where the subfamily I adhesin gene was always present.

DISCUSSION

Two approaches, one phenotypic (SDS-PAGE, immunodetection, hemagglutination, and in vitro adhesion) (3, 19) and one genotypic (hybridization with DNA probes) (25), have been used previously to detect the presence of the F17-related fimbriae (F17a, F17b, F17c/G, and F111 fimbriae). The purpose of this work was to evaluate an easy, quick, and convenient PCR method for the detection and identification of the F17-related fimbriae. We developed two multiplex primer sets (MXP1 and MXP2) which can be applied successfully (i) to detect and identify each structural subunit gene and (ii) to discriminate between two distinct subfamilies of F17-related adhesin genes. This protocol should be useful for both large-scale studies and the occasional analysis of clinical strains of interest.

The multiplex PCR protocol was tested on 166 *E. coli* strains including 129 strains producing F17-related fimbriae at the cell surface. The results show that detection of fimbrial genes by the PCR method is highly specific and at least as sensitive as immunoblotting and hybridization methods. Analysis of the results revealed that pathogenic *E. coli* strains producing F17c subtype fimbriae were prominent in the bovine and ovine isolates tested (47%), whereas F17a, F17b, and F111 subtype fimbriae were detected on 30, 11, and 11% of the bacterial strains tested, respectively. Besides a clear organization of F17-positive *E. coli* strains into four groups producing F17a, F17b, F17c, or F111 fimbriae, we observed few strains that did not belong to this general classification: three of them were PCR negative for the F17-related adhesin genes and five strains possessed two complete operons of either F17c and F111 or F17b and F111.

The plasmid-encoded antigen CS31A (12) was strictly associated with the F17c subtype fimbriae. The F17b-A structural subunit gene was prominent among the CNF2-producing *E. coli* strains (56%), whereas the F17a subtype fimbriae were associated with the bovine isolates producing neither the CS31A antigen nor the CNF2 toxin. Interestingly, five CNF2-producing strains were genetically associated with two distinct F17-related structural subunits (F17b-A/F111-A or F17c-A/F111-A genes) while only the subfamily II adhesin genes were detected. Phenotypic and PCR assays revealed that two of them produced two distinct structural subunits at the bacterial cell surface and harbored two complete and separate gene clusters. The three remaining strains for which the F17c-A and F111-A genes were detected produced only one structural subunit at the cell surface. However, these three strains possessed two separate and complete gene clusters, each containing a gene of subfamily II adhesins. This suggested that (i) wide deletion or insertion within a gene cluster was not responsible for the loss of fimbrial production, and (ii) either one of the operons was not expressed in the culture medium used or the two pilins had a similar apparent molecular weight. The F17-related gene clusters showed high similarity in the adhesin and structural subunit genes. Moreover, *trans*-complementation analysis has shown that the accessory gene products are closely related to each other (10, 22). As demonstrated for a human uropathogenic *E. coli* strain expressing two related Pap adhesins (21), it seems likely that the two F17-related gene clusters have evolved from each other in these bacterial strains by duplication.

Subfamily II adhesin genes (F17b-G and F17c-G/*gafD*) were prominent among the strains studied (64%). Moreover, F17b-A and F17c-A structural subunit genes were strictly associated with subfamily II adhesin genes, and F111-A was associated with subfamily I adhesin genes except when two gene clusters were present in the cell. The F17a-A structural subunit genetically associated with subfamily I adhesin on bovine diarrheic isolates was found associated with subfamily II adhesins on most of the *E. coli* strains isolated from the digestive tract of lambs with nephropathy. Therefore 81% of the ovine *E. coli* strains tested were positive for the subfamily II adhesin genes, suggesting that the ovine GlcNac receptors could contain an exposed oligosaccharide sequence strongly recognized by subfamily II adhesins. Hence, our results strongly suggest that (i) the fimbrial gene clusters are mosaic operons consisting of F17-related pilin and adhesin genes, and (ii) horizontal gene transfer may occur among different *E. coli* strains isolated from different animal species.

The multiplex PCR method is specific and sensitive for the genotypic detection of most of the F17-related adhesins (98.5%). Therefore, to rapidly detect the F17-related adhesins among bovine and ovine diarrheic or septicemic *E. coli*, we propose a first PCR run with the three oligonucleotide primers (P7, P8, and P9) designated as specific to the two subfamilies of F17 adhesin genes. Subsequently, the MXP1 and MXP2

multiplex cocktails can be used as a rapid diagnostic method to identify each of the F17-related fimbriae.

In the present study, we demonstrated that the CS31A antigen was strictly associated with the F17c subtype fimbriae. *E. coli* strains producing F17c subtype fimbriae show an in vitro GlcNac-inhibitable adhesion to both bovine intestinal villi and cultured human Caco-2 cells (3). The CS31A antigen promotes adhesion to Caco-2 cells, but this adhesion is *N*-acetyl neuraminic acid inhibitable (9). This suggests a strategy used by the pathogen to adhere to different host tissues expressing different receptors. It is well documented that most ETEC strains can express simultaneously in vitro several adhesive factors at the bacterial cell surface. However, little is known about the in vivo synthesis of these adhesins. The adhesive factors are probably not produced simultaneously since expression of their genes is subject to regulation by environmental conditions and by phase variation. It is therefore advantageous for the bacteria to possess two or more clusters of genes that can be turned on or off depending on the host and the tissue colonized.

Our results suggest that the F17 family is very large and that other variants exist that are not yet characterized. Five bacterial strains (3.8%) were PCR positive by using the F17 family primer set but failed to produce an amplified fragment when primer sets able to discriminate between each F17-related structural subunit gene were used. These new variants should be closely related to F17-related fimbriae since (i) nucleotide sequences located on the 3′ and 5′ ends of open reading frames encoding structural subunits hybridized with the P1 and P2 primers (corresponding to the highly conserved nucleotide sequences on F17-related genes) and (ii) four of the five bacterial strains produced a PCR product specific to the F17-related adhesins. Identification of new variants and their screening by widened versions of this PCR method could be of great interest in the development of strategies for preventing adhesion of pathogenic bacteria to mucosal tissues.

ACKNOWLEDGMENTS

We thank Annie Garrivier for excellent technical assistance, Timo Khoronen for providing *E. coli* HB101(pRR5), and Henri de Greve for providing *E. coli* 111KH86 and anti-F17a and anti-F111 antibodies.

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