JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2522–2527
0095-1137/91/112522-06$02.00/0

Vol. 29, No. 11

Virulence Factors Associated with Cytotoxic Necrotizing Factor Type Two in Bovine Diarrheic and Septicemic Strains of *Escherichia coli*

E. OSWALD,[1]†* J. DE RYCKE,[1] P. LINTERMANS,[2] K. VAN MUYLEM,[2] J. MAINIL,[3] G. DAUBE,[3] AND P. POHL[2]

Laboratoire de Pathologie Infectieuse et d'Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France,[1] and Institut National de Recherches Vétérinaires, Groeselenberg 99, B-1180 Brussels,[2] and Faculté de Médecine Vétérinaire, Université de Liège, B-1070 Brussels,[3] Belgium

Received 15 March 1991/Accepted 9 August 1991

Forty-three bovine isolates of *Escherichia coli* producing a second type of cytotoxic necrotizing factor (CNF2) and three K-12 strains carrying different Vir plasmids coding for CNF2 were tested for the presence of several virulence factors. Most of the strains were serum resistant (79%), produced an aerobactin (70%), and adhered to calf villi (53%); some of them produced a colicin (32%) and a hemolysin (9%). These strains were also tested by a colony hybridization assay with gene probes for six toxins (classical heat-stable [STaP and STb] and heat-labile [LT-I and LT-IIa] enterotoxins and Shiga-like toxins [SLT-I and SLT-II]) and five adhesion factors (K99, K88, 987P, F17, and F41). Only two gene probes, LT-IIa (9%) and F17A (53%), hybridized with the CNF2 strains. However, antibodies raised against F17 fimbriae did not agglutinate the strains hybridizing with the F17A probe. In contrast, all except one of these strains adhered to calf villi. Interestingly, these two properties, F17A positivity and adherence to calf villi, were the only ones expressed by the K-12 strains carrying different Vir plasmids. In conclusion, this study confirmed that CNF2-producing strains are unrelated to previously described toxigenic *E. coli* strains and also demonstrated that in half of the strains the production of CNF2 was associated with an adhesion factor genetically related to, but different from, F17, which is more than likely encoded by Vir plasmids.

We have recently described the production of a second type of cytotoxic necrotizing factor (CNF2) by clinical isolates of *Escherichia coli* (12). Like CNF1, CNF2 causes cell multinucleation in tissue cultures and induces necrosis in the skin of rabbits (5, 11). However, in contrast to CNF1, CNF2 induces necrosis in the mouse footpad and moderate fluid accumulation in the rabbit intestinal loop (11, 13). CNF2 is produced by isolates from diarrheic calves (4, 12) and by septicemic *E. coli* strains of the Vir group (30). The Vir strains of *E. coli* are characterized by the association of the production of a toxin lethal for chickens and mice (as yet unidentified, but named Vir toxin) with a specific surface antigen(s) (Vir antigen[s]) (22, 38). Both factors are encoded by transmissible plasmids termed Vir (23, 38). Since Vir^+ transconjugants also produce CNF2 (29, 30) and since the lesions observed in chickens after inoculation with CNF2 were identical to those associated with the Vir toxin (12), this toxin is more than likely CNF2. However, in more recently described isolates of *E. coli* from calf diarrhea, CNF2 production was not constantly associated with the possession of the Vir surface antigen (30), in contrast to the constant association of the Vir toxin with this antigen in previously described Vir strains of *E. coli* (22, 38).

In this study, the characterization of CNF2-producing strains was carried out with two main goals. The first was to estimate the association of CNF2 with other virulence factors, and the second was to determine whether the CNF2-producing strains constitute a homogeneous group of *E. coli* strains with a specific pathotype. Thus, we investigated the incidence of several virulence factors associated with intestinal or extraintestinal infections in 43 bovine isolates producing CNF2 and in three K-12 strains carrying different Vir plasmids. All of these strains were tested for the production of colicin, hemolysin, and aerobactin, resistance to sera, adhesion to calf villi, and expression of F17, F111, and Vir surface antigens (the last of which mediates adhesion to calf villi) and were examined by a colony hybridization assay with gene probes for six toxins and five adhesion factors: classical heat-stable (STaP and STb) and heat-labile (LT-I and LT-IIa enterotoxins, Shiga-like toxins (SLT-I and SLT-II), and F4 (K88), F5 (K99), F6 (987P), F17, and F41 adhesin subunits.

MATERIALS AND METHODS

***E. coli* strains.** Most CNF2-producing strains were isolated from the feces of calves with diarrhea (36 strains). Seven strains came from extraintestinal sites, i.e., blood or fetus. In addition to these *E. coli* field isolates, three Vir transconjugant K-12 strains producing CNF2 were also included in this study: 711(pVir), obtained from H. W. Smith (Houghton Poultry Research Station, Huntingdon, England), and 712(pJL1) and 712(pJL2), obtained from J. Lopez-Alvarez (Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario, Canada). The three Vir donor strains were ovine septicemic strain S5 (38) and bovine septicemic strains JL21 and B177 (23); the K-12 recipient strains were 711 (38) and 712 (23).

Cell culture assays. The different *E. coli* isolates were inoculated into 100-ml flasks containing 10 ml of Trypticase soy broth (Biomérieux, Charbonnières-les-Bains, France). Cultures were incubated at 37°C for 24 h with shaking (160 rpm) and then centrifuged at 10,000 × *g* for 20 min. The

* Corresponding author.
† Present address: Department of Microbiology, Uniformed Services University of Health Science, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

TABLE 1. Derivation of the 11 DNA probes from *E. coli* K-12

DNA probe				Origin		
Name	Size (bases)	Endonuclease	Recombinant plasmid	*E. coli* strain	Species	Reference
STaP	157	*Hin*fI	pRIT10130	P310	Porcine	24
STb	460	*Hin*fI	pRAS1	P307	Porcine	19
LT-I	850	*Hin*cII	pEWD299	P307	Porcine	9
LT-IIa	800	*Hin*dIII-*Pst*I	pCP2725	SA53	Water buffalo	31
SLT-I	1,142	*Bam*HI	pJN37-19	933	Bovine	28
SLT-II	842	*Pst*I	pNN111-19	933	Bovine	28
K99	1,700	*Hpa*I	pFK99	B41	Bovine	26
987P	1,100	*Hpa*I-*Bgl*II	pPK150	H868	Porcine	10
K88	1,300	*Eco*RI	pMK005	G7	Porcine	18
F41	700	*Hin*cII-*Pst*I	pDGA17	VAC-1676	Porcine	1
F17A	1,200	*Hin*dIII-*Bam*HI	pPLHD2	25KHO9st	Bovine	21

resulting pellet was resuspended in 1 ml of phosphate-buffered saline (pH 7.2) (PBS) containing gentamicin (100 μg/ml). Extracts were produced from bacterial suspensions by subjecting the latter to two freeze-thaw cycles from 37 to −20°C. After the second freeze-thaw cycle, the lysates were centrifuged (10,000 × *g*, 15 min) and the supernatant was tested in the HeLa and Vero cell culture assays previously described (12). In brief, a 200-μl volume of a HeLa cell suspension (1.7×10^4 cells per ml) or a Vero cell suspension (5×10^4 cells per ml) was distributed into microtiter wells (Falcon 3072; Becton Dickinson, Oxnard, Calif.), and 22.5 μl of a twofold dilution of the bacterial extracts in PBS was added. The multinucleating effect of toxic material was observed after 72 h of incubation.

O serogroup determination. Determination of the O serogroups of *E. coli* was restricted to O serogroups 2, 4, 6, 8, 11, 15, 75, 78, 88, 123, 139, and 153. This test was carried out as previously described (33). In brief, broth cultures of each of the strains heated for 1 h at 100°C were used as antigens. A portion of antiserum was added to a suspension of the antigen. The mixture was examined after 2 h for agglutination. The three strains belonging to O serogroups 76, 88, and 168 were previously serotyped by F. Ørskov and I. Ørskov (Statens Seruminstitut, Copenhagen, Denmark).

Hybridization and derivation of DNA probes. DNA colony hybridizations were performed as previously described (25). In brief, the colonies were transferred to paper filters (Whatman 541; Whatman Inc., Pleuger, Belgium) treated to lyse the cells and denature the DNA and were hybridized overnight with DNA probes at 65°C. After being washed, the filters were autoradiographed for 1 to 4 days. The 11 probes were derived from multicopy recombinant plasmids carrying toxin- or adhesion factor-encoding genes (Table 1). The STaP and LT-I probes were from N. Harford (Smith-kline-RIT, Rixensart, Belgium), the LT-IIa probe was from R. K. Holmes (Uniformed Services University of Health Science, Bethesda, Md.), the SLT-I and SLT-II probes were from J. W. Newland (Walter Reed Army Institute of Research, Washington, D.C.), the STb and K99 probes were from H. W. Moon (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa), the K88 and F41 probes were from S. L. Moseley (University of Washington, Seattle), the 987P probe was from F. K. de Graaf (Vrije Universiteit, Amsterdam, The Netherlands), and the F17A probe was subcloned from the F17 genes which were cloned at the Institut National de Recherches Vétérinaires. All probes were linear DNA fragments isolated by gel electrophoresis and electroelution (35) and were radiolabeled with [α-^{32}P]deoxynucleotide triphosphates by use of a multiprimer DNA labeling kit (Boehringer, Brussels, Belgium).

In vitro adhesion assay. Intestinal villi were prepared by the method of Girardeau (16) with some minor modifications described previously (20). Approximately 50 calf intestinal villi (from the duodenum, jejunum, and ileum) were mixed in PBS with about 10^9 *E. coli* cells from a culture grown at 37°C on Luria-Bertani (LB) agar plates. α-Methyl-D-mannoside (0.1 mM) was added in each experiment to avoid adherence mediated by F1 fimbriae. The mixture was allowed to incubate in plastic cups at room temperature for 15 min with gentle agitation (30 rpm, Gerhardt Ro 10 apparatus). Adhesion was monitored with a phase-contrast microscope at a magnification of ×600.

Phenotypic detection of F17 and F111 fimbriae. The strains were cultivated at 37°C for 18 h on Minca agar plates (17). The presence of F17 fimbriae was determined by agglutination with a rabbit antiserum prepared against the purified pilin of the F17 fimbriae from strain 25KH9 (20). The presence of F111 fimbriae, variants of F17 fimbriae (the F17A probe cross-hybridizes with F111 genes), was determined by agglutination with a rabbit antiserum prepared against the purified pilin of the F111 fimbriae from strain 111KH86 (2).

Vir surface antigen detection. The strains were cultivated at 37°C for 18 h on Trypticase soy agar plates (22). Slide agglutinations were performed with a rabbit antiserum raised against the cells of a Vir$^+$ transconjugant expressing the Vir surface antigen and preadsorbed with the nonfimbriated K-12 recipient strain (27). One colony was suspended in 1 drop of a 1:50 dilution of Vir antiserum. Agglutination due to the Vir antigen was almost immediate.

Colicin production. Colicin production was measured by the agar overlay method (15) with *E. coli* K-12 Row as the susceptible strain. Zones of inhibition were examined after overnight incubation at 37°C. Strains inhibiting the growth of *E. coli* K-12 Row were considered colicin positive. *E. coli* K-12 Row and *E. coli* K-12 Row ColVré were used, respectively, as negative and positive controls.

Aerobactin production. Aerobactin production was identified by use of the assay of Schoch and Lebek (36), except that the strains were grown in a synthetic iron-depleted broth (40). Strains that secreted aerobactin were identified by a halo of growth around strain WO987 (Iut$^+$ Iuc$^-$) in Chelex agar.

Serum resistance assay. The ability of strains to multiply in the presence of sheep serum was determined by use of the

spot assay of Fierer et al. (14). A log-phase culture diluted 1:500 was applied to Trypticase soy agar plates. Fresh sheep serum was dropped on the plates. Inhibition was examined after overnight incubation at 37°C.

Hemolysin assay. For the detection of hemolysis, bacteria were grown on tryptose blood agar base (Difco) with 5% defibrinated washed sheep blood in PBS. Hemolysis was defined as a distinct zone of clearing around and under isolated bacterial colonies after overnight culturing. For the quantitative determination of the hemolytic titer, *E. coli* strains were grown in brain heart infusion at 37°C with aeration (8). After 3 and 6 h, the supernatant was collected by centrifugation (6,000 × *g*, 30 min) and filtered on 0.22-μm-pore-size filters, and serial twofold dilutions (100 μl) were made in PBS and placed in the wells of a 96-well microdilution tray. A 5% sheep erythrocyte suspension in PBS (50 μl) was added to each well, and the tray was incubated for 24 h at 37°C. The hemolytic titer was defined as the highest dilution in which the supernatant was visibly red because of hemoglobin release. *E. coli* WAF270 carrying plasmid pSF4000 encoding alpha-hemolysin was used as the positive control (41).

RESULTS

The 43 bovine isolates and the three Vir^+ transconjugants produced the specific CNF2 effect of multinucleation in a cell culture assay, whereas the K-12 recipient strains were not cytotoxic. Twenty-nine strains were typeable and belonged to 10 different O serogroups (Table 2). The three main O serogroups were 78 (eight strains), 123 (four strains), and 4 (four strains). All strains were examined for resistance to serum, for production of aerobactin, colicin, and hemolysin, and for adherence to calf villi and then tested by a colony hybridization assay with gene probes for the six toxins and the five adhesion factors. The characterization of these strains is summarized in Tables 2 and 3.

Thirty-four strains (79%) were resistant to serum, 30 (70%) produced aerobactin, 14 (32%) produced a colicin, 4 (9%) produced a hemolysin, and 23 (53%) adhered to calf villi. Of note, the four hemolytic strains released the hemolysin into the liquid medium. The hemolytic activity of these strains was two to four time weaker than that of the control strain, WAF270, which produces alpha-hemolysin. No virulence property other than adherence to calf villi was expressed by the Vir^+ transconjugants.

In the colony hybridization assay, only two gene probes, the LT-IIa probe and the F17A probe, reacted with CNF2-producing strains. The LT-IIa probe hybridized with four strains (9%) but did not react with the Vir^+ transconjugants. In contrast, the F17A probe hybridized with 23 strains (53%) and, interestingly, with all Vir^+ transconjugants carrying the Vir plasmids. Furthermore, the F17A probe-positive strains were significantly more adherent to calf villi than were the F17A probe-negative strains. Indeed, almost all F17A probe-positive strains (except one) tested positive in the adherence assay. In contrast, only one F17A probe-negative strain tested positive (Tables 2 and 3).

These results indicated that the DNA sequence recognized by the F17A probe and the sequence present on the Vir plasmids were strongly associated with the ability of the strains to adhere in vitro to calf villi. To further substantiate the possible association of F17 fimbriae with CNF2-producing strains, we tested all strains by agglutination with antisera raised against the purified pilins of F17 and F111 fimbriae. Neither F17 nor F111 antisera agglutinated the CNF2-producing strains. All strains were tested by agglutination with antisera raised against the Vir surface antigen. Among CNF2-producing strains, 12 strains, including the Vir^+ transconjugants, possessed the Vir surface antigen, as shown by slide agglutination. All of these strains were recognized by the F17A probe. Nevertheless, 15 CNF2-producing strains did not possess the Vir surface antigen, although they hybridized with the F17A probe (Tables 2 and 3).

DISCUSSION

The characterization of 43 *E. coli* strains isolated in different countries has shown that CNF2-producing strains do not constitute a homogeneous group of *E. coli* strains. CNF2-producing strains belonged to at least 10 different O serogroups, and the results of hybridization with the F17A fimbrial probe allowed us to distinguish two groups of CNF2-producing strains. The strains of one group, expressing the F17-like fimbriae, were adherent to calf villi, whereas the strains of the other group were not adherent. Nevertheless, we cannot associate one of these groups with a specific pathology. Although the majority of CNF2-producing strians in this study were isolated from diarrheal diseases, it seems that almost all strains expressed virulence properties previously associated with the ability to cause extraintestinal infections: lipopolysaccharide, O serogroup 78, aerobactin, and serum resistance (39). Moreover, the Vir strains of *E. coli* and the Vir plasmids were classically associated with *E. coli* strain from animals with septicemia (22, 38). Taken together, these results suggest that CNF2-producing strains could also act as opportunistic extraintestinal pathogens, like some CNF1-producing strains isolated from diarrhea in Italy (3).

Besides the ability to cause septicemia, we also observed among the CNF2-producing strains four strains with typical enterotoxigenic *E. coli*-like characteristics. These strains hybridized with both the F17A gene probe and the LT-IIa gene probe and adhered to isolated intestinal villi. This putative new class of enterotoxigenic *E. coli* was previously found in Belgium in calves with systemic or enteritic colibacillosis (32). However, the production of LT-IIa by these four strains was not investigated in the rabbit intestinal loop assay. It should be noted that four other CNF2-producing strains, S5, B9S2, LM1-6, and BM2-10, were previously shown to cause fluid accumulation in the rabbit intestinal loop (11). Interestingly, these strains did not hybridize with gene probes for STaP, STb, LT-I, and LT-IIa. This observation supports the hypothesis that the enterotoxigenic effect of crude lysates from CNF2-producing strains was due to CNF2 itself.

F17 fimbriae, also called Att25 (Belgium) or F(Y) (France), were previously associated with both neonatal calf diarrhea and septicemia (7, 33, 34, 37). In this study, 53% of CNF2-producing strains hybridized with the F17A probe, a *Hin*dIII-*Bam*HI DNA fragment of 1.2 kb including the sequence coding for the pilin of F17 fimbriae (21). Interestingly, hybridization with F17A was correlated with the ability of CNF2-producing strains to adhere to calf villi. However, CNF2-producing strains were not agglutinated with antisera raised against F17 fimbriae or F111 fimbriae, variants of F17 which cross-hybridize with the F17A probe (2). Therefore, CNF2-producing strains did not express the classical F17 or F111 adhesion factor but most probably expressed an F17-like adhesion factor which also mediated the adhesion of *E. coli* to calf villi. This F17-like adhesion

TABLE 2. Distribution of virulence characteristics in bovine *E. coli* producing CNF2[a]

Strain	Origin[b]	O serogroup[c]	Serum resistance	Aerobactin production	Colicin production	Hemolysin production	Ability to adhere to calf villi	Hybridization to probes for:		Vir antigen
								Toxins	Adhesion factors	
B177	E	78	+	+	+	−	+	−	F17A	+
1404	E	78	−	+	−	−	+	−	F17A	+
JL21	E	78	−	−	−	−	+	−	F17A	+
JL10	E	78	+	+	−	−	+	−	F17A	+
JL9	E	78	−	+	−	−	+	−	F17A	+
GB240	I	NT	+	+	+	−	−	−	F17A	+
BM2-10	I	88	+	+	−	−	+	−	F17A	(+)
B20A	I	15	+	+	−	−	+	−	F17A	(+)
B28b	I	123	+	+	−	−	+	−	F17A	−
GB228	I	Rough	+	+	−	−	+	−	F17A	−
88-612-1	I	4	+	+	−	−	+	−	F17A	−
GB212	I	78	+	+	−	−	+	−	F17A	−
MV291-1	I	NT	−	+	−	−	+	−	F17A	−
GB205	I	NT	+	−	−	−	+	−	F17A	−
GB207	I	NT	+	−	−	−	+	−	F17A	−
GB208	I	NT	+	−	−	−	+	−	F17A	−
GB229	I	NT	+	−	−	−	+	−	F17A	−
B9S2	I	168	+	−	+	−	+	−	F17A	−
GB236	I	78/123	+	−	+	−	+	−	F17A	−
GB211	I	8/78	−	+	−	−	+	LTII-a	F17A	−
GB227	I	11	−	+	−	−	+	LTII-a	F17A	−
GB206	I	78	−	−	−	−	+	LTII-a	F17A	−
GB230	I	NT	+	−	−	−	+	LTII-a	F17A	−
236KH88	I	2	+	+	−	+	−	−	−	−
33KH89	E	2	+	+	−	+	−	−	−	−
B24c	I	76	+	+	−	+	−	−	−	−
224KH88	I	8	−	+	−	+	+	−	−	−
MV358-2	I	4	+	+	+	−	−	−	−	−
GB213	I	15	+	+	−	−	−	−	−	−
B26a	I	78	+	+	+	−	−	−	−	−
89-201-2/3	I	123	+	+	+	−	−	−	−	−
GB221	I	123	+	+	+	−	−	−	−	−
GB209	I	NT	+	+	−	−	−	−	−	−
GB219	I	NT	+	+	−	−	−	−	−	−
GB220	I	NT	+	+	−	−	−	−	−	−
GB242	I	Rough	+	+	−	−	−	−	−	−
B4A1	E	NT	+	+	−	−	−	−	−	−
LM1-6	I	NT	+	+	+	−	−	−	−	−
88-388-2	I	4	+	−	+	−	−	−	−	−
GB232	I	4	−	+	−	−	−	−	−	−
GB238	I	123	+	−	+	−	−	−	−	−
GB210	I	NT	+	−	+	−	−	−	−	−
GB244	I	NT	+	−	+	−	−	−	−	−

[a] −, negative response; (+), weak positive response; +, clear positive response.
[b] E, extraintestinal infection; I, intestinal infection.
[c] NT, not typeable by our O antisera.

factor seemed to be closely associated with CNF2-producing strains. To date, none of the CNF1-producing strains tested by colony hybridization reacted with the F17A probe (unpublished data).

Since the Vir adhesion factor is encoded by the Vir plasmid (27) and since 12 (44%) of the F17A probe-positive strains, including all Vir^+ transconjugants, possessed the Vir surface antigen, as shown by slide agglutination, we suggest that the Vir adhesion factor could be the F17 variant detected by the F17A probe. In addition to the hybridization of Vir plasmids with the F17A probe, further features support this hypothesis. Pilins with the same molecular mass of 20 kDa are associated with both Vir and F17 (6, 20, 37). Moreover, the adhesion of the Vir adhesion and F17 fimbriae seems to be directed to closely related or identical intestinal receptors, since the adhesion of Vir^+ *E. coli* to calf brush borders was inhibited by *N*-acetylglucosamine (27), as was that of *E. coli* with F17 fimbriae (2, 20). On other hand, the fact that 15 F17A probe-positive strains were not agglutinated by the Vir antiserum could be explained by at least two hypotheses. First, there may be a lack of expression of the Vir antigen in these strains when grown on Trypticase soy agar. This hypothesis is supported by the fact that the results of agglutination with the Vir antigen were not homogeneous; some wild-type strains of *E. coli* produced very fine agglutination with the Vir antiserum (Table 2). Second, these strains may possess another antigenic variant of F17. Further studies to investigate the possibility of common surface antigens among the other CNF2-producing strains will be useful.

TABLE 3. Distribution of virulence characteristics in the Vir donor strains, the Vir transconjugant strains, and the recipient strains[a]

Strain	Origin (reference)	O serogroup	Hybridization to probe	CNF2 cytotoxic effect	Ability to adhere to calf villi	Vir antigen	Hemolysin production	Aerobactin production	Serum resistance	Colicin production
S5	Ovine strain carrying pVir (38)	78	F17A	+	+	+	−	+	+	−
711(pVir)	K-12 transformed with pVir (38)	Rough	F17A	+	+	+	−	−	−	−
711	K-12 strain used as recipient (38)	Rough	−	−	−	−	−	−	−	−
B177	Bovine strain carrying pJL1 (23)	78	F17A	+	+	+	−	+	+	+
712(pJL1)	K-12 transformed with pJL1 (23)	Rough	F17A	+	+	+	−	−	−	−
712	K-12 strain used as recipient (23)	Rough	−	−	−	−	−	−	−	−
JL21	Bovine strain carrying pJL2 (23)	78	F17A	+	+	+	−	−	−	−
712(pJL2)	K-12 transformed with pJL2 (23)	Rough	F17A	+	+	+	−	−	−	−
712	K-12 strain used as recipient (23)	Rough	−	−	−	−	−	−	−	−

[a] −, negative response; +, positive response.

In conclusion, CNF2-producing strains of *E. coli* have been associated with intestinal and extraintestinal diseases in animals, but the pathogenic mechanism and the role of CNF2 in these diseases are still unclear. However, interaction with mucosal surfaces should be a common initial step in infection. Interestingly, this study has shown that CNF2 is associated on Vir plasmids with an adhesion factor genetically related to F17. The fact that about 50% of CNF2-producing strains hybridized at high stringency with the F17A probe suggests the presence of a common plasmid in these strains. Molecular analysis of Vir plasmids is now in progress to further characterize the Vir adhesion factor and investigate the relationship between CNF2 and the Vir adhesin in the virulence of *E. coli*.

ACKNOWLEDGMENTS

We thank E. Jacquemin and R. Boivin for excellent technical assistance. We are also grateful to the following individuals for providing some of the strains used in this research: M. Contrepois, E. González, C. L. Gyles, H. Navetat, and F. Schelcher.

REFERENCES

1. **Anderson, D. G., and S. L. Moseley.** 1988. *Escherichia coli* F41 adhesin: genetic organization, nucleotide sequence, and homology with the K88 determinant. J. Bacteriol. **170:**4890–4896.
2. **Bertels, A., P. Pohl, C. Schlicker, E. Van Driessche, G. Charlier, H. De Greve, and P. Lintermans.** 1989. Isolatie van het Att111 fimbrieel antigen op *Escherichia coli* geisoleerd uit kalverdiarree: karakterizatie en evaluatie van de noodzaak tot aanpassing van de vaccins ter bestrijding van neonatale colidiarree. Vlaams Diergeneeskd. Tijdschr. **58:**118–122.
3. **Bisicchia, R., R. Ciammarughi, A. Caprioli, V. Falbo, and F. M. Ruggeri.** 1985. Toxin production and haemagglutination in strains of *Escherichia coli* from diarrhoea in Brescia, Italy. J. Hyg. **95:**353–361.
4. **Blanco, J., E. A. González, M. Blanco, B. Regueiro, and I. Bernadez.** 1988. Production of toxins by *Escherichia coli* strains isolated from calves with diarrhoea in Galicia (north-western Spain). Vet. Microbiol. **18:**297–311.
5. **Caprioli, A., V. Falbo, L. G. Roda, F. M. Ruggeri, and C. Zona.** 1983. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. Infect. Immun. **39:**1300–1306.
6. **Contrepois, M., H. C. Dubourguier, A. L. Parodi, J. P. Girardeau, and J. L. Ollier.** 1986. Septicaemic *Escherichia coli* and experimental infection of calves. Vet. Microbiol. **12:**109–118.
7. **Contrepois, M., J. L. Martel, C. Bordas, F. Hayers, A. Millet, J. Ramisse, and R. Sendral.** 1985. Fréquence des pili FY et K99 parmi des souches de *Escherichia coli* isolées de veaux diarrhéiques en France. Ann. Rech. Vet. **16:**25–28.
8. **Cross, M. A., V. Koronakis, P. L. D. Stanley, and C. Hughes.** 1990. HlyB-dependent secretion of hemolysin by uropathogenic *Escherichia coli* requires conserved sequences flanking the chromosomal *hly* determinant. J. Bacteriol. **172:**1217–1224.
9. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139:**850–858.
10. **De Graaf, F. K., and P. Klaasen.** 1986. Organization and expression involved in the biosynthesis of 987P fimbriae. Mol. Gen. Genet. **204:**75–81.
11. **De Rycke, J., E. A. González, J. Blanco, E. Oswald, M. Blanco, and R. Boivin.** 1990. Evidence of two types of cytotoxic necrotizing factors (CNF1 and CNF2) in human and animal clinical isolates of *Escherichia coli*. J. Clin. Microbiol. **28:**694–699.
12. **De Rycke, J., J. F. Guillot, and R. Boivin.** 1987. Cytotoxins in non-enterotoxigenic strains of *Escherichia coli* isolated from feces of diarrheic calves. Vet. Microbiol. **15:**137–157.

13. **De Rycke, J., E. Oswald, and R. Boivin.** 1989. An *in vivo* assay for the detection of cytotoxic strains of *Escherichia coli*. Ann. Rech. Vet. **20**:39–46.
14. **Fierer, J., F. Finlay, and A. I. Braune.** 1972. A plaque assay on agar for detection of gram-negative bacilli sensitive to complement. J. Immunol. **109**:1156–1158.
15. **Fredericq, P.** 1957. Colicins. Annu. Rev. Microbiol. **11**:7–22.
16. **Girardeau, J. P.** 1980. A new *in vitro* technique for attachment to intestinal villi using enteropathogenic *Escherichia coli*. Ann. Microbiol. (Inst. Pasteur) **131B**:31–37.
17. **Guinée, P. A. M., J. Veldkamp, and W. H. Jansen.** 1977. Improved Minca medium for the detection of K99 antigen in calf enterotoxigenic strains of *Escherichia coli*. Infect. Immun. **15**:676–678.
18. **Kehoe, M., R. Sellwood, P. Shipley, and G. Dougan.** 1981. Genetic analysis of K88 mediated adhesion of enterotoxigenic *Escherichia coli*. Nature (London) **291**:122–126.
19. **Lee, C. H., S. L. Moseley, H. W. Moon, S. C. Whipp, C. L. Gyles, and M. So.** 1983. Characterization of the gene encoding heat-stable toxin II and preliminary molecular epidemiological studies of enterotoxigenic *Escherichia coli* heat-stable toxin II producers. Infect. Immun. **42**:264–268.
20. **Lintermans, P. F., P. Pohl, A. Bertels, G. Charlier, J. Vandekerckhove, J. Van Damme, J. Schoup, C. Schlicker, T. Korhonen, H. De Greve, and M. Van Montagu.** 1988. Characterization and purification of the F17 adhesin on the surface of bovine enteropathogenic and septicemic *Escherichia coli*. Am. J. Vet. Res. **49**:1794–1799.
21. **Lintermans, P. F., P. Pohl, F. Deboeck, A. Bertels, C. Schlicker, J. Vandekerckhove, J. Van Damme, H. De Greve, and M. Van Montagu.** 1988. Isolation and nucleotide sequence of the F17-A gene encoding the structural protein of the F17 fimbriae in bovine enterotoxigenic *Escherichia coli*. Infect. Immun. **456**: 1475–1484.
22. **Lopez-Alvarez, J., and C. L. Gyles.** 1980. Occurrence of the Vir plasmid among animal and human strains of invasive *Escherichia coli*. Am. J. Vet. Res. **41**:769–774.
23. **Lopez-Alvarez, J., C. L. Gyles, P. Shipley, and S. Falkow.** 1980. Genetic and molecular characteristics of the Vir plasmids of bovine septicemic *Escherichia coli*. J. Bacteriol. **141**:758–769.
24. **Mainil, J. G., F. Bex, M. Couturier, and A. Kaeckenbeeck.** 1985. Hybridization on bovine enterotoxigenic *Escherichia coli* with two heat-stable enterotoxin gene probes. Am. J. Vet. Res. **46**:2582–2584.
25. **Mainil, J. G., F. Bex, E. Jacquemin, P. Pohl, M. Couturier, and A. Kaeckenbeeck.** 1990. Prevalence of four enterotoxin (STaP, STaH, STb, and LT) and four adhesin subunit (K99, K88, 987P, and F41) genes among *Escherichia coli* isolates from cattle. Am. J. Vet. Res. **51**:187–190.
26. **Mainil, J. G., S. L. Moseley, R. A. Schneider, K. Sutch, T. A. Casey, and H. W. Moon.** 1986. Hybridization of bovine *Escherichia coli* isolates with gene probes for four enterotoxins (STaP, STaH, STb, and LT) and one adhesion factor (K99). Am. J. Vet. Res. **47**:1145–1148.
27. **Morris, J. A., C. J. Thorns, A. C. Scott, and W. J. Sojka.** 1982. Adhesive properties associated with the Vir plasmid: a transmissible pathogenic characteristic associated with strains of invasive *Escherichia coli*. J. Gen. Microbiol. **128**:2097–2103.
28. **Newland, J. W., and R. Neill.** 1988. DNA probe for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26**:1292–1297.
29. **Oswald, E., and J. De Rycke.** 1990. A single protein of 110 kDa is associated with the multinucleating and necrotizing activity coded by the Vir plasmid of *Escherichia coli*. FEMS Microbiol. Lett. **68**:279–284.
30. **Oswald, E., J. De Rycke, J. F. Guillot, and R. Boivin.** 1989. Cytotoxic effect of multinucleation in HeLa cell cultures associated with the presence of Vir plasmid in *Escherichia coli*. FEMS Microbiol. Lett. **58**:95–100.
31. **Pickett, C. L., E. M. Twiddy, B. W. Belisle, and R. K. Holmes.** 1986. Cloning of genes that encode a new heat-labile enterotoxin of *Escherichia coli*. J. Bacteriol. **165**:348–352.
32. **Pohl, P., P. Lintermans, J. G. Mainil, G. Daube, and A. Kaeckenbeeck.** 1989. ETEC-like strains from cattle. Vet. Rec. **125**:382.
33. **Pohl, P., P. Lintermans, J. Moury, K. Van Muylen, and M. Marin.** 1986. Facteurs de virulence chez les *Escherichia coli* septicémiques et saprophytes du veau. Ann. Méd. Vét. **130**:515–520.
34. **Pohl, P., P. Lintermans, and K. Van Muylen.** 1984. Fréquence des adhésines K99 et ATT25 chez les *Escherichia coli* du veau. Ann. Méd. Vét. **128**:555–558.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Schoch, C., and G. Lebek.** 1984. Nachweiss von plasmidisch kodierten Eisen. Transportmechanismen in Enterobacteriaceae. Hyg. Med. **9**:409–412.
37. **Shimizu, M., T. Sakano, J. Yamamoto, and K. Kitajima.** 1987. Incidence and some characteristics of fimbriae FY and 31A of *Escherichia coli* isolates from calves with diarrhea in Japan. Microbiol. Immunol. **31**:417–426.
38. **Smith, H. W.** 1974. A search for transmissible pathogenic characters in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical, with colicine V. J. Gen. Microbiol. **83**:95–111.
39. **Sussman, M.** 1985. The virulence of *Escherichia coli*: reviews and methods. Academic Press, London.
40. **Valvano, M. A., and J. H. Crosa.** 1984. Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of *Escherichia coli* K1. Infect. Immun. **46**:159–167.
41. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Haemolysin contributes to virulence of extra-intestinal *E. coli* infection. Nature (London) **294**:665–667.