Detection of Genes for Fimbrial Antigens and Enterotoxins Associated with *Escherichia coli* Serogroups Isolated from Pigs with Diarrhea

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A total of 1,226 *Escherichia coli* strains isolated from 1979 to 1989 from pigs with diarrhea were examined for serogroup and fimbrial antigen F4 (K88) production. Four main patterns of isolation of the various serogroups were observed, depending on the ages of the pigs from which isolates were obtained and the production of F4. In pattern I, serogroups O8:K"S16", O9:K35, O9/O101:K30, O9/O101:K103, O9 (group), O20:K101, and O64:K"V142" were predominant in pigs aged 0 to 6 days (41.9% of isolates) and were less frequent in pigs aged 7 to 27 days (24.6% of isolates) but were rarely found in pigs aged 28 to 60 days (4.0% of isolates). In pattern II, the F4-associated serogroups O8:K"4627", O157:K"V17", O149:K91, and O147:K89 were predominant in pigs aged 7 to 27 days (29.8% of isolates) and in pigs aged 28 to 60 days (35.0% of isolates). In pattern III, serogroups O8 (group), O115:K"V165", and O147:K89 were rarely isolated from pigs aged 0 to 6 days but were equally distributed in pigs aged 7 to 27 days (10.1% of isolates) and in pigs aged 28 to 60 days (10.9% of isolates). In pattern IV, serogroups O138:K81, O139:K82, O141:K85ac, O45:K"E65", and O26:K60 were most frequently isolated in pigs aged 28 to 60 days (19.3% isolates). Over the period from 1979 to 1989, the proportion of isolates belonging to serogroups of pattern II and the proportion of F4 isolates within the serogroup O157:K"V17" declined, whereas the proportion of isolates of serogroups O147:K89, O8:K"S16", and O9:K35 increased. For 228 isolates selected from the most important serogroups, good agreement was observed between the results of gene probes and immunofluorescence for the detection of fimbrial antigens F4 (K88), F5 (K99), F6 (987P), and F41 and between the results of gene probes and biological assays for the detection of heat-labile enterotoxin (LT) and heat-stable enterotoxins a and b (STa and STb). The STa gene was mostly associated with isolates of pattern I serogroups, which had the F5, F6, and F41 genes alone or in various combinations. The LT and/or STb genes, with the F4 gene, mostly were observed in isolates of pattern II serogroups. The STb gene alone was observed mostly in isolates of pattern III serogroups, although isolates were negative for all fimbrial antigen genes. Similarly, isolates of pattern IV serogroups were negative for all fimbrial antigen genes and rarely positive for the enterotoxin genes. However, verotoxin production was associated with isolates of serogroups O138:K81 and O139:K82. The most important pathotypes among enterotoxigenic isolates in this study were F4:LT:STb, F5:STa, STb, F5:F41:STa, F4:STb, F6, STa, and LT.

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in the newborn and weaned pig (37). These strains colonize the small intestine by means of one or more of the fimbrial adhesins F4 (K88), F5 (K99), F6 (987P), and F41 and produce one or more of the heat-stable enterotoxins a and b (STa and STb) and heat-labile enterotoxin (LT) (14). Although a large number of serogroups of *E. coli* have been described, only a restricted number of these serogroups have been associated with neonatal diarrhea. In early studies, the classical serogroups O8:K87, O147:K89, O149:K91, and O157:K"V17", usually associated with fimbrial adhesin F4, have been implicated most commonly in colibacillary diarrhea of pigs (47). More recently, the fimbrial adhesins F5, F6, and F41 have been found in porcine ETEC strains of such serogroups as O9, O64, and O101 (13, 18, 34, 50).

Most studies of the prevalence of fimbrial adhesins and enterotoxins of porcine ETEC isolates have been conducted

until recently by the use of serological tests and biological activity assays, respectively (13, 16, 46, 50). The genes for each of the fimbrial antigens F4 (22), F5 (7), F6 (6), and F41 (1) and the enterotoxins STa (45), STb (26), and LT (4) have now been well characterized. Thus, more recently DNA colony hybridization assays have been used to detect the genes encoding certain fimbriae (30, 33, 40) and, to a greater extent, enterotoxins (5, 10, 11, 26, 28-30, 33, 35, 38, 40, 51) in *E. coli* isolates of human, bovine, and porcine origin.

However, few studies have compared the sensitivities and specificities of gene probes and serological and biological assays for the detection of fimbriae and enterotoxins in a wide range of serogroups of porcine isolates. Thus, the objectives of the study reported here were to determine the frequency of isolates of *E. coli* of various serogroups from diarrheic pigs in Québec with respect to the ages of the pigs and the year of isolation; to compare the sensitivity and specificity of DNA probe assays with those of immunofluorescence for the detection of fimbrial antigens F4, F5, F6, and F41 and with those of biological assays for the detection of enterotoxins STa, STb, and LT; and to identify the

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virulence attributes associated with various serogroups by using these DNA probes.

MATERIALS AND METHODS

Bacterial strains. A total of 1,226 strains of *E. coli* were isolated at the Faculté de médecine vétérinaire, St-Hyacinthe, Québec, Canada, from the intestinal contents of 1- to 60-day-old pigs with diarrhea during the period from 1979 to 1989. These strains were serotyped with antisera for the *E. coli* OK serogroups (24, 25) and examined for the production of fimbrial antigen F4 by slide agglutination (13). Two hundred twenty-eight of these strains were selected at random from the most commonly encountered serogroups for further detection of fimbrial antigens and enterotoxins by gene probes, serological tests, and biological assays. Strains were stored on Dorset egg medium until this investigation was carried out. *E. coli* K-12(pRIT10036) (STap+), K-12(pRAS-1) (STb+), K-12(pEWD299) (LT+), K-12(pMK005) (F4+), K-12(pFK99) (F5+), K-12(pPK150) (F6+), K-12(pDGA12) (F41+), and HB101 (K-12) and reference strains B41 (STa, F5), P80.3539 (STb), P80.7169 (STb, LT, F4), Meyers (F5), 603A (F6, STa), and HB101 were used for the preparation of probes or as controls.

Preparation of DNA probes. Gene probes for enterotoxins STap, STb, and LT and fimbriae F5 and F6 were derived from recombinant *E. coli* K-12 strains containing the plasmids pRIT10036 (46), pRAS1 (26), pEWD299 (4), pFK99 (7), and pPK150 (6), respectively, as described previously (3). Two different gene probes for the F4 fimbriae were derived from the recombinant strain *E. coli* K-12(pMK005) (22). The first probe was the 1.4-kb fragment generated by digestion with *EcoRI* (3), and the second probe was the 382-bp fragment generated by digestion with *EcoRI-HincII* (1). The gene probe for the F41 fimbriae was derived from the recombinant strain *E. coli* K-12(pDGA17) (1) (kindly supplied by J. Mainil) and was a 617-bp fragment generated by digestion with *HincII-PstI*.

Plasmid DNA was extracted and purified by ultracentrifugation in a cesium chloride gradient (31). Plasmids were digested with the appropriate restriction endonucleases under conditions specified by the manufacturer (Pharmacia LKB Biotechnology Inc., Baie d'Urfé, Québec, Canada). The resulting fragments were separated by agarose gel electrophoresis or 8% polyacrylamide gels for smaller fragments according to the method of Maniatis et al. (31). Appropriate fragments were cut from the gel, recovered by the low-melting-temperature agarose gel technique or the "crush and soak" technique, and concentrated by ethanol precipitation. DNA fragments were labeled with [α - ^{32} P] dCTP by using a multiprimer DNA-labeling kit (Amersham Corp., Arlington Heights, Ill.) according to the instructions of the manufacturer.

Detection of fimbrial adhesins. Strains were tested for the production of fimbrial adhesins F4, F5, F6, and F41 by indirect immunofluorescence as described previously (13). Tests for F4 were performed on colonies grown on blood agar base plus 5% sheep blood. Tests for F5 and F41 were performed on colonies grown on Minca agar plus IsoVitaleX (Minca Is). Tests for F6 were performed on strains grown in stationary tryptic soy broth at 37°C for 24 h and pelleted by centrifugation.

Detection of enterotoxins and cytotoxins. Isolates were examined for production of the enterotoxins STa (by the infant mouse test [43]) and LT (in tissue culture assays with Y1, CHO, and Vero cells [20]) and for production of vero-

cytotoxin (VT) and cytolethal distending toxin in Vero and CHO cells (20).

Certain strains were tested for production of STb in the ligated gut loop technique in 5- to 6-week-old pigs (24) or in 6-week-old rats as described by Whipp (49) with some modifications (9). Briefly, a volume of 8 ml of 0.85% saline containing 300 μ g of trypsin inhibitor (soybean; Boehringer, Mannheim, Federal Republic of Germany) per ml was injected into the small intestine of anesthetized rats and removed to the cecum by gentle massage after 5 min. A series of eight ligated segments (loops), each 5 cm long, were made in the small intestine starting approximately 5 cm from the ileum-cecum junction. The anterior 30% of the small intestine was not used. Loops were inoculated with 0.5% culture supernatant containing 300 μ g of trypsin inhibitor per ml and sterilized by passage through a 0.22- μ m-pore-size membrane (Acrodisc 13; Gelman). Each sample was tested in at least two rats, in loops in different positions in the small intestine. The abdominal incision was closed, and the rat was allowed to regain consciousness. Four hours later, the rats were sacrificed and the volume of liquid in each loop was measured. Results were expressed as the volume of liquid (in milliliters) per intestinal measurement (length times diameter, both in centimeters) and were considered positive if they were greater than 0.05.

Colony hybridization. Strains were spotted onto Luria-Bertani agar and incubated at 37°C for several hours. Colonies were then transferred to Whatman 541 filter paper (Whatman, Inc., Clifton, N.J.), and the filters were processed, hybridized, and revealed by autoradiography as described previously (3).

Briefly, strains were kept inoculated on L agar (L broth containing 15 g of agar per liter) and incubated at 37°C for 18 h. Colonies were replicated by placing filter paper (541; Whatman, Inc.) on the surface of the agar for 2 h. The paper filters were then peeled off and consecutively placed colony side up onto Whatman 3MM filter papers saturated with the following solutions: (i) 10% sodium dodecyl sulfate (SDS) for 3 min, (ii) 0.5 M NaOH–1.5 M NaCl for 15 min, and (iii) 0.5 M Tris hydrochloride (pH 7.5)–1.5 M NaCl twice for 15 min each time. Filters were then air dried and stored at room temperature. A method for increasing the yield of immobilized DNA from bacterial colonies has been described elsewhere (28). This method involves the steaming in alkali of filter replicas of colony arrays. The method is particularly useful when the genes in question, such as STap, occur in large single-copy plasmids.

For hybridization, filters were placed in hybridization buffer which consisted of 3 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10 $\times$ Denhardt solution (1 $\times$ Denhardt solution is 0.02% Ficoll [molecular weight, 400,000; Pharmacia Fine Chemicals, Piscataway, N.J.] plus 0.02% bovine serum albumin), 1% heat-denatured salmon sperm DNA, and 0.01% SDS (31) for 4 h at 65°C and then transferred to fresh hybridization solution containing about 10⁶ cpm of the appropriate heat-denatured, ^{32} P-labeled DNA probe per filter. After hybridization under agitation at 65°C overnight, the filters were washed in 3 $\times$ SSC with 0.1% SDS three times for 30 min each time at 65°C and air dried. The filters were exposed to X-ray film (X-Omat-R; Eastman Kodak Co., Rochester, N.Y.) several hours. The film was developed according to the instructions of the manufacturer.

Statistical methods. Agreement between genotypic and phenotypic test results was examined by using the kappa statistic, and the symmetry was examined by using the McNemar chi square (48). The reported values for the kappa

TABLE 1. Relationship among OK serogroup, F4 production, and age of pig among *E. coli* isolates from pigs with diarrhea

OK serogroup	No. of isolates			
	Total (F4 positive)	From pigs of age (days):		
		0-6	7-27	28-60
Pattern I				
O8:K"S16"	39	12	26	1
O9:K35	37	9	27	1
O9/O101:K30	71	21	44	6
O9/O101:K103	14	2	12	0
O9 (group)	28 (5)	12	15	1
O20:K101	31 (2)	14	15	2
O64:K"V142"	48 (1)	10	36	2
O10:K"V50"	18	6	7	5
Pattern II				
O8:K"4627"	75 (66)	9	46	20
O157:K"V17"	88 (41)	10	45	33
O147:K89	36 (28)	2	18	16
O149:K91	138 (130)	1	104	33
Pattern III				
O147:K"1285"	22	1	13	8
O115:K"V165"	22	1	14	7
O8 (group)	70 (3)	7	43	20
Pattern IV				
O138:K81	23 (2)	2	8	13
O139:K82	23 (2)	1	6	16
O141:K85ac	31 (2)	2	18	11
O45:K"E65"	17 (1)	0	6	11
O26:K60	9	4	3	2
No pattern				
O9:K28	32	5	19	8
O119:K"V113"	5	1	0	4
Others				
O15 and O108:K"V189"	5	0	5	0
Rough	26	8	10	8
Nontypable	318 (4)	44	184	90

test were found to be always statistically significant, with $P \leq 0.01$. The calculations were done by procedure 4F (8) from BMDP Statistical Software, Inc., Los Angeles, Calif.

RESULTS

OK serogroups and F4 production among *E. coli* strains from pigs with diarrhea. Of the 1,226 isolates examined, 872 (71.1%) were serotypable with the described antisera (Table 1). Two hundred eighty-seven (23.4%) of all isolates were F4 positive by slide agglutination. Most of the F4-positive isolates belonged to serogroups O149:K91, O157:K"V17", O8:K"4627", and O147:K89. The proportion of isolates belonging to these serogroups declined over the period from 1979 to 1989 (Fig. 1). The proportion of F4-positive isolates within each serogroup decreased during the same period, most noticeably for serogroup O157:K"V17". In contrast, the proportion of O147:K"1285", O8:K"S16", and O9:K35 isolates increased over the period from 1979 to 1989.

Four main patterns of isolation of the various serogroups were observed, depending on the ages of the pigs from which isolates were obtained and the production of F4. Serogroups of pattern I were predominant in pigs aged 0 to 6 days (41.9%

FIG. 1. Frequency of OK serogroups in diarrheic pigs during the time periods 1979 to 1982 (I), 1983 to 1986 (II), and 1987 to 1989 (III). Black areas represent F4-positive isolates, and white areas represent F4-negative isolates.

of isolates) and were less frequent in pigs aged 7 to 27 days (24.6% of isolates) but were rarely found in pigs aged 28 to 60 days (4.0% of isolates) (Table 1). The F4-associated serogroups of pattern II were predominant in pigs aged 7 to 27 days (29.8% of isolates) and in pigs aged 28 to 60 days (35.0% of isolates). In pattern III, serogroups not associated with F4 were also rarely isolated from pigs aged 0 to 6 days and were equally distributed in pigs aged 7 to 27 days (10.1% of isolates) and in pigs aged 28 to 60 days (10.9% of isolates). Serogroups of pattern IV were most frequently isolated in pigs aged 28 to 60 days (19.3% of isolates).

Comparison of gene probes and phenotypic assays for detection of fimbrial antigens and enterotoxins. A total of 228 isolates belonging to the most important serogroups were selected at random for comparison of gene probes and indirect immunofluorescence for the detection of fimbrial antigens F4, F5, F6, and F41 and for comparison of gene probes and the infant mouse test, the pig gut loop assay, and tissue culture assays for the detection of STa, STb, and LT, respectively. Tests for the detection of F4 fimbrial antigen and genes showed perfect agreement, and very good agreement was found between the genotypic and phenotypic tests for the detection of fimbrial antigens F5 and F6 (kappa values, 0.915 and 0.898, respectively) (Table 2). Less agreement was found between the tests for the detection of F41, but it was still statistically significant (kappa value, 0.312). The McNemar test ($\chi^2 = 13.72$; $P < 0.01$) implied that asymmetry was statistically significant. The phenotypic test

TABLE 2. Comparison of genotypic and phenotypic assays for the detection of enterotoxins and fimbrial antigens in *E. coli* isolates from pigs with diarrhea

Virulence attribute	No. of isolates				
	Total	Probe positive		Probe negative	
		Phenotype positive ^a	Phenotype negative	Phenotype positive	Phenotype negative
F4	223	23	0	0	200
F5	218	32	2	3	181
F6	204	20	2	2	180
F41	161	6	2	19	134
STap	228	57	0	1	170
STb	83	20	0	2	61
LT	170	16	2	2	150

^a Production of fimbrial antigens F4, F5, F6, and F41 was detected by indirect immunofluorescence; production of enterotoxins STa, STb, and LT was detected by the infant mouse, pig gut loop, and tissue culture assays, respectively.

TABLE 3. Relationship between OK serogroup and presence of genes for enterotoxins STap, STb, and LT among *E. coli* isolates from pigs with diarrhea

OK serogroup	No. of isolates						
	Total	With genes for enterotoxin					
		STap	STb	LT	STap and STb	LT and STb	None
Pattern I							
O8:K``S16``	9	5		1			3
O9:K35	3	1		1			1
O9/O101:K30	13	9					4
O9/O101:K103	8	4					4
O9 (group)	9	2		1			6
O20:K101	19	2			1		16
O64:K``V142``	41	21	1		1		18
Pattern II							
O8:K``4627``	3		1			2	
O157:K``V17``	11	1	2			4	4
O147:K89	8		1		1	3	3
O149:K91	11		4	1		5	1
Pattern III							
O147:K``1285``	3		2				1
O115:K``V165``	25		17				8
O8 (group)	6		1			3	2
Pattern IV							
O138:K81	4		1	1	1		1
O139:K82	6		1				5
O141:K85ac	3						3
O45:K``E65``	4						4
No pattern							
O9:K28	9						9
O108:K``V189``	10						10
O119:K``V113``	5						5

detected more F41-positive isolates, most of which belonged to the serogroups O9 and O64:K"V142", than the probe did.

Genotypic and phenotypic tests for the detection of enterotoxins LT and STap showed very good agreement (kappa values, 0.988 and 0.876, respectively) (Table 2). Eighty-three STa-negative and LT-negative isolates were selected for comparison of the gene probe for STb and the pig ligated gut loop test for the detection of STb production. Good agreement between the two assays was observed (kappa value, 0.936). In addition, 45 STa-negative and LT-negative isolates were examined by the STb gene probe and in the rat gut loop test, and good agreement was observed (kappa value, 0.706). The McNemar test ($\chi^2 = 1$; $P = 0.3$) implied that asymmetry was statistically significant. Good agreement was observed between the pig gut loop and rat gut loop assays (kappa value, 0.704) for 24 isolates examined in the two assays.

Relationship of presence of enterotoxin and fimbrial genes with serogroup. Of 211 of the above isolates tested by gene probes for all of the virulence attributes F4, F5, F6, F41, STap, STb, and LT, 102 possessed the genes for one or more enterotoxins (Table 3). The presence of the gene for STa was mostly associated with serogroups of pattern I. Isolates of these serogroups possessed the genes for one or more of the fimbrial antigens F5, F6, and F41 (Table 4). Most of the ETEC isolates with the gene for LT also possessed the gene for STb and belonged to the serogroups of pattern II. All of

TABLE 4. Relationship between OK serogroup and presence of genes for fimbrial antigens F4, F5, F6, and F41 among enterotoxigenic *E. coli* isolates from pigs with diarrhea

		No. of enterotoxigenic isolates								
OK serogroup	Total	With genes for fimbria								
		F4	F5	F6	F5 and F41	F6 and F41	F5 and F6	F4, F5 and F6	None	
Pattern I										
O8:K``S16``	6		2	3						1
O9:K35	2		1							1
O9/O101:K30	9		3		6					0
O9/O101:K103	4			3						1
O9 (group)	3		1	1	1					0
O20:K101	3		1	2						0
O64:K``V142``	23		12	5		1	2			3
Pattern II										
O8:K``4627``	3	2						1		0
O157:K``V17``	7	4								3
O147:K89	5	4	1							0
O149:K91	10	9								1
Pattern III										
O8 (group)	4	1								3
O147:K``1285``	2									2
O115:K``V165``	17									17
Pattern IV										
O138:K81	3									3
O139:K82	1									1

the LT-positive isolates possessed the gene for F4, with the exception of two O8 (group) isolates of pattern III, which were negative for all fimbrial genes (Tables 3 and 4). Eight isolates of pattern II possessed the gene for STb but not that for STa or LT. Five of these isolates, belonging to serogroups O8:K"4627", O147:K89, and O149:K91, were also positive for the F4 gene. The remaining isolates were negative for all fimbrial genes. In addition, many isolates of pattern III serogroups possessed the gene for STb but not that for STa or LT. All of these isolates were negative for all fimbrial genes. Five isolates were positive for the LT gene but negative for the STa and STb genes. One of these isolates, belonging to serogroup O149:K91, was also positive for the F4 gene, whereas the remaining isolates were negative for all fimbrial genes.

The pathotypes most frequently observed among the 102 enterotoxigenic isolates by using gene probes were F4:LT:STb (14 isolates), F5:STa (18 isolates), F6:STa (13 isolates), and STb (25 isolates) (Table 5). Less frequently observed pathotypes were F5:F41:STa (seven isolates), F4:STb (five isolates), F6 (four isolates), STa (five isolates), and LT (four isolates).

Production of cytotoxins. Among the 211 isolates described above, 2 of 3 O138:K81 isolates, 3 of 6 O139:K82 isolates, 1 of 8 O147:K89 isolates, and 1 of 11 O149:K91 isolates produced VT. Two isolates, of serogroups O8:K"S16" and O8 (group), produced cytolethal distending toxin.

DISCUSSION

The most frequently encountered serogroup in our study was O149:K91, in which most of the isolates were F4 positive. Similarly, O149 was the predominant serogroup

TABLE 5. Relationship between the presence of genes for enterotoxins and fimbrial antigens in *E. coli* isolates from pigs with diarrhea

Fimbrial antigen	No. of isolates with genes for enterotoxin					
	STap	STb	LT	STa and STb	LT and STb	None
F4		5	1		14	
F5	18	1		2		1
F6	13			1		4
F5 and F41	7					1
F6 and F41	1					
F5 and F6	2					
F4, F5, and F6					1	
F4, F5, and F41						2
None	5	25	4	1	2	100

found in isolates from pigs with diarrhea in Sweden (46) and the midwestern United States (50). In contrast, we have also found a high proportion of isolates from other F4-associated serogroups. These serogroups were much less commonly or rarely found in those two studies but have also been found more commonly in isolates from diarrheic pigs in the United Kingdom (51). In our study, isolates from F4-positive serogroups, particularly O149, were less frequently found in neonatal pigs (0 to 6 days old) than in older pigs. In the studies of porcine *E. coli* isolates from the midwestern United States and Denmark (46, 50), O149 isolates were also less frequent in neonatal pigs than in older pigs. In the latter study, it had been observed that the frequency of this serogroup in neonatal pigs had decreased from 1976 to 1984. In our study, the frequency of this serogroup, as well as of the other F4-associated serogroups of pattern II, declined from 1979 to 1989. Increased vaccination of sows with *E. coli* bacterins or subunit vaccines and subsequent transfer in the colostrum of passive immunity to the newborn pigs could explain the lower frequency of F4-positive isolates in neonatal pigs and the decrease in frequency of F4-positive isolates during the last 11 years. It is also possible that in the serogroup O157:K"V17" vaccination pressure has encouraged the appearance of F4-negative isolates producing a new fimbrial adhesin.

In the pigs with neonatal diarrhea in our study, the dominant serogroups were those of pattern I, with which the virulence attributes F5, F6, F41, and STap were commonly associated. The frequency of these serogroups decreased with the age of the pigs, and very few were observed in pigs postweaning. Similar trends were observed in the Swedish and midwestern United States studies (46, 50). In contrast, we often observed serogroups of pattern III in pigs with diarrhea between the neonatal and postweaning periods and postweaning. The O147:K"1285" and O115:K"V165" isolates produced only the enterotoxin STb and were negative for all fimbrial adhesins. We have demonstrated that at least some O115:K"V165":STb+ strains may cause diarrhea in experimentally infected newborn pigs (12). It is possible that the role of these isolates in the production of diarrhea is dependent on the age of the pigs and that the newborn pig is not the best model for the reproduction of infection with these isolates. It is interesting to note that all O147 and O157 isolates from pigs with postweaning diarrhea in a Hungarian study (40) were F4 negative and both STa and STb positive, whereas we found very few STa-positive isolates from postweaning pigs. Several O8 (group) isolates in our study belonged to serogroup O8:KX105, produced both enterotox-

ins LT and STb, and were negative for fimbrial adhesins F4, F5, F6, and F41 (Table 3) but have been found to induce diarrhea in experimentally infected newborn pigs (2).

Isolates of the pattern IV serogroup O45:K"E65" are usually nonenterotoxigenic and F4 negative (36), although Woodward et al. (51) found a high proportion positive for F4 and enterotoxins LT and STb. Most of the O45:K"E65" isolates in our study were nonenterotoxigenic and negative for all fimbrial adhesins. In fact, many of these isolates originated from pigs with attaching-effacing lesions in the small and large intestines, and at least some of these strains adhered to the intestinal mucosa of experimentally infected newborn pigs and induced the typical attaching-effacing lesions (19). This could be an important mechanism in the development of postweaning diarrhea.

E. coli strains of serogroups O138:K81, O139:K82, and O141:K85ac are commonly associated with edema disease in weaned pigs (15, 17, 32) and often produce a variant of the cytotoxin VT II. VT-positive isolates of these serogroups have also been found in pigs with postweaning diarrhea (15, 40). Similarly, we have found that the production of VT is almost exclusively associated with these serogroups. Some of these isolates, at least in the serogroup O138:K81, were also enterotoxigenic, but no known fimbrial adhesins were observed. The role of VT in the development of diarrhea in weanling pigs has not yet been determined.

ETEC strains are usually identified in the diagnostic laboratory by testing isolates for enterotoxin production in bioassays or serological tests that detect phenotypic expression of the genes encoding for heat-labile and heat-stable enterotoxins. Alternatively, ETEC can be identified by genes encoding for these enterotoxins by DNA hybridization (5, 10, 11, 28, 30). This method is particularly useful in detecting ETEC in large numbers of specimens. ETEC identification by DNA hybridization was compared with immunofluorescence for F4, F5, F6, and F41 fimbrial antigens. The correlation between the results of hybridization with F4, F5, F6, and F41 probes and phenotypic testing for fimbrial antigen production confirmed the suitability of the probe for detecting fimbrial genes in porcine ETEC, as reported for some fimbriae (23, 30). Tests for the detection of F4 fimbrial antigen and genes showed perfect agreement. Previous results (1) and our preliminary findings showed that the 1.4-kb *EcoRI* probe for the F4 genetic determinant cross-reacted with F41 genetic determinants. The 382-bp *EcoRI-HincII* fragment of pMK005 which is internal to the F4 subunit gene did not hybridize with F41 strains. Conversely, the 617-bp *HincII-PstI* fragment of pDGA17 (1) which is internal to the F41 subunit did hybridize to F41 strains specifically. Thus, in the present work we have preferred to use the 382-bp *EcoRI-HincII* fragment of pMK005 for the routine detection of F4 and the 617-bp *HincII-PstI* from pDGA17 for the detection of F41. The tests for the detection of F5 and F6 showed very good agreement. Less agreement was found for tests for the detection of F41, but it was still statistically significant. The phenotypic test detected more F41-positive isolates than the probe did. It is interesting to note that most of these isolates belonged to serogroups O9 and O64, were nonenterotoxigenic, and could produce an additional fimbrial antigen having common epitopes with F41 antigen but no genetic relationship.

Phenotypic and genotypic tests for the detection of LT and STap showed very good agreement. For LT-producing strains, we found good agreement between gene probing and biological activity (Y-1 adrenal cell assay). However, preliminary work using the 850-bp *HincII* probe corresponding

to part of the A and B cistrons of LT from pEWD299 (4) detected isolates positive by biological testing as well as some negative by biological testing. The use of the 800-bp *Hind*III probe from pEWD299 covering the LT B cistron and a portion of the LT A cistron (4, 33) eliminated these false-positives.

In preliminary results for the detection of STap-positive isolates, we found that although isolates negative in the biological assay (infant mouse test) were also negative with the gene probe, only 40 to 50% of the infant mouse test-positive strains were positive with the STap gene probe. Similarly, bovine isolates were reported to produce STa but not to hybridize with the STap probe (30). However, when we used the modified procedure for lysis as described by Maas et al. (28), we could detect most of the strains that were positive in the infant mouse assay. Thus, addition of a steaming step during lysis of the cells considerably increased the sensitivity of the genotypic assay for the detection of STa-positive colonies (5, 28).

For STb toxin testing, we found good agreement between the pig gut loop assay and the rat gut loop assay. Two more strains were ST positive when tested in the pig gut loop assay than in the rat gut loop assay. It is possible that there are more variations in the detection of STb from one rat to another than in pigs, that the rat assay is less sensitive, or that the isolates tested in rats had lost their plasmids carrying STb. For STb toxin testing, it is impractical to handle large numbers of isolates requiring large numbers of susceptible animals. STb toxin testing in pig or rat gut loops is particularly impractical and expensive, yet it had been the only available test for one of the toxins most commonly associated with porcine diarrhea.

The tests for the detection of STb production, phenotypically (in pig or rat ligated ileal loops) and genotypically, showed good agreement. A comparison between the DNA hybridization and pig gut ileal loop assay results revealed that more strains were detected by the phenotypic assay. The differences seen between phenotypic and genotypic tests could be explained in the case of probe-positive phenotype-negative isolates by the possibility that STb toxin genes are not always expressed *in vivo*. In the case of probe-negative and phenotype-positive isolates, the differences could be due to the action of other toxins or other components of the culture filtrates on the gut loops.

The major pathotypes observed in our study were LT+STb+F4+, STap+F5+, STap+F6+, and STb+. A similar pattern was found in isolates from diarrheic piglets in the midwestern United States (50), although a much higher proportion of LT+STb-F4+ isolates were observed in that study. On the other hand, Moon et al. (35) found predominantly LT+STb+ and STb+ isolates but no LT+STb- isolates in a study of the prevalence of enterotoxin genes among *E. coli* from swine in the United States. It is possible that the LT+STb-F4+ isolates observed by Wilson and Francis (50) possessed the STb gene but did not produce the enterotoxin in large enough quantities to be detected in the pig gut loop assay. It is interesting to note that whereas in North America and Sweden the STap+F5+, STap+F6+, and STap+F5+F41+ pathotypes have been more frequently observed, in other countries, such as the United Kingdom (51) and Australia (39), certain of these pathotypes appear to be still relatively uncommon in porcine isolates. In addition, most studies of the prevalence of fimbrial adhesins in porcine isolates have been based on tests for the phenotypic detection of their production (39, 46, 50, 51). We have demonstrated that many F6-positive isolates are not detected by the

routine slide agglutination test but are identified by more sensitive tests such as indirect immunofluorescence (13). Because in the present work a good correlation between indirect immunofluorescence and the gene probe for detection of F6 was observed, use of the latter technique will more accurately reflect the prevalence of F6 in porcine isolates.

A large number of isolates (18%) belonged to the pathotype STb+. It has not yet been determined whether the STb toxin genes are associated with hitherto uncharacterized fimbrial adhesins, as it has been suggested previously (33, 37). Using experimental conditions similar to those of the present study, Moon et al. (35) observed that ETEC strains producing only STb and lacking the F4, F5, and F6 fimbrial antigens neither colonized the small intestine nor caused diarrhea in experimentally inoculated piglets. On the other hand, Kashiwazaki et al. (21) have suggested that such strains may be diarrheagenic. Our own results also confirm that certain ETEC strains producing only STb and lacking the F4, F5, F6, and F41 fimbrial antigens may be diarrheagenic in piglets (12).

Thus, DNA hybridization offers a reliable and practical alternative for the determination of a battery of the important fimbrial adhesins and toxins in porcine ETEC strains. It appears to be as sensitive as conventional serological tests such as immunofluorescence for the detection of fimbrial adhesins whose expression is variable and influenced by culture conditions. DNA hybridization may be used to screen large numbers of pigs in a farrowing house for the detection of ETEC present in the feces at lower, subclinical levels, thus allowing the rapid implementation of preventive measures (32).

More recently, several studies (27, 41, 42) have demonstrated that use of synthetic oligonucleotide primers in the polymerase chain reaction amplification (44) is highly specific and sensitive for the detection of the genes coding for enterotoxins in clinical and reference *E. coli* isolates. In the future, use of this technique will allow even more accurate and inexpensive detection of virulence genes in clinical material.

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