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Virulence Factors in *Escherichia coli* Strains Isolated from Swedish Piglets with Diarrhea

OLOF SÖDERLIND,[1]* BERNT THAFVELIN,[2] AND ROLAND MÖLLBY[3]

National Veterinary Institute, S-750 07 Uppsala,[1] Animal Health Service, S-121 86 Stockholm,[2] and National Bacteriological Laboratory, S-105 21 Stockholm,[3] Sweden

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Parenteral vaccination of sows against *Escherichia coli* diarrhea in their newborn piglets has become more common during the last decade in Sweden, and the vaccination has generally had positive effects. For more than 20 years we have investigated *E. coli* strains isolated from piglets and weaned pigs with enteric disorders, noting the presence of O groups, enterotoxins, and adhesins. There has been a continuous change in the frequency of these virulence factors. The present study was performed during 1983 and 1984 to follow this change, since such information is essential for the proper choice of vaccines. A total of 856 *E. coli* strains were obtained from 683 herds divided into three age groups: 1 to 6 days old, 1 to 6 weeks old, and weaned pigs. O group 149 still dominated in the last two age groups, while O group 101 was, for the first time, the most frequent O group in neonatal piglets. All but four O149 strains carried the K88 antigen, which was found in only one other strain (O group 8). K99 antigen was most often found in O groups 101 and 64, and among all the K99 strains ST mouse was the most common (44 of 57), followed by ST mouse-ST pig strains (12 of 57). The 987P antigen was demonstrated in 26 strains belonging to O groups 141 and OX46 and nontypable strains. Two strains belonging to O group 101 were positive for F41 antigen; one of them also carried the K99 antigen. Among all non-O149 strains, ST mouse was the most common type of enterotoxigenic *E. coli* ($n = 88$), followed in decreasing order by ST mouse-ST pig strains ($n = 69$) and ST pig strains ($n = 33$). In 114 strains producing enterotoxins no adhesive factor was found. Thus, vaccination of the Swedish sow population for more than 5 years with vaccines containing O149 and K88 antigens has apparently changed the pattern of enterotoxigenic *E. coli* in neonatal diarrhea. The frequency of O149:K88 strains has been reduced, and O101:K99:ST mouse strains now dominate. However, O149 strains remain the dominant O group in piglets older than 1 week. In spite of all our diagnostic efforts, no virulence factors were detected in about one third of the piglets and weaned pigs with enteric disorders.

In Sweden today, sows are often treated parenterally with vaccines containing O149 and K88 antigens to protect their newborn piglets against *Escherichia coli* diarrhea. During recent years vaccines containing K99 and 987P antigens have also been used, especially in herds in which the older types of vaccines have become ineffective and the isolated *E. coli* strains are not K88 positive. To be able to develop vaccines with adequate immunogenic components and when searching for other important etiological factors, it is necessary to follow the shift in the frequency of O groups, adhesive factors, and enterotoxins in the *E. coli* strains isolated from piglets and newly weaned pigs suffering from outbreaks of enteric disorders.

E. coli diarrhea is one of the most frequent types of disease in young piglets. *E. coli* strains must be able to adhere, proliferate, and produce enterotoxin in the small intestine (26). The enteropathogenic *E. coli* bacteria adhere to the intestinal epithelium with the help of adhesive factors (28). The production of enterotoxin and K88 fimbriae is governed by transmissible plasmids (23, 27), making it necessary to assay for these properties for routine clinical diagnoses.

The number of recognized enterotoxins and adhesins in porcine *E. coli* has increased since Smith and Gyles (25) described heat-stable (ST) and heat-labile (LT) enterotoxins. Moon and Whipp (17) reported two classes of enterotoxinogenic *E. coli* (ETEC) strains. Later, two ST enterotoxins have been demonstrated in porcine strains in different studies designated as ST_a and ST_b (1), ST1 and ST2 (11), and ST mouse and ST pig (22). Franklin and Möllby (4) reported that the ST pig determinants appear to be linked exclusively to the LT plasmid in *E. coli* strains of O group 149, and that the ST pig enterotoxin, in contrast to the ST_b toxin, was active in both older and neonatal pigs.

In 1975, K99 was established as a transmissible K antigen with adhesive properties in calves and lambs (24). It was previously called Kco by Smith and Linggood (29). Moon et al. (15) and Guinée et al. (8) identified K99 antigen on porcine ETEC. In 1977, Nagy et al. (20) presented 987P as another adhesive factor in porcine ETEC in the United States. F41 antigen was described by Morris et al. (18) in the United Kingdom in 1982. All these antigens exist as fimbrial structures on the bacterial surface. For further references, see Gaastra and de Graaf (7).

Since 1964 we have followed the frequency of O groups and virulence factors in *E. coli* strains isolated from pigs with diarrhea. The aim of the present study was to provide corresponding data on *E. coli* strains isolated during 1983 and 1984 from piglets with enteric disorders. Animals were divided into three age groups, i.e., piglets 1 to 6 days old, piglets 1 to 6 weeks old, and weaned pigs, to follow the changes in frequency in the Swedish pig population. Since, in earlier studies, all strains belonging to O group 149 and possessing K88 antigen were enterotoxin producing, these strains were not tested for enterotoxicity in the present study.

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* Corresponding author.

gress of the International Pig Veterinary Society, 1986, Barcelona, Spain, abstract, p. 131.)

MATERIALS AND METHODS

Isolation of *E. coli*. The 856 *E. coli* strains investigated were isolated from 683 herds during 1983 and 1984. The material consisted of either feces from piglets with diarrhea or the contents of the proximal part of the small intestine. The latter piglets were autopsied at the National Veterinary Institute, Uppsala, or at various regional laboratories.

The material was divided according to the three age groups from which it was obtained: group 1, 1- to 6-day-old piglets; group 2, 1- to 6-weeks old piglets; and group 3, weaned pigs, i.e., older than 6 weeks.

In age group 1, 349 *E. coli* strains from 292 herds were investigated; in age group 2, 410 strains from 314 herds were studied; and in age group 3, 97 strains from 77 herds were examined.

Fecal samples were generally taken with a sterile swab and placed in a transport medium (Culturette; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.). In the laboratory the samples were plated on beef extract agar with lactose and bromocresol purple indicator (E. Merck AG, Darmstadt, Federal Republic of Germany). Two *E. coli*-like isolates from one or two pigs in each herd were investigated further. In most cases these isolates turned out to be identical. Biochemical analyses were performed at 37°C with fermentations in glucose, adonitol, and raffinose and by the indole test. Identified *E. coli* strains were stored in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 5% glycerol at −70°C.

O-antigen determination. Antisera for O-antigen determination were prepared as described earlier (31). Tube agglutination tests were carried out with 18 antisera specific for the O serogroups 2, 6, 8, 9, 20, 45, 64, 98, 101, 115, 138, 139, 140, 141, 147, 149, 157, and OX46. Nontoxigenic isolates that did not react with the 18 antisera were termed in-house nontypable. Enterotoxin-producing strains not belonging to any of the abovementioned O serogroups were serotyped at the Collaborative Centre for Reference and Research on Escherichia (World Health Organization), Statens Seruminstitut, Copenhagen, Denmark. Strains not belonging to any recognized O group were designated as not belonging to any established O group. Only one strain of a particular O group and only one nontypable nonenterotoxinogenic strain were counted from each herd.

K88, K99, 987P, and F41 determination. All determinations of K88, K99, 987P, and F41 were performed by the slide agglutination test. All isolates were first screened for the presence of K88 antigen. K88 antisera were produced as described earlier (31). Tests were performed with bacteria grown on 5% horse blood agar at 37°C for 18 h. K88-negative strains were subsequently tested for K99, 987P, and F41 antigens.

The K99 antiserum was produced as described by Smyth et al. (30). Isolates were grown on Minca-IsoVitaleX (BBL Microbiology Systems) agar medium for 18 h at 37 and 22°C (10, 30). ST mouse-producing strains which tested negative for K99 were retested in the same way.

Anti-987P serum was produced by the method of Söderlind et al. (35). Tests were made with bacteria grown on 5% sheep blood agar plates. After 18 h of incubation, small, transparent colonies were tested. Isolates not agglutinated by antiserum were regrown in Trypticase soy broth at 37°C until a definite pellicle was formed or for at least 4 to 6 days. Material from the pellicle or from the surface was again grown on 5% sheep blood agar plates. Negative strains were inoculated from the pellicle or from the surface of Trypticase soy broth to new Trypticase soy broth and blood agar plates and retested at least 5 times.

F41 antiserum was provided by J. A. Morris, Weybridge, United Kingdom. Tests for F41 antigen were done on colonies grown on 5% sheep blood agar plates.

Enterotoxin tests. Three assays for enterotoxin were used: (i) the Y1 adrenal cell test for LT, using culture supernatant fluids (33); (ii) the infant mouse test for ST in 2- to 3-day-old mice, using heat-treated (80°C, 20 min) culture supernatants of Casamino Acids (Difco Laboratories, Detroit, Mich.)–yeast extract medium (22); and (iii) the intestinal loop test in 3- to 7-week-old piglets, using bacterial suspensions (32). The enterotoxigenicity findings were interpreted as shown in Table 1.

Strains belonging to O group 149 possessing K88 antigen were not tested for enterotoxigenicity in the present study, since in earlier studies (33, 34) all such strains were found to be enterotoxin producers.

Hemolysin test. The hemolysin test was carried out on blood-agar with approximately 5% horse blood.

RESULTS

O-antigen distribution. When the 222 nonenterotoxinogenic in-house nontypable strains were excluded, O group 149 dominated (more than 40%) in piglets older than 1 week; while together the relative frequencies of O groups 101, 9, and 64 decreased with age, from 42 to 8% (Fig. 1). Furthermore, O group 141 was the second most common O group in piglets in the oldest age group (15%).

Of the 222 nonenterotoxinogenic strains not included in Fig. 1, 97 strains belonged to piglets in the youngest age group, 108 to the 1- to 6-week-old piglets, and 17 to the weaned piglets.

The frequency of hemolysis varied among O groups and was 75% for the O149 strains and 40 to 50% for the less frequently occurring O groups 141, 139, 6, 138, and 147. Only one strain each of O groups 8, 64, 101, and 157 produced hemolysin.

Fimbriae distribution. The distribution of adhesive factors found in piglets in the different age groups is shown in Table 2. In Table 3 the number of different fimbriae in O groups 101, 9, 64, 20, 8, and 141 and among strains not belonging to any established O group is summarized.

The frequency of K88 antigen was 98% among the O149 strains; thus, it was much more frequent in age group 2 than in age group 1. Aside from O group 149, K88 fimbriae were only found in one strain, which belonged to O group 8.

K99 antigen was demonstrated in 57 strains and was found most frequently in O groups 101 (31 strains) and 64 (15 strains). One or a few strains with K99 were demonstrated in

TABLE 1. Enterotoxin patterns of porcine *E. coli*

Strain	Enterotoxigenicity test result by:		
	Adrenal Y1 cell	Infant mouse	Pig loop (3–7 weeks)
LT + ST pig	+	−	+
LT + ST pig-ST mouse	+	+	+
ST pig	−	−	+
ST pig-ST mouse	−	+	+
ST mouse	−	+	−

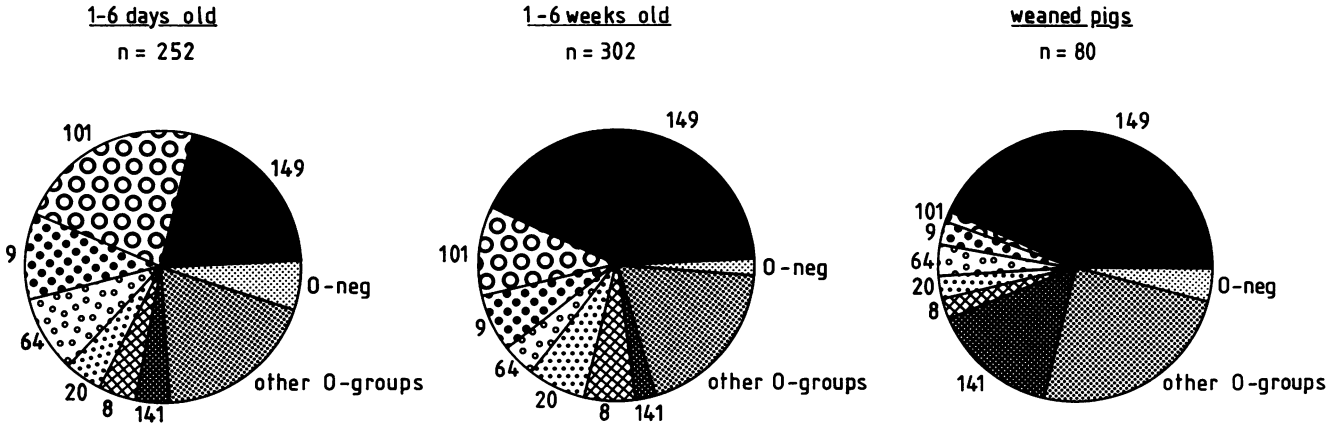

FIG. 1. Distribution of O groups among 634 enterotoxigenic strains from piglets in the different age groups. The 222 nonenterotoxigenic strains that were in-house nontypable were excluded. The blackened area represents O group 149; and thereafter, in counterclockwise order, are given results for O groups 101, 9, 64, 20, 8, 141, other O groups, and enterotoxinogenic strains not belonging to any established O group (O-neg).

O groups 9, 8, 140, and 141. Four K99$^+$ ST mouse-producing strains did not belong to any of the established O groups. A total of 44 K99$^+$ strains produced ST mouse only, while only 12 K99$^+$ strains produced ST mouse-ST pig and one K99$^+$ strain produced LT-ST mouse-ST pig.

Of the 57 K99$^+$ strains, 48 were found in piglets in the youngest age group, and of the 7 strains in age group 2, all except 1 were isolated from 1- to 2-week-old piglets.

987P antigen was demonstrated in 26 strains, most of which belonged to O groups 141 and OX46 and enterotoxinogenic strains not belonging to any established O group. 987P fimbriae were only detected in pigs belonging to the two younger age groups, with 16 and 10 strains from age groups 1 and 2, respectively. The latter 10 strains all originated from piglets that were only 1 to 2 weeks old.

F41 antigen was found in only two strains (O group 101); in one of them it was found together with K99 antigen.

In up to 58% (114 of 198) of the enterotoxinogenic strains no adhesive factor could be demonstrated.

TABLE 2. Adhesion factors and enterotoxin production in 420 strains from piglets in different age groups, with the addition of all 214 strains belonging to O-group 149[a]

Age group and O group	No. of strains					
	O149	ST pig	ST pig-ST mouse	ST mouse	Nonentero-toxinogenic	Total
Group 1						
K88	49	0	0	0	0	49
K99	0	0	9	39	0	48
987P	0	0	13	3	0	16
F41	0	0	0	1	0	1
None	2[b]	12	14	19	91	138
Total	51	12	36	62	91	252
Group 2						
K88	126	0 (1)[c]	0	0	0	127
K99	0	0	2 (1)	4	0	7
987P	0	0	5	5	0	10
F41	0	0	0	(1) K99$^+$	0	0
None	2[b]	14 (5)	12 (1)	13	111	158
Total	128	14 (6)	19 (2)	22	111	302
Group 3						
K88	35	0	0	0	0	35
K99	0	0	1	1	0	2
987P	0	0	0	0	0	0
F41	0	0	0	0	0	0
None	0	7	13 (1)	3	19	43
Total	35	7	14 (1)	4	19	80

[a] Nonenterotoxinogenic strains of unknown O group (n = 222) were not included.

[b] K88-negative strains belonging to O group 149.

[c] Values in parentheses indicate the number of strains that produced LT as well.

Distribution of enterotoxinogenic strains. The distribution of enterotoxin-producing strains in different age groups and in the most common O groups is given in Tables 2 and 3, respectively. The frequency of ETEC strains was high in some O groups. Aside from strains in O group 149, which were not investigated in this study, there was a high percentage of such strains in O groups 101, 64, and 141. In age group 1 the frequency of ETEC strains was greater than 60% in O groups 101, 64, and 141. Of the ETEC strains in O group 101 producing ST mouse enterotoxin, 75% carried K99, which is in agreement with results of earlier studies done in Sweden (30).

Of the 22 enterotoxic strains that did not belong to any of the established O groups, 15 strains produced ST pig-ST mouse, 6 strains produced ST mouse only, and one strain produced ST pig only.

Altogether, 33 enterotoxinogenic strains produced ST pig only, 88 strains produced ST mouse only, and 69 strains produced ST pig-ST mouse. LT was produced by a total of nine strains and was always produced together with ST pig, and in three of these strains ST mouse was also present. Strains producing ST pig only were most common in O groups 101, 64, 141, 157, and 32 and were distributed among pigs in all age groups. On the contrary, ST mouse was most frequent in piglets belonging to age group 1. No adhesive factor could be demonstrated in any of the strains producing ST pig only.

Of the 114 enterotoxinogenic strains that lacked demonstrable adhesin, 33 produced ST pig only, 35 produced ST mouse only, and 39 produced ST pig-ST mouse. In addition, seven strains produced LT together with ST pig, two of which also produced ST mouse. ETEC strains lacking adhesin were most frequent in O groups 141, 101, and 64; and many did not belong to any of the established O groups.

TABLE 3. Adhesion factors and enterotoxin production in the most common O groups except 149[a]

O group	No. of strains				
	ST pig	ST pig-ST mouse	ST mouse	Nonentero-toxinogenic	Total
101					
K99		3	28		31
987P			1		1
F41			1 (1)[b]		1
None	1	5	6	46	58
Total	1	8	36	46	91
9					
K99			4		4
987P		1			1
None	2	1	2	35	40
Total	2	2	6	35	45
64					
K99		8	7		15
None	1	3	5	13	22
Total	1	11	12	13	37
20					
987P			1		1
None		1	4	28	33
Total		1	5	28	34
8					
K99		1			1
987P		1	1		2
None	3 (3)[c]	1	2	23	29
Total	3	3	3	23	32
141					
K99		1 (1)			1
987P		7	1		8
None	6 (1)	8	4	3	21
Total	6	16	5	3	30
O-neg[d]					
K99			4		4
987P		8			8
None	1	7	2	0	10
Total	1	15	6	0	22

[a] All age groups are included.
[b] This strain had both K99 and F41 fimbriae.
[c] Values in parentheses indicate strains that produced LT as well.
[d] Strains not belonging to any established O group.

DISCUSSION

The frequency of the various O groups in Swedish investigations has changed substantially since the study done in 1964 to 1967, when O group 8 was most common in 1- to 10-day-old piglets, followed in decreasing order by 141, 147, and 149 (31) (Fig. 2). In the material from 1975 and 1976 to 1980, O group 149 was most frequent among neonatal piglets (33, 35), while in the present study O101 was the most frequent in piglets in this age group.

Among the 1- to 6-week-old piglets, O149 was still the dominant O group in the present study, followed by O groups 101, 9, 20, and 8, in decreasing order. This finding is interpreted here as the fact that passive protection by the vaccinated sow is diminished when the piglets are older than 1 week (35). Fewer strains were investigated in weaned pigs; still, O group O149 also occurred very frequently in these older animals (Fig. 1).

In studies from other countries, O group 149 was most frequent in Norway (13), the German Democratic Republic (36), and the midwestern United States (37) and was among the most common of the O groups in reports from Brazil (2) and Australia (19). In a study by Wilson and Francis (37) of *E. coli* strains from 1- to 7-week-old pigs with colibacillosis in the United States, O groups 149, 20, 141, 157, 8, 109, and 9 were the most common.

Thus, in Sweden, O group O149 was dominant among the neonatal piglet population with diarrhea for more than 15 years, until sow vaccination became common. However, this O group is still dominant in piglets over 1 week of age. During the same period, O groups 8 and 141 decreased in frequency, while O groups 101 and, possibly, 64 increased (Fig. 2). Despite this decrease, O group 141 was still the second most frequently found O group in weaned pigs (Fig. 1).

With the aid of biochemical fingerprinting in the Phenoplate system, Kühn et al. (12) demonstrated that the O149 strains isolated in Sweden between 1964 and 1984 have been remarkably stable during this 20-year period and seem to have had a common origin. Also, the earlier dominating O8 strains appear to have had a common origin; but with time the clone apparently started to deteriorate; i.e., it lost virulence factors and showed alterations in biochemical properties. The fact that the O149 strains occurred less frequently among the neonatal piglets in the present study than in the material from 1975 (33) and in an investigation from 1976 to 1980 (35) seems to be a result of the widespread treatment of sows with vaccines containing O149 and K88 antigens during the last 5 to 10 years. However, during 1985 and 1986, isolated strains of O149 showed signs of unstable genetic characters (unpublished data); thus, this phenotype may have started to deteriorate as well.

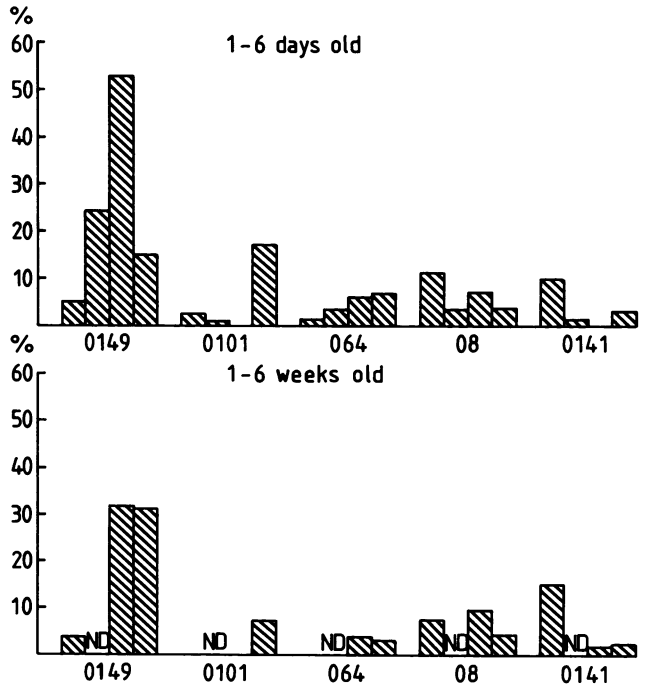

FIG. 2. Relative frequency of O groups 149, 101, 64, 8, and 141 in piglets in age groups 1 (1 to 6 days old) and 2 (1 to 6 weeks old) in various Swedish investigations. The four columns represent results from material collected during the following different time periods, from left to right, respectively: 1964 to 1967, 1975, 1976 to 1977, and 1983 to 1984. ND, Not done.

When all of the various kinds of enterotoxin-producing and fimbriae-positive strains are considered together, their combined frequencies showed great variations among O groups, in agreement with results of other studies (see Gaastra and de Graaf [7] and Wilson and Francis [37]). In the present study, 98% of the O149 strains produced K88 antigen. Franklin et al. (5) showed that the determinants for LT, ST mouse, and K88 reside on different plasmids in the O149 strains and that the stable content of two or three of these virulence plasmids in the O149 strains may explain their epidemiological importance. In addition to O group 149, high frequencies of enterotoxinogenic strains were found in O groups 101, 64, and 141. ST mouse was the dominating enterotoxin produced among the O101 strains, and interestingly, a higher percentage of enterotoxin-producing strains (38 of 59) was isolated from the youngest piglets (1 to 6 days old) than from among strains isolated from older piglets (6 of 31). In O groups 9 and 64, the tendency was the same. In the present study, as well as in the investigation by Moon et al. (16), ST mouse strains were much more frequent than ST pig strains in piglets of up to 1 week of age.

Moon et al. (14) and Francis et al. (3) both found 987P to be the most frequent fimbrial antigen in strains from piglets less than 1 week old in the United States. In the present investigation, K99 was more frequent than 987P in this age group, but in older pigs both adhesins were found in low numbers.

Why were adhesins apparently absent in so many ETEC strains? Guinée and Jansen (9), like Nakazawa et al. (21), also found a high percentage of enterotoxinogenic porcine strains that did not carry K88, K99, or 987P. The agglutination method for determination of fimbriae may be insensitive, but it may also be a question of phase variation, as described for 987P by Fusco et al. (6). Moon et al. (14) found most of their 987P isolates only after they were grown in vivo. In the same study, most ST pig strains without detectable adhesin did not cause diarrhea in orally challenged neonatal piglets. In the present study, all LT^- ST $mouse^-$ ST pig^+ strains lacked demonstrable adhesin in vitro; and according to the results of Moon et al. (14), all of these strains should be tested in vivo for 987P antigen and for enteropathogenicity. So far, we have infected newborn piglets that had suckled their mother for 2 to 3 h with two of these strains without inducing any symptoms. Have the ST pig strains without adhesive factor increased because they have been cured of the plasmid responsible for adhesin production by vaccinating the sows?

In conclusion, vaccination of the Swedish sow population for 5 to 10 years with vaccines containing O149 and K88 antigens has apparently changed the pattern of virulent *E. coli*-inducing neonatal diarrhea during the first week of life; i.e., the frequency of O group 149 has been reduced, while that of O101 has increased to the same level as that of O149. K99:ST mouse strains now appear with an increased frequency during the first week of life. The most common identifiable phenotypes in 1- to 6-day-old piglets are O101:K99:ST mouse strains and O149:K88 strains. In piglets in both of the older age groups, O149:K88 strains dominate. In spite of all efforts, about one third of piglets and weaned pigs with diarrhea subjected to the battery of diagnostic procedures tested negative for enteropathogenic *E. coli*.

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