INFECTION AND IMMUNITY, Feb. 1977, p. 549-555

Vol. 15, No. 2
Printed in U.S.A.

Serological Identification of Pig Enterotoxigenic *Escherichia coli* Strains Not Belonging to the Classical Serotypes

P. A. M. GUINÉE,* C. M. AGTERBERG, W. H. JANSEN, AND J. F. FRIK

National Institute for Public Health, P. O. Box 1, Bilthoven, and Faculty of Veterinary Medicine, Utrecht, The Netherlands*

Received for publication 31 August 1976

Escherichia coli strains isolated from pigs suspected to have succumbed to *E. coli* enterotoxicosis and not belonging to the commonly incriminated (classical) serotypes (O8:K87:K88, O45:K88, O138:K81:K88, O141:K85:K88, O147:K89:K88, O149:K91:K88, and O157:K88) were tested for enterotoxigenicity in the ligated gut test (LGT) using pig intestine. Of 202 strains tested, 54 strains belonging to 13 different O groups were positive in the LGT. Four of these strains had K88 antigen and one possessed K99 antigen. The majority of the strains was not agglutinated by any of the standard OK antisera. Four new K antigens ("K200," "K442," "K2346," and "K2347") were provisionally designated. K200 was found in pig enterotoxigenic strains belonging to O group 8 and carrying flagellar antigen H31 and in non-enterotoxigenic non-motile strains of O group 8, as well as in O group 20 strains isolated from calves succumbing to *E. coli* septicemia in two countries. The provisional antigen K2346 was encountered in 18 enterotoxigenic strains with various O antigens from two countries. It is proposed to include these two K antigens into the international *E. coli* antigenic scheme. Attempts to demonstrate a common antigen in the nonclassical enterotoxigenic strains lacking K88 and K99 antigens failed.

Particular serotypes or OK antigen types of *Escherichia coli* may cause severe diarrhea in pigs. Commonly incriminated (classical) OK types are O8:K87:K88, O45:K88, O138:K81:K88, O141:K85:K88, O147:K89:K88, O149:K91:K88, and O157:K88. Enterotoxigenic strains of these OK types also occur without the K88 antigen, with the exception of O45:K88 and O157:K88 (24). The K88 antigen enables the bacteria to attach to the intestinal wall and to reach large numbers in the anterior small intestine (7). Production of K88 antigen is plasmid controlled (14). The diarrhea is caused by the production of enterotoxins (6, 11, 19). Two types of enterotoxins are distinguished. The heat-stable enterotoxin is produced by all classical pig enteropathogenic *E. coli* strains; the heat-labile enterotoxin is produced only by strains possessing the K88 antigen (4, 17). Production of both types of enterotoxin is controlled by plasmids termed *Ent* (17, 20). The ligated gut test (LGT) in 6-week-old pigs has become the main criterion for enterotoxigenicity of porcine *E. coli* strains (5, 12, 18). Heat-stable as well as heat-labile enterotoxins were found to dilate pig intestine (17). Another type of enterotoxin found in so-called atypical pig strains failed to dilate intestine of 6-week-old pigs, but dilated the intestine of pigs under 1 week of age. This enterotoxin is also plasmid controlled (21).

The prerequisites for enteropathogenicity, K88 antigen, and enterotoxin production are plasmid controlled. This leads to the expectation that strains with serotypes not commonly associated with diarrhea in pigs may also possess one or both of the plasmids *Ent* and K88. During recent years, we received about 400 *E. coli* strains that were suspected to have caused enterotoxicosis in pigs, but did not belong to any of the classical serotypes. The aim of the present study was to characterize such strains in terms of their O, K, and H antigens and to test them for enterotoxigenicity and the presence of K88 antigen. The presence of the recently established K antigen K99 (3, 15), being a common antigen of calf enterotoxigenic *E. coli* strains, was also sought. Also tested was whether the strains possessed an undescribed common antigen.

MATERIALS AND METHODS

Strains. The strains studied were selected from strains that had been isolated from newly born piglets suspected of *E. coli* enterotoxicosis at 10 Dutch veterinary diagnostic laboratories, which had been sent to us for serotyping during 1971 through 1973. The strains do not reflect the true frequency distribution of serotypes in our country, because *E. coli* OK antisera required for the identification of classical serotypes are commercially available and so these strains are usually not sent for serotyping.

Cultures belonging to the classical pig enteropathogenic serotypes were not included in this study. Strains used in this study were selected from the routine material when: (i) their serotype or O group was relatively frequently encountered; (ii) they belonged to O groups that have incidentally been reported to be associated with intestinal disease in pigs such as O9, O10, O108, and O119 (24), O7 and O93 (22), O101 (9, 10), O35, O64, and O115 (22, 24), and OX46 (a provisional O group) (23); or (iii) they belonged to O or OK groups associated with infantile diarrhea (1) or diarrhea in other animals (16). All these strains have been given the prefix H to distinguish them from the standard O and K antigens kindly provided by F. Ørskov (Statens Seruminstitut, Copenhagen, Denmark). The type strain OX46 (23), as well as its O antiserum, were kindly supplied by O. Söderlind (National Veterinary Institute, Stockholm, Sweden). Strain N2361 (8) was kindly supplied by J. Manz (Bavarian Veterinary Investigation Institute, Nürnberg, Germany).

Media and serological procedure. Nutrient broth, nutrient agar, Minca, and D5 medium were prepared as previously described (3). Serotyping of *E. coli* and the recording of typing results, preparation and absorption of antisera, immunoelectrophoresis (IE), and double diffusion in gel were performed as previously described (2, 3). Two types of antigens were used in IE and double-diffusion tests. Aqueous extracts of cells were used to establish whether a strain contained a special thermostable K polysaccharide. For this purpose, cells were grown on D5 medium, harvested in 0.85% NaCl solution, heated at 60°C for 20 min, and centrifuged at 15,000 × *g* for 20 min, and the supernatant was heated at 100°C for 60 min, as described by Ørskov and Ørskov (13). This type of antigen will be referred to as crude heat extract (CHE). The other type of antigen was the supernatant of ultrasonically treated cells and was used to establish whether strains with different serotypes shared a common antigen. Ultrasonically treated materials were prepared from cells grown on Minca medium at 37°C and termed US37. Details of this procedure were described previously (3). Antisera for the latter type of study were prepared like OK antisera (2), with the exception that the number of injections was not limited to 4 but was extended to 10 over a period of 5 weeks. The additional injections were made with approximately 10^9 living cells intraveneously. Blood samples were taken after the injections 4, 6, and 10.

Enterotoxigenicity test. Enterotoxigenicity was established by means of the LGT. The surgical technique used was that described by Smith and Halls (19). Pigs used were 5 to 6 weeks old. Each strain was tested at least three times in different animals. The first ligated segment (10 cm in length) at about 40 cm distal of the stomach was inoculated with a known enterotoxigenic (Ent^+) strain with serotype O149:K91:K88:H10. The second segment was inoculated with a known non-enterotoxigenic (Ent^-) strain with serotype O2:K-:NM, which had been isolated from the feces of a healthy man. Positive and negative controls were repeated every 10 segments. A reaction in a particular segment was considered to be reliable when the distal and proximal positive and negative controls gave the proper reaction. About 40 segments per animal separated by noninoculated segments of 5 cm in length were prepared and inoculated with 3 ml of an overnight (37°C) aerated broth culture. The animals were autopsied about 20 h after inoculation. A reaction was recorded as positive if the LGT index (amount of fluid in milliliters/length in centimeters) was ≥1, provided that the strain inoculated could be reisolated from the segment at autopsy.

RESULTS

Enterotoxigenicity tests and serotyping. In total, 202 isolates belonging to 26 O antigen groups, including OX46, were tested for enterotoxigenicity. Fifty-four strains belonging to 13 O groups gave a positive reaction in the LGT. The LGT index observed was found to depend on the site of the segment in the small intestine. The anterior part of the small intestine was more sensitive than the posterior part. Strains evoking an LGT index of 2 or less were always retested in a more anterior segment in another animal. The results are summarized in Table 1. Only a few of the LGT-positive (Ent^+) strains reacted in the standard OK antisera, e.g., strains with seroformula O9:K55, O101:K28, and O101:K30. The strains belong-

TABLE 1. *O antigen of porcine E. coli strains tested for enterotoxigenicity by means of the LGT in pigs*

O antigen	No. of strains positive in LGT/no. of strains tested	LGT index (fluid in ml/length in cm)
O1	0/1	
O6	0/4	
O7	1/4	5–7
O8	16/49	4–12
O9	10/37	1–7
O10	0/2	
O16	3/3	4–10
O20	1/19	9
O25	1/11	2–4
O35	3/3	6–8
O54	2/11	0–1.5
O64	0/1	
O75	0/5	
O88	0/4	
O98	0/1	
O101	2/7	1
O108	11/11	1–2
O112	0/2	
O114	0/1	
O126	0/6	
O137	1/2	6
O142	1/2	4
O152	0/5	
O153	0/5	
O154	0/4	
OX46	2/2	2–5

TABLE 2. *Serotypes of nonclassical pig enterotoxigenic strains of E. coli*

Serotype[a]	No. of strains positive in LGT/no. of strains tested	LGT index (fluid in ml/ length)
O7:K-H4[a]	1/1	5-7
O8:K83:H21	5/5	5-7
O8:K200:H31	7/7	9-12
O8:K200:NM[b]	0/5	
O8:K442:H14	4/4	4-10
O9:K(A)2346:NM	6/7	1-6
OX46:K(A)2346:NM	2/2	2-5
O9:K(A)2347:K88:NM	3/3	5-7
O9:K55:NM	1/1	6
O16:K83:H20	3/3	4-10
O20:K?:K88:NM[c]	1/1	9
O25:K?:H23[c]	1/11	2
O35:K-:H6	3/3	6-8
O54:K-:H21	2/2	0-1.5
O101:K28:K99:NM	1/1	1
O101:K28:NM	0/1	
O101:K30:NM	1/2	1
O108:K-:H9	11/11	1-2
O137:K-:NM	1/2	6
O142:K-:NM	1/2	4

[a] K-, No K antigen.
[b] NM, Nonmotile.
[c] K?, Strain is not agglutinated in the living state by any of the O or OK antisera.

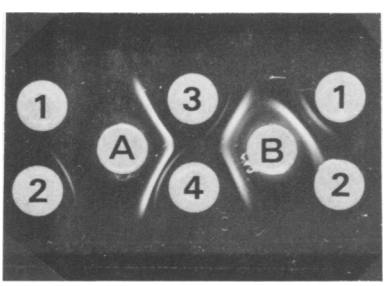

FIG. 1. *Double-diffusion tests with CHE of standard strains O8, O20, and O20:K83, strain O8:K83, and antisera prepared with O20:K83 and O8:K83. 1, CHE of O20; 2, CHE of O8; 3, CHE of O20:K83; 4, CHE of O8:K83; A, O8:K83 antiserum; B, O20:K83 antiserum.*

ing to O groups 7, 35, 54, 108, 137, and 142 were agglutinated in the living state in their homologous O antisera and apparently had no K antigen. None of the other strains agglutinated in the standard OK antisera nor in their homologous antiserum in the living state (Table 2). None of the strains belonging to O group 8 and found to be Ent$^+$ possessed a K antigen known to occur among O group 8 strains (1). Five O group 8 strains were strongly agglutinated by K83 antiserum. The K83 antigen was hitherto found only in combination with O antigen 20 (1). The three strains belonging to O group 16 also reacted strongly in K83 antiserum. Standard O20:K83 antiserum absorbed with either an O8:K83 strain or an O16:K83 strain no longer agglutinated the latter two strains nor its homologous strain O20:K83 in the living state. Double-diffusion and IE tests using standard O20:K83 antiserum and O8:K83 antiserum, and aqueous extracts of the standard strains O8, O20, and O20:K83 and strain O8:K83, confirmed that the K83 antigen is shared by the two strains (Fig. 1). The strains were therefore designated O8:K83:H21 and O16:K83:H20, respectively. All eight strains belonging to these two serotypes were strongly positive in the LGT. None of the other Ent$^+$, O group 8 strains reacted in any of the standard OK antisera nor in O8 antiserum as living culture. This might indicate that the O antigen was masked by a hitherto unknown K antigen. An OK antiserum was prepared with one of these strains (strain H200). The unknown K antigen was provisionally designated "K200." Twelve strains, including strain H200, were agglutinated by this antiserum, seven being Ent$^+$ and five Ent$^-$. All seven Ent$^+$ strains possessed flagellar antigen H31, whereas all five Ent$^-$ strains were nonmotile. Each of the four remaining O group 8 strains that were Ent$^+$ agglutinated only in an OK antiserum prepared with one of these strains (H442) and were provisionally designated O8:"K442":H14. Similar studies on O antigen 9 strains led to the

designation of two provisional K antigens: "K2346" and "K2347." These K antigens were only inactivated by heating at 120°C for 2.5 h, indicating that they belong to the A variety (1). One of the O9:K2346:NM strains was Ent$^-$; all other strains were Ent$^+$. Two strains with K2346 antigen did not react in any of the standard O antisera after heating at 120°C and were agglutinated to titer by OX46 O antiserum and termed OX46:"K2346":NM. The K antigen of strain OX46 was found to be identical to K2346 in absorption tests and double-diffusion tests (Fig. 2). Among twelve O antigen 25 strains tested, one was found to be Ent$^+$ (Table 1). The two Ent$^+$ strains of O group 101 carried K28 or K30 antigen. They gave only weak reactions in the LGT, even when tested in the most anterior segment (Table 2).

Immunoelectrophoresis with provisional K antigens. CHEs of strains possessing provisionally designated K antigens were studied by means of IE to establish whether these K antigens were represented by specific K polysaccharide. CHE of each strain was tested in comparison to CHE of the standard O antigen strain against their homologous O antiserum and OK antiserum prepared with the strain under test. The results are shown in Fig. 3. Specific polysaccharides were found for all provisionally designated K antigens. CHE of O9:K2346 failed to precipitate with O9 antiserum. However, CHE of standard strain O9 precipitated with O9:K2346 antiserum. This indicates that the latter antiserum indeed contains O9 antibodies, but that CHE of O9:K2346 strains does not contain enough O antigen to give a visible precipitation line. Similar observations were made with CHE of strains with OK type O9:K2347.

Presence of K88 and K99 antigen. All strains mentioned in Table 1 were tested for the presence of K88 and K99 antigen by means of the slide agglutination test after subculturing three times on Minca medium (3). K88 antigen was found in all three strains belonging to the provisional serotype O9:K2347:NM and in one strain belonging to O antigen group 20. K99 antigen was found in one strain having serotype O101:K28:NM. All five strains having K88 or K99 antigen were Ent$^+$.

Prevalence of provisional K antigens among strains isolated in 1974 and 1975. The OK antisera prepared with strains with provisionally designated K antigens were included in the set of OK antisera used for routine typing of strains isolated in 1974 and 1975. The results are summarized in Table 3. Of the provisional OK types, only O8:K83:H21, O8:K200:H31, and O9:K2346:NM were encountered among Ent$^+$ strains of pigs. A strain isolated from the lungs of the piglet and found to be Ent$^-$ and belong to O group 20 was strongly agglutinated in K200 antiserum. Absorption tests as well as double-diffusion tests using O8:K200 and O20:K200 antisera and aqueous extracts of the two strains and of standard strains O8 and O20 proved that the two first-mentioned strains share the provisionally designated K antigen K200 (Fig. 4). Strains with serotype O20:K200:NM were repeatedly isolated from calves that succumbed to *E. coli* septicemia. Strain N2361, described by Manz as the cause of *E. coli* septicemia in calves (8), was found to belong to the provisional serotype O20:K200:NM. The provisional antigen K2346 was not only found in Ent$^+$ strains with O group 9 from pigs, but also in O group 101 strains from calves.

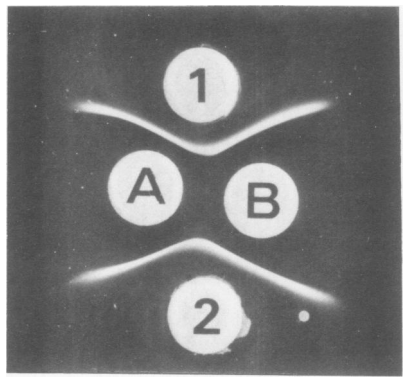

FIG. 2. *Double-diffusion test with CHE from a provisional K2346 type strain and strain OX46 (18) and homologous antisera. 1, OK antiserum prepared with O9:K2346; 2, OK antiserum prepared with OX46; A, CHE of O9:K2346; B, CHE of OX46.*

Attempts to demonstrate a common K antigen in nonclassical pig enterotoxigenic strains. Ultrasonically treated materials prepared from all Ent$^+$ and Ent$^-$ strains mentioned in Table 1, as well as the standard O antigen strains 8, 9, 16, 20, and 25, were tested in double diffusion and in IE against OK antisera prepared with the provisionally designated OK antigen type strains. At least six thermolabile antigens shared by many of the strains could thus be detected. They were shared not only by the Ent$^+$ strains, but also by many of the Ent$^-$ and the standard O antigen strains. Antigenic relationships were also detected by means of the slide agglutination test, using undiluted OK antisera. Thus, Ent$^+$ strains could not be separated from Ent$^-$ strains in this way. Absorption of O8:K200 antiserum (prepared with an Ent$^+$ strain) with an Ent$^-$ strain of serotype O8:K200:NM (Table 2) removed all precipitating antibodies when tested in IE.

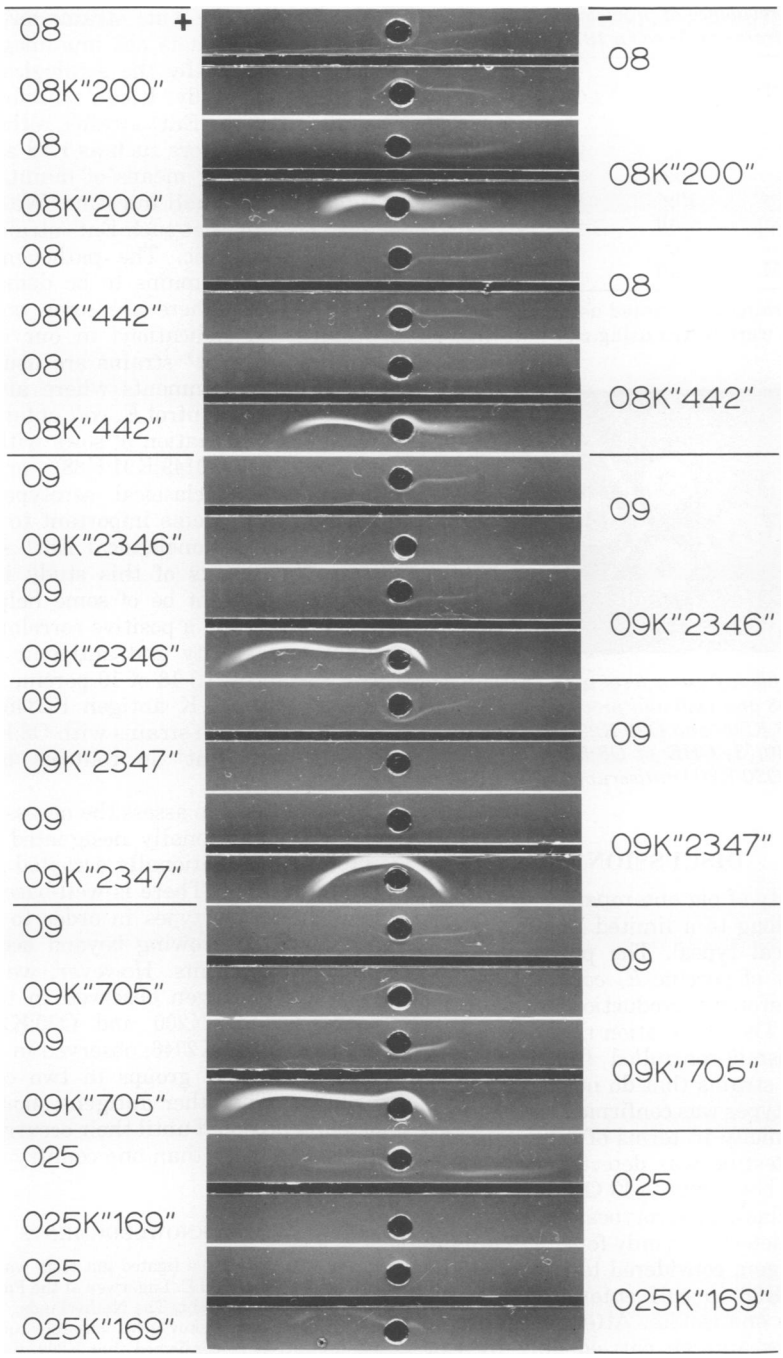

FIG. 3. *Immunoelectrophoretic patterns obtained with CHE of standard O strains and provisional K antigen type strains using homologous O antisera and autologous OK antisera. Antigens are given on the left-hand side, and antisera are given on the right-hand side.*

Moreover, the absorbed antiserum no longer agglutinated either of the two types of strains. Similar results were obtained when using O9:K2346 antiserum (prepared with an Ent^+ strain) absorbed with an Ent^- strain of serotype O9:K2346:NM (Table 2). Thus, no antigen could be detected that was carried only by Ent^+ strains.

TABLE 3. *Prevalence of provisional K antigens among strains isolated in 1974 and 1975*

(Provisional) serotype	Source	LGT[a]	No. of strains encountered
O8:K83:H21	Pig	+	2
O8:K200:H31	Pig	+	2
O20:K200:NM	Pig	−	1
O20:K200:NM	Calf	−	25
O9:K2346:NM	Pig	+	10
O101:K2346:NM	Calf	−	5

[a] Porcine strains were tested using pig intestine; bovine strains were tested using calf intestine.

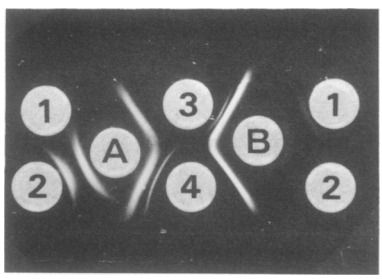

FIG. 4. *Double-diffusion tests with CHE of standard strains O8 and O20 and provisional K antigen type strains O8:K200 and O20:K200. 1, CHE of O8; 2, CHE of O20; 3, CHE of O8:K200; 4, CHE of O20:K200; A, O20:K200 antiserum; B, O8:K200 antiserum.*

DISCUSSION

The majority of pig enteropathogenic strains of *E. coli* belong to a limited number of serotypes (classical types). The prerequisites for pathogenicity of porcine *E. coli* strains, e.g., K88, and enterotoxin production are controlled by plasmids. The expectation that one or more of these plasmid-controlled functions might also occur in strains that do not belong to the classical serotypes was confirmed by this study. Enterotoxigenicity in terms of dilatation of ligated pig intestine was detected in 54 of 202 (27%) strains belonging to 13 O groups not covered by the classical serotypes. However, K88 antigen was detected in only four (2%) isolates, and K99 antigen, considered to have a similar function as K88 in calf enterotoxicosis, was found only in one isolate. Attempts to demonstrate another antigen carried only by Ent$^+$ strains and not by Ent$^-$ strains failed. We found in IE experiments at least six different thermolabile antigens that were shared by many of the strains, Ent$^+$ as well as Ent$^-$ strains. If one of the antigens represented an attachment factor, many Ent$^-$ strains would also possess the attachment factor. It might also be that the Ent$^+$ strains have an attachment factor that is not immunogenic and escapes detection by the serological techniques used in this study. It can therefore not be excluded that the Ent$^+$ strains, although lacking attachment factors such as K88 and K99 antigen, have other means of maintaining themselves in the small intestine. We would therefore consider that such Ent$^+$ strains are potentially pathogenic. The pathogenicity of the Ent$^+$ strains remains to be demonstrated by future work. There are some not very well-documented indications in our country that nonclassical Ent$^+$ strains are usually encountered in environments where attempts have been made to control *E. coli* enterotoxicosis by means of vaccination of sows with heat-killed monovalent (O149:K91:K88) or polyvalent (more or all classical serotypes) vaccines. Therefore, it seems important to monitor the prevalence of nonclassical Ent$^+$ strains of *E. coli*. The results of this study indicate that serotyping might be of some help in this respect. We found a positive correlation between enterotoxigenicity and antigenic structure in two instances: (i) 18 of 19 porcine strains with the provisional K antigen K2346 were Ent$^+$; and (ii) all seven strains with O8:K200:H31 antigens were Ent$^+$ in contrast to nonmotile O8:K200 strains.

It is difficult to assess the necessity of including the provisionally designated K antigens into the internationally accepted *E. coli* serotyping scheme. There is well-based reluctance to include new types in order to prevent the scheme from growing beyond acceptable and manageable limits. However, we recommend that K200 antigen observed in two different pathogens, O8:K200 and O20:K200, in two countries, and K2346, observed in Ent$^+$ strains with various O groups in two countries, be included. The other antigens should be provisionally accepted until their occurrence in Ent$^+$ strains in more than one country has been established.

ACKNOWLEDGMENTS

The surgery for the ligated gut tests was performed by M. van der Velde and E. Lagerwey at the Faculty of Veterinary Medicine, Utrecht, The Netherlands.

Frits and Ida Ørskov gave valuable comments on the manuscript. They confirmed that K200, K442, and K2347 are new K antigens, whereas K2346 is identical to a new but hitherto undescribed K antigen, K103. They also confirmed the serotypes O8:K83:H21 and O16:K83:H20.

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