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Behavior of *Escherichia coli* K Antigens K88ab, K88ac, and K88ad in Immunoelectrophoresis, Double Diffusion, and Hemagglutination

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Porcine enteropathogenic *Escherichia coli* strains were found to possess a variant of the K88 antigen provisionally termed K88ad. We propose to include this antigen into the international *E. coli* typing scheme. Ultrasonic extracts of field strains of *E. coli* possessing the K88ab, K88ac, or K88ad antigen and their *E. coli* K-12 K88$^+$ transconjugants showed a specific K88 precipitation line in immunoelectrophoresis and double diffusion only when grown at 37°C, but not when grown at 18°C. By using agarose gels, K88ab, K88ac, and K88ad antigens showed anodic mobility in immunoelectrophoresis. When using Difco Noble agar gels, K88ad was not mobile or anodic, K88ab was cathodic; K88ac of 17 strains was cathodic and of 24 strains was anodic. The immunoelectrophoretic behavior of a K88 antigen (K88ab, K88ac, or K88ad) did not alter after transfer of the corresponding plasmid to *E. coli* K-12. Anodic and cathodic K88ac antigens could not be distinguished serologically. The differences between the results obtained in Noble agar gels and agarose gels are due to electro-endosmotic flow. We describe a procedure which increases the detection level of K88$^+$ transconjugants in a mating mixture. It is based on the specific mannose-resistant attachment of K88$^+$ cells to guinea pig erythrocytes.

The detection of the K88 antigen in porcine *Escherichia coli* strains is indicative for the enteropathogenicity of the strains, because K88$^+$ strains are nearly always also enterotoxigenic (15). Two serological variants of the K88 antigen, K88ab and K88ac, have been accepted internationally. Additional, minor variations of the K88ac antigen have been observed, but these have not been taken into the diagnostic scheme (9).

E. coli infections in neonatal piglets in The Netherlands are controlled by immunization of sows with vaccines containing K88 antigen. Cases of neonatal diarrhea are monitored at veterinary diagnostic laboratories, particularly when the disease occurs in vaccinated herds. Suspected enteropathogenic *E. coli* strains are serotyped with commerically available OK antisera at these laboratories. Strains which cannot be fully typed are sent to us for confirmation. The strains thus received can be subdivided into two groups: (i) nonclassical OK types producing enterotoxin (4) and (ii) classical or nonclassical OK types which react in K88ab and K88ac antisera, but not in monofactor K88b or K88c antisera.

This report describes the behavior of the K88ad antigen in hemagglutination (HA), immunoelectrophoresis (IE), and double diffusion, in comparison with that of the K88ab and K88ac antigens. We describe how the detection level of K88$^+$ transconjugants in a mating mixture can be increased by adhesion to erythrocytes of the K88$^+$ cells.

MATERIALS AND METHODS

Bacterial strain and media. The *E. coli* K-12 strain used was a nalidixic acid-resistant mutant of the lactose-negative C600. *E. coli* standard strains and field isolates, collected in 1972 through 1978, are listed in Table 1. The strains were kept on cork-stoppered Dorset slopes at room temperature. Liquid medium was Trypticase soy broth (TSB; Biomérieux, France). Solid medium was TSB agar (TSB plus 1.2% agar, Oxoid). Raffinose medium consisted of a medium with the formula of MacConkey medium (as from Difco) except that lactose was replaced by 1% raffinose (Merck) and neutral red was added to a final concentration of 0.009% instead of 0.003%.

Serological procedures. The preparation of ultrasonic extracts (US) from cells grown at 18°C (US18) or at 37°C (US37) was as previously described (3), except that the cells were harvested in 0.022 M Sörensen phosphate buffer (pH 7.3) in a concentration of 10^{11} cells per ml. Preliminary investigations indicated that ultrasonic oscillation yielded more K88 antigen than other techniques, such as heating at 60°C and shearing (16), or freezing and thawing. IE was per-

TABLE 1. *Standard strains and field isolates of porcine E. coli used*

OK type	Designation of standard strain (reference)	No. of field isolates (reference)
O8:K87:K88ab	G7[a] (9)	1
O8:K87:K88ac	G205[a] (9)	4
O8:K87:K88?[b]	H56[c]	7
O8:K?:K88ac		1
O8:K?:K88?[b, d]	H70[c]	1
O9:K"2347":K88?[b, e]		1 (4)
O9:K(A)?:K88?[b, d]	H110[c]	
O20:K?:K88ac[d]		1 (4)
O45:K88ac		7
O138:K81:K88ac	G491[a] (9) H1360[c]	14
O141:K85:K88ab	E68[a] (9)	
O147:K89:K88ac	G1253[a] (9)	2
O149:K91:K88ac	A1[a] (9)	2
O157:K88ac	A2[a] (9)	4

[a] Reference strains obtained from the World Health Organization Collaborative Centre for Reference and Research on *Escherichia*, Copenhagen.

[b] K88? = strain agglutinates in K88ab and K88ac antisera, but not in monofactor K88b and K88c antisera. Strains with formula O8:K87:K88? were isolated for the first time in 1973.

[c] Standard strains used in this laboratory for the production of K88 antisera. Strains H70 and H110 were isolated in 1978.

[d] K? = strain does not agglutinate in the living state in any of the *E. coli* O or OK antisera except K88. K(A)? = inactivated only after heating at 120°C.

[e] K"2347" is a provisional K antigen (4).

formed by the method of Scheidegger (11) as described by Ørskov et al. (7) with a modification previously described (4) of gels prepared with 1.5% Difco Noble agar or with 1% type 1 low EEO (electroendosmosis) agarose from Sigma. Double diffusion was done as described (4). Antisera were prepared with the methods previously described for the production of OK antisera (2). The rabbits were selected for the absence of antibodies to the standard and reference strains listed in Table 1 by testing proof bleedings in double diffusion tests against US37 of these strains before immunization. Antigens for immunization were preferably prepared from *E. coli* K-12 K88$^+$ transconjugants.

Specific K88 antisera could then be obtained by absorption with *E. coli* K-12 grown at 37°C. Properly absorbed K88 antisera showed no precipitation lines with US18 in double diffusion and did not agglutinate with strains grown at 18°C. The antisera used are listed in Table 2.

HA. The finally adopted procedure of HA was based on those described by Jones and Rutter (6) and Jones and Freter (5) with slight modifications. Whole blood from guinea pigs was freshly collected with sodium citrate (3.8%, wt/vol, in distilled water) and washed three times in phosphate-buffered saline ($Na_2HPO_4 \cdot 2H_2O$, 1.44 g; KH_2PO_4, 0.27 g; NaCl 8.7 g in 1,000 ml of distilled water). The packed cells were suspended in a modified Krebs-Ringer-tris(hydroxymethyl)aminomethane solution, pH 7.4 (KRT, 6) or in KRT plus 0.5% mannose (KRTM) to give 1% (vol/vol) suspensions. The HA was done in 8-mm-ID glass tubes with round bottoms in melting ice (0°C). Bacterial cells grown on solid medium or in liquid medium with aeration (250 strokes per min) were collected and suspended in KRT or KRTM to give 5 $\times 10^9$ cells per ml. The cells were serially diluted to 1:1,024 in KRT or KRTM. To 0.2 ml of this antigen, 0.2 ml of 1% erythrocyte solution was added. The control tube contained 0.2 ml KRT or KRTM instead of antigen. The HA was read after 3 h of incubation at 0°C.

Transfer of K88 and enrichment of K88$^+$ transconjugants. K88 antigen can be transferred along with the ability to ferment raffinose (*raf*). K88$^+$ transconjugants can be obtained by testing raffinose-fermenting (*raf*$^+$) transconjugants for the presence of K88 antigen (12, 14). The mating procedure was basically that described by Smith and Halls (13) by using the nalidixic acid resistance marker of the recipient for selection. Ten milliliters of TSB was seeded with 0.2 ml of a 24-h culture in TSB of the prospective donor and 0.2 ml of a similar culture of the *E. coli* K-12 recipient. The mixed culture was incubated at 37°C for 24 h, and then 0.2 ml was inoculated into 10 ml of TSB containing 30 μg of nalidixic acid per ml. After 24 h of incubation at 37°C, 0.2 ml of this material was inoculated into another 10 ml of the nalidixic acid-containing TSB. After 24 h of incubation at 37°C, 0.2 ml was subcultured into 10 ml of plain TSB. This culture is referred to as the final mating mixture. If the donor was *raf*$^+$, the final mating mixture was subcultured onto raffinose medium containing 30 μg of nalidixic acid per ml. *raf*$^+$ colonies were tested for the K88 antigen by slide agglutination and confirmed to be *lac*$^-$. The transfer efficiency was estimated from the ratio between *raf*$^+$ and *raf*$^-$ colonies.

If the donor was *raf*$^-$, the final mating mixture was subcultured onto TSB agar with 30 μg of nalidixic acid per ml. This will be referred to as the direct method. For enrichment of K88$^+$ transconjugants, 10 ml of the final mating mixture was centrifuged at 3,000 $\times$ *g* at 4°C for 10 min. The sediment was suspended in 1 ml of KRTM, to which was added 0.5 ml of a 10% solution

TABLE 2. *List of absorbed K88 antisera*

Designation of antiserum[a]	Prepared with antigen[b]	Absorbed with:
K88ab(G7)	G7	K-12, 37°C[c]
K88ac(G205)	G205	K-12, 37°C
K88ac(H1360)	H1360	H1360, 18°C
K88ad(H56)	H56	K-12, 37°C
K88ad(H70)	H70	K-12, 37°C
K88ad(H110)	H110	H110, 18°C

[a] The designations K88ab and K88ac have been accepted internationally (8). The designation K88ad is preliminary and hypothetical.

[b] All strains used for immunization were *E. coli* K-12 K88$^+$ transconjugants of the strains listed, except H1360 and H110.

[c] K-12, 37°C = *E. coli* K-12 grown at 37°C.

of mannose in KRT and 1 ml of 1% guinea pig erythrocytes in KRTM. The mixtures were gently shaken at 4°C for 2 h and then carefully laid upon 6 ml of a 15% Ficoll solution in KRTM (Ficoll 400, Pharmacia, Uppsala, Sweden) in a polyallomer tube (Beckmann no. 326814). The tubes were centrifuged in a swing-out rotor for 5 min at 400 × *g* at 4°C and then frozen at −30°C. After freezing, the bottom of the tube containing the sedimented erythrocytes and, expectedly, the K-12 K88$^+$ cells attached to them, was cut off and transferred into 4 ml of sterile 0.4% NaCl solution prewarmed at 37°C. Incubation at 37°C for 10 min resulted in complete hemolysis, and the mixture was then loop-streaked onto TSB agar and/or raffinose medium containing 30 μg of nalidixic acid per ml. This method is referred to as the Ficoll sedimentation method.

RESULTS

HA. Our results confirmed earlier published observations (5, 6) that HA of guinea pig erythrocytes by K88 antigen is completely mannose resistant. The highest HA titers were obtained with cultures grown on solid media and varied from 1/32 to 1/1,024 per strain. There was no correlation between HA titers and the serological variant of the K88 antigen. Erythrocytes from five different guinea pigs gave the same HA titers per strain. There was no difference in HA titer between standard or field strains and their K-12 K88$^+$ transconjugants. When US37s of K88$^+$ strains were tested in HA, titers varying from 1/2 to 1/8 were obtained. K88$^+$ cultures grown at 18°C gave always titers of <1/2. The HA titers were much lower when the cultures under test had been grown in the presence of nalidixic acid or streptomycin.

Transfer of K88 antigen to *E. coli* K-12. It was attempted to transfer the K88 antigen of 21 standard and field strains to *E. coli* K-12 because of two reasons: (i) a number of K88$^+$ transconjugants was needed for production of K88 antisera, and (ii) US37s of a number of K88$^+$ transconjugants were tested in IE to compare their electrophoretic behavior to that of their parent strains (see below). Of the 21 strains tested, 19 were *raf*$^+$. K88$^+$ transconjugants were looked for by testing *raf*$^+$ transconjugants for K88 antigen. All *raf*$^+$ colonies were found to possess the K88 antigen. Sixteen of the 19 *raf*$^+$ strains transferred their K88 antigen. Strain G1253 (O147:K89:K88ac) transferred its K88 antigen very efficiently, because about 10% of the colonies grown on the selective plate were *raf*$^+$ and K88$^+$, using the direct method. The other 15 *raf*$^+$ strains transferred their K88 antigen with much lower efficiency. The number of K88$^+$ transconjugants obtained with the Ficoll sedimentation method was 50- to 100-fold higher than that obtained with the direct method which yielded usually not more than one or two *raf*$^+$ colonies per plate. The *raf*$^+$ strains with formula O8:K?:K88ac, O20:K?:K88ac and O9:K(A)?:K88? (= strain H110) did not transfer their K88 antigen with the two methods. The two type O149:K91:K88ac field isolates were *raf*$^-$. With the direct method, 0/50 colonies tested in each mating was K88$^+$. With the Ficoll sedimentation method, 10/12 and 11/12 colonies, respectively, were K88$^+$.

Evaluation of antisera in double diffusion and establishment of the K88d antigen. US37s of the strains G7, G205, H56, H70, and H110 were tested in double diffusion tests against their K88 antisera listed in Table 2. The sera were further cross-absorbed and tested in double diffusion and slide agglutination. The

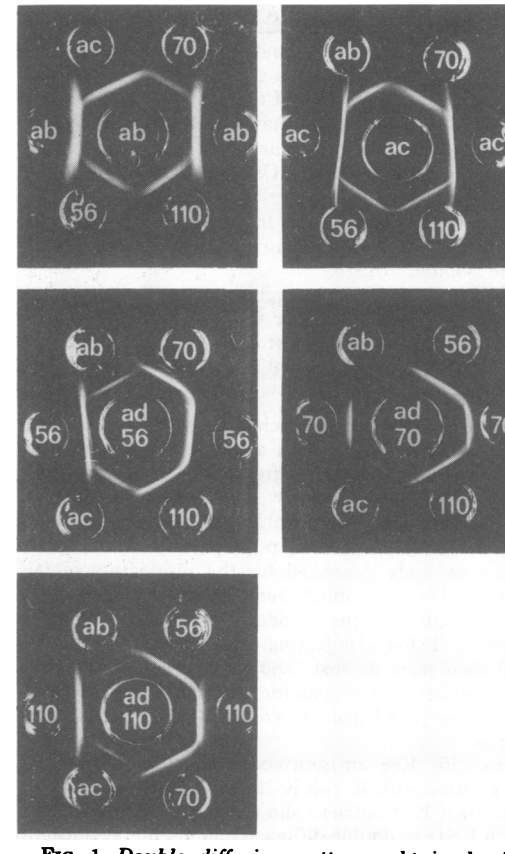

FIG. 1. *Double diffusion patterns obtained with K88 antigens and antisera. Outer wells contain US37 of: ab = strain G7 (O8:K87:K88ab); ac = G205 (O8:K87:K88ac); 70 = H70 (O8:K?:K88ad); 110 = H110 (O9:K(A?):K88ad); 56 = H56 (O8:K87:K88ad). K88ad is a preliminary and hypothetical designation. Antisera in center wells: ab = K88ab(G7); ac = K88ac(G205) or K88ac(H1360); ad56 = K88ad(H56); ad70 = K88ad(H70); ad110 = K88ad(H110) (see Table 2).*

TABLE 3. *Reactions of cross-absorbed K88 antisera with K88⁺ standard strains of E. coli*

Designation of antiserum	Absorbed with	Results of double diffusion and slide agglutination tests with strains:					
		G7 K88ab	G205 K88ac	H1360 K88ac	H56 K88ad	H70 K88ad	H110 K88ad
K88ab(G7)	G205 or H1360	+	−	−	−	−	−
	H56, H70, or H110	+	−	−	−	−	−
K88ac(G205)	G7	−	+	+	−	−	−
	H56, H70, or H110	−	+	+	−	−	−
K88ac(H1360)	G7	−	+	+	−	−	−
	H56, H70, or H110	−	+	+	−	−	−
K88ad(H56)	G7, G205, or H1360	−	−	−	+	+	+
	H70 or H110	−	−	−	−	−	−
K88ad(H70)	G7, G205, or H1360	−	−	−	+	+	+
	H56 or H110	−	−	−	−	±	−
K88ad(H110)	G7, G205, or H1360	−	−	−	+	+	+
	H56 or H70	−	−	−	−	−	±

results, presented in Fig. 1 and Table 3, indicate that all five strains have one K88 antigen in common: K88a. Strain G7 and G205 each have one additional specific determinant that has been termed previously K88b and K88c, respectively. The strains H56, H70, and H110 do not possess K88b or K88c antigen, but have another K88 antigen in common, preliminarily termed K88d. The K88ad antisera contain less K88a antibodies than the K88ab and K88ac antisera. Absorption of the K88ad antisera with autologous strains removed all antibodies. Absorption of the antisera K88ad(H70) and K88ad(H110) with heterologous K88ad antigens yielded antisera which reacted undiluted very weakly with their homologous antigens only. All strains written as K88? in Table 1 reacted in K88d antiserum.

IE in Noble agar and in agarose. US37s of the strains G7, G205, H1360, H56, H70, and H110 were tested immunoelectrophoretically in Noble agar (NIE) against their K88 antisera listed in Table 2. Against all antisera, G7 and H1360 showed a cathodic line, and G205, H70, and H110 showed an anodic line, whereas the K88 antigen of H56 did not move in NIE and formed a center line. A representative result is given in Fig. 2. US37 of all strains listed in Table 1 and US37 of the *E. coli* K-12 K88⁺ transconjugants were tested in NIE. All three K88ab⁺ strains formed a cathodic line. The K88ad⁺ strains formed a center line or an anodic line. The K88ac⁺ strains formed an anodic or a cathodic line. There was no correlation between the serotype of the strain from which K88 had been isolated and the NIE behavior of the K88 antigen (Table 4). The behavior in NIE of a K88 antigen did not change after transfer to *E. coli* K-12. US37s of strains with anodic or cathodic K88ac antigens were identical in double diffusion tests against K88ac antisera prepared with an anodic (strain H1360) or a cathodic (strain G205) K88 antigen. When K88ac antiserum prepared with H1360 (anodic) was absorbed with strain G205 (cathodic), or inversely, all antibodies were removed. None of the strains with K88ac antigen reacted in K88d antisera. Thus, we found no indication that anodic and cathodic K88ac antigens are antigenically different. There was no correlation between the behavior in IE

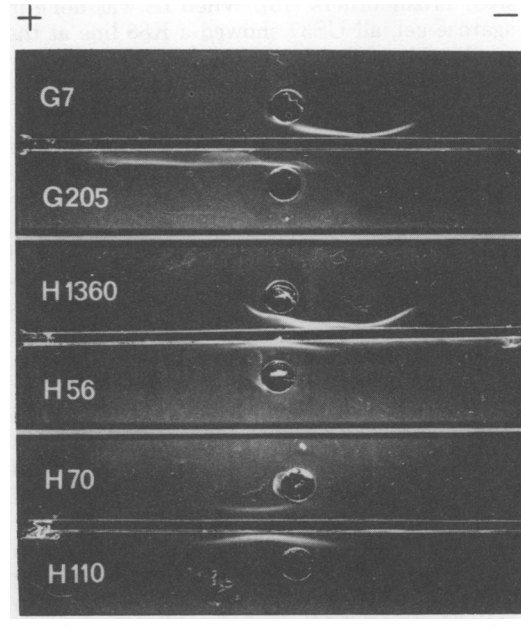

FIG. 2. *IE patterns obtained with K88 antigens and antiserum K88ab(G7) in Noble agar gel. Antigens are US37 of: G7 = O8:K87:K88ab; G205 = O8:K87:K88ac; H1360 = O138:K81:K88ac; H56 = O8:K87:K88ad; H70 = O8:K?:K88ad; H110 = O9:K(A)?:K88ad.*

TABLE 4. *Results of NIE tests with the K88⁺ strains listed in Table 1*

Strains with K88 antigen type:	Site of K88 precipitation line		
	Anodic	Center	Cathodic
ab			3
ac	24		17[a]
ad	2[b]	10	

[a] Four field isolates of O157:K88ac; strain H1360 and 11 field isolates of O138:K81:K88ac; and strain O8:K?:K88ac.

[b] The strains H70 and H110 possess an anodic K88ad antigen.

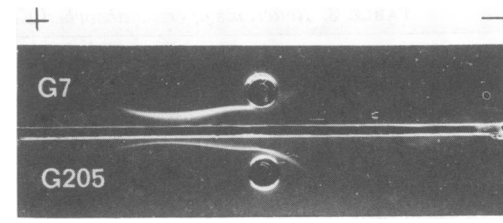

FIG. 3. *IE patterns obtained with K88 antigens and antiserum K88ab(G7) in agarose gel. Antigens are US37 of G7 (O8:K87:K88ab) and G205 (O8:K87:K88ac).*

and HA titers. Gibbons et al. (personal communication) reported that all types of K88 antigen adhered equally well to known "adhesive" brushborders and did not adhere to "nonadhesive" brushborders (10). When IE was done in agarose gel, all US37 showed a K88 line at the anodic side of the application well (Fig. 3).

DISCUSSION

K88ac antigens with different NIE mobilities could not be distinguished serologically. The K88ac antigen of a given strain retained its type of NIE mobility after transfer to *E. coli* K-12. This indicates that the NIE behavior of a K88 antigen is plasmid determined and not influenced by properties of the host. The NIE behavior was not dependent on the method of isolation of the K88 antigen. There was no correlation between the NIE behavior of the K88 antigen and the year of isolation of the strain. In agarose immunoelectrophoresis, all K88 antigens showed anodic mobility. It has been reported that Noble agar gel shows a higher level of electroosmotic flow than agarose (17). The different behavior of a number of K88 antigens in agarose and NIE can be explained by varying sensitivity to electro-endosmotic flow. Thus, in addition to serological variation, K88 antigens may vary in sensitivity to electro-endosmotic flow, which might indicate a difference in structure. It was recently reported (1) that the K88 antigen of the strains A1, A2, G7, and E68 gave an anodic line in NIE, whereas G7 and E68 gave a cathodic line in our experiments. Comparison of the techniques used in the two studies does not readily offer an explanation for these differences. Cahill and Glantz (1) reported that K88 antigen is not entirely inactivated at 100°C. K88 antigen could no longer be demonstrated after heating in 100°C in other work (9, 16) as well as in our experiments. Strains possessing the preliminarily designated K88ad antigen were not entirely identical. Similar slight serological variations were observed also among the K88ac antigens of different strains by Ørskov et al. (9). Slight variations were also observed in the plasmids coding for K88 antigen (12). These variations might be partly due to the immunological pressure upon the bacterial K88⁺ population as a result of the large-scale vaccination with K88 antigen-containing vaccines. The new variant K88ad might then be considered as an attempt of the pathogen to escape this pressure. Strains with K88ad antigen emerged for the first time in 1973. It seems important to monitor the prevalence of such aberrant K88 antigens. We therefore propose to include the antigen provisionally designated K88ad into the international *E. coli* typing scheme.

The Ficoll sedimentation method enhanced the detection level of K88⁺ transconjugants 50- to 100-fold. This proved very useful, expecially when *raf*⁻ donor strains were used, since no selection marker for the detection for K88 antigen was available in such matings. The method might also be useful for the detection of transconjugants carrying adhesive pilus antigens other than K88.

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ADDENDUM IN PROOF

I. and F. Ørskov, World Health Organization Collaborative Centre for Reference and Research on *Escherichia*, Copenhagen, confirmed our findings on K88ad antigen and will establish it as a new *E. coli* K antigen.

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