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REPORT NUMBER II

INTESTINAL COLONIZATION BY ENTEROTOXIGENIC

Escherichia coli

ANNUAL REPORT

HARLEY W. MOON

December, 1976

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20315

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National Animal Disease Center
U.S. Department of Agriculture, and
Department of Pathology, Iowa State University
Ames, Iowa 50010

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Summary

This study was designed to test the hypothesis that: (1) enterotoxigenic E. coli characteristically colonize mammalian small intestine by adhering to the epithelial surface; (2) that adhesion, referred to above and involving strains lacking K88 antigen, occurs between pili on the bacterial surface and microvilli on villous absorptive cells of the host.

1. Selection of Piliated Forms in vivo and Adhesion of Piliated forms in vitro

Porcine enterotoxigenic (ETEC) E. coli strain 987 (serotype 09:K103:NM) was previously found to be piliated and consistently adhere to intestinal villi in pig small intestine, colonize pig small intestine and cause profuse diarrhea. Nonpiliated variants or mutants of the strain lacked these virulence attributes. In the study reported here, it was found that growth of strain 987 in pig intestine in vivo yielded a greater percentage of piliated cells than growth in vitro. This increase in piliated forms was demonstrable by electron microscopy, by colonial morphology and by agglutination in specific antisera against the pili of strain 987. Non-piliated or lowly piliated forms of strain 987 do not adhere to isolated intestinal epithelial cells in vitro, heavily piliated forms do. These data are consistent with the hypothesis that the pili of strain 987 facilitate intestinal adhesion and colonization by this strain.

This work is presented in detail in the manuscript in the Body of the Report, "Colonization of Porcine Intestine by Enterotoxigenic Escherichia coli: Selection of Piliated Forms in vivo, Adhesion of Piliated Forms to Epithelial Cells in vitro, Incidence of a Pilus Antigen Among Porcine Enteropathogenic E. coli," which has been submitted for publication in Infection and Immunity.

2. Characterization of K99 Surface Antigen of Escherichia coli

This antigen has previously been shown to be common among ETEC isolated from cattle and sheep and to facilitate intestinal colonization in these species. In the study reported here it was found to be a pilus or pilus-like structure. Some of the physical and chemical characteristics of this virulence attribute are presented. This extends the data indicating that pili of ETEC are virulence attributes which facilitate intestinal colonization.

This work is presented in detail in the manuscript in the Body of the Report, "Studies on the K99 Surface Antigen of Escherichia coli: Purification and Partial Characterization," which has been submitted for publication in Infection and Immunity.

3. Colonization of Pig Ileum by K99⁺ Enterotoxigenic E. coli

Colonization of pig small intestine has previously been considered to be species specific; i.e., confined to ETEC of pig origin. Furthermore, K99 antigen was considered to be species specific; i.e., occur only on strains of bovine and ovine origin. In contrast, the study reported here demonstrates that K99 antigen occurs among pig ETEC, that K99⁺ ETEC both of pig and of bovine origin colonize pig small intestine by adhesion, and that K99⁺ ETEC produce K99 antigen in pig small intestine in vivo. These observations along with those in 1 and 2 above indicate that there are at least 3 distinct pili or pilus-like structures among porcine and ruminant ETEC which facilitate intestinal colonization (probably by adhesion). These are the K88 antigen previously well described in the world literature. The pili of strain 987 and the pilus designated as K99 antigen.

This work is presented in detail in the manuscript in the Body of the Report, "The Occurrence of K99 Antigen on Escherichia coli Isolated from Pigs and Colonization of Pig Ileum by K99⁺ Enterotoxigenic E. coli from Calves and Pigs," which was submitted for publication in Infection and Immunity.

Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

Colonization of Porcine Intestine by Enterotoxigenic
Escherichia coli: Selection of Piliated Forms in vivo,
Adhesion of Piliated Forms to Epithelial Cells in vitro,
Incidence of a Pilus Antigen Among Porcine Enteropathogenic E. coli

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Running head: Adherence of E. coli

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ABSTRACT

In contrast to K88 positive porcine enterotoxigenic E. coli (ETEC), K88 negative porcine ETEC strains did not adhere to isolated intestinal epithelial cells in vitro. However, they did adhere to intestinal epithelium in vivo. Growth of one such ETEC (strain 987) in pig small intestine consistently yielded a greater percentage of piliated cells than growth in vitro. This increase was demonstrable by electron microscopy, by change in colonial morphology, and by agglutination in specific antisera against the pili of strain 987. In contrast to the stored stock culture (which contained very few piliated cells), richly piliated forms of strain 987 did adhere to isolated intestinal epithelial cells in vitro. A series of porcine E. coli strains were tested for agglutinability in antiserum against the pili of strain 987 and several K88 negative ETEC strains were agglutinated. These data are consistent with the hypothesis that pili facilitate intestinal adhesion and colonization by K88 negative ETEC strains.

INTRODUCTION

Colonization of mammalian small intestine by enterotoxigenic Escherichia coli (ETEC) is apparently dependent upon the ability of such strains to adhere to villous epithelium in the small intestine (1,2,5,13,16). There is evidence that this adhesive attribute is conferred by pili or pilus-like structures produced by the ETEC (5,8,9,14). An in vitro system for studying adhesion to intestinal epithelial cells has been developed for porcine ETEC strains which have the pilus-like K88 antigen (14,18). However, other porcine ETEC strains which lacked the K88 antigen, and nonenterotoxigenic E. coli (NETEC) did not adhere to intestinal epithelial cells in vitro. The objectives of the work reported here were: i. to adopt or develop an in vitro system for the study of the adhesive abilities of porcine ETEC strains which lack K88 antigen, and ii. to determine the role of pili in the in vitro and in vivo adhesion of one such ETEC strain to intestinal epithelium.

MATERIALS AND METHODS

E. coli. Strains used for in vitro adhesion tests were all isolated from pig intestine and are listed in Table 1. The colonizing and adhesive attributes, for pig intestine in vivo as well as enterotoxigenicity, pilus production, and hemagglutinating abilities of these strains have been reported previously (8,13). Bacteria were grown and maintained in trypticase soy broth (TSB) or on trypticase soy agar (TSA) slants (BBL) and stored at 4 C unless otherwise stated.

The in vitro adhesion test, using isolated intestinal epithelial cells, was performed as described by Wilson and Hohmann (18).

Adherent ETEC and intestinal mucus in vivo. The effect of washing on mucus removal and adhesion of E. coli was tested in ligated intestinal loops for strains 431, 123, and in intact pigs for strain 987. Ligated intestinal loops were created in hysterectomy-derived, colostrum-deprived (HDCD) pigs (20-30 h old) as described earlier (2), inoculated with 10^7 bacteria of the test strains in 1 ml fresh TSB. Pigs were killed 6 h post exposure. Intragastric inoculation of (HDCD) pigs to study colonizing abilities of E. coli strain 987 was also described before (2,13). The following procedure was done on intestinal segments from both ligated loops and intact pigs. A 5 cm piece of the intestinal segment was everted onto a plastic pipette and washed in 100 mM Clealand's reagent (Baker) with strong stirring (magnetic stirring device) for 10 min to remove mucus (17). The effect of washing on tissue morphology and on viable bacterial counts was studied. For microscopic investigation,

samples were fixed in formaldehyde, embedded in paraffin, sectioned and stained either with Giemsa or with periodic-acid-Schiff's (PAS) reagent. This washing procedure removed almost all the PAS positive amorphous mucus from the villous surface so that the microvilli could be visualized as a PAS stained thin border (Fig. 1a, b). Each strain was tested in 3 intestinal segments from 3 different pigs. For viable bacterial counts, 4 cm of washed and unwashed everted intestine were transferred into cold, .3% peptone water, homogenized and viable bacterial counts determined (13). Association of bacteria to the mucosal epithelium of the intestinal segments was determined by microscopic evaluation of fluorescent antibody stained frozen sections as described earlier (2,13).

Electron microscopy. Samples for transmission electron microscopy were prepared as for the in vitro adhesion test (18), then processed and stained as described earlier (12). For direct examination of bacteria, negatively stained preparations were used as described (8).

Preparation of anti-pilus serum. Rabbits were injected i.v. with phenolized suspension of a piliated form of E. coli strain 987 grown on blood agar, according to Edwards and Ewing (6). The O and K antibodies were removed from the serum by absorption using both an uncapsulated (K^-), pilus-lacking and a capsulated (K^+), pilus-lacking variant of this strain (8) as described for O and K absorption (6). With this absorbed anti-pilus serum, (anti 987-P), colonies of E. coli 987 containing richly piliated cells were detected by agglutination of bacteria on a glass slide at a serum dilution of 1:10.

Agglutinability of richly piliated cultures in anti 987-P serum was destroyed by heating the cultures 10 min at 100 C. The anti 987-P serum did not agglutinate strains carrying K88. Neither did it agglutinate colonies of the human enterotoxigenic H-10407 which were agglutinable by the absorbed anti-colonizing factor serum kindly sent us by Dr. D. J. Evans (5).

Serological survey for incidence of 987 pilus antigen. The E. coli strains tested were isolated from pig intestine and maintained on TSA slants at room temperature in the dark for periods of 2 weeks to 10 years. For the purpose of these studies, strains isolated from baby pigs diagnosed as having enteric colibacillosis (11) were classified as enteropathogenic E. coli (EEC). Many of them were tested and found to produce enterotoxins. Strains isolated from normal pigs or pigs with diseases other than enteric colibacillosis were classified as nonenteropathogenic E. coli (NEEC). For the serological survey, bacteria were inoculated into TSB and incubated at 37 C for 4-6 days until a definite pellicle was formed. From this pellicle, 5% sheep blood agar plates were inoculated and after 24 h incubation, 5-10 colonies (when available, small, transparent) were tested for agglutinability in the anti 987-P serum, using normal rabbit serum as negative control. Detection of colonies agglutinable in anti 987-P serum required careful inspection of the pellicle derived blood agar culture. Usually the small, more transparent colonies were agglutinable while the larger, less transparent colonies were not.

RESULTS

In vitro adhesion tests were carried out using isolated epithelial cells from both ileum and jejunum of three pigs. As the results in Table 1 show, the K88 negative ETEC did not adhere to any of these cells. Two of the NETEC strains did not adhere either, but one was moderately adherent to both cell types in all three experiments. In contrast, the K88⁺ strain adhered intensively as described (18).

The fact that the K88 negative ETEC strains used in these experiments had not lost their in vivo adhesive abilities prior to the above in vitro experiments was proved by subsequent intragastric inoculation of HD CD pigs and by inoculation of ligated intestinal loops of HD CD pigs. The K88 negative ETEC colonized and adhered to the ileal epithelium of intragastrically inoculated pigs and adhered to the wall of both ileal and jejunal loops as reported earlier (13).

Some pigs are congenitally resistant to adhesion of K88⁺ ETEC (2,15). Experiments were conducted to see if the above failure of K88 negative ETEC to adhere in vitro was caused by the use of cells from pigs resistant to K88 negative ETEC. E. coli strains 431, 987, and 74-5208 were inoculated into ligated intestinal loops of 2 HD CD pigs. After 6 h of incubation, the pigs were killed, the loops were examined by immunofluorescent microscopy for adherent E. coli (Association Index) (13). The remaining unexposed small intestinal segments from these pigs were used to prepare isolated intestinal epithelial cells which were used for in vitro adhesion tests with the same strains. All three K88 negative

EPEC adhered intensively in the loops of both pigs 6 h post exposure but none of the E. coli strains adhered to the isolated intestinal epithelial cells of the same pigs in vitro. We concluded that the principal strains would not adhere in vitro even when the epithelial cells used came from pigs susceptible to adhesion in vivo.

Subsequently, several trials were made to modify the in vitro adhesion test so that it would demonstrate adhesiveness of the K88 negative EPEC. Calcium and Mg^{++} ions, bile salt, sodium deoxycholate E. coli enterotoxins (heat stable and heat labile), and fluid from positive ligated intestinal loops after exposure to EPEC were added to the system, but without any major improvement in the adhesiveness of the K88 negative EPEC over the NETEC strains. In further experiments, the isolated intestinal epithelial cells were replaced by the following cultured mammalian cell lines from the American Type Culture Collection: "Y1" (mouse adrenal cell), "Intestine 407," and "HeLa." The K88 carrying strain (used as positive control) had a tendency to adhere to the above cells but the K88 negative EPEC were less adherent and indistinguishable from the NETEC strain which was used as a negative control.

In vivo adhesion of K88 negative EPEC and intestinal mucus.

Since the K88 negative EPEC did not adhere to epithelial cells in vitro under the above conditions, the question was raised: Do they adhere to epithelial cells in vivo? Alternatively, are they located in the mucopolysaccharide layer covering the epithelial cells? The vigorous

washing of everted intestinal segments from ligated intestinal loops and intact intragastrically exposed pigs with Clealand's reagent removed most of the mucopolysaccharide layer from the villous epithelium (Fig. 1a, and 1b). The association indexes of intestinal segments colonized by K88 negative ETEC 987 or 431, however, were not significantly affected; there were still large numbers of bacteria on the washed mucosal surface remaining adherent to the epithelium (Fig. 1c). Using the same washing procedure, the NETEC strain 123 was removed (Table 2).

Differences in colonial morphology and agglutinability in anti 987-P serum. During the experiments reported here, we realized that there were at least 2 types of colonial morphology of strain 987 on blood agar plates. Some colonies were smaller and more transparent in direct transmitted light than most of the colonies of that strain when the stock was grown on sheep grown on sheep blood agar at 37 C (Fig. 2). Seven representative colonies from each of these two types were tested in different cultures on two separate days. Twenty bacterial cells from each colony were observed under the electron microscope and cells from the same colonies were tested for agglutinability in anti 987-P serum on a glass slide at a serum dilution of 1:10. Based on electron microscopic investigation, 80-100% of the cells in these small colonies were richly piliated (Fig. 3). The anti 987-P serum agglutinated bacteria from 7/7 of these small colonies, containing richly piliated cells of strain 987 (P^{++}). In repeated cultures of stock 987 strain on blood agar plates, the P^{++} colonies were consistently in the minority ranging from about < 1% to 5% of the total.

The majority of blood agar plate colonies from the stock culture of strain 987 was consistently larger and more opaque than the P^{++} colonies. Electron microscopy revealed that only 0-20% of the cells are piliated in these colonies and when piliated, these had few pili per cell. The anti 987-P serum agglutinated bacteria from 0/7 of these large colonies, containing non-, or poorly piliated cells ($P^{\pm}$). The above observations on colonial differences for piliated and non-piliated phase of E. coli are in good agreement with those of Brinton (3).

In vivo selection of piliated forms. Based on the differences in colonial morphology and serological reaction between P^{++} and $P^{\pm}$ forms of E. coli 987, we compared the percentage of the P^{++} colonies in 6 in vitro TSB grown inocula with that in the population isolated from the ilea of 16 HD CD pigs colonized by E. coli 987, 16 h post exposure. The results in Table 3 show a 20-fold increase in the percentage of P^{++} colonies in vivo compared to that in vitro.

In vitro adhesiveness of the P^{++} form of E. coli 987. We isolated one strain ($987P^{++}$) from one of the P^{++} colonies and tested it for in vitro adhesiveness using isolated intestinal epithelial cells (18) along with the stock E. coli 987 and with positive (E. coli 263) and negative (E. coli 123) as control strains. As the results in Table 4 show, the in vitro adhesiveness of this richly piliated variant of E. coli 987 was significantly ($P < .01$) higher than that of the parent strain and of the negative control but was significantly less ($P < .01$) than adhesiveness of the K88 carrying positive control strain. These richly piliated

variants of 987 tended to aggregate in vitro and these aggregates adhered to the isolated intestinal epithelial cells (Fig. 4). Transmission electron microscopic sections of these bacteria-epithelial cell aggregates usually revealed large numbers of bacteria with several surface appendages resembling pili, reaching between the bacteria and the microvilli of the epithelial cells (Fig. 5).

When tested for hemagglutinating abilities against guinea pig red blood cells (8,10), the P^{++} form of 987 remained nonhemagglutinating just like the E. coli 987 strain (Table 4).

The piliated variant of 987 ($987 P^{++}$) lost its in vitro adhesiveness during laboratory storage of 2 months at 4 C and lost its characteristic colonial morphology and agglutinability in anti 987 P-serum as well. Subsequently, 3 more richly piliated variants ($987P^{++}$ -1, -2, and -3) were isolated from the ileum of E. coli 987-colonized newborn pigs based on colonial morphology and agglutinability in anti 987-P serum. The fresh piliated isolates contained a high (32.5-98.9) percent of small colonies with agglutinable cells while only a very low (< 1.0-2.6) percent of these colonies was found in the cultures of 987 and $987P^{++}$. Variants 1, 2, and 3 were tested for in vitro adhesiveness in three experiments parallel with the once richly piliated isolate ($987P^{++}$). All three fresh isolates adhered significantly ($P < .01$) more than the negative control NETEC, or the parent E. coli 987. $987P^{++}$ was indistinguishable from the negative control or parental E. coli 987 strain (Table 5). The parental E. coli 987 and once richly piliated $987P^{++}$ forms were different from the fresh, richly piliated isolates ($987P^{++}$ -1, -2 and -3) in terms of colonial morphology as well.

Incidence of 987 pilus antigen among porcine EEC strains. Using the absorbed anti-987-pilus serum, we tested 119 EEC and 54 NEEC strains for agglutinability in this serum by slide agglutination. As the results of Table 6 show, 50% of the 09 and 14% of the 020 EEC strains contained colonies which were agglutinated by the anti 987-P serum; whereas, none of the EEC from serogroup 0101 or from serogroups (08, 0138, 0139, 0141 did so. All the NEEC strains tested were negative as well.

DISCUSSION

The in vitro adhesion tests confirmed the report of Wilson and Hohmann (18) that K88 carrying E. coli strains adhere while K88 negative ETEC and NETEC do not. Trials to modify the test so that it could be used to demonstrate in vitro adhesiveness of stock cultures of K88 negative ETEC were unsuccessful. Based on the observation that K88 negative EEC colonized ileum but not jejunum in vivo (13), we expected some adhesion to ileal cells in vitro; however, this was not the case. The data indicate that the lack of in vitro adhesiveness by K88 negative EEC was not due to the loss of in vivo colonizing and adhesive abilities of these strains or to the resistance of epithelial cells used. Furthermore, as the results of Table 2 show, the principal K88 negative

ETEC were strongly attached to the epithelial cells in vivo and remained there in spite of removal of the surface mucus layer.

The differences between in vitro and in vivo adhesiveness of the E. coli 987 strain could be explained by the difference in pilus production under these two conditions. Data in Table 3 indicate that growth in pig intestine either selects for, or promotes the development of, piliated forms. A relatively higher growth rate of piliated than of nonpiliated forms in vivo could be the result of limited oxygen present in the intestine, as suggested by Brinton (4). It is logical to assume that an essential part of the adherent bacterial layer formed on the small intestinal epithelium in vivo is composed of richly piliated forms of the bacteria. There is ultrastructural evidence to support that assumption (12). Richly piliated forms had increased in vitro adhesiveness to isolated intestinal epithelial cells and the surface appendages reaching from the bacteria towards the isolated epithelial cells can be best interpreted as pili (Fig. 5).

However, the P^{++} colonies, isolated from colonized ileum of newborn pigs tended to lose their characteristic colonial morphology during laboratory storage in TSB or on TSA and during subsequent cultures on sheep blood agar and tended to revert back to P^+ form. The loss of in vitro adhesiveness of the strain 987 P^{++} was probably due to the loss of its piliated form during 2 months of laboratory storage as indicated

by the low percentage of P^{++} colonies in blood agar cultures of this strain compared to the adherent, fresh isolates 987 P^{++} -1, -2, and -3 (Table 5). This line of evidence together with the lack of in vivo colonizing and adhesive abilities of pilus-lacking variants of E. coli 987 (8) indicates the importance of pili in the virulence of this strain.

It could be that under circumstances similar to those of 987 P^{++} , NETEC strains would also form large numbers of pili and adhere in vitro. In fact, one of our NETEC strains (E. coli 252) was moderately adhesive (Table 1). In earlier studies however, when HD CD pigs were intragastrically inoculated with 3 different NETEC strains, none of them (including strain 252) colonized or adhered to the small intestinal epithelium (13). These NETEC strains were all piliated (8) but none of them agglutinated with the absorbed anti-987-pilus serum. Furthermore, all three NEEC strains showed mannose sensitive hemagglutination of guinea pig red blood cells indicating a different kind of pili from that of 987. Functional differences among pili could be one explanation for the lack of in vivo adhesiveness by these NETEC strains. On the other hand, pili may be necessary but not the only factor for effective colonization of the small intestine.

The fact that several EEC strains from serogroups 09 and 020 reacted with the anti 987-P serum leads us to suggest that this pilus antigen is not confined to strain 987. Further studies are needed to determine the degree of antigenic relatedness, anatomic nature and functional significance of these antigens on the other strains.

ACKNOWLEDGMENTS

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FIG. 1. A. Thick PAS stained mucopolysaccharide layer before washing. Frozen section, from a villous tip in the small intestine. PAS, X 300. B. Frozen section from a villous tip in the small intestine comparable to A. Lack of the PAS positive mucus layer after washing with 100 mM Clealand's reagent. PAS, X 300. C. Bacteria adherent to the intestinal epithelium of the same villus as B after washing with Clealand's reagent. Giemsa, X 300.

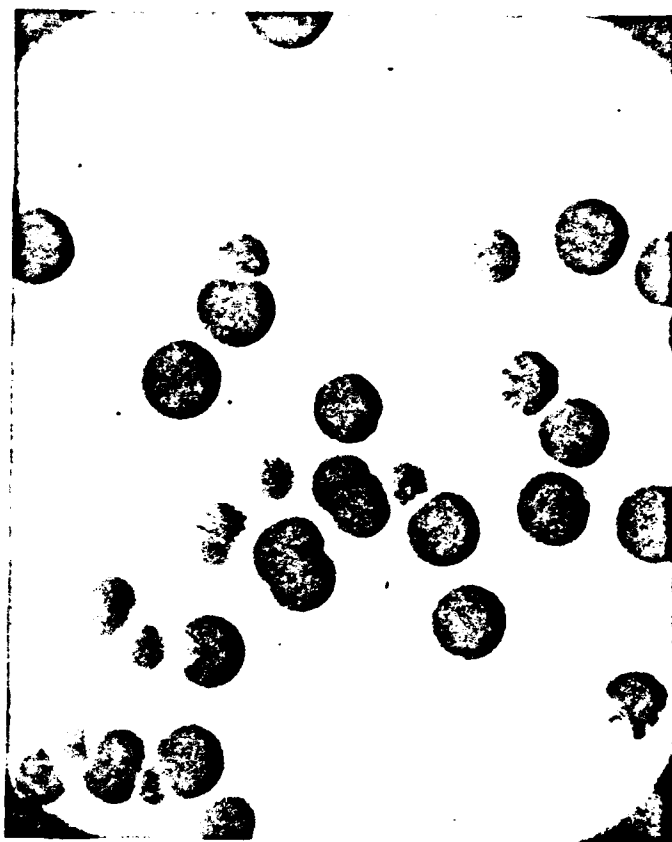


FIG. 2. Blood agar culture of richly piliated small (P^{++}) and poorly piliated large (P^+) colonies of *E. coli* 987 derived from the ileum of an HD CD pig 16 h post exposure.



FIG. 3. Part of the piliated surface of a richly piliated variant of E. coli 987 (987P⁺⁺).

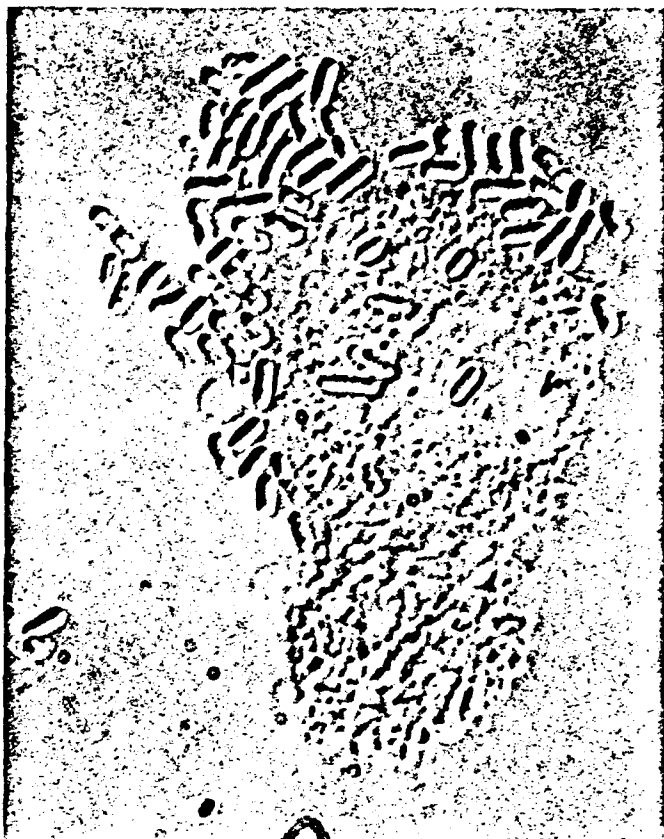


FIG. 4. Bacteria of a richly piliated variant of *E. coli* 987
(987P⁺⁺) adherent to an isolated intestinal epithelial cell in vitro.



FIG. 5. Richly piliated variant of *E. coli* 987 (987P⁺⁺) with surface appendages extending to the microvilli of isolated intestinal epithelial cell in vitro. Transmission electron micrograph of an ultrathin section. Bar equals 100 nm.

TABLE 1. E. coli strains used and their in vitro adhesion to isolated
intestinal epithelial cells of pigs

Strain	Serotype	Designation	Adherent bacteria per cell	
			Jejunal	Ileal
263	08:K87,88ab:H19	ETEC ^a	16.8	10.1
1351	0101:K30:NM	ETEC	< 1.0	< 1.0
613	0101:K30:NM	ETEC	< 1.0	< 1.0
431	0101:K30:NM	ETEC	< 1.0	< 1.0
125	0101:K-:NM	ETEC	< 1.0	< 1.0
987	09:K103:NM ^b	ETEC	< 1.0	< 1.0
340	09:K+:NM	ETEC	< 1.0	< 1.0
74-5208	020:K101:NM ^b	ETEC	< 1.0	< 1.0
381	020:K+:NM	ETEC	< 1.0	< 1.0
123	043:K-:H28	NETEC ^c	< 1.0	< 1.0
252	013:K-:H11	NETEC	5.9	5.9
124	08:K50:H19	NETEC	< 1.0	< 1.0

^aEnterotoxigenic for pigs.

^bDetermination of K-type by Drs. Frits and Ida Ørskov, Statens Serum Institute, Copenhagen.

^cNonenterotoxigenic for pig.

TABLE 2. Effect of mucus removal by Clealand's reagent on association indexes and on E. coli counts of everted intestinal segments, colonized by K88 negative ETEC and NETEC

E. coli strains	No. of pigs tested	Test system used	Association Index ^a			Significance t test	Log ₁₀ E. coli	
			Unwashed	Washed ^b			Unwashed	Washed
987	4	Intact pig	4.2	4.1	P < 0.05	8.26	8.37	
431	3	Loop	4.8	4.8	P < 0.05	8.88	8.07	
123	3	Loop	3.6	2.0	P > 0.01	8.24	7.54	

^a Degree of adhesion of bacteria to intestinal epithelium as determined by immunofluorescence microscopy (2,13).

^b Washed with 100 mM Clealand's reagent for 10 minutes.

TABLE 3. Prevalence of P^{++a} and $P^{\pm}$ colonies
in the in vitro grown inoculum of E. coli 987 compared to the population
recovered from the small intestine of newborn pigs 16 h post exposure

	P^{++} Small colonies		$P^{\pm}$ Large colonies		No. of tests ^b
	%	No. aggl./ no. tested ^c	%	No. aggl./ no. tested ^c	
Inoculated	2.4	9/9	97.6	0/9	6
Recovered	48.3	95/96	51.7	1/98	16

^a P^{++} small, translucent colonies, containing bacteria agglutinable in anti 987-P serum and of the type shown previously to contain numerous richly piliated cells. $P^{\pm}$ large opaque colonies, containing bacteria not agglutinable in anti 987-P serum and of the type shown previously to contain no or few piliated cells.

^bNo. of inocula or pigs tested.

^cNo. of colonies with bacteria agglutinable in anti-987-P serum over Number tested.

TABLE 4. In vitro adhesion and hemagglutination of E. coli 987
and its richly piliated form in comparison with positive
and negative control E. coli strains

<u>E. coli</u> strains	Designation	Hemagglutination ^a of GP red blood cells	Adherent bacteria/ isolated intestinal ^b epithelial cells	
			Mean	Range
263	ETEC, K88 ⁺	MR	17.6	(15.3-18.7)
123	NETEC	MS	1.7	(1.0-4.2)
987	ETEC, K88 ⁻	-	2.1	(1.0-5.1)
987P ⁺⁺	ETEC, K88 ⁻	-	6.0	(3.0-9.7)

^aMR = mannose resistant hemagglutination, MS = mannose sensitive
hemagglutination, - = no hemagglutination.

^bResults of duplicate experiments on 3 different days.

TABLE 5. In vitro adhesion of piliated forms of E. coli 987
comparison of stored cultures with fresh isolates

Status of cultures	<u>E. coli</u> strains	Adherent bacteria/cell		% of small (p ⁺⁺) colonies	
		Mean	Standard error	Mean	Range
Stored	263	19.24	1.37	NT ^a	-
"	123	.17	.18	NT	-
"	987	.11	.08	< 1.	-
"	987P ^{++b}	.51	.91	2.1	(1.8-2.6)
"	987P ^{++c}	1.89	1.70	5.7	(4.6-6.7)
Fresh	987 ⁺⁺ -1	5.28	2.97	98.4	(98.0-98.9)
"	987P ⁺⁺ -2	3.73	1.57	38.2	(32.5-49.5)
"	987P ⁺⁺ -3	2.90	1.90	52.9	(42.6-66.7)

^aNot tested.

^bOnce richly piliated variant of E. coli 987 stored on TS-agar slant
for 2 months.

^cOnce richly piliated variant of E. coli 987 stored in TSB for 2 months.

TABLE 6. Results of serological survey among porcine E. coli
strains with anti 987-P serum

Serogroup	<u>No. of strains agglutinable over the no. tested</u>	
	Enterotoxigenic	Nonenterotoxigenic
09	11/22	0/9
020	3/21	0/12
0101	0/16	0/2
Others	0/60	0/31

Studies on the K99 Surface Antigen of Escherichia coli:

Purification and Partial Characterization

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Running Head: Purification of K99 Antigen

ABSTRACT

K99, a presumed colonizing factor of enterotoxigenic Escherichia coli of calf origin, has been purified. K99 was removed from K99+ bacteria by salt extraction and subsequently purified by ammonium sulfate precipitation and column chromatography on DEAE-Sephadex. The purified material was homogenous in size having an $s_{20,w}$ of 13-15 S. It is composed of two subunits: a major component with a molecular weight of 22,500 and a minor component of 29,500. When observed in the electron microscope, K99 appears to be rod-shaped with a strong tendency for self-aggregation. At concentrations where aggregation is minimized, individual rods were observed with diameters of 8.4 nm and mean lengths of 130 nm. Based on the subunit structure, exterior location, and rod-like shape of K99, it is concluded that it is a pilus or pilus-like structure. Chemically, K99 is composed primarily of protein and has an isoelectric point of greater than 10. Purified K99 does not hemagglutinate guinea pig red blood cells.

INTRODUCTION

Colonization of the small intestine is a necessary prerequisite for enterotoxigenic Escherichia coli to cause diarrheal disease. Smith and Linggood (17) reported on the identification of a transmissible, plasmid mediated K antigen which was commonly found on calf and lamb enterotoxigenic E. coli strains and demonstrated it to be a colonization factor and an adhesive factor. This antigen has been designated K99 (16). The removal of the K99 plasmid and thus the K99 antigen from an enterotoxigenic calf strain resulted in a nondiarrhea inducing organism. Reintroduction of the K99 plasmid into the same strain resulted in a virulent organism. Several more recent studies (9,14,15) have demonstrated the presence of K99 on a high percentage (76-95%) of enterotoxigenic E. coli strains isolated from calves and on a low percentage (0-14%) of nonenterotoxigenic E. coli strains isolated from calves.

These data are consistent with K99 being a colonization factor. The purification of K99 was undertaken as a prelude to the determination of the role that it plays in colonization of the calf small intestine. The results of K99 purification and partial characterization are presented here.

MATERIALS AND METHODS

E. coli strains and growth conditions. E. coli strain B41 (0101;K-,99:NM) which has been established as the K99 reference strain (16), was obtained from W. J. Sojka. E. coli strains 1474 (K99+) and 1475 (K99-) are isogenic K12 strains which were obtained from C. L. Gyles. Bacteria were grown in 1-liter batches of trypticase soy broth (BBL) in 2-liter Erlenmeyer flasks at 37 C with shaking (200 rpm).

Assay of K99. K99 was detected by a double diffusion technique (Ouchterlony) in 1% agar (in 0.15 M sodium chloride). Wells were punched into the agar 4-5 mm apart and filled with 15 μ l of either sample or antibody. Absorbed K99 antiserum was previously described (15). K99- antiserum was prepared from strain 1475 (K99-) by the method of Edwards and Ewing (). K99 activity was quantitated by preparing serial 2-fold dilutions of the sample and determining the most dilute sample which would result in a precipitin arc when reacted with absorbed K99 antiserum after 24 h. The reciprocal of that dilution was used to designate K99 activity/15 μ l.

Chemicals. Reagents for polyacrylamide gel electrophoresis were obtained from Eastman Kodak. Diethylaminoethane (DEAE)-Sephadex A50, G50, and G100 were obtained from Pharmacia. Biogel P60, A1.5, A5 and A15 were obtained from Bio Rad. Carboxymethyl cellulose, phosphocellulose and molecular weight protein standards were purchased from Sigma.

Chemical assays. Protein was determined by the method of Lowry (13) using bovine serum albumin as standard. Neutral sugars were determined by the indole test (1) using glucose as standard. Lipid was estimated by direct weight measurement after extraction with alcohol-ether (6). Hexosamines were determined in the amino acid analyzer.

Polyacrylamide gel electrophoresis. Polyacrylamide stacking gels were prepared as described by Davis (3) and electrophoresed at pH 8.9. Sodium dodecyl sulfate polyacrylamide gels (SDS-gels) were prepared by the method of Weber and Osborn (19) and stained by the method of Fairbanks et al. (8). Molecular weight determinations in SDS-gels has been previously described (19).

Isoelectric focusing. Isoelectric focusing at 4 C was performed in an LKB Ampholine column (model 8101). The column was prepared as described in the LKB manual and contained 1% carrier ampholytes (pH 3-10). K99 in distilled water was added to either the light or heavy sucrose solution prior to preparing the density gradient. Isoelectric focusing was carried out for 72 h at a constant voltage of 300 volts.

Amino acid analysis. Purified K99 was dialyzed exhaustively against distilled water, lyophilized, and dissolved in 6N hydrochloric acid. After evacuating and sealing, individual samples were hydrolyzed at 110 C for 24, 48 and 72 h. After hydrolysis, the samples were taken to dryness and amino acid composition determined in a Beckman 121 automated amino acid analyzer.

Hemagglutination. Microhemagglutination of guinea pig red blood cells has been previously described (12).

Electron microscopy. K99 (1.5 mg/ml) was exhaustively dialyzed against distilled water. This material was then stained with potassium phosphotungstate as previously described (11).

RESULTS

K99 assay. It has previously been shown that a double diffusion technique (Ouchterlony) can be useful in the detection of K99 activity (9). The specificity of this precipitin reaction is demonstrated in Fig. 1 and 2. Materials in wells B-E of Fig. 1 (all containing K99) have a very slow diffusing component which is precipitated by absorbed K99 antiserum, but not by antiserum prepared against the isogenic K99- parent. The materials in well A, which were prepared from strain 1475 (K99-) do not have this slow diffusing component when reacted with either sera. After extraction, but prior to purification, materials from K99+ or K99- strains contain a rapidly diffusing component which is precipitated by both sera. This component must therefore be an antigen common to both K12 strains and thus is not K99. Figure 2 demonstrates that the slow diffusing component obtained from 1474 (K99+) is identical to the slow diffusing component extracted from the K99 reference strain B41. This is demonstrated by the lines of identity formed by the precipitin arcs of the two K99+ strains. No spurring was observed. Guinée (9) previously showed that this B41 component was K99. The double diffusion technique thus appeared to be a reliable and specific assay for K99 and was therefore adapted to monitor K99 activity during purification.

Purification of K99:

I. Extraction of K99. Initial attempts to remove K99 from bacterial cells made use of a short, high speed homogenization: 2 min at a setting of 10 in a Sorvall omnimixer. After homogenization, bacteria and large debris were removed by centrifugation. K99 activity could be detected in the resultant supernatant. However, a 2- to 4-fold increase in K99 yield could be achieved by using the following salt extraction. Cells from 12 liters of 18- to 24-h old cultures were harvested by centrifugation (17,000 xg for 10 min) and resuspended in 200-300 ml of 0.05 M sodium phosphate buffer, pH 7.2 (PB) containing 1.0 M sodium chloride. K99 was extracted at 4 C by homogenization in a Sorvall omnimixer (setting of 4) for 30 min. Bacteria were removed by centrifugation at 17,000 xg for 10 min at 4 C and reextracted. The two cleared supernatants were pooled and stored at -20 C for further use. When the extracted bacteria were assayed for K99 activity by slide agglutination, residual activity was detected. Additional extraction of the bacteria did not substantially increase the amount of K99 liberated and therefore two extractions were adapted for the purification schema. The amounts of K99 and protein extracted, and the specific activity of the K99 are shown in Table 1. Fig 3 shows an SDS-gel of the extracted material.

II. Ammonium sulfate precipitation. All subsequent steps were performed at 4 C. Ammonium sulfate (10.6 g/100 ml) was slowly added to the pooled extract with constant stirring. After 30 min, the insoluble material was removed by centrifugation (20,000 xg for 10 min) and discarded. Ammonium sulfate (11.3 g/100 ml) was again added to the supernatant. After another 30 min of stirring, the insoluble material was collected by centrifugation, resuspended in PB, and dialyzed overnight against PB. As shown in Table 1, the ammonium sulfate precipitation resulted in a recovery of 44% of the total extracted K99 activity and a 3.2-fold increase in specific activity. SDS-gels of this material (Fig 3) looked similar to the pattern observed from the extract with the exception that there was a substantial increase in the amount of material in band I with respect to the other bands, and band II was absent. Assay for K99 activity (Fig 1) showed the presence of the slow diffusing K99 component and the rapid diffusing non-K99 component.

III. DEAE-sephadex column chromatography. The sample from the ammonium sulfate precipitation step was next applied to a DEAE-Sephadex A50 column (2.5 x 40 cm) which was equilibrated with PB and developed with 300 ml of PB. A 400 ml 0-1.0 M NaCl gradient (in PB) was applied to the column. Fractions were assayed for absorbance at 280 nm and for K99 activity (Fig 4). All recovered K99 activity was located in fractions 14-42. Material pooled from this peak contained 26% of the original K99 activity (Table 1) and showed an additional 2.45-fold increase in specific activity. SDS-gels (Fig 3) showed that material from the DEAE-Sephadex column contained only bands I and III. Assay of K99 activity (Fig 1) showed that the DEAE-Sephadex step removed the rapidly diffusing material and only the slow diffusing K99 component was present.

IV. Cation-exchange columns. Aliquots from the pooled DEAE-Sephadex step were applied to columns of carboxymethyl cellulose or phosphocellulose, both equilibrated and eluted with PB. With each column, a single peak was obtained and it contained K99 activity. No increase in specific activity was detected and SDS-gels demonstrated the presence of bands I and III. The K99 obtained from these steps behaved identically to the DEAE-Sephadex derived K99 in the double diffusion assay (Fig 1).

V. Gel filtration chromatography. Columns of Sephadex G-50, G-100, Biogel P60, A1.5, A5 and A15 in PB were employed in an attempt to separate bands I and III from the DEAE-Sephadex purified K99. In each case, both bands (I and III) co-migrated in the column void volume and could not be separated. This indicated that K99 is very large (Biogel A15 excludes particles of 15×10^6 daltons or larger). The cation exchange and gel filtration columns were therefore not included in the purification schema since they did not result in any increase in purification.

At this point, it is of interest to note that SDS-gel bands I and III are not present in materials extracted from 1475 (K99-) which lacks the K99 plasmid (Fig. 5). Therefore, the presence of these two components is conferred on the strain carrying the K99 plasmid only. Also, treatment of absorbed K99 antiserum with purified K99 results in the loss of the ability of the antiserum to agglutinate the two K99+ strains. These data along with the data demonstrating the specificity of K99 precipitation in the double diffusion assay are convincing evidence that the purified material is indeed K99. All subsequent reference to purified K99 will refer to the material obtained from the DEAE-Sephadex column chromatography step.

Isoelectric focusing. In an attempt to separate bands I and III and to characterize these components, purified K99 was subjected to isoelectric focusing in a pH 3-10 gradient. In Fig 6, it can be seen that isoelectric focusing resulted in a single A_{280} absorbing peak with a corresponding pI of 10.1. Assay of K99 activity also resulted in a single peak located at the identical location as the A_{280} absorbing material. SDS-gels of this material showed the presence of both bands I and III. The basic nature of K99 was also confirmed by its migration toward the cathode during electrophoresis in polyacrylamide stacking gels. K99 freely electrophoresed through the 3% polyacrylamide portion but barely entered the 7-1/2% portion at the time that the tracking dye had migrated to the bottom of the gel.

Ultracentrifugation. The sedimentation coefficient of K99 was determined by two methods. The first involved the cosedimentation of purified K99 with 16s and 23s ribosomal ribonucleic acid through a neutral 5-20% sucrose density gradient. An $s_{20,w}$ of 15.2 was calculated. K99 sedimented as a single symmetrical peak which contained all of the measurable K99 activity. Fractions from various peak locations were analyzed in SDS-gels and were shown to contain bands I and III. In the second method, the sedimentation coefficient of purified K99 (in 0.05 M PB) was determined by sedimentation at 56,000 rpm at 20 C in a 2° , 12 mm Kel-F aluminum cell in a Spinco analytical ultracentrifuge. An $s_{20,w}$ of 12.96 was calculated. In the analytical ultracentrifuge, a single symmetrical peak was observed (Fig 7). A concentration dependence was indicated since the peak appeared to be hyper-sharp.

Molecular weight determination. The method of Weber and Osborn (19) which employs the use of SDS-gels was used to determine the molecular weights of the two K99 components. The results are shown in Fig 8 and indicate that band I has a molecular weight of 22,500 and band III has a molecular weight of 29,500. Incubation and electrophoresis of K99 in 2% SDS-gels yielded the same molecular weights. Also, omission of 2-mercaptoethanol from the incubation mixture does not affect the band patterns or molecular weights. The molecular weights obtained from SDS-gels are considerably lower than would be expected for a particle of 13-15s. This discrepancy probably indicates that native K99 is composed of subunits. The SDS-gels were scanned at 550 nm in a Gilford recording spectrophotometer and the results indicate that there was approximately 5 times more material in band I than in band III. Assuming each component stains identically with coomassie brilliant blue, there are 5 band I subunits for every one band III subunit.

Chemical analysis. Less than 7 μ g of neutral sugar and 66 μ g of lipid were detected per mg of K99 protein. The relative amino acid composition of K99 is given in Table 2. Calculation of the number of residues of each amino acid was based on a molecule of approximately 22,500. It should be noted that at this stage of purity, two proteins are present and therefore the results of amino acid analysis should be interpreted as such. K99 is very low in aromatic amino acids with 3 tryptophan residues and no tyrosine or phenylalanine residues. Three residues of an unusual amino acid, hydroxylysine, were found. Eight percent of the amino acids are basic (lysine, hydroxylysine, and

arginine). Based on the experimentally measured pI, it is assumed that all or most aspartic and glutamic acid residues are present as asparagine and glutamine prior to hydrolysis. No hexosamines were detected.

Hemagglutination. Strain 1474 (K99+) and 1475 (K99-) when grown in Trypticase soy broth and concentrated to 10^{10} cells/ml were capable of hemagglutinating guinea pig red blood cells in a D-mannose resistant manner. The ammonium sulfate precipitated 1474 (K99+) material also retained this capability. However, purified K99 no longer hemagglutinated guinea pig red blood cells. The hemagglutination activity was recovered from the DEAE-sephadex column in the material eluting after application of the salt gradient but containing no K99 activity.

Electron microscopy. Purified K99 was negatively stained and examined in an electron microscope (Fig 9). Numerous rod-like structures were observed. At high protein concentration (1.5 mg/ml), the rods had a strong tendency to aggregate and form long thick, somewhat irregular filamentous structures (Fig 8A). When the K99 concentration was diluted several fold, aggregates were no longer observed and instead short rod structures were seen (Fig 8B). These shorter structures had an average diameter of 8.4 nm (range = 7.0-9.8 nm) and a length of 130 nm (range = 84-183 nm). It is probable that the large, filamentous aggregates seen at high protein concentration are composed of the shorter rod structures.

DISCUSSION

K99 has been purified and has been shown to be composed primarily of protein. Less than 0.6% is carbohydrate and 6.6% is lipid. Data obtained from ultracentrifugation, gel filtration, double diffusion precipitation and isoelectric focusing indicate that K99 has been purified to homogeneity. However, under strong denaturing conditions (treatment with SDS), K99 can be dissociated into two protein components or subunits (SDS-gel bands I and III) having molecular weights of 22,500 and 29,500 respectively. In the native state, it is assumed that the two subunits are aggregated in an orderly manner to form a long filamentous structure. This interpretation is confirmed by observation of purified K99 in the electron microscope. The data from double diffusion, ultracentrifugation, and gel filtration which indicate a very large size for native K99 are also consistent with this interpretation. It is also possible that K99 is composed of a single subunit and that the other component is a K99 plasmid specific protein which co-purifies with K99.

The native filamentous structure of K99, its subunit structure, and its physical location on the bacterial cell envelope are consistent with it being a pilus or pilus-like structure. Pili are elongated or filamentous protein containing structures which reside on the outside of the bacterial cell envelope (2). Like K99, pili are aggregates of smaller subunits, called pilin. The postulated role of K99 is as a colonizing factor. In this regard, several other pili or pilus-like structures have been shown to be involved in the colonization of the small intestine by enterotoxigenic *E. coli* (7,11,18). By analogy, it can be hypothesized that K99 plays a similar role.

Isoelectric focusing showed that K99 has a pI of greater than 10 and therefore at the physiological pH of the small intestine it has a positive charge. The positive charge makes K99 particularly suitable as an adhesive structure involved in colonization of the small intestine. Besides the possibility of K99 attaching to specific intestinal epithelial cell receptors, it would also be expected to stick avidly in a non-specific manner to the acidic mucopolysaccharides found in intestinal mucus. One can envision a two-step process for K99+ E. coli strains to colonized calf small intestines. The first step is based on a charge attraction to intestinal mucus. Once the bacteria are immobilized, a highly specific interaction between K99 and an epithelial cell receptor occurs, further anchoring the bacteria in place. The second step would be required since it would be expected that with time and increase in intestinal secretion due to the induced diarrhea, that a substantial amount of mucus would be washed out of the host. Therefore, a secondary attachment would be necessary.

The role that K99 plays in colonization is at present uncertain. With the availability of pure K99, it will now be possible to study interactions of K99 with intestinal epithelium. Such experiments will be useful in the elucidation of the role that K99 plays in the colonization of the calf small intestine.

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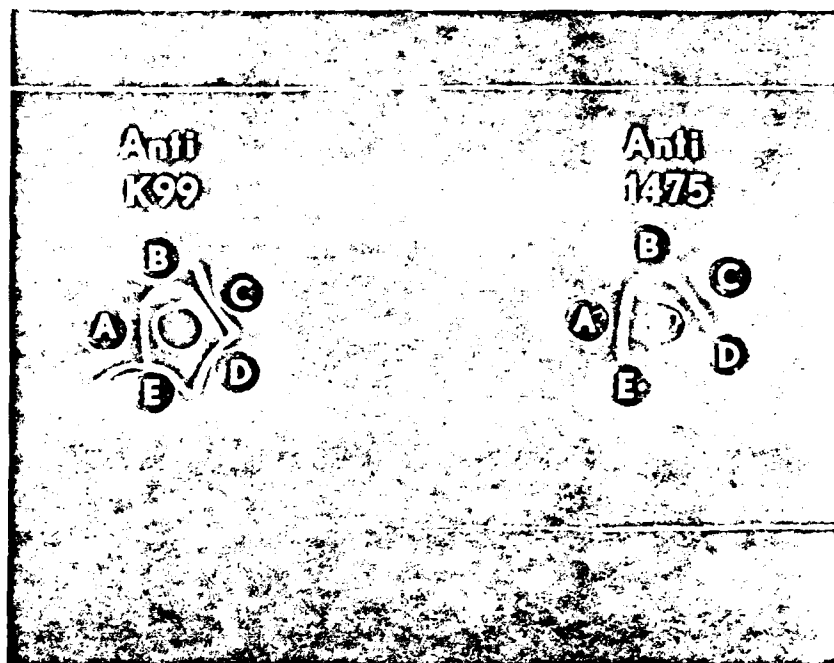


Fig. 1. Assay of K99 from 1474 ($K99^+$) at various stages of purity and 1475 ($K99^-$) using absorbed K99 antiserum and antiserum prepared against 1475 ($K99^-$). In well A is 1475 ($K99^-$) extract, B is 1474 ($K99^+$) extract, C is K99 after ammonium sulfate precipitation, D is K99 after DEAE-Sephadex, and E is K99 after carboxymethyl cellulose.

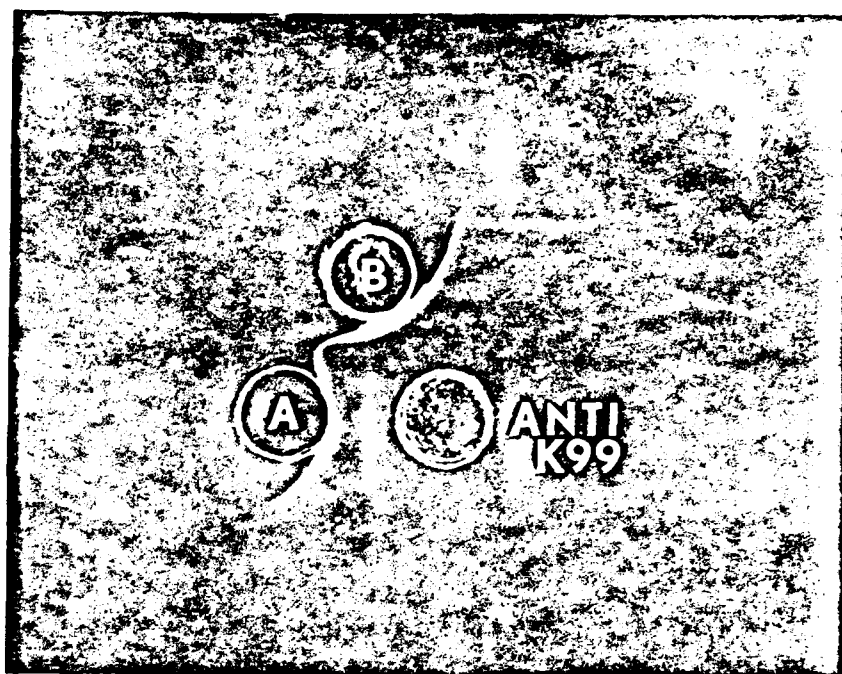


Fig. 2. Comparison of K99 activity from B41 extract (A) and purified K99 from 1474 (K99⁺) (B).

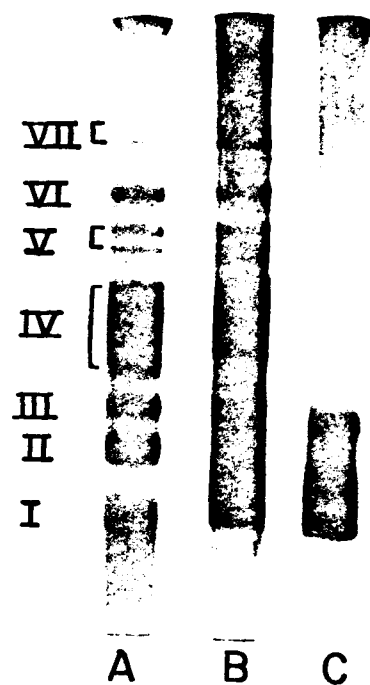


Fig. 3. SDS-gels of 1474 ($K99^+$) extract (A), ammonium sulfate precipitate (B), and DEAE-Sephadex purified K99 (C). Bands or band clumps are numbered with Roman numerals.

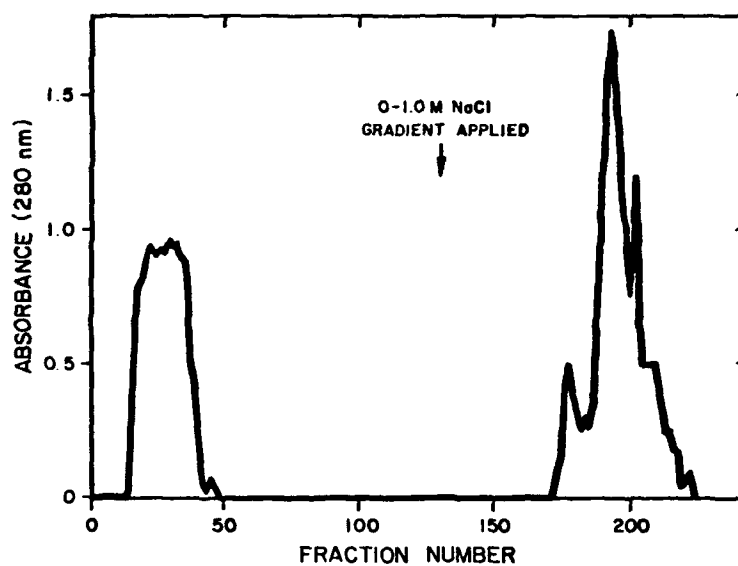


Fig. 4. DEAE-Sephadex chromatography profile of ammonium sulfate precipitate from 1474 (K99⁺). Conditions are described in Results.

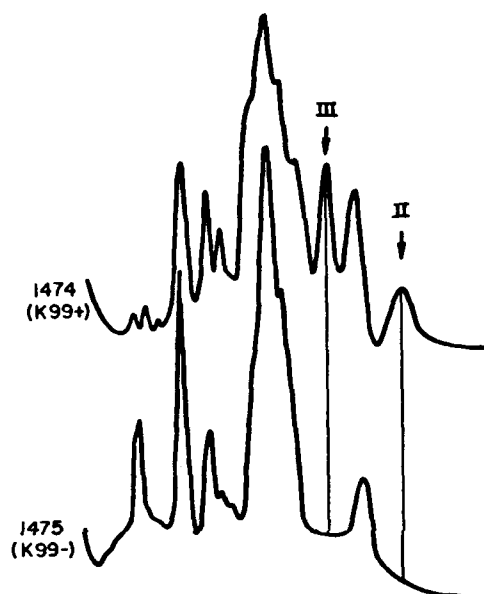


Fig. 5. Scan of 550 nm coomassie blue stained SDS-gels of extract from 1474 ($K99^+$) and 1475 ($K99^-$). Scan was performed using a Gilford recording spectrophotometer.

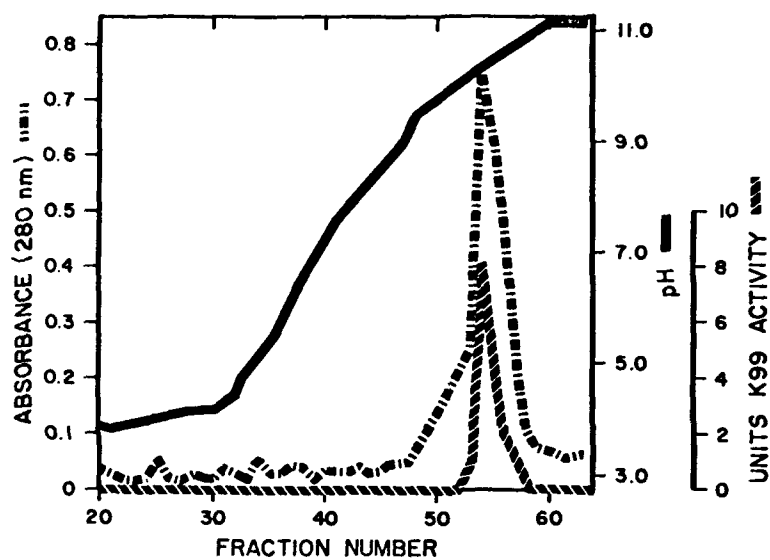


Fig. 6. Isoelectric focusing of purified K99. Conditions are described in Materials and Methods.

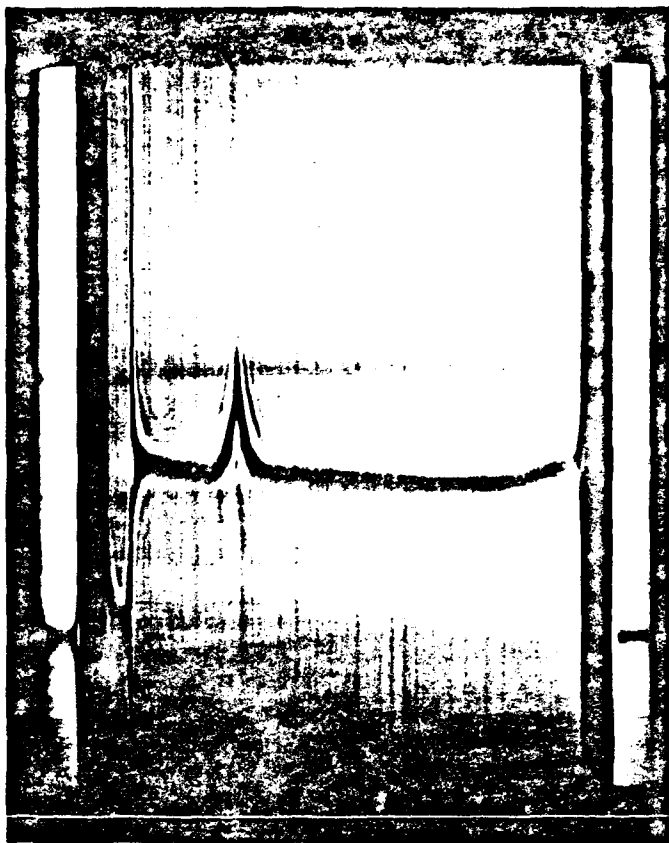


Fig. 7. Schlieren pattern obtained for purified K99 in the analytical ultracentrifuge. Conditions for centrifugation are described in Results. Concentration of K99 is 1.5 mg/ml.

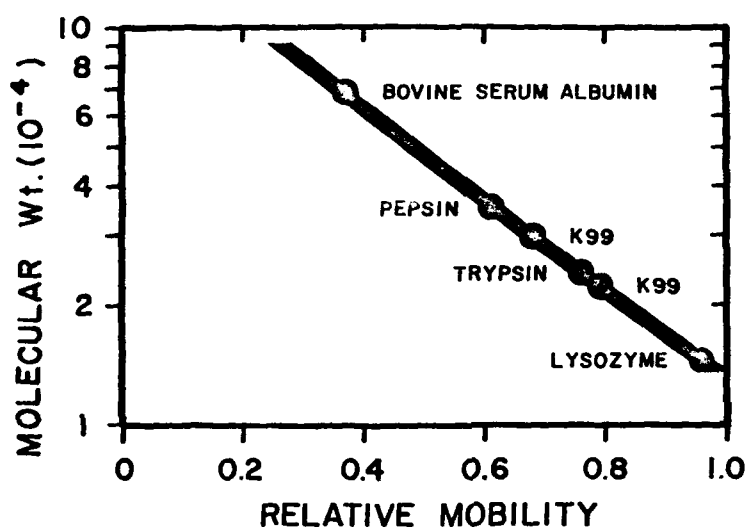


Fig. 8. Determination of K99 molecular weights in SDS-gels. Conditions for electrophoresis are described in Materials and Methods. The molecular weights of marker proteins are bovine serum albumin, 68,000; pepsin, 35,000; trypsin, 24,000; lysozyme, 14,600. The relative distance of migration is expressed as the distance of migration with respect to the bromophenol blue tracking dye.

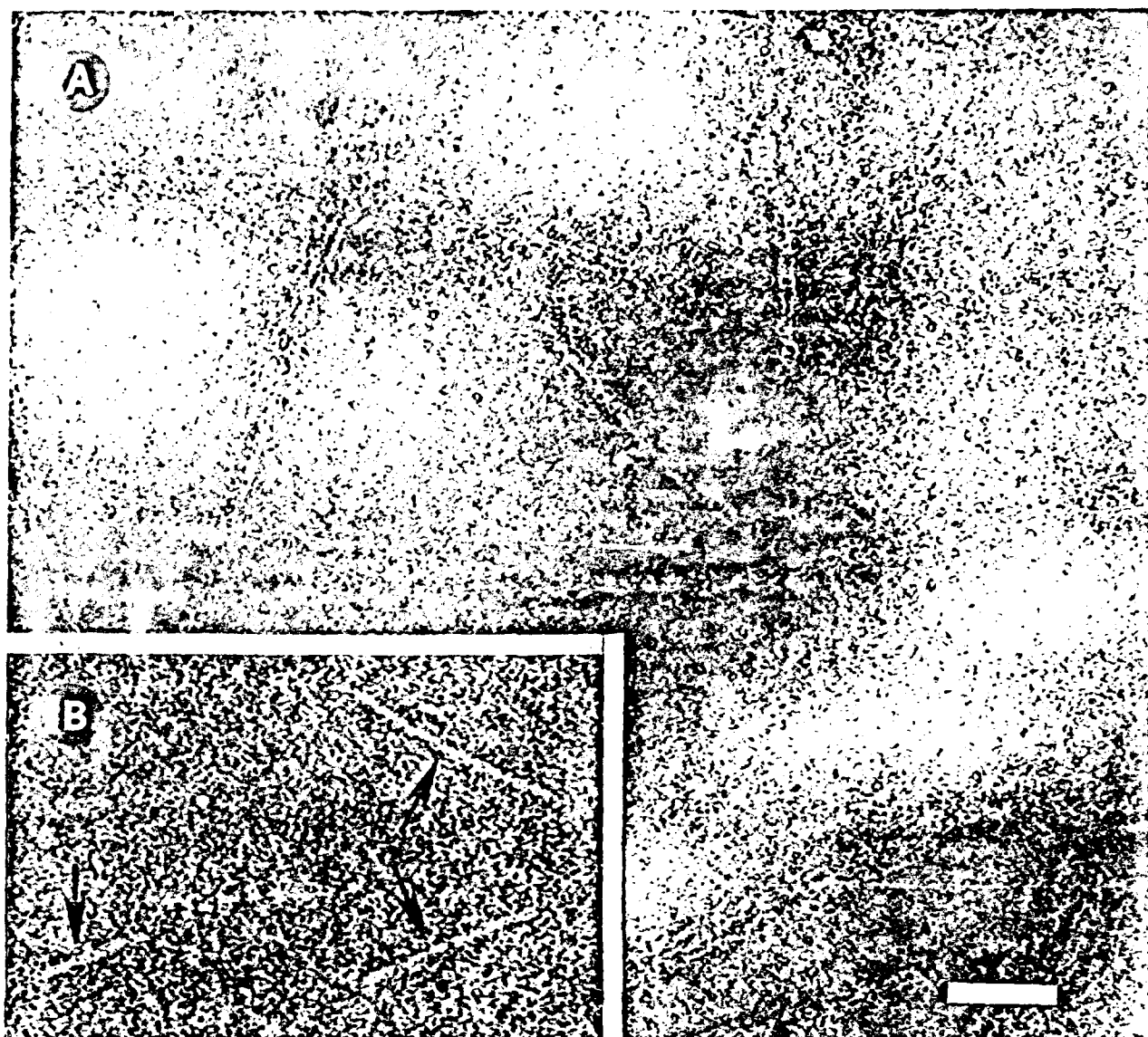


Fig. 9. Electron microscopy of purified K99 negatively stained as described in Materials and Methods with 1% potassium phosphotungstate. Concentration in A is 1.5 mg/ml, and in B is 150 μ g/ml. Magnification: 177,000 X. Bar equals: 100 nm.

Table 1. Purification of K99^a

Step	Protein (mg)	K99 (units)	Specific activity (units/mg protein)	Yield ^b (%)
Crude extract	3887	207,000	53	100
Ammonium sulfate	526	92,000	174	44
DEAE-Sephadex	125	53,360	427	26

^aData is based on purification from 72 liters of broth grown 1474 (K99+).

^bYield based on the amount extracted from whole cells.

Table 2. Amino acid composition of K99

Amino acid	Calculated ^{a,b}	Assumed
Cysteine ^c	2.0	2
Aspartic	25.5	25
Threonine ^d	20.2	20
Serine ^{d,f}	21.6	22
Proline	2.6	3
Glutamic	7.2	7
Glycine	15.2	15
Alanine	46.4	46
Valine	13.6	14
Isoleucine	13.1	13
Leucine	7.5	7
Lysine	9.4	9
Histidine	3.4	3
Arginine	4.2	4
Hydroxylysine	2.8	3
Tryptophan ^e	2.9	3

^aBased on 2.0 cysteine.

^bAverage values for 24, 48, and 72 h hydrolysates except where noted.

^cDetermined as cysteic acid after oxidation by performic acid (10).

^dValues obtained by extrapolation to zero time.

^eDetermined spectrophotometrically (4).

^fSerine and methionine sulfone could not be resolved on the amino acid analyzer and therefore both are expressed serine only.

The Occurrence of K99 Antigen on Escherichia coli
Isolated from Pigs and Colonization of Pig Ileum by K99⁺

Enterotoxigenic E. coli from Calves and Pigs

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Several strains of enterotoxigenic E. coli (ETEC) isolated from pigs were found to have an antigen (K99) previously reported only on strains of calf and lamb origin, and which facilitates intestinal colonization in the latter 2 species. Several human ETEC were also tested for K99, however, none were positive. Each of four K99 positive ETEC strains of calf origin and one of pig origin produced K99 in pig ileum in vivo, adhered to villous epithelium in pig ileum, colonized pig ileum, and caused profuse diarrhea in newborn pigs. In contrast to the K99 positive strains above, 4 K99 negative ETEC from humans and chickens and one K99 positive ETEC from a calf, either did not colonize pig ileum or did so inconsistently. When the K99 negative strains did colonize, they had little or no tendency to adhere to intestinal villi. These results are consistent with the hypothesis that K99 facilitates adhesion to and colonization of pig ileum by some ETEC.

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Running Head: Intestinal Colonization by E. coli.

Antigen K99 is common among enterotoxigenic Escherichia coli (ETEC) isolated from calves and lambs, and facilitates colonization of calf and lamb small intestine (11,14). Intestinal colonization by K99 positive ETEC appeared to be host species specific, in that, such strains intensively colonized lamb but not pig small intestine and K99 was not reported among ETEC from other species. Host species specificity appears to be a general characteristic of enterotoxigenic E. coli, in that, calves, lambs, monkeys, and humans were not intensively colonized by pig ETEC fed to them (2,12). Although some serotypes of E. coli have been isolated from cases of enteric disease in more than 1 host species, in general those isolated from different host species tend to be serologically different as well (15). Furthermore K88 antigen, which is common among pig ETEC and facilitates colonization of pig small intestine, has not been reported among ETEC from other species. In contrast there is evidence that some ETEC have the ability to colonize the small intestine of more than 1 host species. Evans et al., reported colonization of rabbit small intestine by an ETEC from humans (4) and Davidson et al. (1), presented indirect evidence that K83 carrying ETEC of porcine origin colonize the small intestine of newborn mice.

The objectives of this manuscript are to report that: (1) Some ETEC isolated from pigs have K99 antigen. (2) Some K99 positive ETEC isolated from calves have the ability to colonize the ileum and adhere to villous epithelium in pigs. (3) K99 antigen can be produced in pig ileum in vivo.

MATERIALS AND METHODS

E. coli strains. The K99⁺ calf ETEC reference strains were B41, B44 and B117 (11). Additional K99⁺ calf ETEC used were strains 665, 1439, E90B and 990A. The latter 2 were supplied by Dr. Steve Acres. Dr. Carlton Gyles supplied the laboratory strain K12 and a derivative of it which carried the K99 plasmid from strain B41 (K12-K99) and another derivative of it which carried a K88 plasmid (K12-K88). He also supplied the non-enterotoxigenic chicken strain F11 and a derivative of it carrying the enterotoxin plasmid from strain P155 [F11 (P155-Ent⁺)]. Dr. Sam Formal supplied human ETEC strains H10402, H13634, H10400, H10401, H10407, B7A and B2C. The pig ETEC were the K88 negative strains 431, 613, 1351, 987, 340, 74-5208 and 381 described previously (10) plus TR75211 (Troyer) (9). In addition, 65 E. coli isolated from the intestine of newborn pigs with diarrhea were tested for K99 antigen. The pigs from which these isolates were taken came from several different herds in Minnesota, Iowa, Ohio and South Dakota. Many, but not all, of the isolates had previously been shown to produce enterotoxin.

K99 antiserum. This was produced in rabbits according to the method for producing O:K antiserum (3). The antigen was strain K12-K99. The resulting hyperimmune serum was absorbed (3) with the parent K12 strain which lacked the K99 plasmid. The resultant absorbed serum did not agglutinate the K12 parent, but did agglutinate the K12-K99 used for immunization and the wild type, K99⁺ calf ETEC reference strains. E. coli agglutinins in the absorbed serum were considered to be mono-specific for K99. It was used in agglutination tests after a further 10-fold dilution and in indirect immunofluorescence tests after a further 100-fold dilution.

Demonstration of K99 on bacteria grown in vitro. This was done by slide agglutination tests using live bacteria. Bacteria were grown aerobically at 37 C (except when stated otherwise below) on trypticase soy agar (BBL) or Minca agar (5) for 24 or 48 h. When available, individual small translucent colonies were selected as recommended (11). When small translucent colonies were not available, areas of confluent growth or individual colonies were selected at random for testing. Bacteria from a single colony or area of confluent growth were suspended in 2 separate drops of saline on a glass slide, K99 antiserum was added to 1 drop and normal rabbit serum to the other. All tests were replicated on 3 or 10 colonies or areas per plate. Suspensions which underwent grossly apparent agglutination in K99 antiserum and were not agglutinated in the normal rabbit antiserum, were recorded as K99 positive.

Immunofluorescence tests for K99 were done by the indirect method using K99 antiserum as the first antibody and fluorescein-labeled goat immunoglobulin G prepared against rabbit immunoglobulin G (Miles Labs., Inc.) as the second antibody. These were controlled by conducting parallel tests in which normal rabbit serum and/or K88 antiserum was (were) used as the first antibody on the same material tested for K99. The K88 antiserum was prepared the same way as K99 antiserum using strain K12-K88.

Cross agglutination and absorption. The methods for absorption of serum and production of 431-0:K antiserum, shown in Table 1 were those of Edwards and Ewing (3). The antigens were grown on Minca for 24 h except strain K12-K99, which was grown on trypticase soy agar. In order to be sure that the colonies used for the tests were agglutinable in K99 antiserum, the tests were conducted such that portions of a single colony were tested in all 4 or 5 of the serums shown (Table 1).

Colonization of pig small intestine. Newborn hysterectomy derived colostrum deprived pigs were exposed intragastrically to 1 ml of an overnight broth culture of E. coli as previously (10). The pigs were killed 16 h post-exposure and observations on weight loss, numbers of E. coli/segment and layers of E. coli adherent to villous epithelium in ileum were made as previously (10). Pigs having $\geq 10^8$ viable test strain E. coli/segment were considered to be colonized. Each strain was tested in 6 pigs (2 pigs from each of 3 different litters). The numbers of E. coli and the extent to which they were adherent to intestinal villi were determined for 4 pigs per strain. Layers of adherent E. coli were evaluated by the association index method (microscopic examination of immunofluorescent stained sections from colonized ileal segments) (10). Fluorescent antibody conjugates using the direct immunofluorescence method and antisera prepared against strains 431 and K12K99 were both used to determine the association indices of the K99 positive strains. Some sections were also examined by the indirect fluorescent antibody method. Antisera against the K99 negative strains were not available.

Therefore, the association indices for these strains were determined using Giemsa stained sections.

RESULTS

K99 positive pig ETEC. Four of the 8 K88 negative pig ETEC initially tested were positive for K99. These were strains 431, 613, 1351 and Troyer which had previously been shown to adhere to epithelium in and colonize pig ileum. Like some calf strains, K99 was sometimes difficult to demonstrate on these pig strains (5,11) and results were not consistent day to day. The percentage of positive reactions was increased by selecting small translucent colonies and growing on Minca rather than on trypticase soy agar. For example, in 1 set of tests with strain 431, none of 10 small colonies from trypticase soy agar were positive, while 2/10 large and 6/10 small colonies grown on Minca were positive. The numbers of positive colonies/number tested for the Troyer strain in the same set of tests were, 3/10 for trypticase soy agar, 3/10 for large and 7/10 for small colonies grown on Minca. Strains B41, 117, 431 and Troyer grown 48 h on Minca were agglutinated by K99 antiserum when grown at 37 C but not when grown at 18 C.

There were no differences among the K99 antigens of the pig and calf strains tested by reciprocal cross agglutination and cross absorption tests (Table 1). Calf strains B41, B117 and 665 as well as pig strain Troyer and laboratory strain K12-K99 were all agglutinated in O:K antiserum to strain 431, demonstrating that the cross reaction between K99 and strain 431 was 2-way. Absorption of K99 antiserum with 431 antigen removed the K99 agglutinins. Furthermore, absorption of 431-O:K antiserum with K12-K99 antigen removed its B117, 665 and Troyer agglutinins. Strains 431 and 1459 were also examined at the WHO Collaborative Centre for Reference and Research on Escherichia, Copenhagen, Denmark, and their serotypes found to be: 0101:K30,K99:H⁻ and 09:K35,K99:H⁻.

Some of the 65 additional E. coli isolates from the intestines of newborn pigs with diarrhea also carried K99. At least 1 agglutinable colony was detected in 10 of 18 isolates of serogroup 0101:K30(A), in 4/4 isolates of 064, in 0/8 isolates of 0101:K+(A), in 0/18 isolates of 09, and in 0/17 isolates of 020. The human ETEC strains were all negative.

Colonization of pig small intestine by calf ETEC. Five K99 positive bovine strains and five K99 negative strains of chicken or human origin were tested in parallel (Table 2). Four of the 5 calf strains (B44, E90B, 665 and 1439) consistently colonized pig ileum (more than 10^8 E. coli/segment) and had high association indices (layers of E. coli adherent to villous epithelium) (Table 2 and Fig. 1). The adherent E. coli stained with both fluorescent antisera (anti-K12-K99 and anti-431) and both antisera gave the same association indices.

The colonizing and adhesive attributes of these 4 strains were comparable to those for pig enteropathogens tested in this system previously (10). The incidence of diarrhea and severity of weight loss caused by these 4 strains was also comparable to that caused by pig enteropathogens (10). Two of the pigs exposed to strain E90B did not develop diarrhea; unfortunately, the numbers of E. coli and the association indices for these 2 pigs were not determined.

In contrast to the other 4 calf K99 positive ETEC, strain 990A did not colonize the ileum of any of the pigs, nor did the enterotoxigenic chicken strain F11 (P155 - Ent positive) nor human strain B7A. Chicken strain F11 and human strains H10407 and B2C colonized (attained more than 10^8 per segment) 5 of the 12 pigs that were examined bacteriologically. However, even in these 5 colonized segments, these strains had little or no tendency to adhere to epithelium (association indices 2-1) (Table 2).

Diarrhea occurred in 22 of 24 (92%) pigs exposed to the 4 consistently colonizing strains (Table 2) and the mean weight loss of these 24 pigs was 17.5%. Diarrhea occurred in 24 of 30 (80%) pigs exposed to the 5 noncolonizing or inconsistently colonizing ETEC, and these 30 had a mean weight loss of 9%. None of the pigs exposed to the nonenterotoxigenic strain F11 developed diarrhea and these had a mean weight loss of 4%.

In vivo production of K99. Presumptive evidence that calf strains can produce K99 in pig ileum was provided by the observation that layers of strains B44, E90B, 665 and 1439, adherent to ileal villi in pigs 16 hours post exposure, consistently stained with fluorescent antibody prepared against strain K12-K99 and against strain 431 (Table 2, Fig. 1). The only antigen known to be common to all 6 of these strains is K99. Sections from the ilea of pigs exposed to the 4 calf strains above were recut and stained with the K99 antiserum (from which K12 antibodies had been removed by absorption) via the indirect immunofluorescent method, using normal rabbit serum as the control. All sections exposed to K99 antiserum were positive (comparable to Fig. 1) and those exposed to normal rabbit serum were negative (the adherent layers of bacteria were not stained).

Sections of ileum from 3 pigs used in previous experiments (10) and shown to have layers of strain 431 adherent to their villi were recut (3 slides per pig) and examined by the indirect immunofluorescence method using K99 antiserum as the first antibody for 1 slide, K88 antiserum as the first antibody for the second slide and normal rabbit serum as the first antibody for the third slide from each pig. Layers of strain 431 adherent to villi were demonstrable in all 3 sections exposed to K99 antiserum (Figs. 2, 3). These were not visible in sections exposed to K88 antiserum or normal rabbit serum.

DISCUSSION

The pig ETEC strains 431 and Troyer were shown by serum agglutination and cross absorption tests to have an antigen either closely related or identical with the K99 antigen of a K12 strain to which the K99 plasmid from a calf strain had been transferred. Like K99 antigen, that on the pig ETEC was also: (a) more readily demonstrable in small than in large colonial forms, (b) more readily demonstrable in colonies grown on Minca than on trypticase soy agar, and (c) suppressed when grown at 18 C (11). We interpret these results to mean that the antigen on the pig ETEC is identical to K99. In view of the preliminary survey conducted it seems probable that K99 occurs rather commonly on pig strains of O groups 101 and 64, occasionally on those in O group 9, and may occur in other O groups as well. Our failure to demonstrate it on human ETEC could be because it is not carried by human ETEC. Alternatively, it may be carried by human ETEC other than those tested, or it could have been present on the human ETEC tested but not detected. The latter possibility warrants consideration in view of the difficulty in consistently demonstrating this antigen on some strains.

Presumably the K99 demonstrated in vivo was produced in vivo as the inocula contained approximately 10^9 cells and the segments examined for K99 contained 10^8 - 10^9 /10 cm by 16 h post exposure. There is evidence that K99 facilitates colonization of calf and lamb small intestine and it was suggested that K99 might act as an adhesin to

facilitate such colonization (14). Studies of adhesion by K99 positive strains have not previously been reported. The data reported here demonstrate that K99 positive ETEC can adhere in pig ileum. However, they do not permit conclusions as to whether or not the adhesion was mediated by K99. In contrast to the results reported here, K99 positive calf and lamb ETEC apparently did not colonize pig small intestine (14). The reasons for these different results between laboratories, using different strains and different pigs, are unknown. Strain 990A did not colonize pigs in this study, demonstrating that not all K99 positive ETEC colonize pig ileum in the model used here. Furthermore (even though present), K99 may not have facilitated colonization of pig ileum by the other 4 strains which were consistent colonizers (Table 2). That is, the latter strains may have had attributes other than K99 which allowed them to adhere to epithelium and colonize pig ileum. This would imply that such strains possess K99 which facilitates colonization of calf and lamb small intestine, and an additional attribute (or attributes) which facilitates colonization of ileum in the newborn pig. However, both pig and calf ETEC produced K99 in pig ileum in vivo. For this reason we are more attracted to the hypothesis that the ileum of the newborn pig provides an environment comparable to calf intestine with regard to colonization by ETEC and that K99 facilitates colonization in this environment. Assuming this hypothesis is correct, the K99 of the noncolonizing strain 990A, as used here, could be quantitatively or

qualitatively defective, or the remainder of the bacterial cell could lack other unknown characteristics which are necessary for colonization.

Pigs exposed to ETEC developed diarrhea even though not colonized (less than 10^8 E. coli/10 cm ileum). These pigs may have had more than 10^8 E. coli/10 cm at other sites in small intestine. Alternatively fewer than 10^8 ETEC/10 cm may be sufficient to cause diarrhea. Weight loss of pigs colonized by ETEC exceeded that of those exposed to but not consistently colonized by ETEC (Table 2). This indicates that diarrhea was more profuse in the colonized pigs and is additional evidence that the consistent colonizers were more virulent than the inconsistent and noncolonizing ETEC.

K88 antigen is a pilus, or pilus-like structure (16), produced by many pig ETEC and which facilitates adhesion to and colonization of pig small intestine (8,13). There is evidence that pili such as those on pig ETEC strain 987 (distinct from K88 and K99 and designated as 987-P) also facilitate adhesion and colonization in pig small intestine (6). There is evidence that K99 is also a pilus (7). These observations and the data reported here are consistent with the hypothesis that K99 is 1 of several pili occurring among pig ETEC which act as adhesins to facilitate intestinal colonization. However, before acceptance of the hypothesis, the role of K99 and 987-P, both in adhesion and in colonization of pig small intestine should be investigated directly. Information as to how

many and which pili of ETEC are involved in intestinal colonization and their host species specificity will have useful diagnostic, epidemiologic and preventative implications for enteric infections with enterotoxigenic E. coli.

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Fig. 1. E. coli of K99 positive calf strain B44, adherent to villous epithelium in ileum of newborn pig (association index 5.0). Frozen section stained directly with fluorescent antibody prepared against E. coli strain 431.

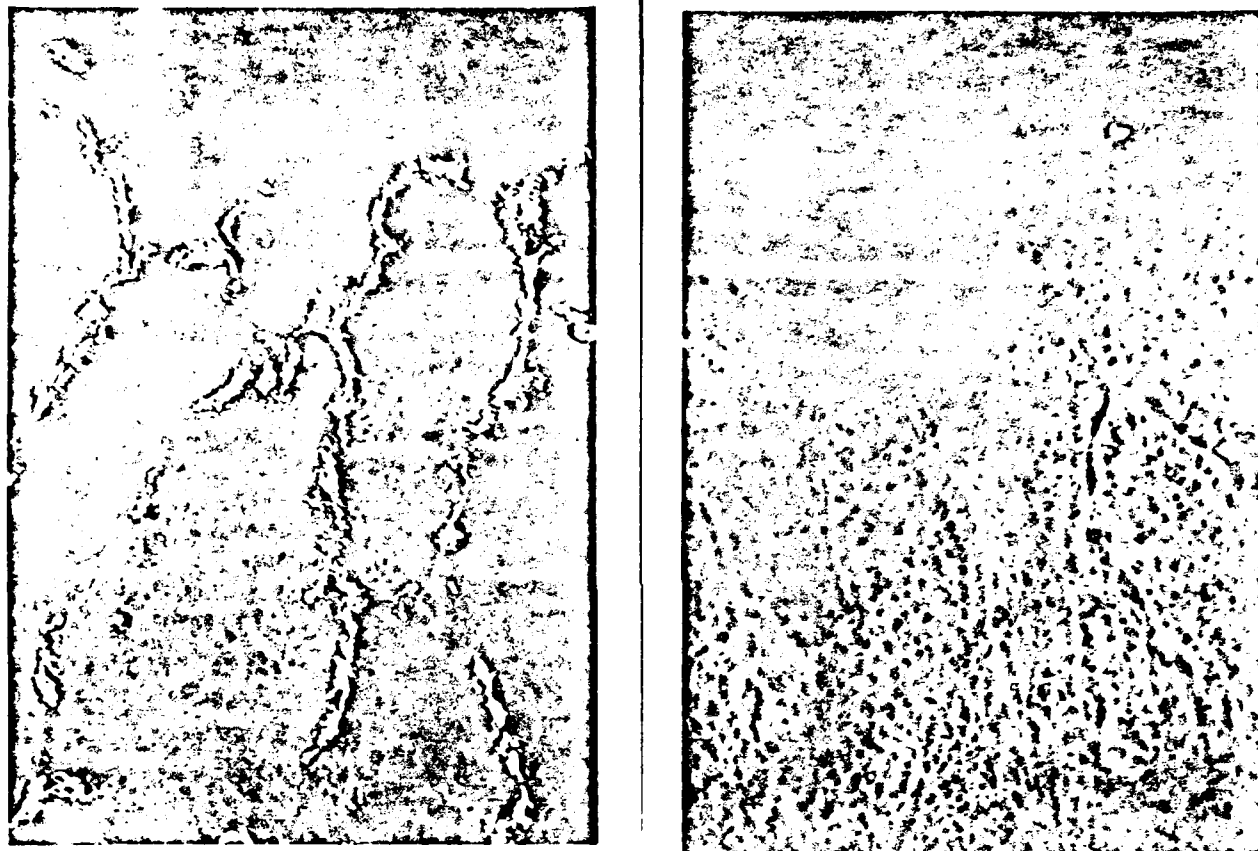


Fig. 2. K99 antigen produced by pig E. coli strain 431 in pig ileum in vivo. (a) Layers of bacteria adherent to villi were stained for K99 by the indirect immunofluorescent method using specific K99 antiserum. (c) Section from same segment of ileum as (a) but stained via specific K88 antiserum. The K99 positive bacteria are not stained. Indirect immunofluorescence.



Fig. 3. K99 antigen produced by pig E. coli strain 431 in pig ileum in vivo, as Fig. 2 except (a) higher magnification to demonstrate that the antigen occurs as a ring of peribacterial fluorescence. (b) Section from same segment of ileum as (a) but stained with K88 antiserum. The K99 positive bacteria are not stained. Indirect immunofluorescence.

TABLE 1. Results of slide agglutination tests using living Escherichia coli antigens and rabbit serums as agglutinins

Strain	Antigen Serotype	Origin	Antiserum				Unimmunized rabbit
			K99	431:O:K	K99/absorbed by 431 antigen	431-O:K/Absorbed by K12-K99 antigen	
B41	0101:K99:NM	Calf	+	+	0	NT ^a	0
B117	08:K85,99:NM	Calf	+	+	0	0	0
665	014:K99:NM ^b	Calf	+	+	0	0	0
K12-K99	K12:K99 ^c	Laboratory	+	+	0	0	0
431	0101:K30(A):NM	Pig	+	+	0	NT	0
Troyer	09:K35(A)	Pig	+	+	0	0	0

^aNot tested.

^bIt is not known whether or not strain 665 has a K antigen in addition to K99.

^cThe antigens of strain K12-K99 other than K99 are unknown and indicated as K12.

TABLE 2. Diarrhea and intestinal colonization of newborn pigs exposed to Escherichia coli from different sources

E. coli inoculum		Pigs 16 hours post exposure				Ileal E. coli ^a	
Strain	Source	Serotype	Diarrhea	% Weight loss (mean)	Numbers ^b	Range	Association index (mean) ^c
B44	Calf	09:K30,99:NM	6/6	17	7x10 ⁸	10 ⁸ -10 ⁹	4.5
E90B	Calf	K99 ^d	4/6	14	2x10 ⁹	10 ⁹	4.8
665	Calf	014:k99 ^d	6/6	20	2x10 ⁹	10 ⁸ -10 ⁹	4.4
1439	Calf	K99 ^d	6/6	19	1x10 ⁹	10 ⁸ -10 ⁹	4.4
990A	Calf	08:K85,99:H27	4/6	9	9x10 ⁶	10 ⁵ -10 ⁷	-
H10407	Human	078:K80:H11	5/6	6	1x10 ⁸	10 ⁷ -10 ⁸	1.0
B7A	Human	0148:H25 ^d	6/6	13	2x10 ⁶	10 ⁶	-
B2C	Human	06:H16 ^d	6/6	9	2x10 ⁸	<10 ⁵ -10 ⁸	1.0
F11(P155-Ent+)	Chicken	? ^d	3/6	8	5x10 ⁶	10 ⁵ -10 ⁷	-
F11	Chicken	? ^d	0/6	4	7x10 ⁷	10 ⁷ -10 ⁸	2.0

^aFour pigs per strain.

^bNumber of viable test strain E. coli per 10 cm segment of ileum.

^cMicroscopic evaluation of intensity of adhesion to intestinal villi in pigs with $\geq 10^8$ E. coli per ileal segment (5.0 is maximal, 1.0 is minimal).

^dSerotype unknown.

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1. Growth of enterotoxigenic <u>E. coli</u> in porcine small intestine selects for piliated forms which adhere to the intestinal epithelium. 2. Surface antigen K99 on enterotoxigenic <u>E. coli</u> is a pilus. 3. Antigen K99 occurs on porcine enterotoxigenic <u>E. coli</u> strains and is produced in pig small intestine.		

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