Detection of *Escherichia coli* Adhesins with DNA Probes

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DNA hybridization assays for genes encoding the *Escherichia coli* adhesins K88 and K99 were developed. These assays were used to screen a variety of animal *E. coli* strains, and the results were compared with results obtained by serological methods. All methods compared well for determining the presence of adhesin K99. However, the DNA hybridization detected more K88-positive strains than did either enzyme immunoassay or slide agglutination. Agreement was improved when strains were grown on blood or nutrient agar instead of Minca medium.

Enterotoxigenic *Escherichia coli* strains are a significant cause of diarrheal disease in animals and humans. Standard techniques for determining toxin production by *E. coli* have involved the use of suckling mouse assays for the heat-stable toxin (ST1) and enzyme immunoassay (EIA) and Y1 adrenal cell lines for heat-labile toxins. Recently, DNA hybridization techniques have become available for the identification of those *E. coli* strains which possess the genes for toxin production (8, 13, 14, 17-19). Several workers have subsequently used these more efficient techniques to study the epidemiology of enterotoxigenic *E. coli* strains in various populations (12, 16).

Another virulence factor associated with enterotoxigenic *E. coli* strains is the ability to attach to mucosal surfaces. Pili (fimbriae) enable *E. coli* strains to adhere to the gut epithelium, thus facilitating release of the enterotoxins at their site of action. Serological methods for typing adhesins K88, K99, and 987P have been described previously (4, 6, 15). Genes specifying some of these attachment antigens or adhesins have recently been cloned into plasmid vectors (2, 10, 11, 20), and the genes involved have been physically mapped on the DNA. From the cloned genes specifying K88 and K99 we have developed DNA probes for the identification of strains possessing these genes and have compared the technique with serological assays.

MATERIALS AND METHODS

E. coli strains carrying recombinant plasmids pFM205 and pRI9906-1 containing the genes for K88 and K99, respectively, were obtained from Frits de Graaf, Department of Microbiology, Free University, Amsterdam, through P. A. Manning, Department of Immunology and Microbiology, University of Adelaide, Adelaide, Australia.

Plasmids were purified from their host *E. coli* cells by a modification of the alkaline lysis method of Birnboim and Doly (1). A 50-ml portion of an overnight culture of bacteria in nutrient broth was added to 1 liter of L broth (9) and incubated with shaking at 37°C. At mid-log phase, usually after about 4 h, chloramphenicol was added to a concentration of 150 mg/liter to amplify the plasmid, and incubation was continued overnight. The bacteria were pelleted at 5,000 × g for 10 min. The pellet from each 500 ml of culture was resuspended in 4 ml of 25 mM Tris (pH 8.0)-10 mM EDTA-15% sucrose-2 mg of lysozyme per ml. The suspension was kept on ice for 20 to 40 min, after which 8 ml of 0.2 M sodium hydroxide-0.1% sodium dodecyl sulfate was

added. The tubes were mixed gently by inversion and then left on ice for 10 min (pH 12.5). A 5-ml portion of 3 M sodium acetate (pH 4.6) was added, and the tubes were stood on ice for 40 min. The tubes were then spun at 15,000 rpm for 15 min, and the supernatant was removed very carefully. Heat-inactivated RNase A was added to the supernatant (100 µg per liter of original culture), which was incubated at 37°C for 2 h. One phenol-chloroform (1:1, vol/vol) extraction was carried out. The volume of DNA was then reduced to approximately 5 ml by two butanol extractions. The phenol and butanol were removed by three or four ether extractions, and the ether was subsequently removed by heating in a 68°C water bath for 10 min. The DNA was dialyzed against 0.1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) for at least 4 h and then precipitated overnight at -20°C. The following day the precipitate was collected by centrifugation at 10,000 × g for 30 min, and the supernatant was discarded. The DNA pellet was lyophilized. If too much salt had precipitated, the pellet was washed with 70% ethanol and freeze-dried again. The purified DNA pellet was reconstituted in 1 ml of TE (10 mM Tris-hydrochloride, 1 mM EDTA [pH 8]).

Plasmids were digested with the restriction enzymes *EcoRI* (for K88) and *SmaI* and *BglIII* (for K99) obtained from Boehringer Mannheim Biochemicals and used according to specified reaction conditions. The digests were fractionated by 0.8% agarose gel electrophoresis.

The smaller DNA band derived from each digest contained the required fragments. For both K88 and K99 these fragments were part of the structural genes encoding the subunits of the fimbriae (10, 20). Each band slice was cut out of the gel, placed in dialysis tubing, and eluted by electrophoresis from the agarose into buffer within the dialysis tubing. The DNA was phenol-chloroform extracted and ethanol precipitated. The probes were labeled with ³²P-phosphate by the nick-translation method described by Maniatis et al. (9).

All strains were typed for O antigen by standard methods at the Salmonella Reference Laboratory, Institute of Medical and Veterinary Sciences. A total of 101 wild-type *E. coli* strains previously typed by slide agglutination with absorbed K88 and K99 antisera were obtained from Keith McKechnie through Parry Monckton, Veterinary Laboratory, Bendigo, Victoria, Australia. Organisms for slide agglutination had been grown on blood agar. In addition, 544 *E. coli* strains were obtained from the Salmonella Reference Laboratory. These strains had been submitted to the Reference Labora-

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TABLE 1. Comparison of DNA hybridization with EIA for detecting adhesins in 645 strains of *E. coli* grown on Minca agar

EIA reaction	No. of strains tested by DNA hybridization for ^a :			
	Adhesin K88		Adhesin K99	
	+	–	+	–
+	125	0	33	0
–	23 ^b	497	0	612

^a +, Positive for adhesins by DNA hybridization; –, negative for adhesins by DNA hybridization.

^b A total of 21 of 23 strains found to be EIA positive when subsequently grown on nutrient agar.

tory for O, H, and K serotyping and had been isolated from a variety of animals, including sheep, birds, pigs, and calves.

All strains were typed for the K88ab and K88ac variants of the K88 antigen and for K99 by an EIA kit supplied by Biotechnology Australia. Strains were grown overnight at 37°C on a Minca plate (7) with the following formulation: KH₂PO₄, 1.36 g; Na₂HPO₄ · 2H₂O, 10.1 g; glucose, 1 g; Casamino Acids (Difco Laboratories), 1 g; agar (Difco), 12 g; trace salts solution (MgSO₄ · 7H₂O, 10 g/liter; MnCl₂ · 4H₂O, 1 g/liter; FeCl₃ · 6H₂O, 0.135 g/liter; CaCl₂ · 2H₂O, 0.4 g/liter), 1 ml; Vitox SR90 (Oxoid Ltd.), 10 ml; distilled water, 1 liter. Later in the study, organisms were grown on 5% sheep blood agar (sheep blood, 50 ml; Oxoid Agar no. 3, 12 g; Lab Lemco [Oxoid], 10 g; peptone [Oxoid], 10 g; sodium chloride [BDH], 7.5 g; yeast extract [GIBCO Laboratories], 5 g/liter of water) and nutrient agar with the same formulation as the blood agar without the blood and yeast extract.

The cells were removed from the plate with a swab and suspended in 1 ml of phosphate-buffered saline (pH 7.2). Pili were separated from the cells by incubation at 60°C for 20 min followed by centrifugation at 3,000 rpm for 20 to 30 min. The pili remained in the supernatant, which was taken off and stored at –20°C.

The EIA was performed on the supernatant as follows. Each well of a microtiter tray was coated with one of each purified specific K88ac, K88ab, and K99 rabbit antiserum. The test antigen solution was then added, the trays were washed, and a conjugate of the appropriate K antiserum linked to horseradish peroxidase was then added. The trays were again washed, and the substrate was added. Reactions were read visually.

Isolates for DNA hybridization were spotted onto filters and incubated at 37°C. The following day the cells were lysed, and the DNA was denatured and immobilized to filters by standard techniques (9). Hybridization was carried out at 42°C overnight in a solution containing 50% formamide, 5× Denhardt solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% sodium dodecyl sulfate, 5× SSPE, and 250 µg of denatured salmon sperm DNA per ml. Approximately 10⁵ CPM of radiolabeled probe per ml was added to the hybridization reaction.

Filters hybridized with the K88 probe were washed in 0.2× SSC–0.1% sodium dodecyl sulfate, and filters hybridized with K99 probe were washed in 1× SSC–0.1% sodium dodecyl sulfate for 1 h at 68°C. The filters were autoradiographed overnight at –70°C.

RESULTS

Table 1 is a comparison of the number of strains found to be positive for K88 and K99 by DNA hybridization and the number that were positive by EIA. There was excellent

TABLE 2. Comparison of DNA hybridization with slide agglutination for detecting *E. coli*-containing adhesins^a

Slide agglutination reaction	No. of strains tested by DNA hybridization for ^b :			
	Adhesin K88		Adhesin K99	
	+	–	+	–
+	16	0	17	0
–	13	72	0	84

^a A total of 101 strains were tested.

^b +, Positive for adhesins by DNA hybridization; –, negative for adhesins by DNA hybridization.

agreement between the two methods for K99 typing. However, the agreement was not so close for K88. The DNA probe detected 148 K88-positive strains compared with 125 identified by EIA. None was detected by EIA only.

Table 2 is a comparison of slide agglutination with DNA hybridization. Fewer K88-containing strains were detected by slide agglutination than by DNA hybridization. When these strains were tested by EIA, all DNA probe-positive strains were also positive by EIA and no additional K88-containing strains were detected. The agreement between the methods for K99 was good.

Of the 23 K88 strains which were identified by DNA hybridization but not by EIA, 18 were O149, two were O40 strains, one was an O64 strain, one was an O140 strain, and one was an O141 strain. All 23 strains were isolated from piglets with diarrhea. The O149 strains were heat-labile toxin positive by both DNA hybridization with heat-labile-toxin-specific probe and by EIA. The other four O types did not produce heat-labile or heat-stable toxin.

Strains for K88 testing by EIA were grown on Minca plates, which are optimal for K99 production (7) as a matter of convenience. The suggestion was made that in fact the culture medium may have an effect on K88 production. To test this theory, isolates previously probe positive but EIA negative were cultured on 5% sheep blood and nutrient agar. The EIA became positive for all but two *E. coli* strains grown on these media but remained negative for organisms grown on Minca medium and tested in parallel. This suggests that the type of culture medium can affect production of K88 pili.

TABLE 3. *E. coli* O serotypes associated with K88 and K99 adhesins

O Type	No. of strains	
	K88 positive	K99 positive
O5	4	
O8	8	
O20	30	2
O40	7	
O45	15	
O56	1	
O64	15	26
O102	2	2
O112	2	
O115	1	
O120	6	
O140	1	
O141	4	2
O149	49	
Not typable	3	1

Table 3 lists the variety of *E. coli* O serotypes associated with K88 and K99 in this series of Australian strains.

DISCUSSION

This study demonstrates that gene probes may be used to detect the adhesin-producing strains of enterotoxigenic *E. coli*. There was excellent agreement between the K99 gene probe results and the corresponding serological results.

However, the agreement among different typing methods for K88 was not as close. The DNA hybridization technique appeared to be more sensitive than EIA for K88. EIA, in turn, was more sensitive than the slide agglutination test.

One criticism of the use of DNA probes for diagnostic purposes is that strains may be detected which contain homologous DNA fragments but do not express the gene for some reason. We had suspected that this was the reason that more positive reactions for K88 among the strains were detected by DNA hybridization than by EIA. However, we were able to show that the gene was expressed so poorly on Minca plates that EIA was not sufficiently sensitive to detect it. Expression of genes has been shown to be affected by culture conditions such as temperature of incubation (3). However, the production of K88 pili was reported to be unaffected by the type of medium on which strains for testing were cultured (5). This study suggests that this is not so and that K88 production is improved when strains are grown on blood agar or nutrient agar.

Expression *in vitro* did not correlate completely with possession of the gene. On subculture on C.L.E.D. medium (Oxoid), three O40 strains which were originally probe and EIA positive for K88 were shown to lose the K88 antigens as determined by EIA. However, two of these retained the ability to hybridize with the K88 probe. The third O40 strain also lost the gene on subculture. These three strains showed a similar, colonial morphology on nutrient agar when expressing the K88 antigen but became larger and rougher with loss of seroreactivity.

An interesting further investigation of these DNA probe-positive EIA-negative strains might involve infecting piglets with them to see whether they could still produce disease.

In summary, DNA hybridization for K88 and K99 adhesins was found to be a sensitive and specific technique. When combined with toxin typing by DNA hybridization it should greatly facilitate the investigation of outbreaks of disease in animals. In addition, this study clearly demonstrates its potential use as a reference technique to ensure that serological tests are used at conditions optimal for the production of the antigens under investigation.

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