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Hemadsorption and Enzyme-Linked Immunosorbent Assay Nitrocellulose Replica Methods for Identification of Colonization Factor Antigen (CFA)-Positive *Escherichia coli* Colonies and for Isolation of CFA-Negative Mutants

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Methods were developed that allow demonstration of individual colonies carrying colonization factor antigen (CFA) I or CFA/II or E8775-type antigen in mixed bacterial cultures on solid media. These methods are based on mannose-resistant hemadsorption or CFA enzyme-linked immunosorbent assay (ELISA) on nitrocellulose replicas of the cultures allowing simultaneous analysis of up to 200 colonies per plate. The sensitivity and specificity of the CFA ELISA nitrocellulose replica method were 97 and 99%, respectively, for CFA/I-carrying colonies and 99 and 100% for CFA/II-positive colonies; corresponding figures for the quicker and simpler hemadsorption modification were somewhat lower. Both methods seem to be useful for studying excretion of CFA-carrying bacteria in feces, as indicated by studies in rabbits infected with enterotoxin-producing *Escherichia coli* in a nonligated-intestine model. By initially absorbing CFA-carrying bacteria on erythrocytes and then performing nitrocellulose replicas of agar colonies of the nonabsorbed bacteria, CFA-deficient mutants could be identified by the hemadsorption method, as well as by the CFA ELISA. Treatment of CFA-carrying bacteria with antiserum against CFA and complement also resulted in enrichment of spontaneous CFA-deficient mutants that could be identified by the replica methods. Several stable CFA-deficient mutants from enterotoxin-producing *E. coli* carrying CFA/I, CFA/II, or E8775 were isolated by these approaches.

Enterotoxin-producing *Escherichia coli* seem to colonize the small intestine by means of different types of fimbriae (4, 5). Three major presumed fimbrial adhesins that are associated with human enterotoxin-producing *E. coli* isolates have been identified. These are the colonization factor antigens (CFA) CFA/I and CFA/II and the E8775-type antigen (8, 6, 17). Bacteria carrying any of these structures are capable of agglutinating different species of erythrocytes in the presence of mannose to prevent type 1 pilus-mediated hemagglutination, i.e., they give mannose-resistant hemagglutination. Thus, CFA/I-positive strains agglutinate human A and bovine erythrocytes, CFA/II-positive bacteria agglutinate bovine but not human A erythrocytes, and bacteria with E8775-type antigen agglutinate human A erythrocytes and bovine erythrocytes (9, 17).

These three adhesins do not cross-react immunologically and can be differentiated by agglutination with specific antisera or by immunodiffusion analyses (1, 3). For identification of CFA-carrying strains in stool cultures, mannose-resistant hemagglutination or slide agglutination with specific anti-CFA sera are standard methods (1, 3, 9). However, these methods do not allow estimation of the proportion of CFA-positive colonies in the cultures unless all the colonies are studied.

We here describe methods to identify the expression of CFA by individual colonies in cultures on solid media either by adsorption of erythrocytes on nitrocellulose replicas of the colonies or by CFA enzyme-linked immunosorbent assay (ELISA) on such replicas. These methods proved to be useful both for determining the excretion of CFA-positive bacteria in the feces of rabbits infected with CFA-bearing enterotoxin-producing *E. coli* strains in a nonligated-intestine model, the RITARD model (16), and for isolating spontaneous CFA-negative mutants of CFA-carrying strains.

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MATERIALS AND METHODS

Bacteria. The following *E. coli* reference strains were used: H10407 (O78, K80, H11, CFA/I, and ST/LT) provided by D. G. Evans, Houston, Tex.; 258909-3 (O128, K-H?, CFA/I, and ST/LT [11]) and 408-3 (O78, K-nontypable, H12, CFA/II, and ST/LT) provided by B. Sack, Baltimore, Md.; and 1392-75 (O6, K15, H16, CFA/II, and ST/LT) and E8775 (O25, H42, E8775, and ST/LT) kindly provided by B. Rowe, London, United Kingdom. In some experiments, CFA-deficient mutants of strains H10407, 258909-3, 408-3, and 1392-75, i.e., H10407P (provided by D. G. Evans) and 258909-3M, 408-3M, and 1392-75P (isolated in our department) were used. In addition, a hemagglutination-negative *E. coli* K6 control strain was used.

Culture of bacteria. Stock cultures in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 15% (wt/vol) glycerol were stored in aliquots at −70°C. The strains were grown from these cultures directly on CFA agar (7) or blood agar at 37°C for 18 h. After harvesting, the bacteria were washed with physiological saline (0.85% NaCl), and the suspension was adjusted to 10^9 bacteria per ml by optical density. After the *E. coli* were diluted in saline to less than 1,000 bacteria per ml, they were inoculated on CFA agar and cultured at 37°C for 18 h.

Antisera. CFA antisera were produced by giving rabbits three subcutaneous injections of 100 μg of either purified CFA/I or purified CFA/II at intervals of 2 to 3 weeks; the first two immunizations were given with Freund complete

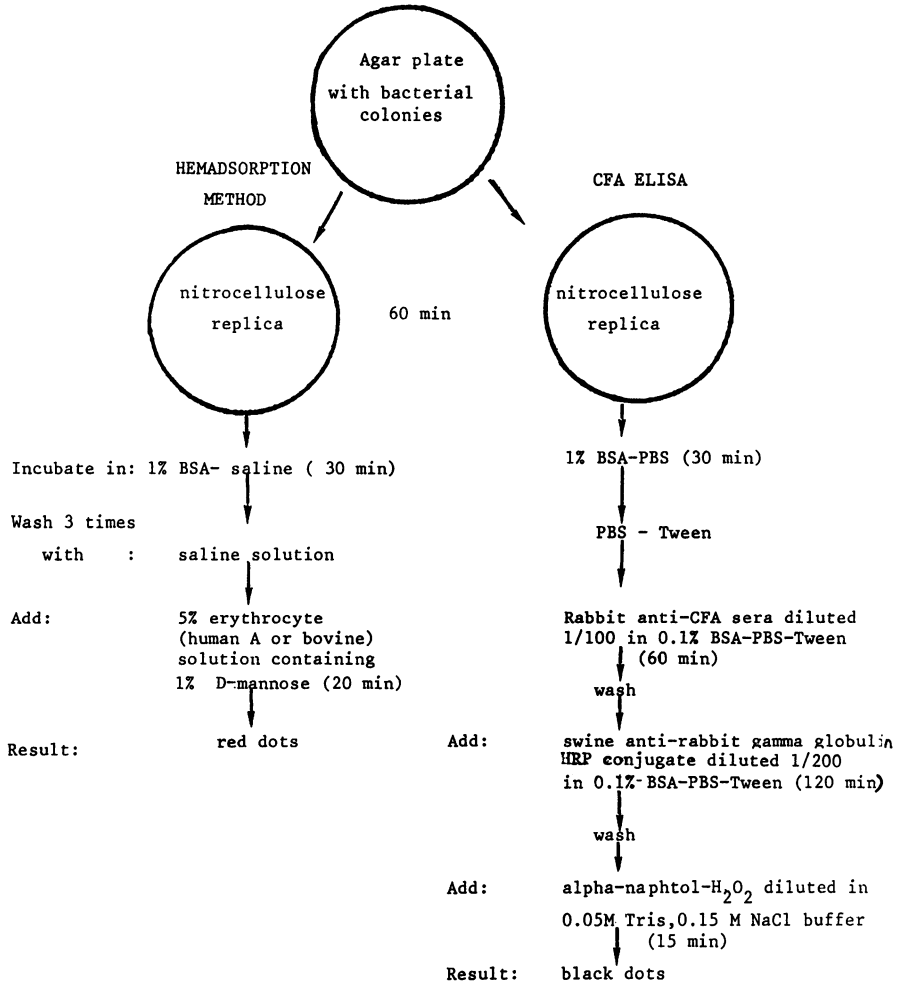

FIG. 1. Schematic diagram of nitrocellulose replica methods for demonstration of CFA-positive colonies.

adjuvant. The animals were bled by heart puncture 1 to 2 weeks after the last immunization.

Replica methods. The replica methods are outlined in Fig. 1. Nitrocellulose replicas of outgrown colonies were performed essentially as described by Connor and Loeb (2). Nitrocellulose filter paper (HAWP; 0.45-μm pore size; Millipore Corp., Bedford, Mass.) was cut to fit standard petri dishes. Each paper disk was soaked in saline solution and marked with ink to indicate a reference point. The disk was carefully applied on the surface of an agar plate with colonies and left on the plate for different times (15 min to 2 h) at room temperature; usually two replicas were performed on each plate. Replicas were then carefully removed to avoid smearing of the colonies and placed colony-side-up in a clean petri dish containing 10 ml of phosphate-buffered saline (PBS) (pH 7.2) with 1% bovine serum albumin (BSA). After gentle agitation on a shaker (Giratory Rocker Plattform; Heidolph, Kelheim, Federal Republic of Germany) for 30 min, the replica was rinsed three times by using 20 ml of PBS in each washing and gentle agitation for a total of 10 min. Visualization of CFA-positive colonies on the replicas was performed by the hemadsorption method or by an ELISA.

For the hemadsorption method, the nitrocellulose replica was incubated for 20 min in 5 ml of a 5% (vol/vol) erythrocyte suspension (for CFA/I- and E8775 antigen-positive strains, human A erythrocytes were used, and for CFA/II-positive strains, bovine erythrocytes or, in some experiments, chicken erythrocytes were used) with 1% D-mannose. After the disk was rinsed twice with saline solution, red dots developed at the sites of replicated CFA-positive colonies. For the ELISA, the rinsed and BSA-blocked nitrocellulose replicas were incubated for 1 h in 5 ml of specific rabbit anti-CFA serum diluted in PBS containing 0.05% Tween and 0.1% BSA. After it was washed three times with PBS-Tween, the replica was incubated for 2 h in 5 ml of swine anti-rabbit γ-globulin-horse radish peroxidase conjugate (Dakopatts, Copenhagen, Denmark) diluted in PBS-Tween-BSA. After repeated washings in PBS-Tween, the disk was incubated for 15 min in chromogen-enzyme substrate solution (1 mg of alpha-naphtol per ml, 0.01% H_2O_2) diluted in 0.05 M Tris–0.15M NaCl buffer (pH 7.5); black dots developed at the site of replicated CFA-positive colonies. All incubations of the replicas were performed with gentle shaking at room temperature.

Preparation of bacteria for selecting CFA-deficient mutants. CFA-carrying bacteria were grown on CFA agar at 37°C for 18 h. Cells were scraped off the agar plates, washed with saline solution, and adjusted to 10^9 bacteria per ml by optical density. The bacteria were then extensively absorbed with an equal volume of a 3% (vol/vol) erythrocyte solution (for

CFA/I- and E8775-carrying strains, human A erythrocytes were used, and for CFA/II-carrying strains, bovine erythrocytes were used) with 1% D-mannose for 20 min at room temperature. The erythrocytes were then removed by centrifugation at 160 × *g* for 2 min, and the supernatant was absorbed three times more with erythrocytes. The final supernatants, after centrifugation at 160 × *g*, were further diluted 100- to 10,000-fold, and 0.1 ml was plated on CFA agar. Alternatively, CFA/II-carrying bacteria (200 bacteria in 0.1 ml of PBS) were incubated with 0.1 ml of rabbit serum against the homologous CFA/II (diluted 1/200 in PBS) at 37°C for 2 h, after which 0.1 ml of guinea pig complement was added and incubation was continued at 37°C for 1 h. The mixture was then centrifuged at 160 × *g* for 5 min, and 0.1 ml of the supernatant was incubated on CFA agar plates. After the plates were incubated at 37°C for 18 h, nitrocellulose replicas were made and developed with the hemadsorption assay and the CFA ELISA as described above.

RESULTS

The optimal conditions for transferring colonies from agar plates to the nitrocellulose replicas were evaluated. Transfer was most efficient when the nitrocellulose disk was left on the agar for a minimum of 60 min at room temperature; longer incubation times or higher temperatures did not result in more effective transfer. Incubation for less than 1 h gave incomplete transfer of colonies. To avoid nonspecific binding of erythrocytes or antiserum to the nitrocellulose paper, the disks were incubated in a 1% BSA solution; addition of erythrocytes or anti-CFA antiserum to nonblocked disks resulted in unacceptable background activities.

The optimal concentration of specific antiserum used in the ELISA had to be determined individually for each serum. In our studies, the rabbit anti-CFA/I serum was diluted 1:100, and the anti-CFA/II serum was diluted 1:150.

The sensitivity and specificity of the replica methods for detecting colonies carrying CFA/I or CFA/II were evaluated. Several replicas of the CFA/I-carrying strains H10407 and 258909-3, as well as of CFA-deficient mutants of these strains, were tested by the hemadsorption method with human A erythrocytes or by CFA/I ELISA, and the results of these tests were compared with those obtained by slide agglutination of individual colonies with anti-CFA/I serum (Table 1). The sensitivity and specificity of the hemadsorption method for detection of CFA/I were 93 and 92%, and for the ELISA they were 97 and 99%, respectively. A similar analysis for individual colonies of the CFA/II-carrying strains 1392-75 and 408-3, as well as for the CFA-deficient mutant of strain 1392-75, showed that the sensitivity and specificity of the hemadsorption method with bovine erythrocytes were 56 and 95%, and for the ELISA they were 99 and 100%, respectively, as compared with slide agglutination with anti-CFA/II serum (anti-1392 CFA/II) (Table 1). Based on previous observations in Bangladesh (1) that hemagglutination with chicken erythrocytes was far more sensitive than hemagglutination with bovine erythrocytes in identifying CFA/II-carrying bacteria, we also developed replicas of strains 1392-75 and 408-3 with chicken erythrocytes from different animals. In no instance was either strain detected with higher sensitivity than 60% when using chicken erythrocytes for the development of the replicas.

The specificity of the replica ELISA for detection of CFA was further supported by the fact that antiserum against CFA/I from strain H10407 (O78) did not give any positive reaction with replicas of different CFA/II-positive strains, including a CFA/II-positive strain of, in this regard, the unusual O group 78 (12, 13).

The two nitrocellulose replica assays were used to study fecal excretion of CFA-carrying *E. coli* from rabbits after infection with such bacteria in the RITARD model (16). Both replica methods detected individual colonies of the challenge strain in stool cultures for several days after infection (Fig. 2); the sensitivity and specificity of the ELISA replica method using anti-1392 CFA/II serum were 99 and 100%, respectively, compared with the standard testing of individual colonies. During the initial 1 to 3 days, most or all of the colonies in the fecal cultures could be visualized by the ELISA as well as by the hemadsorption method. Later after infection, however, a lower proportion of the colonies of the fecal cultures were CFA positive by the replica methods as well as by standard testing. Occasionally, CFA-carrying colonies could be detected on replicas made on fecal cultures of specimens taken as late as 10 days after infection.

The possibility of using the replica methods for identifying CFA-deficient mutants from CFA-carrying strains was also tested. After a culture of strain 258909-3 was repeatedly absorbed with human A erythrocytes and the nonabsorbed bacteria were inoculated on CFA agar, nitrocellulose replicas were taken and developed by either the hemadsorption method or the CFA/I ELISA. Colonies that were not visible on the replicas after development with both methods and that continued to be negative on nitrocellulose replicas after repeated passage on CFA agar were considered to be CFA-negative mutants. These results were confirmed by slide agglutination and immunodiffusion with specific anti-CFA/I serum. As an example, the individual colony indicated by an arrow in Fig. 3 that was repeatedly negative on ELISA and hemadsorption replicas was shown to be a stable CFA-deficient mutant on follow-up testing. By a similar approach, CFA-deficient mutants from a CFA/II-carrying strain (408-3)

TABLE 1. Comparison of hemadsorption and ELISA nitrocellulose replica methods with slide agglutination in specific antiserum for detection of CFA/I or CFA/II on individual *E. coli* colonies

CFA detection by replica methods	CFA detection by slide agglutination test (no. of colonies)	
	+	−
CFA/I[a]		
Hemadsorption		
+	297	12
−	21	137
ELISA		
+	308	1
−	10	148
CFA/II[b]		
Hemadsorption		
+	78	3
−	61	57
ELISA		
+	138	0
−	1	60

[a] Of the colonies studied, 42% represented strain 258909-3 (CFA/I), 26% were H10407 (CFA/I), 15% were 258909-3M (CFA negative), and 17% were H10407P (CFA negative). Hemadsorption was performed with human A erythrocytes, and for the ELISA and slide agglutination test, antiserum against purified CFA/I (from strain H10407) was used.

[b] Of the colonies studied, 48% represented strain 408-3 (CS2 plus CS3), 22% were strain 1392-75 (CS1 plus CS3), 20% were strain 408-3:M (CS negative), and 10% were strain 1392:75P (CS negative). Hemadsorption was performed with bovine erythrocytes, and for the ELISA and slide agglutination test, antiserum against purified CFA/II (from strain 1392) was used.

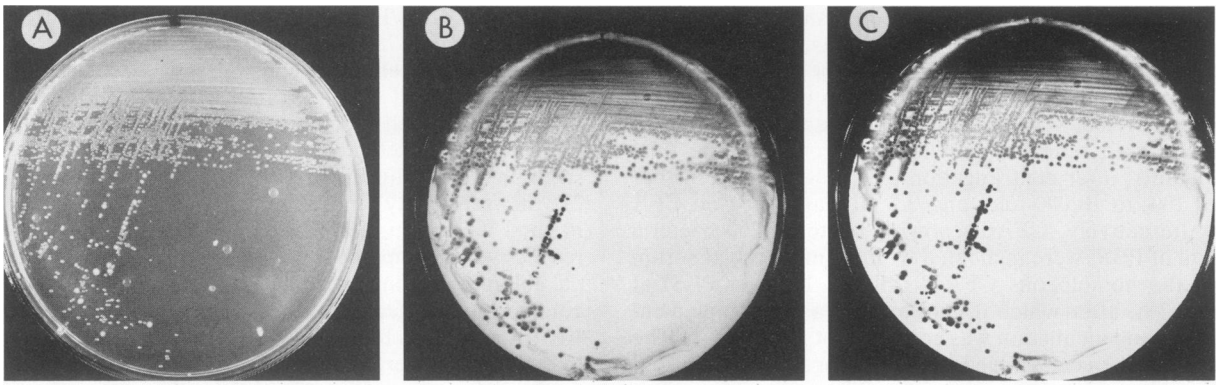

FIG. 2. Stool culture from a rabbit (A) and nitrocellulose replicas of the culture developed by the hemadsorption method (B) and CFA ELISA (C). The rabbit was infected with the CFA/I-positive strain H10407 by the RITARD model (16), and the fecal specimen was collected 36 h after challenge.

and an E8775-antigen-positive strain (E8775) were also isolated after repeated absorption of cultures of these strains with bovine and human A erythrocytes, respectively. After CFA/II-carrying bacteria were treated with serum against partially purified homologous CFA/II and complement, as many as 0.2% of the original number of bacteria were identified as mutants, either lacking CS3 or CS3 and CS1 (from strain 1392) or lacking CS3 and CS2 (from strain 408-3) when the replicas were developed with antiserum against the homologous and the heterologous CFA/IIs, respectively.

DISCUSSION

Two methods for identification and quantitative estimation of CFA-carrying colonies in stool cultures were developed. When nitrocellulose replicas of the cultures were made and the paper disks were developed by the hemadsorption method or CFA ELISA, individual CFA/I- or CFA/II-positive colonies could be identified with high sensitivity and specificity. The ELISA was somewhat more specific and was considerably more sensitive, particularly for detecting CFA/II-carrying colonies, than was the hemadsorption test. This was not unexpected because other studies have shown considerably higher sensitivities of immunoassays than of hemagglutination tests for detecting CFA/I or CFA/II. For instance, in a recent study we found that hemagglutination with bovine erythrocytes is less sensitive than agglutination with specific serum raised against purified CFA/II—85 versus 100% sensitivity in detecting CFA/II-carrying strains (1). Cravioto et al. (3) also reported that CFA/II-carrying strains may differ from one another in the strength of mannose-resistant hemagglutination of bovine erythrocytes. Our finding in a recent study (1, 11) in Bangladesh that agglutination of chicken erythrocytes is more sensitive for detection of CFA/II-positive bacteria than is agglutination with bovine erythrocytes is not supported by the present study. Thus, the development of the nitrocellulose replicas with erythrocytes from different chickens did not result in a more sensitive test than was obtained when bovine erythrocytes were used.

One major reason for using the hemadsorption method in spite of its lower sensitivity and specificity is that in most laboratories erythrocytes are more readily available than the specific antisera and other reagents that are necessary for the ELISA. Also, the hemadsorption procedure is faster and easier to perform than the ELISA.

With regard to the sensitivity of the replica methods for CFA/II, visualization by hemadsorption seems equally effective irrespective of whether the *E. coli* strains carried the coli surface antigen pattern CS1 plus CS3 of CFA/II (strain 1392-75) or CS2 plus CS3 (strain 408-3) (3, 13, 15). In the ELISA, a good antiserum against the CS3 component seemed to be sufficient to demonstrate all CFA/II-positive colonies irrespective of the CS pattern. Thus, antiserum against purified CFA/II from strain 1392 (i.e., containing

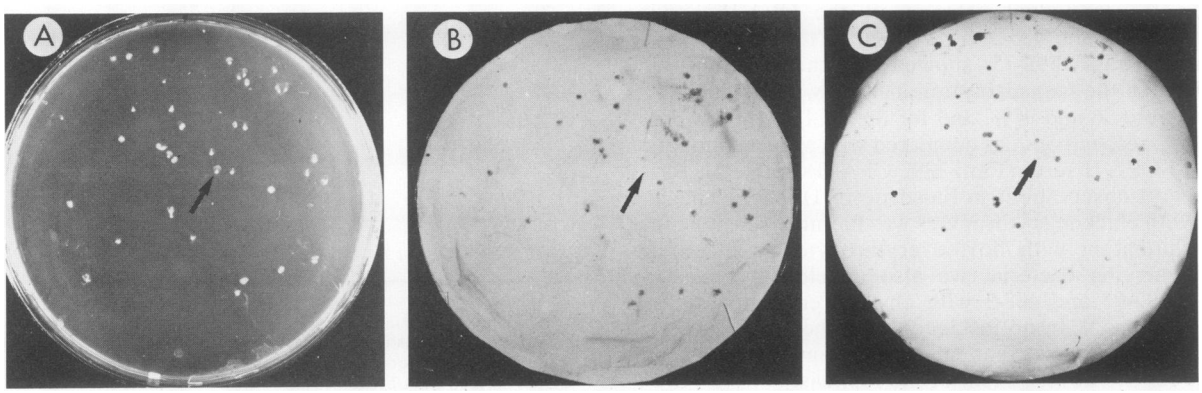

FIG. 3. Identification of a CFA-deficient mutant of the CFA/I-positive strain 258909-3 by means of nitrocellulose replicas developed by the hemadsorption method and the CFA ELISA. (A) Original culture on CFA agar after repeated absorption of the culture with human A erythrocytes. Nitrocellulose replicas of the CFA agar colonies developed by the hemadsorption method (B) and CFA/I ELISA (C) are shown. The arrows indicate the position of the colony for the deficient mutant.

anti-CS1 and anti-CS3) not only gave a positive result for replicas of the homologous strain but also for replicas of strain 408-3 carrying CS2 and CS3 on its surface (unpublished results).

The high sensitivity of the ELISA method for detecting individual CFA-carrying colonies on agar plates made it well suited for identifying potentially CFA-deficient mutants from CFA-carrying enterotoxin-producing *E. coli* strains. Initial absorbtion of cultures of CFA-carrying bacteria with appropriate species of erythrocytes enabled spontaneous hemaglutination-negative mutants to be enriched in the supernatant, after which the mutants could be identified by the replica methods. A similar enrichment of CFA-deficient mutants seemed to be obtained after treatment of CFA-carrying bacteria with antiserum against partially purified homologous CFA and complement. By these approaches we were able to identify CFA-deficient mutants from CFA/I- as well as CFA/II-positive strains.

The finding that none of the CFA-negative mutants expressed CFA after repeated culture on CFA agar or as tested, in some instances, even after repeated passage in vivo in rabbit intestine (Y. López-Vidal, C. M. Åhrén, and A.-M. Svennerholm, submitted for publication) suggests actual loss of the CFA-encoding plasmids. This is further supported by the fact that most of the mutants lost the capacity to produce heat-stable enterotoxin, since the genes coding for CFA and heat-stable enterotoxin are often located close to each other on the same plasmid (14). The replica methods may also be used to identify genetically manipulated colonies, either those lacking CFA or those engineered to produce them. We also modified the ELISA replica method to permit detection of colonies producing heat-labile enterotoxin in stool cultures. This was done after the release of periplasmic heat-labile enterotoxin by treatment of the colonies with polymyxin B (10) and by using monoclonal anti-heat-labile enterotoxin antibodies in the immunodetection step (unpublished data).

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