JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1993, p. 2163–2166
0095-1137/93/082163-04$02.00/0

Vol. 31, No. 8

Evaluation of Conventional Media for Detection of Colonization Factor Antigens of Enterotoxigenic *Escherichia coli*

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Received 5 February 1993/Accepted 12 May 1993

Strains of enterotoxigenic *Escherichia coli* producing either colonization factor antigen (CFA) I or II were tested for expression of CFA when grown on 16 different agar media by using agglutination and coagglutination with monoclonal antibodies, mannose-resistant hemagglutination, and a salt aggregation assay. CFA was detected from the CFA-positive strains when CFA agar was used, and it was also detected when other commercially available media were used, notably nutrient agar. CFA was not detected when other commercial media such as MacConkey agar were used. The use of nutrient agar with monoclonal antibody-based coagglutination reagents offers a potentially simple and rapid method for detecting *E. coli* which express CFA I or II.

Colonization of the small intestine and production of enterotoxin by certain strains of *Escherichia coli* leads to severe diarrhea in humans and domestic animals. The attachment of human strains of enterotoxigenic *E. coli* (ETEC) to the epithelial cells of the small intestine is frequently mediated by protein fimbrial antigens called colonization factor antigens (CFAs), including CFA I, CFA II complex, CFA III, PCF 8775 (CFA IV), and others. The CFA II complex is further divided into strains which produce colisurface antigen 1, 2, and 3, abbreviated as CS1, CS2, and CS3, respectively. Some strains express more than one CS antigen. Since CFAs are immunogenic and are important virulence factors, these antigens have stimulated considerable interest as potential vaccine antigens (7, 14).

Evans et al. (3, 4) first detected colonization factors with a hemagglutination (HA) assay using CFA agar, a medium composed of Casamino Acids, yeast extract, trace salts, and agar. When comparing MacConkey agar (MAC), Tergitol, and 2% peptone agar (PA), Evans et al. (3) concluded that 2% PA was also an efficient medium for detecting CFA I (other CFAs were not known at that time) from *E. coli* isolates. Except for these early evaluations of agar media for the production of CFA, there have been no reports comparing different media for the detection of these fimbrial antigens. The goal of our study was to reevaluate the conventional plating media for efficient and simple detection of CFAs and to use simplified methods with a coagglutination (COA) technique with monoclonal antibodies (MAbs).

MATERIALS AND METHODS

The presence and quantitation of CFA I were determined by HA and a salt aggregation test (SAT), while the presence and quantitation of CFA II subgroups were determined by HA, SAT, and agglutination with MAbs and their respective COA reagents. The SAT measures hydrophobicity of the bacteria and is thought to correlate with expression of pilus antigens. All of the comparative assays were performed under code by a blinded investigator.

Bacterial strains. The strains used in the present study are listed in Table 1. All the strains were stored in tryptic soy broth (TSB) with 15% glycerol at −70°C.

Media used. Sixteen different media were used (Table 2). These included CFA agar, nutrient agar (NA; Difco), 2% PA (Difco), Mueller-Hinton agar (MHA; Difco), antibiotic medium 3 (ABM; Difco), MAC (Difco), brain heart infusion agar (BHI; BBL), tryptic soy agar (TSA; BBL), and 5% sheep blood agar (BA; BBL). Except for CFA and BA, each of the other agars was tested with and without the addition of 0.6% yeast extract (Difco) because yeast extract is commonly used in broth media for the production of enterotoxins of *E. coli*. All bacteria were grown at 37°C.

Antisera used. MAbs for CS1 (MAbs 12:4), CS2 (MAbs 10:3), and CS3 (MAbs 10:2) were kindly supplied by A.-M. Svennerholm, Goteborg University (14). COA reagents that use these same MAbs were prepared in our laboratory. In brief, *Staphylococcus aureus* Cowan I strain was grown on BA (BBL). Six colonies were inoculated into 50 ml of TSB and were incubated at 37°C for 4 h, as a starter culture, for inoculation into 500 ml of TSB, which was incubated at 37°C for 20 h by shaking (120 rpm; New Brunswick Gyrator Incubator). The broth was centrifuged at 5,000 rpm for 15 min, and the pellet was suspended in 60 ml of sterile phosphate-buffered saline (PBS; 0.15 M, pH 7.3) containing 0.1% sodium azide and was washed three times. The washed cells were resuspended in 50 ml of PBS containing 0.5% formaldehyde and were incubated at room temperature for 3 h with shaking every 15 min. These cells were centrifuged, after which the pellet was resuspended in 50 ml of PBS and heated at 80°C for 1 h. This suspension was then centrifuged again, with the pellet being resuspended in PBS to make a 10% (vol/vol) *S. aureus* cell suspension.

The stable *S. aureus* cells were sensitized separately with MAbs of CS1, CS2, or CS3 or normal rabbit serum (negative control). One milliliter of 10% cells was mixed with 0.1 ml of each serum sample, and the mixture was incubated at room temperature for 4 h with gentle shaking every 30 min. Sensitized cells were centrifuged, washed three times, and finally resuspended in PBS containing 0.02% bovine serum

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albumin and 0.001% thimerosal to make a 1% cell suspension as a working reagent.

Bacterial agglutination with MAbs and COA reagents. Screening tests were performed by microscopic slide agglutination by using either a hand lens or a colony counter (American Optical Company). *E. coli* isolates grown on 16 different media were tested for CFA by bacterial agglutination with the MAbs and the COA reagents. For the measurement of the time taken for agglutination, selected colonies from different media were agglutinated with PBS, MAbs, and the respective COA reagents.

Quantitation of COA. Quantitation was carried out by using serial dilutions of the COA reagents in a microtiter plate. *E. coli* isolates from the various media were harvested with 0.1 M PBS, and the bacterial suspension was calibrated to an optical density at 600 nm of 2.0 with a spectrophotometer (Gilford Spectrophotometer 250). Serial twofold dilutions of the COA reagents were prepared in microtiter plates with PBS containing 0.02% bovine serum albumin. A 25-μl drop of the bacterial suspension was added to each well containing diluted COA reagents, sealed, and incubated for 20 min at 37°C. COA was determined visually or with a colony counting lens, using the COA reagent with PBS and negative bacteria as negative controls.

HA. HA and mannose-resistant HA were tested as described by Evans et al. (4). Blood of group A, drawn from a volunteer donor, was placed into a tube containing 1.0 ml of 3.8% citric acid in distilled water per 9.0 ml of blood. Bovine blood was supplied by Cocalico Biologicals in Alsever solution. Both types of erythrocytes were diluted with PBS (with 1% D-mannose for mannose-resistant HA) to make a 3% cell suspension. Twenty-five microliters of the bacterial suspension and 25 μl of the erythrocyte suspension were mixed well and rocked on a microscopic glass slide, and the reactions were read either visually or through a colony counter lens.

Quantitation of HA. Tenfold dilutions of bacterial cells grown on different media were prepared for quantitating HA. The number of bacterial cells in the suspension was determined by the plate counting technique, and HA activity was then expressed as HA units (4). One HA unit is defined as the reciprocal of the smallest number of bacterial cells producing HA times a factor of 10^5, i.e., 1 HA unit = 10^5/limiting bacterial colony count. Thus, a culture with a limiting dilution of 1×10^5 would be assigned 1 HA unit, while another with a limiting dilution of 5×10^5 would be assigned 0.2 HA units.

SAT. The SAT for hydrophobicity was performed as described by Rozgonyi et al. (10) by using different molar solutions of ammonium sulfate. The SAT value is determined by observing the limiting molar concentration of ammonium sulfate in which aggregation is not observed. Thus, lower SAT values correspond to a higher hydrophobicity. The bacterial suspension for the HA test was also used for the SAT test. A 25-μl drop of bacterial suspension was mixed with an equal amount of ammonium sulfate solution and rocked on a glass slide. Bacterial aggregation was read visually or through a colony counter lens.

RESULTS

When grown on CFA agar, the CFA II-producing strains of *E. coli* agglutinated with the MAbs, as shown in Table 1, and reacted with COA reagents consistent with their CS antigens. The CFA-negative control strain did not agglutinate with the MAb or the COA reagents, nor did it show HA activity.

TABLE 1. Sources and descriptions of *E. coli* strains used in the present study

Strain	Serotype	Colonization factor	Enterotoxin[a]
E1392-2A	O6:H16	II (CS1, CS2, CS3)	No LT or ST
R 13	O139:H28	II (CS1, CS3)	ST
VM64761	O6:H16	II (CS2, CS3)	ST
B2C	O6:H16	II (CS3)	LT, ST
H10407	O78:H11	I	LT, ST
E1392-7A	O6:H16	Negative	Negative

[a] LT, heat-labile enterotoxin; ST, heat-stable enterotoxin.

Evaluation of conventional agar media for production of CFA by agglutination and COA. Sixteen different conventional media were used to select the best one for the production of CFA. Of the 16 media used in the present study, *E. coli* strains grown on six different media demonstrated CFA production with MAbs and COA reagents. As shown in Table 2, these were CFA agar, NA, NA-yeast extract, PA, PA-yeast extract, and MHA-yeast extract. The agglutination titers ranged between 1:8 and 1:4,096. The other media used, i.e., MHA, ABM, ABM-yeast extract, BHI, BHI-yeast extract, TSA, TSA-yeast extract, BA, MAC, and MAC-yeast extract, demonstrated either very low or undetectable titers of CFA. Cultures grown on CFA agar and NA both agglutinated with high titers of COA reagents. For strain E1392-2A, titers of CS1, CS2, and CS3 were 4,096, 2,048, and 1,024, respectively, for both agars.

As shown in Table 3, bacteria grown on CFA agar and NA also demonstrated COA in a shorter time relative to the other agars. Strains grown on NA and CFA agar showed nearly instantaneous reactions, while strains grown on yeast extract-supplemented NA and PA took longer, and those grown on PA and MHA plus yeast extract took even longer.

Effect of culture medium on the detection and quantitation of CFAs of *E. coli* by mannose-resistant HA. Cultures from the six media that produced the highest titers of agglutination with antisera and COA reagents were also positive for HA.

TABLE 2. COA titers of strain E1392-2A grown on different agar media

Culture medium	Agglutination titer		
	CS1	CS2	CS3
CFA	1:4,096	1:2,048	1:1,024
NA	1:4,096	1:2,048	1:1,024
NA-YE[a]	1:1,024	1:1,024	1:256
PA	1:512	1:256	1:128
PA-YE	1:1,024	1:256	1:128
MHA-YE	1:256	1:64	1:8
MHA	1:2	>1:2	>1:2
ABM	1:4	>1:2	NA[b]
ABM-YE	1:2	>1:2	NA
BHI	1:2	NA	NA
BHI-YE	>1:2	NA	NA
TSA	NA	NA	NA
TSA-YE	>1:2	NA	NA
MAC	NA	NA	NA
MAC-YE	NA	NA	NA
BA	>1:2	NA	NA

[a] YE, yeast extract.
[b] NA, no agglutination.

TABLE 3. Kinetics of agglutination with COA

Antiserum	Strain	Inoculum from the following medium[a]:					
		CFA	PA	PAY	NA	NAY	MHAY
CS1	E1392-2A	2	5	4	1	2	3
	R13	1	5	2	1	4	3
	E1392-7A	NA	NA	NA	NA	NA	NA
CS2	E1392-2A	1	1	2	1	4	3
	VM64761	2	2	3	1	2	3
	E1392-7A	NA	NA	NA	NA	NA	NA
CS3	E1392-2A	1	5	4	2	3	NA
	R13	3	5	3	1	2	3
	VM64761	1	5	3	1	2	5
	E1392-7A	NA	NA	NA	NA	NA	NA

[a] 1, ≤30 s; 2, ≤60 s; 3, ≤90 s; 4, ≤120 s; 5, >150 s; NA, no agglutination. The other media tested showed either weak or no agglutination. PBS showed no agglutination with any of the antisera or strains tested. Abbreviations: PA, 0.2% peptone agar; PAY, 0.2% peptone agar plus 0.6% yeast extract; NA, nutrient agar; NAY, nutrient agar plus 0.6% yeast extract; MHAY, Mueller-Hinton agar plus 0.6% yeast extract.

Among those cultures that gave positive assay results there were clear differences among the agar media, as shown in Table 4, with CFA agar and NA again yielding higher values.

Effect of culture medium on hydrophobicity. Table 5 shows the SAT values of the strains when grown on different media. Cultures grown on CFA agar and NA gave consistently lower SAT values for all strains tested.

DISCUSSION

In the study described here, we tested various commonly used agar media for expression of the CFAs associated with ETEC and confirmed that CFA is an optimal medium for CFA expression, but also demonstrated that NA is equivalent to CFA agar. CFA agar is not difficult to prepare, but it is not a standard agar for most laboratories; hence, NA may have an advantage since it is a standard, inexpensive, and readily available agar.

In the present study, we also found that a COA test with preserved *S. aureus* cells coated with CS-specific MAbs is a convenient and simple method for detecting CFA. The reagents were found to be sensitive and specific and to show a positive reaction by slide agglutination in less than 1 min with positive strains. COA reagents could be highly diluted and still demonstrated agglutination, suggesting that such reagents might be very efficient diagnostically.

This study is an extension of the study of Evans et al. (3, 4). Focusing on CFA I with HA, Evans et al. (3, 4) found that

TABLE 4. Effect of culture medium on titers of mannose-resistant HA by CFA-producing *E. coli*

Culture medium	Mannose-resistant HA (HA unit) by strain:				
	E1392-2a	R13	VM64761	B2C	H10407
CFA agar	0.833	0.476	1.235	0.303	0.526
NA	0.909	0.400	1.087	0.322	0.526
NA-yeast extract[a]	0.833	0.164	0.222	0.256	0.164
PA	0.011	0.322	0.238	0.015	0.012
PA-yeast extract	0.014	0.286	0.020	0.322	0.143
MHA-yeast extract	0.020	0.016	0.023	0.020	0.019

[a] Yeast extract was used at 0.6%.

TABLE 5. Effect of agar medium on salt aggregation test results with CFA-producing *E. coli*

Culture medium	Limiting molarity with aggregation by strain:				
	E1392-2A	R13	VM64761	B2C	H10407
CFA agar	0.05	0.05	0.25	1.5	0.05
NA	0.05	0.05	0.25	1.5	0.05
NA-yeast extract[a]	0.10	0.05	0.50	1.5	0.05
PA	0.05	0.10	0.70	2.0	0.10
PA-yeast extract	0.10	0.10	0.50	2.0	0.10
MHA-yeast extract	0.10	0.10	0.50	1.5	0.10

[a] Yeast extract was used at 0.6%.

either CFA agar (Casamino Acids, yeast extract, trace salts, and agar) or 2% PA was considerably more effective for the detection of CFAs than was either MAC or Tergitol agar medium. Our study confirms the results of the study of Evans et al. (3, 4) and extends these observations by finding that several agars are acceptable and others are unsuitable. Unsuitable agars included MAC, TSA, MHA, ABM, BHI, and the corresponding yeast extract (0.6%)-supplemented agars (except MHA–0.6% yeast extract) and BA. These unsuitable agars were associated with either no agglutination or low agglutination titers with either the MAbs or HA.

A standard medium for the detection of enterotoxin is TSB supplemented with 0.6% yeast extract, and since CFAs and enterotoxin(s) are frequently associated with the same plasmid (12, 13), enhancement of CFA expression might be expected with yeast extract. However, the addition of yeast extract in the present study was found to have a minimal effect on CFA expression, although it did enhance production in a few media (Tables 2 to 5). Thus, of 16 different media, from only 6 could CFA be detected, and in only 2 (CFA and NA) did expression appear to be optimal. Expression of CFA was found with an equal frequency with these two media and frequently, but with a lower titer, with PA and MHA with or without yeast extract.

The different assays for CFA correlated well; e.g., CFA agar and NA were consistently superior whether the assay used bacterial agglutination, COA, HA, or SAT, suggesting that these assays measured the same attributes of the CFA. Furthermore, correlation of the quantitative assays with COA, HA, and SAT was also consistent. In each case, the CFA agar and NA performed in an identical fashion and were superior to the other agars. In a comparison of the two "optimal" media, NA is somewhat easier to obtain, but bacteria grow somewhat more luxuriantly on the CFA agar.

Results of the present study with a limited number of strains also suggest that the optimal medium and conditions for expression of CFA I and CS antigens of CFA II are similar. CFA agar was developed while evaluating its usefulness for the expression of CFA I by the HA assay. Our study extends these observations to CFA II and their subtypes CS1, CS2, and CS3 by using MAbs, HA, and SAT. Shipley et al. (11) used either NA or Luria agar for testing of colonization antigen K88, suggesting that CFA of animal ETEC strains are also detected with NA.

The present study provides evidence that a simple medium can be used for the detection of CFA from human ETEC isolates but also offers a simple method of using COA with MAbs for each of the CS antigens. Other investigators have used DNA probes or enzyme-linked immunosorbent assays to detect CFA I and CFA II (1, 2, 6, 8); however, the assay described here may have advantages over the others in that

it is rapid and monospecific. The COA reagents for CFA I and CFA II have been developed for the detection of CFA I and CFA II (5), but our study used monospecific CS-specific reagents for the specific detection of different subgroups within CFA II. The major advantage of using MAbs over polyclonal antisera for the demonstration of CFAs is not only the higher specificity of MAbs but also the fact that MAbs are homogeneous (9). A potential limitation of agglutination tests such as the one described here is the possibility of spontaneous agglutination; hence, appropriate controls need to be included to rule this out.

From the present study, the most efficient approach to the detection of the different CS components of ETEC isolates is to grow the strains on NA and to use the MAb-specific COA reagents in a slide agglutination test, a test which takes less than a minute. This report suggests that the anti-CS MAb COA reagents produced may be useful for identifying CFA II and its different subcomponents from clinical ETEC isolates. Such analyses may be important for epidemiological studies of ETEC outbreaks or to monitor ETEC transmission. Since CFA antigens may be important antigens for use in vaccines, it will be important to discern the geographic distributions of ETEC isolates with different CFA patterns.

ACKNOWLEDGMENTS

This work was supported by grant RO3AI30993 from the National Institutes of Health.

We gratefully acknowledge the monoclonal antibodies provided by Ann Marie Svennerholm of the University of Gothenburg, the genetically defined strains of *E. coli* provided by James Kaper of the University of Maryland, and the assistance of Evangaline Sowers at the Centers for Disease Control and Prevention, Atlanta, Ga., who kindly performed the serotyping assays.

REFERENCES

1. **Ahren, C. M., L. Gothefors, B. J. Stoll, M. A. Salek, and A. M. Svennerholm.** 1986. Comparison of methods for detection of colonization factor antigens on enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **23**:586–591.
2. **Evans, D. G., D. J. Evans, Jr., and S. Clegg.** 1980. Detection of enterotoxigenic *Escherichia coli* colonization factor antigen I in stool specimen by an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **12**:738–743.
3. **Evans, D. G., D. J. Evans, and H. L. DuPont.** 1977. Virulence factors of enterotoxigenic *Escherichia coli*. J. Infect. Dis. **136**: S118–S123.
4. **Evans, D. G., D. J. Evans, and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
5. **Faris, A., A. Ljungh, and T. Wadstrom.** 1985. Rapid detection of colonization factor antigens (CFA/I and CFA/II) of enterotoxigenic *E. coli* and enteropathogenic *E. coli* (EPEC) by coagglutination test. Zentralbl. Bacteriol. Mikrobiol. Hyg Reihe A **259**:477–484.
6. **Grewal, H. M., H. Sommerfelt, W. Gaastra, A. M. Svennerholm, M. K. Bhan, A. M. Hamers, R. Kumar, G. Wiklund, and B. Bjorvatn.** 1990. Detection of colonization factor antigen I-positive enterotoxigenic *Escherichia coli* with a cloned polynucleotide probe. J. Clin. Microbiol. **28**:2264–2268.
7. **Levine, M. M., J. G. Morris, G. Losonsky, E. Boedeker, and R. Rowe.** 1986. Fimbriae (pili) adhesins as vaccines, p. 143–5. *In* D. Lark, S. Normak, and E. Brent-Uhlin (ed.), Protein-carbohydrate interactions in biological systems. Academic Press, London.
8. **Lopez-Vidal, Y., and A. M. Svennerholm.** 1986. 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. J. Clin. Microbiol. **24**:615–619.
9. **Lopez-Vidal, Y., and A. M. Svennerholm.** 1990. Monoclonal antibodies against the different subcomponents of colonization factor antigen II of enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **28**:1906–1912.
10. **Rozgonyi, F., K. R. Szitha, A. Ljungh, S. B. Baloda, S. Hjerten, and T. Wadstrom.** 1985. Improvement of the salt aggregation test to study bacterial cell-surface hydrophobicity. FEMS Microbiol. Lett. **30**:131–138.
11. **Shipley, P. L., C. L. Gyles, and S. Falkow.** 1978. Characterization of plasmids that encode for the K88 colonization antigen. Infect. Immun. **20**:559–566.
12. **Smith, H. R., S. M. Scotland, and B. Rowe.** 1983. Plasmids that code for production of colonization factor antigen II and enterotoxin production in strains of *Escherichia coli*. Infect. Immun. **4**:1236–1239.
13. **Smith, H. R., G. A. Willshaw, and B. Rowe.** 1982. Mapping of a plasmid, coding for colonization factor antigen I and heat-stable enterotoxin production, isolated from an enterotoxigenic strain of *Escherichia coli*. J. Bacteriol. **149**:264–275.
14. **Svennerholm, A. M., C. Wenneras, J. Holmgren, M. M. McConnell, and B. Rowe.** 1990. Roles of different coli surface antigens of colonization factor antigen II in colonization by and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect. Immun. **58**:341–346.