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Comparison of Two Assay Methods for Patterns of Adherence to HEp-2 Cells of *Escherichia coli* from Patients with Diarrhea

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To determine whether methodological differences in the HEp-2 adherence assay could explain conflicting results of field studies, 244 strains of *Escherichia coli* from Mexican children with diarrhea were tested for patterns of adherence by the method used at the Center for Vaccine Development, University of Maryland (CVD), and at the Center for Infectious Diseases, University of Texas Medical School and School of Public Health (UTH). The CVD assay differentiated three phenotypes of adherent *E. coli*, including localized, diffuse, or aggregative adherence (LA, DA, or AA, respectively). There was agreement on pattern of adherence in 241 of the 244 strains (98.8%) tested by the CVD method in both Baltimore and Houston, and AA$^+$ was the most common phenotype (28.5% of isolates). Among these isolates, the UTH assay detected only two adherent phenotypes (LA and DA), since it did not distinguish the AA pattern. The LA$^+$ strains detected by each assay were compared for positivity with the enteropathogenic *E. coli* adherence factor (EAF) gene probe. Of the 16 strains LA$^+$ by the CVD method, 100% were EAF$^+$; in contrast, only 11 of 22 strains LA$^+$ by the UTH method were EAF$^+$ (P = 0.00074). These results help explain why in pediatric field studies in Mexico where isolates were tested by the UTH method (J. J. Mathewson, R. A. Oberhelman, H. L. Dupont, F. J. de la Cabada, and E. V. Garibay, J. Clin. Microbiol. 25:1917–1919, 1987) LA$^+$ strains often did not belong to enteropathogenic *E. coli* O serogroups and why the AA pattern was not observed; the opposite was found in studies of pediatric diarrhea in Chile in which the CVD assay was used (M. M. Levine, V. Prado, R. M. Robins-Browne, H. Lior, J. B. Kaper, S. Moseley, K. Gicquelais, J. P. Nataro, P. Vial, and B. Tall, J. Infect. Dis. 158:224–228, 1988). Since it appears that both assays identify *E. coli* strains associated with diarrheal illness, the genetic relationships among these strains should be examined in future studies.

The propensity for certain strains of *Escherichia coli* isolated from patients with diarrhea to adhere to HEp-2 cells was first reported by Cravioto et al. (5). They found that 80% of strains of classical enteropathogenic *E. coli* (EPEC) infantile diarrhea serotypes that were tested adhered to HEp-2 cells, while this was an uncommon property among enterotoxigenic and other *E. coli* strains. Baldini et al. (1) showed that a 60-megadalton plasmid was required for EPEC to adhere to HEp-2 cells and that the bacteria attach in clusters or microcolonies, later referred to as localized adherence (LA) (20, 21). Nataro et al. (17) cloned a 1.0-kilobase fragment of DNA from a region of the EPEC adherence factor (EAF) plasmid which was critical for adherence; they demonstrated that the fragment could serve as a sensitive and specific DNA probe to identify *E. coli* colonies that exhibit LA in the HEp-2 cell assay. The strong correlation (circa 98%) between positivity by the EAF probe and LA$^+$ detected by the HEp-2 (or HeLa cell) assay has been observed in several studies (3, 7, 11, 18). Approximately 80 to 90% of strains that are LA$^+$ and EAF$^+$ belong to O:H serotypes considered to be classical (3, 7, 11, 18).

Subsequently, a second pattern of adherence of *E. coli* to HEp-2 cells was described, diffuse adherence (DA), in which the bacteria adhere over the entire surface of the cells (21). DA was not usually associated with strains of classical EPEC O:H serotypes (19, 20). More recently, a third distinct pattern, aggregative adherence (AA), was described (11, 18, 22), in which the bacteria adhere to HEp-2 cells as well as to the glass between the cells in a characteristic "stacked brick" appearance. AA$^+$ strains also do not belong to classical EPEC serotypes (11, 22). AA$^+$ and DA$^+$ *E. coli* isolates do not hybridize with the EAF gene probe (11, 18, 22).

Several different groups of investigators have carried out prospective epidemiological studies to determine the frequency with which HEp-2 cell-adherent *E. coli* exhibiting the different patterns of adherence occurs among children with diarrhea compared with their occurrence in age-matched control children without diarrhea (3, 6, 7, 11, 16). These different studies have yielded many similar findings, but some inconsistencies have also been reported. Mathewson et al. (16) reported that both LA$^+$ and DA$^+$ HEp-2 cell-adherent *E. coli* occurred significantly more often among Mexican children with diarrhea than among controls. Serogrouping revealed that the LA$^+$ isolates were not of classical EPEC O serogroups (16); the isolates were not tested with the EAF gene probe (16). A study of Korean children yielded similar results (8). Mathewson et al. (14) also reported the isolation of LA$^+$ *E. coli* of non-EPEC O serogroups from U.S. travelers with diarrhea in Mexico. One LA$^+$, non-EPEC O serogroup isolate from a traveler, strain 221, caused unequivocal diarrhea when ingested by volunteers (15).

Three other groups of investigators have reported epide-

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miological studies of pediatric diarrhea in Latin America wherein *E. coli* isolates from young children with diarrhea and from age-matched controls without diarrhea were examined for patterns of adherence to HEp-2 cells, for O serogroup, and (in two of the studies) for positivity with the EAF probe. In studies in Chile (11), Mexico (6), and Brazil (7), LA^+ strains were found significantly more often in patients with diarrhea than in controls, and approximately 90% of these strains were of classical EPEC O serogroups. Moreover, in these three studies, AA^+ *E. coli* isolates were common and in the Chilean study were identified significantly more often in patients with diarrhea than in controls.

Levine et al. (11) and Vial et al. (22) hypothesized that technical differences in the performance of the HEp-2 cell assay were likely to be largely responsible for the divergent results reported (6, 7, 11, 16) with respect to the frequency of occurrence of LA^+, non-EPEC O group strains and of *E. coli* manifesting AA. In a recent editorial, Mathewson and Cravioto (13) concurred with this hypothesis. Support for the hypothesis can be derived from the observation that the non-EPEC O serogroup, LA^+ strain 221 of Mathewson et al. (15) proved to be AA^+ in the HEp-2 assay as performed by Vial et al. (22) and Levine et al. (11); moreover, 221 did not hybridize with the EAF probe. This suggested that some proportion of the strains reported by Mathewson et al. as LA^+, non-EPEC O serogroup might in fact be AA^+ when tested by the HEp-2 assay utilized by Vial et al. (22) and Levine et al. (11). In order to test the hypothesis and perhaps clarify the situation, *E. coli* strains from the studies of Mathewson et al. of Mexican children were tested by two different HEp-2 cell assays and with the EAF probe.

MATERIALS AND METHODS

***E. coli* strains.** A total of 244 *E. coli* strains isolated by Mathewson et al. (16) from 122 children with diarrhea in Guadalajara, Mexico, were examined. These included strains previously reported as being LA^+ and DA^+ strains of non-EPEC O serogroups (16). Also tested was strain 221 (O78:H33) isolated from a U.S. student with traveler's diarrhea in Mexico and originally reported as LA^+ (15).

HEp-2 cell assays. The patterns of adherence of the 244 *E. coli* test strains were examined by two separate methods, referred to as the Center for Vaccine Development (CVD) and the University of Texas Medical School, Houston (UTH), techniques. Differences between these assays include the solid surface on which the HEp-2 monolayers are grown, the degree of confluence of the monolayer (50 versus 90%), the number of washes performed (three versus six), the number of incubations of monolayers in the presence of bacteria (one versus two), and the duration of uninterrupted incubation of bacteria on the monolayer (3 versus 1 and 2 h with a wash in between).

The CVD method is essentially the 3-h assay originally described by Cravioto et al. (5) which has been extensively used by investigators at the CVD (1, 11, 17–19, 22). This method was performed with monolayers of HEp-2 cells grown to 50% confluence on circular cover slips in wells of 24-well tissue culture plates. Bacteria were grown in L broth for 16 h statically at 37°C. Twenty microliters of each bacterial culture (2×10^7 bacteria) was incubated with the HEp-2 monolayers in the presence of 1% methyl-α-D-mannoside for 3 h at 37°C in 5% CO_2. Wells were then washed three times with Hanks balanced salt solution, fixed with 70% ethanol, and stained with 10% Giemsa stain. Cover slips were mounted on glass slides and examined under the oil immersion lens of a light microscope.

TABLE 1. Ability of the CVD HEp-2 assay method to detect adherent *E. coli* among 244 isolates tested independently in two separate laboratories

Adherence pattern	No. of isolates positive in:	
	Baltimore	Houston
LA	16	16
DA	21[a]	21[b]
AA	60[c]	60
Nonadherent	147	147

[a] One strain was nonadherent in Houston and DA^+ in Baltimore.
[b] One strain was DA^+ in Houston and AA^+ in Baltimore.
[c] One strain was AA^+ in Houston and nonadherent in Baltimore.

The UTH method (14), derived from the method described by Clausen and Christie (4), was performed with nearly confluent HEp-2 monolayers grown on plastic chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Strains were grown overnight in tryptic soy broth at 37°C with 1% mannose. Twenty microliters of these cultures (circa 10^7 bacteria) was added to the HEp-2 monolayers and incubated for 1 h at 37°C in 5% CO_2 in the presence of mannose. The monolayer was then washed three times, fresh medium was added, and a second incubation was carried out for 2 h. Chamber slides were then washed, fixed with 70% ethanol, stained with carbolfuchsin, and examined under a light microscope.

The slides prepared by the two methods were examined for the presence of LA, DA, or AA according to the descriptions of Nataro et al. (18). The CVD method was performed in parallel at both the CVD and the UTH, while the UTH method was used only in the Houston laboratory. In both laboratories, the specimens were processed under a code and the slides were read blinded. The code was broken and results were compared only after written summaries from each laboratory had been exchanged.

Hybridization with DNA probes. In the Baltimore laboratory, colony blots of the coded strains were hybridized with DNA probes that detect various categories of diarrhea-causing *E. coli* (10, 11). These include probes for EPEC (the EAF probe) (17), enteroinvasive *E. coli* (23), enterotoxigenic *E. coli* (with probes for heat-labile and human and porcine heat-stable enterotoxins [9]), and enterohemorrhagic *E. coli* (12).

RESULTS

HEp-2 cell assays. A comparison of results of the CVD HEp-2 method run independently in the Baltimore and Houston laboratories is summarized in Table 1. There was agreement on 241 of the 244 strains tested by this method (98.8%) in the two laboratories. AA was the most common pattern when the CVD HEp-2 cell assay method was used to test these strains from Mexico. For all further comparisons involving data derived with the CVD method (e.g., as in Table 2), we used results of the 241 isolates for which there was complete agreement between the laboratories.

Table 2 compares results from testing strains by the CVD method and the UTH method. Overall, some pattern of adherence was observed in 95 of the 241 strains (39.4%) tested by the CVD method and in 63 strains (26.1%) tested by the UTH method. An important observation made in this study is that with the UTH method, one does not discern the AA pattern; none of the 241 strains were AA^+ by the UTH method, whereas 59 strains (24.5%) were AA^+ by the CVD

TABLE 2. Comparison of HEp-2 cell adherence patterns of 241 *E. coli* isolates[a] tested by two different HEp-2 assay methods

Adherence pattern	No. (%) of positive isolates by:	
	CVD method	UTH method[b]
LA	16 (6.6)	22 (9.1)
DA	20 (8.3)	41 (17.1)
AA	59 (28.5)	0 (0)
Nonadherent	146 (60.6)	178 (73.9)

[a] These are the 241 strains (out of a total of 244) for which both the Baltimore and Houston laboratories gave identical results by the CVD method.
[b] Results of the UTH method used in the Houston laboratory.

method ($P < 0.00000001$, Fisher's exact test, two tailed). By the latter method, AA was in fact the most frequent adherence pattern. Forty-three percent of the strains reported as LA^+ or DA^+ when tested by the UTH method were found to be AA^+ by the CVD method. Approximately 10% of strains recorded as adherent by the UTH method were nonadherent by the CVD method.

Hybridization with DNA probes. None of the strains hybridized with the probes that detect enterotoxigenic, enteroinvasive, or enterohemorrhagic *E. coli*. A total of 16 strains hybridized with the EAF probe; all 16 strains were LA^+ in the CVD assay, and 11 were LA^+ in the UTH assay. Table 3 lists all strains that were LA^+ in either assay, along with their EAF probe test results. Of the 16 strains LA^+ by the CVD method, 100% were EAF^+, while only 11 of the 22 LA^+ strains detected by the UTH method were EAF^+ (50%) ($P = 0.00074$, Fisher's exact test, two tailed).

TABLE 3. *E. coli* strains that exhibited localized adherence in either the CVD or the UTH HEp-2 cell assay and results of testing with the EAF gene probe

Strain	Pattern[a] in:		Hybridization[b] with EAF probe
	UTH assay	CVD assay	
22A	LA	LA	+
22B	LA	LA	+
37A	LA	LA	+
37B	LA	LA	+
110C	LA	LA	+
116A	LA	LA	+
118A	LA	LA	+
118B	LA	LA	+
144A	LA	LA	+
144B	LA	LA	+
167A	LA	LA	+
14A	NA	LA	+
14B	NA	LA	+
80A	DA	LA	+
80B	DA	LA	+
146A	NA	LA	+
11A	LA	AA	−
13A	LA	AA	−
13B	LA	AA	−
16A	LA	AA	−
19A	LA	AA	−
77B	LA	AA	−
78A	LA	AA	−
79A	LA	DA	−
92A	LA	DA	−
95A	LA	NA	−
128B	LA	AA	−

[a] NA, No adherence.
[b] +, Hybridized with probe; −, did not hybridize with probe.

Results with strain 221. Strain 221 was AA^+ when tested by the CVD method in both Baltimore and Houston but LA^+ when tested by the UTH method. Strain 221 was negative with the EAF probe.

DISCUSSION

Investigations of the capacity of *E. coli* strains from patients with diarrhea and controls to adhere to HEp-2 cells has expanded knowledge of the pathogenesis and etiology of diarrhea but has also inadvertently led to some confusion over interpretation of the results. This laboratory study represents a collaboration wherein investigators who carried out the epidemiological studies that generated divergent results agreed to perform experiments that might clarify the results of the epidemiological studies. The main discrepancies among the epidemiological studies related to the frequency of occurrence in diarrhea cases and controls of LA^+ *E. coli* strains that are not of EPEC O serogroups and the frequency of occurrence of AA^+ *E. coli*. The comparison in this study of *E. coli* tested by two different methods of HEp-2 cell assay helped clarify the previously conflicting data.

With the UTH method, as before (16), only the LA and DA patterns were seen among these isolates; AA was not discerned. In contrast, the CVD HEp-2 assay identified strains exhibiting AA as well as LA or DA in this collection of *E. coli* isolates from Mexican children with diarrhea. Indeed, AA^+ strains were found to be the most common phenotype of HEp-2 cell-adherent *E. coli* among these isolates. A coincidental finding was the notable consistency of the CVD assay when performed in two different laboratories (Table 1), although the UTH assay was not evaluated in both laboratories.

Both assays appear to identify strains of *E. coli* associated with diarrheal illness (2, 11, 16). However, two fundamental questions remain to be resolved. (i) What are the relationships among the various classes of *E. coli* detected by these assays? (ii) Which assay is more predictive of the pathogenicity of strains for humans? Is it more relevant to know whether adhering strains fall into three separate phenotypes (CVD assay) or two phenotypes (UTH assay) or rather simply to score strains as positive or negative for HEp-2 adherence, irrespective of pattern?

Since DNA probes have proven useful for correlating genotypes with phenotypes for other categories of diarrhea-causing *E. coli* (9, 11, 12, 17, 23), such techniques may similarly prove helpful in deciphering the differences between HEp-2 cell-adherent *E. coli* isolates exhibiting different patterns. Efforts to identify a DNA sequence that correlates with a respective adherence phenotype represent a challenging avenue of investigation for proponents of each of the two HEp-2 assay methods. If successful, this could lead to a more practical and objective assay (e.g., DNA probes) in addition to allowing definitive studies of pathogenesis that use engineered mutant strains. A probe to identify *E. coli* strains that exhibit diffuse adherence in the CVD HEp-2 assay has undergone preliminary testing (11). It was satisfactorily specific (96%) but only 75% sensitive. Some progress is also being made to develop probes that detect *E. coli* strains that manifest AA in the CVD assay. Preliminary laboratory standardization of a probe for AA^+ strains has demonstrated high specificity (99%) and acceptable sensitivity (89%) with strains from several geographic areas; the probe is somewhat less sensitive but equally specific with strains from one other geographic area (B.

Baudry, S. J. Savarino, P. Vial, J. B. Kaper, and M. M. Levine, J. Infect. Dis., in press). The AA probe has not yet been used in a prospective epidemiologic study.

There is one well-established example of a high level of correlation between a HEp-2 phenotype and a genotype detected by a DNA probe: approximately 98% of isolates LA^+ by the CVD HEp-2 assay hybridize with a DNA probe derived from the EAF virulence plasmid (3, 7, 11,18). This correlation was again observed in this study, as all 16 strains that were LA^+ by the CVD HEp-2 method were also EAF^+. This elicits optimism that other HEp-2 adherence phenotypes may eventually also be correlated with specific DNA sequences.

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