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Escherichia coli Adherence to HEp-2 Cells with Prefixed Cells

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We describe a new method which uses cold absolute methanol-prefixed cells for adherence of enteropathogenic *Escherichia coli* to HEp-2 cells. We found that a method using bacteria grown in Penassay broth to 10^6 to 10^7 CFU/ml and incubated with prefixed cells for 3 h at 37°C, showed 100% sensitivity and specificity against a method using live cells.

The mechanisms by which enteropathogenic *Escherichia coli* (EPEC) causes diarrhea are uncertain; however, its ability to adhere to small intestine mucosa and efface epithelial microvilli is an important fact (6).

Cravioto et al. (2) found that 80% of EPEC strains presented mannose-resistant adherence to HEp-2 cells. Three distinct patterns of adherence have been described: localized adherence (LA), in which bacteria attach to and form microcolonies in distinct regions of the surface; diffuse adherence (DA), in which bacteria adhere evenly to the whole cell surface, and aggregative adherence (AggA), in which aggregated bacteria attach to the cell in a stacked-brick arrangement (7, 8, 11).

LA is a feature of attaching and effacing EPEC strains (5). The attaching and effacing lesion by EPEC is characterized by intimate attachment to brush border microvilli and actin concentration in this specific site which may be observed by fluorescence actin stain (FAS) (4). The adherence assay uses living cells. For this reason the utility of the method has been limited to some research centers. In addition, it has proven difficult to maintain tissue cultures in ordinary laboratories.

We used strain E2348/69 serotype O127:H6 and strain B171 serotype O111:NM, kindly provided by M. S. Donnenberg and Jorge Giron, from the Center for Vaccine Development, University of Maryland.

We used four LA^+ strains (O111ab:H^-, O119:H2, O114:H2, and O55:H7), one DA^+ strain (O75), two $AggA^+$ strains (O?:H10 and 0136:H33), and one strain without adherence (NA) (040), all of them kindly provided by Alejandro Cravioto, Departamento de Salud Publica, Facultad de Medicina, UNAM, Mexico. We also used 72 fresh isolates of EPEC strains from 72 children hospitalized in Hospital Infantil de Mexico for whom the clinical laboratory did not find another diarrhea causal agent. These were isolated during 6 months from March to August. The HEp-2 cell assay was done as previously described (2). Confluent 70 to 80% growth of HEp-2 cells was obtained on 13-mm diameter glass coverslips placed in the wells of a multiwell tissue culture plate (Nunc, Denmark). Bacteria were grown statically in Penassay broth overnight at 37°C. Twenty microliters of each bacterial culture was mixed with minimal essential medium (Gibco), 1% D-mannose was added to obtain a final concentration of between 10^6 and 10^8 CFU/ml, and the mixture was incubated for 3 h. The coverslips were washed three times with phosphate-buffered saline, and the cultures were fixed with 70% methanol and stained with 10% Giemsa solution. The coverslips were then washed, mounted on glass slides, and examined under the oil immersion objective of a light microscope (magnification, ×1,000). In our method, we used HEp-2 cells grown on coverslips till 70% confluent; after this growth, coverslips were fixed with 70, 80, 90, and 100% cold methanol and 3.7, 5, and 10% formaldehyde. Next, we used the living-cell method for an adherence assay. Each variable was tested with the following conditions: bacterial culture media, Penassay broth, tryptic soy broth, and brain heart infusion broth; bacterial concentrations, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/ml; bacterial growth temperatures, 22 and 37°C; incubation times, 1, 2, and 3 h; and stains, crystal violet, Giemsa, and Wright. All the assays were done in triplicate. The adherence index was obtained by counting the number of cells to which bacteria have adhered.

Our results showed that the best cell-fixing medium for the assay was cold absolute methanol, the best bacterial culture medium was Penassay broth, the optimum bacterial concentration was 10^6 to 10^7 CFU/ml, the best temperature and incubation time were 3 h and 37°C, and the optimum staining procedure was 10% Giemsa solution for 20 min. Our principal indicator for LA was microcolony formation on the cell surface by strain E2348/69, and we found that this strain produced LA on living (Fig. 1A) and prefixed (Fig. 1B) cells in which we could see at least six microcolonies on the apical portion of the cell. Fig. 2 shows strain 075 with DA on living (A) and prefixed (B) cells, and Fig. 3 shows strain O?:H10 with AggA on living (A) and prefixed (B) cells. We observed the same adherence patterns with both living and prefixed cells and all the reference strains. Results for the remaining strains are shown in Table 1. The living-cell method was performed simultaneously with the prefixed-cell method; when the result for LA adherence by the living-cell method was considered the standard, the sensitivity and specificity were 100%. Moreover, we found that one O111 strain that had a DA pattern by the living-cell method showed AggA by the prefixed-cell method; the same thing happened with one 055 strain. In other event, one O26 strain with AggA showed DA by the prefixed-cells method, although the statistical data showed no significant difference (t 0.001) and the result could have been attributed to strong washes. EPEC strains showing LA patterns in HEp-2 cells by the living-cell method were FAS positive, but the same strains with LA pattern by the prefixed-cell method were FAS negative. Other diagnostic methods for EPEC strain detection with LA exist, like that described by Albert et al. (1) in which an

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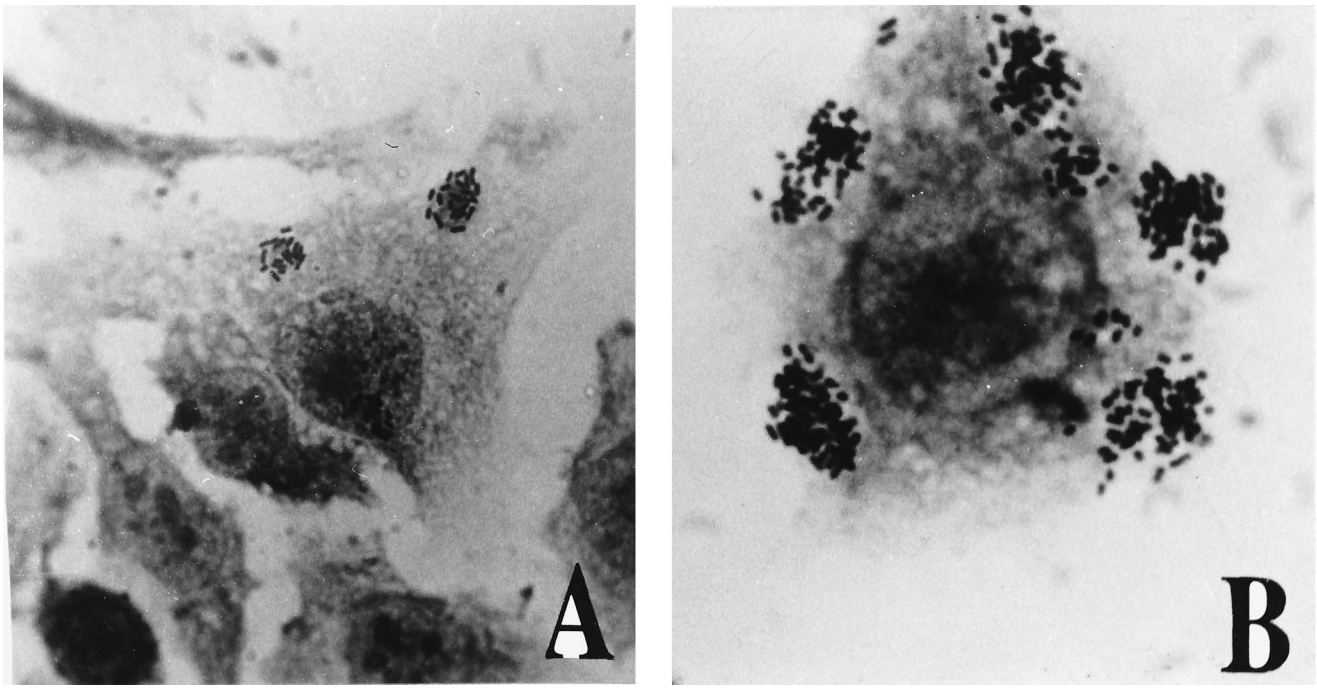

FIG. 1. HEp-2 cell adhesion assay stained with Giemsa showing E2348/69 strain LA on living (A) and prefixed (B) cells. Magnification, ×1,000.

enzyme-linked immunosorbent assay was used, but the factors apparently associated with the *E. coli* adherence factor (EAF) plasmid used in the test are unknown. There have been modifications to the living-cell assay, for instance the CVD and UTH methods (10). Despite those modifications, the cell line maintenance continues to be a problem in ordinary laboratories. Another modification, UTH-M, was done by Haider et al. (3); in this method they increased postwash incubation of en-

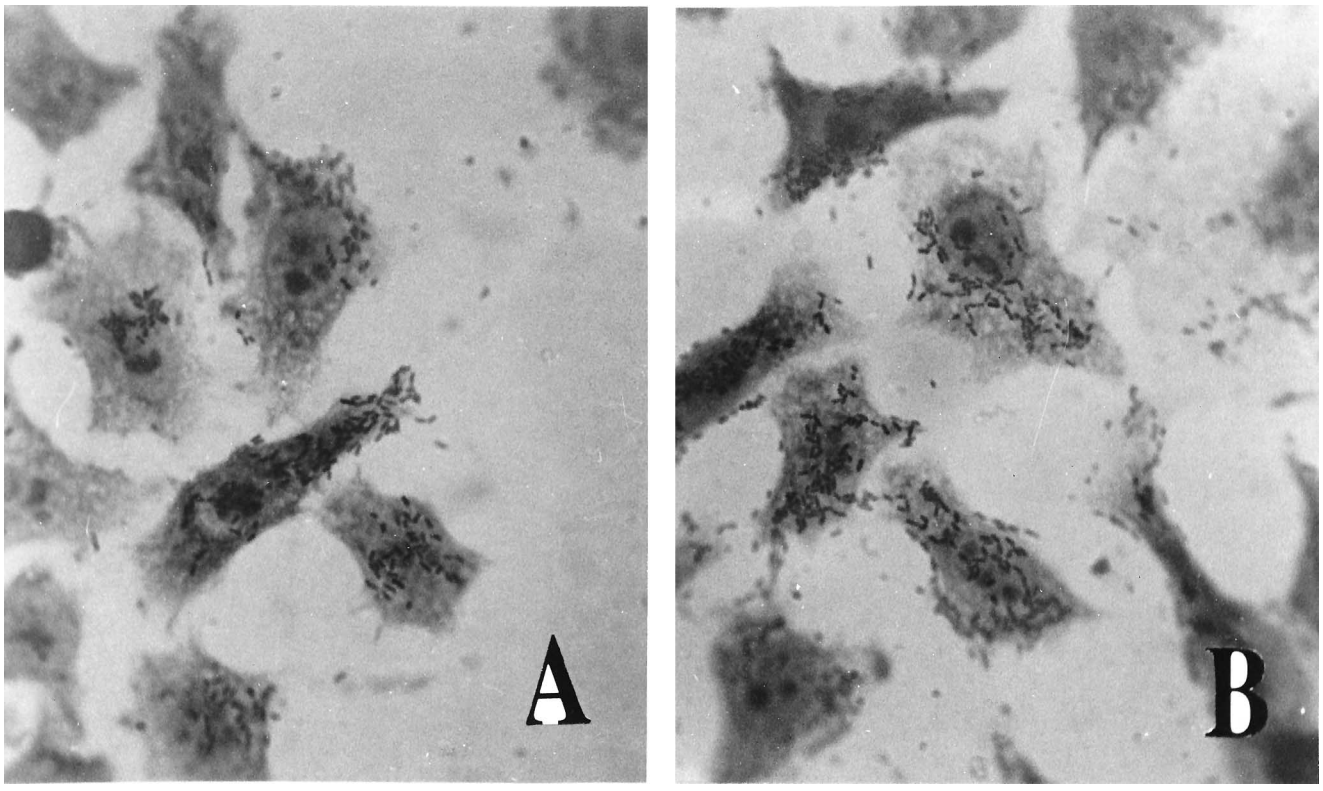

FIG. 2. Light micrographs showing strain 075 DA adhered on living HEp-2 cells (A) and prefixed cells (B). Magnification, ×1,000.

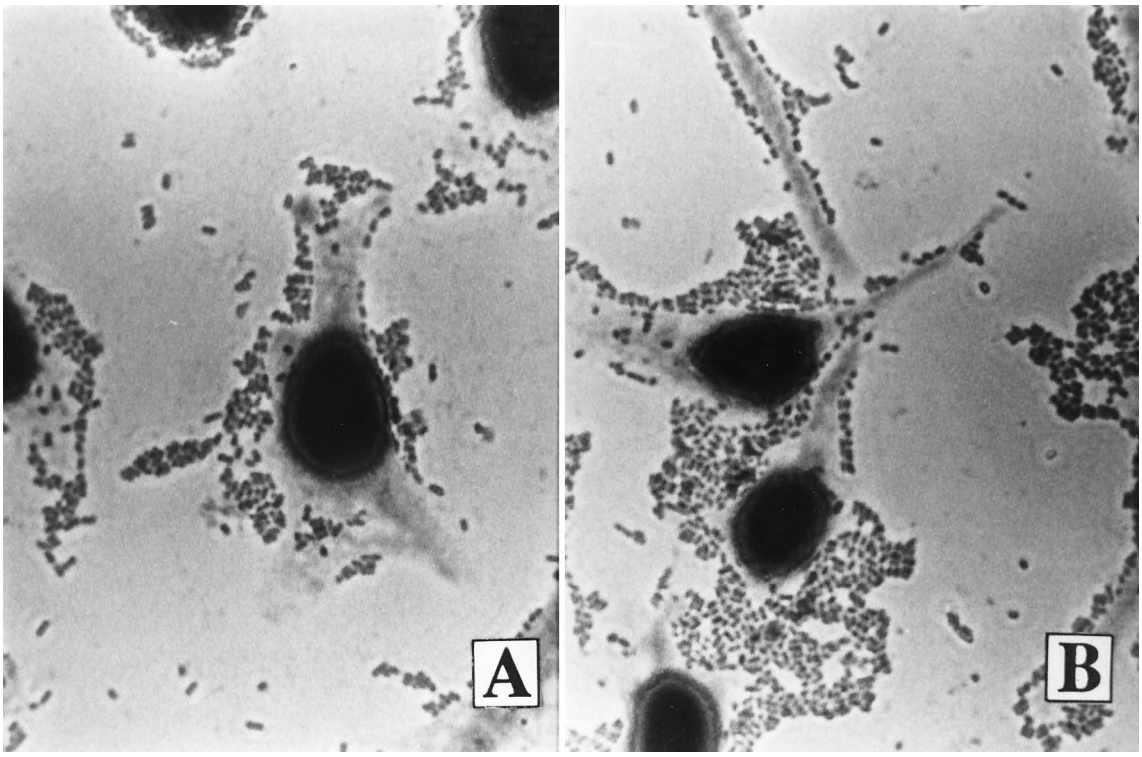

FIG. 3. Giemsa stain showing aggregative adherence pattern of 0?:H10 strain on living cells (A) and prefixed cells (B). Magnification, ×1,000.

teroaggregative *E. coli*. Although there is an assay with the EAF probe, Scotland et al. (9) reported that the use of this probe in epidemiological studies was unsuitable and the best indicator of virulence was the HEp-2 adhesion assay supplemented by the FAS test. Yamamoto et al. (12) also used prefixed HeLa cells with glutaraldehyde or formalin for adherence assay. They found that microcolonies were smaller in prefixed cells than in living cells; we believe this result may have occurred because the technique was not standardized previously. The LA strains become FAS^- because prefixed cells cannot accumulate actin in the union site of the bacteria; thus, LA and FAS are used for separate studies. We propose that this new method could be an important tool in epidemiological studies, and it could also be useful for ordinary laboratories in developing countries. Moreover, this method could be appropriate to study the cellular receptor responsible for LA in HEp-2 cells.

TABLE 1. Relationship between living-cell and prefixed-cell methods in 72 fresh isolates from strains belonging to O serogroups

Serogroup	*n*	Result							
		Living-cell method				Prefixed-cell method			
		LA	DA	AggA	NA	LA	DA	AggA	NA
O111	39	20	9	2	8	20	8	3	8
O26	15	1	4	4	6	1	5	3	6
O112	5	2	2	1	0	2	2	1	0
O44	4	0	2	0	2	0	2	0	2
O125	3	1	1	0	1	1	1	0	1
O55	2	0	2	0	0	0	1	1	0
O119	2	2	0	0	0	2	0	0	0
O128	2	2	0	0	0	2	0	0	0
Total	72	28	20	7	17	28	19	8	17

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