

## Mannose-Resistant Hemagglutination, Enzyme-Linked Immunosorbent Assay, and Immune Electron Microscopy for Detection of K99 Fimbrial Antigen in *Escherichia coli* from Calves

FRANCISCO HERNÁNDEZ,<sup>1</sup> MAGALY CABALLERO,<sup>2</sup> PATRICIA RIVERA,<sup>3</sup> AND DAVID HIRD<sup>4\*</sup>

*Centro de Investigación y Diagnostico en Parasitología, Facultad de Microbiología, Universidad de Costa Rica,<sup>1</sup> and Departamento de Patología, Hospital Nacional de Niños,<sup>3</sup> San José, and Escuela de Medicina Veterinaria, Universidad Nacional, Heredia,<sup>2</sup> Costa Rica, and Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, California 95616<sup>4</sup>*

Received 3 January 1989/Accepted 23 May 1989

**Mannose-resistant hemagglutination (MRHA) was evaluated for identification of *Escherichia coli* with K99 fimbriae. The sensitivity and specificity of MRHA, relative to the enzyme-linked immunosorbent assay, were 21 and 79%, respectively. Disagreement between the tests may have been due in part to separation of pili from the cells, with resultant enzyme-linked immunosorbent assay-positive, MRHA-negative tests. MRHA was not useful as the sole means for the identification of *E. coli* with K99 fimbriae.**

Adhesion and colonization are important first steps in the pathogenesis of disease caused by enterotoxigenic *Escherichia coli*. Adhesion is usually mediated by interactions between adhesins on the surface of the enterotoxigenic *E. coli* and specific receptors on the enterocyte surface (4, 8).

Various adhesins, such as fimbriae or pili, have been reported to be virulence factors in enterotoxigenic *E. coli* isolated from different species of animals. These include colonization factor antigens in human strains (2), K88 antigens in pig strains, and K99, F41, and 987P fimbrial antigens in calf strains (4). These antigens have been identified by serological methods, such as the enzyme-linked immunosorbent assay (ELISA) with specific (e.g., monoclonal) antibodies (9, 10). Because erythrocytes (RBCs) have on their surfaces receptors to fimbriae similar to those found on enterocytes, hemagglutination (HA) can occur with enterotoxigenic *E. coli* strains possessing fimbriae (5). HA has been used successfully to identify fimbriated *E. coli* from human enteric (4) and urinary tract (11) infections.

HA can also be used to classify types of *E. coli* fimbriae. Type I fimbriae, present in many strains of *E. coli*, including nonpathogenic strains, react with receptors which contain mannose (1). In this case, HA is inhibited when mannose is included in the RBC suspension. This phenomenon is referred to as mannose-sensitive HA. Non-type I fimbriae, such as those responsible for the colonization factor for intestinal epithelium, react with different non-mannose-containing receptors. This type of HA is called mannose-resistant HA (MRHA). Fimbriae responsible for MRHA include K88, K99, colonization factor antigen I, and some *E. coli* surface components of colonization factor antigen II, among others (4). These findings suggest that MRHA may be a simple, rapid, and inexpensive method for differentiating bacteria with fimbriae other than type I fimbriae.

This study was part of a larger, multidisciplinary study of dairy calf morbidity and mortality in Costa Rica (D. Hird, E. Perez, M. Caballero, L. Rodriguez, and J. Velazquez, submitted for publication). The objective of this study was to evaluate MRHA as a technique for identifying *E. coli* strains possessing K99 fimbriae, with ELISA as a comparative

method and with electron microscopy and immune electron microscopy to confirm the presence of fimbriae.

A total of 232 strains of *E. coli* were obtained from Holstein and Jersey female dairy calves less than 3 months old and with or without diarrhea on 43 Costa Rican dairy farms. For the isolation of *E. coli*, rectal swabs were streaked on MacConkey agar plates and incubated for 24 h at 37°C. For ELISAs and HA tests, 20 lactose-positive colonies from each strain were inoculated into a Minca broth tube (6) and incubated for 24 h at 37°C. The ELISAs were performed in triplicate for each Minca broth tube after centrifugation and suspension of colonies in buffer diluent of a commercial K99 detection kit (Coli-Tect 99 antigen test kit; Molecular Genetics, Inc., Minnetonka, Minn.) with a monoclonal antibody against K99 (MGA 2BD 4E4 globulin and partially purified K99 pilus antigen as a positive control; both were kindly provided by D. Reed, Molecular Genetics, Inc.). Working buffer was used as a negative control. HA of the Minca broth-cultured bacteria was performed as described by Vaisanen et al. (11). Briefly, the bacterial cells were washed in phosphate buffer, and the cell concentration was adjusted by the McFarland turbidimetric method to approximately  $2 \times 10^{10}$  CFU/ml. Twenty microliters of this cell suspension was mixed with 20  $\mu$ l of a 2% group O human RBC suspension in phosphate buffer with or without 5% mannose. The HA test was done on glass slides supported on an ice bath, and positive reactions were recorded at 30 s. *E. coli* isolated from normal urine samples (MRHA and mannose-sensitive HA negative) was used for the MRHA negative control, and working buffer was used to check the RBC suspension.

A total of 100 strains of *E. coli* from Minca broth were washed with phosphate buffer (0.1 M, pH 7.4) and fixed for 2 h at 4°C in 2.5% glutaraldehyde solution in the same buffer. Cells were then negatively stained with 0.5% phosphotungstic acid and examined under an electron microscope (Hitachi HU 12A).

Three strains of each of the following groups were processed for immune electron microscopy: strains positive in both the MRHA test and ELISA (true-positives), strains negative in both tests (true-negatives), MRHA-positive, ELISA-negative strains (false-positives), and MRHA-nega-

\* Corresponding author.

TABLE 1. Relationship between the MRHA test and the ELISA for detection of K99 fimbrial antigen in *E. coli* from calves

Reaction	No. (%) of calves	
	With diarrhea (n = 122)	Without diarrhea (n = 110)
MRHA positive, ELISA positive	4 (3)	3 (3)
MRHA positive, ELISA negative	24 (19)	17 (15)
MRHA negative, ELISA negative	84 (69)	74 (67)
MRHA negative, ELISA positive	10 (8)	16 (15)

ive, ELISA-positive strains (false-negatives). For the negative control, working buffer was used in place of the monoclonal antibody. The method used for immune electron microscopy was similar to that described for Sendai virus (7). All washings were done with phosphate buffer (0.1 M, pH 7.4), and prior to each of the following steps grids were washed in this buffer for 5 min. Briefly, bacteria were fixed with 1.0% glutaraldehyde for 1 h, adsorbed on grids with Formvar support membranes, incubated with 3% gelatin for 1 h at 37°C, and incubated for 1 h at 37°C with the anti-K99 monoclonal antibody described above at dilutions of 1:500, 1:1,000, and 1:2,000. The grids were incubated with a 1:10 solution of protein A conjugated with 7-nm-diameter colloidal gold (kindly provided by Jollyanna Malavasi, Electron Microscopy Unit, Universidad de Costa Rica); subsequently, a light negative staining with 0.1% phosphotungstic acid for 5 min was performed.

The results of the MRHA test and ELISA were compared (Table 1). Relative to the ELISA, the sensitivity, specificity, predictive value of a positive test, and predictive value of a negative test for MRHA were 7 of 33 (21.2%), 158 of 199 (79.4%), 7 of 48 (14.6%), and 158 of 184 (85.9%), respectively. The kappa statistic (3) was 0.005, indicating very little agreement between the two tests beyond that expected by chance.

During electron microscopy observations of MRHA-negative, ELISA-positive strains, bacterial cells showed only patches of attached fimbriae, but many free fimbriae were seen in the grid background. This finding suggests that the fimbriae could be very fragile and had become separated from the cells. It was previously demonstrated that super-

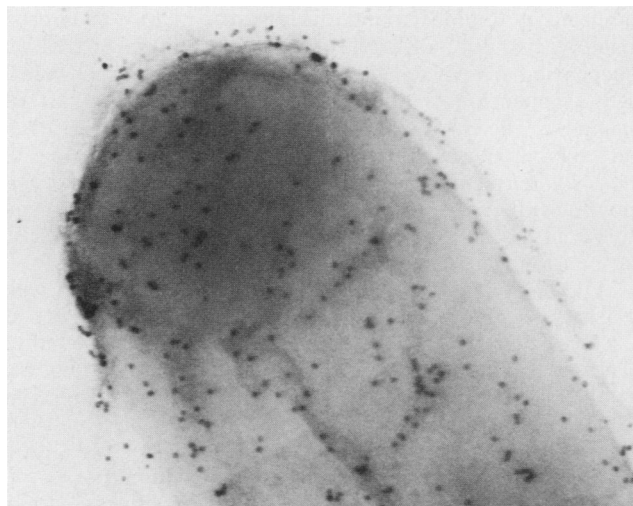


FIG. 1. Immune electron micrograph of *E. coli* showing the pattern of anti-K99 monoclonal antibody-receptor complexes.

natants of fimbriated bacterial suspensions yielded positive ELISA results (10). Therefore, cells from which fimbriae have become separated may yield positive ELISA results but negative MRHA test results.

In immune electron microscopy with the same monoclonal antibody as that used in the ELISA and protein A-colloidal gold, a homogeneous pattern of distribution of immune marker on the bacterial surface was observed (Fig. 1). This result seemed to indicate that even after a fimbria, or part of it, was separated from the bacterium, the cell still retained the K99 antigen, which reacted with the epitope for this monoclonal antibody. This may explain the high proportion of false-negative MRHA tests. False-positive MRHA tests are possibly explained by the presence of fimbriae different from K99 but capable of agglutinating RBCs in the presence of mannose. These non-K99 fimbrial antigens could be important as virulence factors.

In conclusion, we found that MRHA, despite being rapid and inexpensive, was not useful for the identification of *E. coli* K99 antigen. Nevertheless, the test could be useful for presumptive identification of fimbriated bacteria.

We thank Enrique Pérez for assistance in specimen collection, D. Reed, Molecular Genetics, Inc., for providing the anti-K99 monoclonal antibody, and Jollyanna Malavasi for the colloidal gold.

This study was conducted while D. Hird was a Fulbright research scholar in Costa Rica.

#### LITERATURE CITED

1. Brinton, C. C., Jr. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. *Trans. N.Y. Acad. Sci.* 27:1003-1054.
2. Evans, D. G., D. J. Evans, Jr., 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.
3. Fleiss, J. L. 1981. *Statistical methods for rates and proportions*, 2nd ed. John Wiley & Sons, Inc., New York.
4. Gastra, W., and F. de Graff. 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiol. Rev.* 46:129-161.
5. González, E., and J. Blanco. 1985. Comparative study of inhibition of mannose-resistant haemagglutination caused by CFA/I, CFA/II, K88 and K99-positive *Escherichia coli* strains. *FEMS Microbiol. Lett.* 29:115-121.
6. Guinée, P. A. M., J. Veldkamp, and W. H. Jansen. 1977. Improved Minca Medium for the detection of K99 antigen in calf enterotoxigenic strains of *Escherichia coli*. *Infect. Immun.* 15:676-678.
7. Hernández, F., P. Rivera, and Y. Hosaka. 1987. Homogeneous distribution of parainfluenza virus glycoproteins demonstrated by immunogold-labelling and light staining with uranyl acetate in electron microscopy. *J. Virol. Methods* 15:273-277.
8. Levine, M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* 155:377-389.
9. Mills, K. W., and K. L. Tietze. 1984. Monoclonal antibody enzyme-linked immunosorbent assay for identification of K99-positive *Escherichia coli* isolates from calves. *J. Clin. Microbiol.* 19:498-501.
10. Raybould, T. J. G., C. F. Crouch, and S. D. Acres. 1987. Monoclonal antibody passive hemagglutination and capture enzyme-linked immunosorbent assays for direct detection and quantitation of F41 and K99 fimbrial antigens in enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 25:278-284.
11. Vaisanen, V., L. G. Tallgren, P. H. Makela, G. Kallenius, H. Hultberg, J. Elo, A. Siitonen, C. Svanborg-Eden, S. B. Svenson, and T. Korhonen. 1981. Mannose-resistant haemagglutination and P antigen recognition are characteristic of *Escherichia coli* causing primary pyelonephritis. *Lancet* ii:1366-1369.