INDIAN JOURNAL
OF
MEDICAL SCIENCES

VOLUME 62
NUMBER 1
JANUARY 2008

ORIGINAL CONTRIBUTIONS

LETTERS TO EDITOR

PRACTITIONERS' SECTION

Published by
Medknow Publications
A-109, Kanara Business Center, Off Link Road, Ghatkopar (E), Mumbai - 400075, India

IDENTIFICATION OF ENTEROAGGREGATIVE *ESCHERICHIA COLI* IN INFANTS WITH ACUTE DIARRHEA BASED ON BIOFILM PRODUCTION IN MANIPAL, SOUTH INDIA

RAJU BANGAR, BALLAL MAMATHA

ABSTRACT

BACKGROUND: Enteroaggregative Escherichia coli (EAEC) is an emerging enteric pathogen that causes persistent diarrhea among infants, both in developing and industrialized countries. The EAEC strains adhere to epithelial cell surface, to the glass substratum and to each other in a distinctive stacked brick-formation. Thus, gold standard for identification of EAEC remains the HEp-2 cell adherence test, which is time consuming and requires specialized facilities. AIM: To evaluate the usefulness of quantitative biofilm assay to screen for EAEC from children with acute diarrhea. MATERIALS AND METHODS: A total of 100 E. coli strains were collected from acute diarrheal cases from December 2005 to November 2006. The strains were screened for biofilm production using microtiter plate method. The biofilm in the microtiter plate was visualized after staining with crystal violet and was quantified using enzyme immunosorbent assay plate reader. The Aggregative plasmid and Heat stable toxin genes were evaluated by a multiplex polymerase chain reaction. The strains were identified as EAEC with an optical density at 570 nm (OD_{570}) > 0.2. RESULTS: Of the total 100 Escherichia coli strains, 28 were positive by Polymerase Chain Reaction for two genes, AggR and EAST. Of the 28 PCR-positive strains screened for biofilm, 25 (89.2%) showed positive results by microtiter plate method. CONCLUSION: The quantitative biofilm assay using microtiter plate is convenient and economical and can be used as a screening method to screen E. coli isolates from acute diarrheal cases. The best use of this test is to screen large number of isolates quickly, and if positive this can be confirmed by multiplex PCR for AggR and EAST genes. This assay may contribute to demonstrating the true incidence of EAEC with and without AggR among clinically isolated E. coli strains, which can cause acute diarrhea.

Key words: Biofilms, diarrhea/polymerase chain reaction, enteroaggregative escherichia coli

INTRODUCTION

Escherichia coli is the predominant facultative anaerobe of human colonic flora. Some clones of this species are pathogenic. One of the major clinical syndromes to result from infection with pathogenic *E. coli* is enteric/gastrointestinal disease. Five major categories of diarrheagenic *E. coli* have been identified: enteropathogenic (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic. *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC).[1,2]

Department of Microbiology, Kasturba Medical College, Manipal - 576 104, Karnataka, India

Correspondence:
Dr. Mamatha Ballal, Department of Microbiology, Kasturba Medical College, International Center, Manipal - 576 104, Karnataka, India. E-mail: mamatha_98@yahoo.com

These categories are differentiated on the basis of pathogenic mechanisms and clinical manifestations. The importance of EAEC in diarrhea in children and in immunocompromised patients is well documented. Members of EAEC group are defined as *E. coli* strains that adhere to HEp-2 cells in an aggregative pattern. Most *E. coli* strains that belong to EAEC category have been found to be serologically nontypable using standard methods.[3-5]

The pathogenesis of EAEC infection is only partially understood. In several *in vivo* and *in vitro* models, EAEC strains adhere to the small and large bowel mucosal surface in a thick aggregating biofilm. The gold standard for identification of EAEC remains HEp2 cell adherence test, which is time consuming and requires specialized facilities. We evaluated the usefulness of a quantitative biofilm assay to screen for EAEC from a total of 100 *E. coli* strains from children with diarrhea. Although confirmation by multiplex PCR is needed, the biofilm assay is convenient and useful in screening for EAEC among clinical isolates of *E. coli*.

MATERIALS AND METHODS

A total of 100 *E. coli* strains from children with acute/persistent diarrhea were isolated in a bacteriological laboratory from December 2005 to November 2006. Only one stool specimen was tested from each case patient. All specimens were processed by routine microbiologic and biochemical tests in the bacteriological laboratory to identify *E. coli*. Mac Conkey's agar was used as a primary media. All *E. coli*-like colonies from a single stool specimen with identical colony morphology and biochemical properties were assumed to be identical; if isolates were found to be identical, only one colony was analyzed further from that specimen. Strains of *E. coli* were stabbed into semisolid nutrient agar butts and were stored at -20°C till further investigation.

Quantitative biofilm assay

To assess biofilm formation, we inoculated 200 μl of 0.45% glucose-rich Muller-Hinton broth in 96 well-bottom microtiter polystyrene plates (NUNC, Immunomodules) with 5 μl of an overnight Luria broth culture grown at 37°C with shaking. The sample was incubated overnight (18 h) at 37°C and visualized by staining with 0.5% crystal violet for 5 min after washing with water. The biofilm was quantified in duplicate after adding 200 μl of 95% ethanol by an enzyme-linked immunosorbent assay plate reader at 570 nm. Strain 042 was used as positive control, and *E. coli* HB 101 was used as negative control.

Detection of AggR gene by multiplex PCR

All 100 *E. coli* strains were examined for the detection of AggR gene by a PCR. Each PCR tube contained 50 μl of reaction mix (10× PCR buffer with $MgCl_2$; dNTP mix 2.5 mM each; 4 primers 10 mM each), which comprised of *AggR* 5' CTGGCGAAAGACTGTATCAT' 3 + 5'CAATGTATAGAAATCCGCTGTT' 3 and for *east* 5'CACAGTATATCCGAAGGC' 3 + 5' CGAGTGACGGCTTTGTAG' 3, template lysate, sterile water, Taq polymerase (5 U/μl), and total volume was made up to 50 μl. The solutions were then subjected to the following cycling conditions: denaturation 94°C/1 min, annealing 55°C/1 min, extension 72°C/1 min, final extension 72°C/7 min in a thermal cycler. Then 10 μl of the PCR mixture was visualized by ethidium bromide staining after electrophoresis in 2% agarose gel in tris acetate-ethylene diamine tetra acetic acid buffer.

Statistical analysis

Statistical analysis was performed using the Fishers Exact Probability Test. *P*-values <0.05 were considered significant.

RESULTS

Of the total 100 *E. coli* isolates from infants with acute diarrhea, 28 were confirmed to be EAEC due to the fact that they showed the specific bands against AggR (630 bp) and EAST (97 bp) by multiplex PCR using specified primers.

To assess the biofilm formation of these 28 proven EAEC strains, they were inoculated by standard procedure and were assessed for the ability to form biofilm by quantitative microtiter plate assay. Twenty-five (89.2%) out of 28 produced biofilm in the microtiter plate. The EAEC 042 positive control showed a strong biofilm with a + standard deviation optical density at 570 nm (OD_{570}) of 2.0 + 0.34, while the *E. coli* HB 101 negative control did not produce a biofilm. The test strains showed absorbances in the range of 0-1.9. All EAEC strains showed absorbances >0.2. EAEC showed significantly stronger biofilm than did non-EAEC strains (*P* = 0.001).

DISCUSSION

Looking back, research on biofilms has come a long way since the initial characterization of a biofilm by Antoni Von Leeuwenhoek. The pathogenesis of EAEC is presently being defined. The major obstacle in identifying the mechanism of pathogenesis for EAEC is the diversity and heterogeneity of strains.[6-8] EAEC has been clearly associated with diarrhea in some individuals; but in many others, EAEC strains appear to cause sub-clinical infections or intestinal colonization. These bacteria exert a complex pathogen-host immune interaction. The current studies on EAEC pathogenesis suggest the presence of three stages: (1) adherence to intestinal mucosa by aggregative adherence fimbriae (AAF) or other organism-adherence factors; (2) increased production of mucus by the bacteria and the host cell which is deposited as a mucous biofilm encrusted with bacteria on the surface of enterocytes and (3) an inflammatory response with cytokine release, mucosal toxicity and intestinal secretion.[9,10] Numerous virulence factors that allow EAEC to adhere to intestinal mucosa have been reported to be important. The pathogenesis of EAEC involves the production of a mucous layer probably from contributions of both bacteria and intestinal mucosa. This thick mucus-containing biofilm is encrusted with EAEC on the surface of enterocyte. This mucus-containing biofilm may explain why individuals infected with EAEC develop mucoid stools, malnutrition and persistent colonization with prolonged diarrhea.[11-13]

EAEC strains in population around the world are becoming increasingly clear. The EAEC strains have been associated classically with persistent diarrhea (>14 days) and with growth retardation in infants. This pathogen has currently not been identified except in a small number of research laboratories. Therefore, there is a need for convenient assay to detect EAEC.[14-16] We applied the quantitative biofilm assay using microtiter plate to screen for EAEC among *E. coli* isolated from children with acute/persistent

Indian J Med Sci, Vol. 62, No. 1, January 2008

diarrhea.[15-18] All EAEC strains in this study showed an $OD_{570} > 0.2$ in the assay, and the incidence of EAEC among the strains with an $OD_{570} > 0.2$ was 89.2%. Although confirmation by PCR test is needed to identify EAEC, this biofilm assay is convenient and useful in screening of EAEC. In particular, it is more convenient when a large number of strains are examined in clinical and epidemiologic studies.[19-21] This assay may be more useful in developing countries, where EAEC is often a major problem. Furthermore, the test may be available without a spectrophotometer, since a biofilm showing an $OD_{570} > 0.2$ is clearly visible. In addition, this assay may contribute to demonstrating the true incidence of EAEC with and without AggR among clinically isolated *E. coli* strains.

ACKNOWLEDGMENTS

We thank the Centre for Scientific and Industrial Research (CSIR, New Delhi, India) for funding the above work.

REFERENCES

1. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998;11:142-201.
2. Nataro JP, Steiner T, Guerrant RL. Enteroaggregative *Escherichia coli*. Emerg Infect Dis 1998;4:251-61.
3. Bhan MK, Raj P, Levine MM, Kaper JB, Bhandari N, Srivastava R, *et al*. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J Infect Dis 1989;159: 1061-4.
4. Bhan MK, Arora NK, Ghai OP, Ramachandran K. Major factors in diarrhea related mortality among rural children. Indian J Med Res 1986;12:83-9.
5. Miqdady MS, Jiang ZD, Nataro JP, DuPont HL. Detection of Enteroaggregative *Escherichia coli* with formalin-preserved HEp-2 cells. J Clin Microbiol 2002;40:3066-7.
6. Kahali S, Sarkar B, Rajendran K, Khanam J, Yamasaki S, Nandy RK, *et al*. Virulence characteristics and molecular epidemiology of enteroaggregative *Escherichia coli* isolates from hospitalized diarrheal patients in Kolkata, India. J Clin Microbiol 2004;42:4111-20.
7. Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P, Levine MM. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr Infect Dis J 1987;6:829-31.
8. Okeke IN, Nataro JP. Enteroaggregative *Escherichia coli*. Lancet Infect Dis 2001;1:304-13.
9. Cobeljic M, Miljkovic-Selimovic B, Paunovic-Todosijevic D, Velickovic Z, Lepsanovic Z, Zec N, *et al*. Enteroaggregative *Escherichia coli* associated with an outbreak of diarrhoea in a neonatal nursery ward. Epidemiol Infect 1996;117:11-6.
10. Itoh Y, Nagano I, Kunishima M, Ezaki T. Laboratory investigation of enteroaggregative *Escherichia coli* O untype-able: H10 associated with a massive outbreak of gastrointestinal illness. J Clin Microbiol 1997;35:2546-50.
11. Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F, Nataro JP. Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. Infect Immun 1999;67:2692-9.
12. Sheikh J, Hicks S, Dall'Agnol M, Phillips AD, Nataro JP. Roles for Fis and YafK in biofilm formation by Enteroaggregative *Escherichia coli*. Mol Microbiol 2001;41:983-97.
13. Kang G, Mathan MM, Mathan VI. Evaluation of a simplified HEp-2 cell adherence assay for *Escherichia coli* isolated from south Indian children with acute diarrhea and controls. J Clin Microbiol 1995;33:2204-5.
14. Nishi J, Sheikh J, Mizuguchi K, Luisi B, Burland V, Boutin A, *et al*. The export of coat protein from enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system. J Biol Chem 2003;278:45680-9.
15. Sarantuya J, Nishi J, Wakimoto N, Erdene S, Nataro JP, Sheikh J, *et al*. Typical enteroaggregative *Escherichia coli* is the most prevalent pathotype among *E. coli* strains causing diarrhea in Mongolian children. J Clin Microbiol 2004;42:133-9.
16. Cerna JF, Nataro JP, Estrada-Garcia T. Multiplex PCR for detection of three plasmid-borne genes of Enteroaggregative *Escherichia coli* strains. J Clin Microbiol 2003;41:2138-40.
17. Baudry B, Savarino SJ, Vial P, Kaper JB, Levine MM. A sensitive and specific DNA probe to identify Enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J Infect Dis 1990;161:1249-51.
18. Schmidt H, Knop C, Franke S, Aleksic S, Heesemann J, Karch H. Development of PCR for screening of Enteroaggregative *Escherichia coli*. J Clin Microbiol 1995;33:701-5.
19. Iwanaga M, Song T, Higa N, Kakinohana S, Toma C, Nakasone N. Enteroaggregative *Escherichia coli*: Incidence in Japan and usefulness of the clump-formation test. J Infect Chemother 2002;8:345-8.
20. Albert MJ, Qadri F, Haque A, Bhuiyan NA. Bacterial clump formation at the surface of liquid culture as a rapid test for identification of enteroaggregative *Escherichia coli*. J Clin Microbiol 1993;31:1397-9.
21. Wakimoto N, Nishi J, Sheikh J, Nataro JP, Sarantuya J, Iwashita M, *et al*. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. Am J Trop Med Hyg 2004;71:687-90.

Source of Support: CSIR, New Delhi, India, **Conflict of Interest:** None declared.