

Biochemical Identification of Enteroaggregative Escherichia Coli among Infants with Acute Diarrhea from Manipal, India

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Abstract:

Background : The EAEC strains have been associated classically with persistent diarrhea which represents a disproportionate share of diarrheal mortality. EAEC strains have been shown to elicit damage to intestinal mucosa and growth retardation in infants. Detection of EAEC strains can make a significant contribution to public health in many areas.

The use of biofilm assays as a screening method for EAEC from clinical isolates and multiplex PCR as confirmatory method may be useful.

Methods : We evaluated the usefulness of quantitative micro titer plate method for biofilm production and multiplex PCR to screen and confirm EAEC from a total of 100 E.coli strains from children below two years of age with acute diarrhea.

Results : E.coli strains were isolated from fecal specimens from 680 Diarrheic children who attended an out patient clinic or who were admitted to Kasturba Hospital and other peripheral hospitals in and around Manipal, Karnataka-South India. E coli isolates from 50 fecal specimens from infants without diarrhea (controls) who attended the same outpatient clinic and who belonged to the same group as the infants with diarrhea were also examined.

Conclusion : Better diagnostic tools are needed to allow for more standardized laboratory testing on a regular basis. Improved diagnostic tools will help identify epidemiologic patterns of illness and guide treatment recommendations of EAEC illness.

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Introduction

Enteroaggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen that causes persistent diarrhea among infants both in the developing countries and industrialized countries. The defining feature of EAEC is its characteristic aggregative adherence (AA) to human epithelial cell surface, to the glass substratum, and to each other in a distinctive stacked brick formation.^(1,2) Thus the gold standard for EAEC identification remains the HEp-2 cell adherence test. However this test requires specialized facilities, is time consuming to set up and much prone to contamination restricting it to be conducted only in reference laboratories.

Although this pattern provided the first EAEC epidemiologic association with diarrhea, there has long been a desire to base detection on the presence of requisite virulence factors. It is difficult to screen for EAEC among *E. coli* isolates from patients with diarrhea in clinical laboratories. The use of biofilm assays may be useful in overcoming these difficulties. Natarao and Kaper reported that EAEC produces a bacterial film on a polystyrene surface that could be easily visualized with 0.5 % crystal violet or Giemsa^(2,3,4). In this study, we evaluated the usefulness of quantitative biofilm assay to screen for EAEC among clinical isolates of *E. coli*.

Similarly many of EAEC virulence factors have been localized to the 60 to 65 mDa AA plasmid including the transcriptional activator AggR and EAST enterotoxins. These appear to be specific for EAEC and reported to be sensitive and specific for EAEC detection. PCR assay demonstrated to better correlate with clinical findings than the cell adherence assay^(5, 6, 7). Our data suggest that the prevalence and significance of EAEC infections depends on age. Discordant results from other studies may be explained by the strain to strain heterogeneity in other geographical areas.

Methods

Specimens and Strains

E. coli strains were isolated from fecal specimens from 680 Diarrheic children who attended an out patient clinic or who were admitted to Kasturba Hospital and other peripheral hospitals in and around Manipal, Karnataka-South India. *E. coli* isolates from 50 fecal specimens from infants without diarrhea (controls) who attended the same outpatient

clinic and who belonged to the same group as the infants with diarrhea were also examined.

Diarrhea was defined as the occurrence of two or more watery stools in a 24 h period. Neither patients nor controls had been treated with antimicrobials in the 10 days preceding sampling.

Quantitative biofilm assay

To assess biofilm formation, we inoculated 200µl of glucose rich Muller Hinton broth in 96 well flat bottom microtiter polystyrene plates with 5µl of overnight Luria broth culture grown at 37°C with shaking. The sample was incubated overnight (18h) at 37° C and visualized by staining with 0.5% crystal violet for 5 minutes after washing with water. The biofilm was quantified in duplicate after adding 200µl of 95% ethanol by enzyme linked immunosorbent assay reader at 500-600nm. Strain 042 was used as positive control^(8, 9, 10).

Bacterial clump formation on liquid cultures was previously reported as a rapid test to EAEC. This convenient test is also based on the biofilm formation of EAEC and useful for screening but it is not quantitative. Furthermore it may overlook EAEC strains with weak biofilm formation^(11, 12).

Multiplex Polymerase Chain Reaction

The strains isolated were also characterized by a multiplex Polymerase Chain Reaction with below mentioned primers for the detection of two specific genes AggR (630bp) and EAST (97bp). The primers were chosen from a reference protocol and optimized specifically for the present study. For standardization purpose we used positive 042 strain and 044 strains.

Bacterial lysates were prepared by re-suspending a single colony in 1ml of deionized water in a sterile 5ml glass tube followed by boiling for 10 minutes at 95°C. After boiling the suspension is centrifuged at 10,000 rpm for 10 minutes and the supernatant solution is directly used as a template for PCR.

Each PCR tube contained 50µl of reaction mix [(10x PCR buffer with MgCl₂; dNTP mix 2.5mM 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 (5U/l)] and total volume made up to 50µ l.

The solutions were then subjected to the following cycling conditions—denaturation 94 °C/1 mts, annealing 55 °C/1 mts, extension 72° C/1 mts, final extension 72° C/7 min in a thermal cycler. Then 10µl of the PCR mixture was visualized by ethidium bromide staining after electrophoresis in 2% agarose gel in tris acetate –EDTA buffer.

The amplicon sizes are shown in Fig. (1).

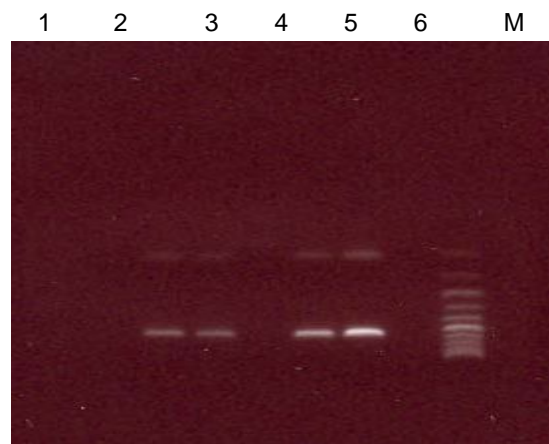


Fig. (1). Multiplex PCR amplification of E. Coli strains from pure cultures. Lane 1, test negative; lane 2, test positive; lane 3, test positive; lane 4, test negative; lane 5, EAEC 042; lane M, marker (100 bp DNA ladder; Bangalore Genie/India).

Only the presence of the correct sized gene products was interpreted as positive. The positive strains 042,044 were found to be positive for the *Aggr* and *east* genes. This multiplex PCR sensitivity was confirmed by the fact that 23 of 100 strains were positive for 2 loci by PCR. Of the total 23 strains positive for PCR, 20(86%) showed positive for biofilm by quantitative microtitre plate method.

Other enteric pathogens like *Vibrio*, *Shigella*, *Salmonella*, *Aeromonas*, *Plesiomonas* were sought by standard methods.

Results

During the study period from (July 2005 to Nov 2006) stool specimens were investigated from a total of 680 children with diarrhea and from a total of 50 randomly selected children of same age group without diarrhea seen at Kasturba Hospital and other peripheral hospitals in and around Manipal, Karnataka, South India.

Isolation of bacterial enteropathogens other than E coli are sought by standard procedures.

Fig. (2) shows the biofilm formation on a microtitre plate. We found the dark wells to be biofilm positive. The EAEC 042 positive control showed a strong biofilm with a mean + SD optical density of 500-600 nm (OD₅₀₀₋₆₀₀) of 0.2 while E coli negative control did not produce biofilm. The test strains showed absorbance's in the range of 1.8 to 2.0. The strains positive for biofilm by Quantitative Microtitre plate assay were then subjected to PCR studies for evaluation of presence of *AggR* and *EAST* genes.

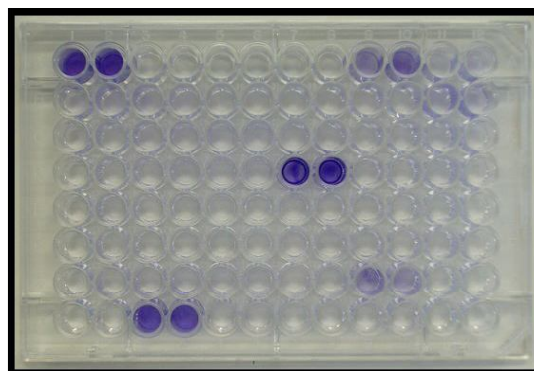


Fig. (2). Biofilm formation on a polystyrene microtiter plate. The blue wells are biofilm positive. The positive control is EAEC 042. The negative control is E.coli HB101.

Of the total 100 E coli isolates, 23 generated positive results with multiplex PCR for two specific genes *AggR* and *EAST*. When these 23 PCR positive strains were studied for biofilm production, 20(86%) strains showed positive results by Quantitative microtitre plate assay. All EAEC strains showed absorbance >0.2. All EAEC strains showed stronger biofilm formation than non-EAEC strains.

Thus EAEC can be screened by identifying an OD >0.2 in this assay. 3 (23%) E.coli strains which were positive for biofilm showed no/negative results with PCR specific primers. None of the 50 control samples collected showed positive results either with biofilm nor generated positive PCR for two specific genes tested.

Compared with cell culture assays and colony hybridization our findings revealed that PCR assay was more rapid, simple, and highly sensitive and can therefore be recommended as a screening method for EAEC in clinical laboratory.

Our data suggest that the prevalence and significance of EAEC infections depends on age. Discordant results from other studies may be explained by the strain to strain heterogeneity in other geographical areas.

Discussion

The importance of EAEC strains in public health around the world is becoming increasingly clear. The EAEC strains have been associated classically with persistent diarrhea (>14 days) and with growth retardation in infants^(2, 3, 4).

EAEC diarrhea involves bacterial aggregation, adherence to intestinal epithelial cells and elaboration of several toxigenic bacterial mediators. EAEC is primarily recognized as a cause of endemic and persistent childhood diarrhea in developing countries.^(5,6,7) EAEC diarrhea is frequently seen in children, attending day care, in travelers and in immunocompromised persons in developed countries. Understanding the pathogenesis of EAEC associated diarrhea, host susceptibility and host pathogen interactions is extremely important. This pathogen is currently not being identified except in a small number of research laboratories. EAEC has also been reported to form a pellicle when growing in Luria broth, a feature proposed as a simple and rapid test^(9,10,11,12).

In the present study a panel of EAEC was examined for phenotypic and genotypic characteristics in an attempt to produce an easy method for differentiating strains of EAEC from other strains of diarrheagenic E coli. A strong correlation between the presence of the specific marker aggR and the biofilm production was found. The EAST gene had a similar distribution between aggregative and localized strains, indicating that this gene could not be considered as a marker of EAEC. We conclude that AggR may be used to identify EAEC, using the PCR method as a screening test.

Better diagnostic tools are needed to allow for more standardized laboratory testing on a regular basis. Improved diagnostic tools will help identify epidemiologic patterns of illness and guide treatment recommendations of EAEC illness. This study has been done to increase awareness of this emerging pathogen, a common cause of diarrhea.

To the best of our knowledge, the present work is the first of its kind that has been reported from Southern part of rural India.

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References

1. Okeke I N, Nataro J P(2001) Enteroaggregative Escherichia coli; Lancet Infec Dis 1:304-313.
2. Nataro JP, Kaper JB(1987) Pattern of adherence of Diarrheagenic Escherichia coli to HEP-2 cells; Pediatr Infec Dis J 6:829-831
3. Bhan MK, Raj P, Levine MM(1989) Enteroaggregative Escherichia coli association with persistent diarrhea in cohort of rural children in India; J Infec Dis 159:1061-4
4. Bhan M.K, Arora N.K, Ghai O.P, Ramachandran K (1986) Major factors in diarrhea related mortality among rural children; Indian J Med Res 12:83-9.
5. World Health Organization: Diarrhoeal Disease Control Programme: Persistent diarrhea in children-research priorities. Geneva: World Health Organization;1985;CDD/DDM/85.1
6. Gascon J M Vargas, D Schellenberg, H Urassa, C Casals, E.Kahigwa, J J Aponte, H Mshinda and J vila (2000) Diarrhoea in children under 5 years of age from Ifakara, Tanzania; a case control study; J Clin. Microbiol 38:4459-4462.
7. Albert M J, A S G Faruque, S M Faruque, R B Sack and D Mahalanabis (1999) Case control study of Enteropathogens associated with childhood diarrhea in Dhaka Bangladesh; J.Clin.Microbiol 37:3458-3464.
8. Sarantuya J, Nishi J, Wakimoto N, Erdene S, Nataro P, Sheik J, Iwashita M, Manago K (2004) Typical Enteroaggregative Escherichia coli is the most prevalent pathotype among E coli strains causing diarrhea in Mangolian children; J Clin Microbiol 42:133-139
9. Wakimoto, Nishi, Nataro P, Sarantuya J, Iwashitha, Monago K, K Thokuda, Kawano y(2004) Quantitative Biofilm assay using a Microtiter plate to screen for Enteroaggregative Escherichia coli ; Am J Trop Med Hyg 71: 687-690.

10. Iwanga M, Song T, Higa N, Kakinohana , S Toma, C Nakasone (2002) Enteroaggregative *Escherichia coli*: Incidence in Japan and usefulness of clump formation test; *J Infect Chemother* 8:345-348.
11. Albert M J, Qadri F, Haque A, Bhuiyan N A(1993) Bacterial clump formation at the surface of liquid cultures as a rapid test for identification of Enteroaggregative *Escherichia coli*; *J Clin Microbiol* 31:1397-1399.
12. Cerna J F,Nataro J P, Estrada-Garcia T, (2003) Multiplex PCR for detection of three plasmid borne genes of Enteroaggregative *Escherichia coli* stains; *J Clin Microbiol* 41:2138-2140.
13. Baudry,Savarino S J,Vial P, Kaper J B, Levine M.M(1990) A Sensitive and specific DNA probe to identify Enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen; *J Infect Dis* 161:1249-1251.
14. Arena K R S, Fagundes Neto, U Scaletsky(2004) Evaluation of Multiplex PCR for diagnosis of infection with Diarrheagenic *Escherichia coli* and *Shigella* sps; *J Clin Microbiol* 42:5849-5853.
15. Kahali, Sarkar, Rajendran, Khanam, Yamasaki, Nandy, Bahattcharya, Ramamurthy (2004) Virulence Characteristics and Molecular Epidemiology of Enteroaggregative *Escherichia coli* isolates from hospitalized diarrheal patients in Kolkata, India; *J Clin Microbiol* 42:4111-4120.
16. Pass,M.A.Odedra.R,Batt.R.M,(2000) Multiplex PCRs for identification of *Escherichia coli* Virulence genes; *J.Clin Microbiol* 38:2001-2004.
17. Elias, W.P, Brros S.F, Moreiram C.G, Trabulis, L.R.Gomes (2002). Enteroaggregative *E.coli* strains among classical enteropathogen. *Escherichia-coli* O serogroups; *J.Clin Microbiol* 40:3540-3541.