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Evaluation of a DNA Probe to Identify Enteroaggregative *Escherichia coli* from Children with diarrhoea in Bangladesh

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ABSTRACT

Six hundred and seventy – five *Escherichia coli* isolates obtained from 225 diarrhoeal children less than five years of age were tested for adherence to HeLa cells and for hybridisation with DNA probes for genes conferring aggregative adherence (AggA), localised adherence (LA) and diffuse adherence (DA) to assess the usefulness of a recently developed DNA probe for AggA of enteroaggregative *E. coli* (EAggEC). The strains were further analysed with the DNA probes for heat – labile enterotoxin (LT), heat – stable enterotoxin (ST), Shiga – like toxins (SLT I and SLT II) and for enteroinvasiveness and adherent strains were all negative for these properties. The HeLa cell assay and DNA probe assays showed excellent agreement in identifying LA and DA positive isolates. However, significant disparities occurred in the case of AggA positive isolates, and the DNA probe failed to identify 31.9% (15 of 47) of the EAggEC identified by the HeLa cell adherence assay. The failure of the DNA probe to identify all the EAggEC indicated that there may be a high degree of genetic heterogeneity for the expression of AggA, and development of more DNA probes is necessary to detect all the possible genetic variants of EAggEC.

Key words: Diarrhoea; Diagnosis; Infantile Diarrhoea; DNA probes, Adherence assay, Enteroaggregative *E. coli*

INTRODUCTION

Diarrhoeagenic *Escherichia coli* are classified into four major categories: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohaemorrhagic (EHEC), and enteroinvasive (EIEC) strains. They are a major cause of diarrhoea in infants and young children in developing countries (1) and a leading cause of travellers' diarrhoea and haemorrhagic colitis worldwide (1 – 3). These *E. coli* produce one or more enterotoxins, cytotoxins, colonisation factors and adhesins, and differ from one another in their pathogenesis, clinical features, epidemiology and antigenic types (1). Diarrhoeagenic *E. coli* can be identified by bioassays including animal models and tissue culture systems (4 – 9), immunoassays (10,11) or by using specific DNA probes (12 – 17). Development of DNA probes for heat – labile enterotoxin (LT) and heat – stable enterotoxin (ST), Shiga – like toxins (SLT I and SLT II), and enteroinvasiveness has greatly facilitated the identification of different categories of diarrhoeagenic *E. coli*, since the conventional assays are time consuming, and not suitable for screening a large number of isolates.

Diarrhoeagenic *E. coli* adheres to HEp – 2 or HeLa cells in three distinct patterns: localised, diffuse and aggregative (7 – 9). Most classical EPEC serotypes associated with infant diarrhoea exhibit localised adherence (LA) which is mediated by a ≈60 megadalton plasmid, the EPEC adherence factor (EAF) plasmid (2,16). *E. coli* exhibiting diffuse adherence (DA) are claimed by some and disputed by others (3,8,18) as causative agents of

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diarrhoea. Strains showing aggregative adherence (AggA) have recently been classified as yet another pathogenic category of *E. coli* (3,19–22). DNA probes have been constructed for both localised and diffuse adhesiveness of *E. coli* (16,23,24) to identify these organisms in a convenient alternative assay. A DNA probe to identify *E. coli* exhibiting the aggregative pattern of adherence has also been constructed recently (19). In initial studies, with a small number of isolates, these probes for adhesiveness have provided encouraging results. However, it is not yet clear what the usefulness of these probes would be in a large scale field study or in clinical diagnosis. We previously reported on the comparative study of some gene probes with standard bioassays (25). The present study was carried out to assess the general usefulness of the recently developed probe for enteroaggregative *E. coli* (EAggEC), using a large number of isolates, and to compare the results with those obtained in adherence assay using HeLa cells.

MATERIALS AND METHODS

Between May 1988 and April 1989, stool samples were collected from 225 children less than 5 years of age with diarrhoea in Matlab, a rural area of Bangladesh, where the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) has been maintaining a field research project since the early 1960s. The stool samples were cultured for enteric pathogens as previously described (26) and 3 *E. coli* isolates from each patient were stored on nutrient agar slants for further analysis.

DNA probes were prepared from recombinant plasmids containing the probe DNAs as inserts. The plasmids were prepared, purified, digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD) and appropriate restriction fragments were purified as described by Moseley *et al.* (15). The EAF probe for LA was a 1–kb *Bam*HI–*Sal*I fragment of pJPN16, derived from pMAR2 (16), the DA probe was a *Pst*I 450–bp fragment of pSLM852 (23,24), and the DNA probe for EAggEC consisted of a 1–kb *Bam*HI digestion fragment of pCVD432 (19). The DNA probes for LT, ST, SLT I, SLTII and invasiveness have been described previously (25).

DNA fragments were labelled by random priming (27) using ($\alpha-^{32}P$)–deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc., U.K.) and a random primers DNA labelling system (BRL). Colony blots were prepared, processed, and hybridised under stringent conditions as described by Echeverria *et al.* (13).

Strains were assayed for their patterns of adherence to tissue culture cells as described by Cravioto *et al.* (7). HeLa cells were used instead of HEp–2 cells (8) and the adhesion was carried out for 3 hours in the presence of 1% methyl–α–D–mannoside. Three distinct patterns of adherence as defined by Nataro *et al.* (9) were identified by two independent observers.

RESULTS

DNA probe analysis of a total of 675 *E. coli* isolates from 225 children (Table) showed that 4.2% of the isolates representing 6.6% of patients were positive for LA; 7.5% of isolates from 11.1% of patients were positive for DA; and 4.7% of isolates from 7.5% of patients were positive for AggA. With the other DNA probes used 5.7 % of the isolates from 7.5% of patients were positive for heat–labile toxin (LT); 0.7% of isolates from 1.3% of patients (5 isolates from 3 patients) were positive for heat–stable toxin (ST); and 1.4% of isolates from 2.2% of patients (10 isolates from 5 patients) were positive for both LT and ST. None of the isolates were positive with the probes for either Shiga–like toxins or invasiveness.

Table. Comparison of detection of diarrhoeagenic *E. coli* in the DNA hybridisation assay versus HeLa cell adherence assay using 675 isolates obtained from 225 children with diarrhoea under five years of age

Categories of results obtained with DNA probe assay and HeLa cell adherence assay	Number of isolates (from patients) exhibiting patterns of		
	LA	DA	AggA
Probe and adherence positive	28 (15)	48 (24)	32 (17)
Probe positive adherence negative	1 (0)*	3 (1)	0 (0)
Probe negative adherence positive	0 (0)	0 (0)	15 (9)
Total probe positive	29 (15)	51 (25)	32 (17)
Total adherence positive	28 (15)	48 (24)	47 (26)
% probe positive**	4.2 (6.6)	7.5 (11.1)	4.7 (7.5)
% adherence positive**	4.1 (6.6)	7.1 (10.6)	6.9 (11.5)

LA = Localized adherence
DA = Diffuse adherence
AggA = Aggregative adherence
* One of two LA probe positive isolates from the same patient was negative in the HeLa cell adherence assay.
** Percentage of total isolates (percentage of total patients)

In the HeLa cell adherence assay of the 675 isolates, 96.5% of the EAF probe positive isolates (28 of 29 isolates obtained from 15 patients) and 94.1% of the DA probe positive isolates (48 of 51 isolates obtained from 24 of 25 DA probe positive patients) were positive respectively for LA and DA. None of the DA and LA probe negative isolates were positive for the respective adhesivenesses in the HeLa cell adherence assay (Table). HeLa cell adherence assay identified 47 EAggEC, which included all of the 32 isolates found positive with the DNA probe for EAggEC. Fifteen isolates from 9 patients showed the aggregative pattern of adherence

(AggA) in the HeLa cell assay but these were negative in the DNA probe assay (Table). None of the isolates hybridised with more than one of the DNA probes for adherence used in this study. All of the isolates found positive for adherence either with the DNA probes or in the HeLa cell assay were negative with the DNA probes for enterotoxins, Shiga – like toxins or invasiveness, and all the isolates which were positive with the DNA probes for either LT or ST or both were non – adherent in the HeLa cell assay.

DISCUSSION

In agreement with previous reports (14,25), strains which were positive with the EAF probe for LA correlated well with the bioassay results (with the exception of one isolate out of 29). A synthetic oligonucleotide probe has recently been described (28) which correlated better with the adherence assay than the EAF probe. However, in several other studies (25,29) the specificity and sensitivity of the EAF probe has also been found to be satisfactory.

In a previous study (25) we found a significantly higher prevalence of LA positive *E. coli* (11.8%) than that found in the present study (4.1%). Similarly the prevalence of enterotoxin producing *E. coli* was also found to be higher in the previous study as compared to the present study. However, *E. coli* isolates in the previous study were obtained from patients less than 1 year of age (25), whereas in the present study patients up to 5 years old were enrolled. The patients were recruited from nearby places, and hence the results suggest that the incidence of infection with these categories of *E. coli* may vary significantly between different age groups.

Although there is controversy about the role of DA *E. coli* in the causation of diarrhoea (3,9,18), both the DNA probe assay and the HeLa cell adherence assay detected a significant number of DA isolates and the two assay methods generally agreed. This also supported our previous report on the DA probe (25). Three DA probe positive isolates from 1 patient, and 1 EAF probe positive isolate were negative in the HeLa cell adherence assay. These probe positive and bioassay negative isolates did not appear to be false positives because the probe reactions with control strains run on each filter were specific, and the probe reactions with the *E. coli* isolates also appeared specific and were easily interpretable. The negative result in the bioassays could be due to impairment of phenotypic expression, possibly due to small deletions in the genes conferring the respective adhesiveness.

As opposed to the LA and DA isolates the detection rate of EAggEC was higher in the HeLa cell assay as compared to the DNA hybridisation assay. Fifteen isolates which were positive for AggA in the Hela cell assay did not hybridise with the DNA probe. However, all of the 32 probe positive EAggEC isolates were also positive in the HeLa cell adherence assay. Therefore, although the DNA probe was very specific the sensitivity was only 68% (32 of 47 isolates). However, the sensitivity of this probe was reported to be 89% by Baudry *et al.* (19) who analysed a small number of isolates from Chile and India. In a recent case – control study by Echeverria *et al* (29), the sensitivity of the EAggEC probe was reported to be only 47% (with 99% specificity) when tested on *E. coli* isolates in Bangkok. This suggests that the sensitivity of the probe may be different when tested on isolates from different geographical locations. In the present study, the failure of the DNA probe to identify all EAggEC isolates suggested that *E. coli* isolates from this locality can produce more than one genetically heterologous adhesins conferring similar aggregative adhesion pattern in the HeLa cell adherence assay.

The EAggEC probe had been derived from a 60 Mdal plasmid necessary for the expression of AggA (19). In the probe – negative isolates which were positive for AggA in the HeLa cell assay, the genetic determinants for the adhesiveness are likely to be considerably different from the probe – positive isolates in terms of DNA sequence. This group of EAggEC isolates needs further studies to characterise the genes responsible for the adhesiveness.

None of the adherence positive isolates were positive with the probes for enterotoxins and invasiveness. This suggested that these isolates are most probably neither toxigenic nor invasive, and possibly do not possess more than one pathogenic mechanism.

In view of several reports (20,21) incriminating EAggEC as a diarrhoeal pathogen, the epidemiologic significance of this category of *E. coli* needs to be assessed. DNA probes have been proven to be useful in screening large numbers of isolates in epidemiologic studies. The present study, however, pointed out that the quality of probe needs extensive testing for sensitivity and specificity before applying as an alternative to conventional bioassay. Although the AggA probe did not show any non – specific hybridisation, the sensitivity was not satisfactory. This type of probe could miss a significant proportion of pathogenic *E. coli* in spite of the other advantages of DNA hybridisation assays. However, at this point it is also important to carry out case control studies to establish whether the probe negative, but HeLa cell positive, isolates are also diarrhoeagenic. It is, therefore, necessary to study more extensively various isolates from different geographical locations exhibiting the AggA phenotype with a view to confirming their diarrhoeagenic ability and developing more sensitive DNA probes for this category of diarrhoeagenic *E. coli*.

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