A comparative study of specific gene probes and standard bioassays to identify diarrhoeagenic *Escherichia coli* in paediatric patients with diarrhoea in Bangladesh

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Summary. We compared the usefulness of gene probes with standard bioassays to identify diarrhoeagenic *Escherichia coli* amongst isolates from Bangladeshi children under 1 year of age with diarrhoea. *E. coli* isolates were analysed with specific gene probes for localised adhesiveness (LA), diffuse adhesiveness (DA), heat-labile toxin (LT), heat-stable toxin (ST), Shiga-like toxins (SLT I and SLT II), and enteroinvasiveness, and in bioassays for production of enterotoxins and cytotoxins, and for cell adherence. With 1136 isolates from 387 patients, there was general agreement between the two assay methods. When there was disparity, gene-probe-positive isolates gave negative results in the corresponding bioassay. In the HeLa cell adherence assay, 94% of the LA probe-positive isolates and 91.6% of the DA probe-positive isolates gave positive bioassay results for LA and DA respectively. Thirty-six of 39 LT probe-positive isolates and 73 of 86 ST probe-positive isolates gave positive results in the bioassays. Of 28 isolates that gave negative results in the suckling mouse assay but were initially positive with the probe for ST, 15 were later found to hybridise with the cloning vector for the ST probe. Addition of denatured vector DNA at a concentration of 10 µg/ml in the hybridisation solution eliminated these false positive results. None of the other probe-positive isolates hybridised with any of the cloning vectors used. The DNA hybridisation assay appeared to be a convenient alternative to bioassays for screening large numbers of isolates in epidemiological investigation.

Introduction

Diarrhoeagenic categories of *Escherichia coli*—enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohaemorrhagic (EHEC), and enteroinvasive (EIEC)—are a major cause of diarrhoea in infants and young children in developing countries and a leading cause of travellers' diarrhoea and haemorrhagic colitis in developed countries.^{1–3} These *E. coli* strains produce one or more enterotoxins, cytotoxins, colonisation factors and adhesins, which contribute to the development of diarrhoea.¹ *E. coli* strains that produce toxins or adhesins, or both, are usually identified in bioassays with live animals⁴ or cultured cells^{5–8} or in immunoassays^{9,10} that require specific antisera. Because these techniques are time-consuming, expensive and not suitable for screening large number of isolates, recombinant DNA technology has been used.

Nucleotide probes have been developed to identify plasmid encoded genes for heat-labile (LT) and heat-stable (ST) enterotoxins,¹¹ genes involved in enteroinvasion,¹² bacteriophage-encoded genes for Shiga-

like toxins (SLT I and SLT II),¹³ and genes involved in localised (LA), diffuse (DA), and aggregative (AggA) adherence.^{14–17} In initial studies, these probes have provided encouraging results in detecting diarrhoeagenic *E. coli* strains.^{11–15,17,18} However, there have not been any studies in which large numbers of clinical isolates have been tested simultaneously with a wide variety of gene probes and bioassays in a single investigation. The present study was done to assess the general usefulness of a wide range of gene probes in identifying diarrhoeagenic *E. coli* in the stools of children with diarrhoea to compare the results with those of standard bioassays, and to investigate the relative importance of the various categories of *E. coli* as causes of early childhood diarrhoea in Bangladesh.

Materials and methods

The Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) has a surveillance system in which every 25th patient seen is entered into the programme for in-depth clinical, microbiological and demographic

investigation.¹⁹ Between March and August of 1988, stools were obtained from 387 children up to 1 year of age with diarrhoea. The stools were cultured¹⁹ and three *E. coli* isolates from each stool were stored on nutrient-agar slants for further analysis.

DNA hybridisation assays

DNA probes were prepared from recombinant plasmids containing the probe DNA fragments as inserts. Plasmids were prepared, purified, and digested with restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, MD, USA) and the appropriate restriction fragments were purified as described by Moseley *et al.*¹¹ The ST probes consisted of a 216-bp *EcoRI* digestion fragment of plasmid pCVD402 derived from the cloned STh gene²⁰ and a 157-bp *PstI* fragment of plasmid pCVD404 derived from the cloned STp gene.²¹ The recombinant plasmids pCVD402 and pCVD404 were constructed at the Centre for Vaccine Development (Baltimore, MD, USA) by cloning the DNA fragments into the vector plasmid pBR325. The origin and construction of the other gene probes have been described previously.^{11-15,22} The LT probe was a 1.3-kb *BamHI* digestion fragment of plasmid pCVD403.^{11,22} The SLT I probe was a 1.1-kb *BamHI* fragment of plasmid pJN37-19 and the SLT II probe was a *SmaI-PstI* 850-bp digestion fragment of plasmid pNN110-18.¹³ The LA probe was a 1-kb *BamHI-SalI* fragment of plasmid pJPN16, derived from plasmid pMAR2,¹⁴ the DA probe was a *PstI* 450-bp fragment of plasmid pSLM852,^{15,16} and the EIEC DNA probe was a 17-kb *EcoRI* digestion fragment of plasmid pRM17.¹²

DNA fragments were labelled by random priming²³ with [α -³²P]-deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc, UK) and a random primers DNA labelling kit (BRL). Colony blots were prepared, processed and hybridised under stringent conditions as described by Echeverria *et al.*²⁴

All probe-positive isolates were screened for false positive results by hybridising with the corresponding vector DNA. Purified plasmids (either obtained from BRL or prepared in our laboratory) were labelled by nick translation²⁵ and hybridised with the colony blots. These isolates were also hybridised for a second time with the corresponding radiolabelled probe DNA; the hybridisation solution was supplemented with 10 μ g/ml of the corresponding unlabelled denatured vector DNA.

Bioassays for toxin production and adherence

All *E. coli* strains were tested for enterotoxin production by the suckling mouse assay for ST⁴ and by the Y1 adrenal cell assay for LT.⁵ Production of cytotoxins was tested in the Vero cell assay.⁶ Strains were assayed for their patterns of adherence to tissue-culture cells as described by Cravioto *et al.*⁷ Instead of HEp-2 cells, HeLa cells were used⁸ and the adhesion test was incubated for 3 h in the presence of methyl- α -D-mannoside 1%. Three distinct patterns of adherence, as defined by Nataro *et al.*²⁶ were assessed and the opinions of two independent observers were considered.

Results

Of 1161 isolates stored for examination, 25 could not be revived; tests were done on 1136 isolates representing all 387 patients. In DNA probe analysis, 3.4% gave positive results for LT, 7.5% gave positive results for ST, and 3.0% gave positive results for both LT and ST; 11.8% gave positive results for LA and 6.3% for DA (table). None of these isolates gave positive results with the probes for enteroinvasiveness or for SLTs and were not tested for invasiveness in a bioassay.

The subsequent bioassay of probe-positive isolates showed that some (5.9–15.1%) isolates which gave positive results with a DNA probe gave negative

Table. Comparison of DNA hybridisation with standard bioassays for the detection of diarrhoeagenic *E. coli* in 1136 isolates from 387 children up to one year old with diarrhoea

Toxins and adhesins	Number of isolates that were			
	Probe and bioassay positive	Probe positive, bioassay negative	Total probe positive (%)	Total bioassay positive (%)
LT	36	3	39 (3.4)	36 (3.1)
ST	73	13	86 (7.5)	73 (6.4)
LT + ST	29	6*	35 (3.0)	29 (2.5)
LA	127	8	135 (11.8)	127 (11.1)
DA	66	6	72 (6.3)	66 (5.8)
AggA	...	...	ND	59 (5.1)

ND, not done.

* Of the 29 isolates that were both LT- and ST-positive in the DNA probe assay, six produced only LT in the bioassay.

results in the corresponding bioassay. Thirty-six (92.3%) of the 39 LT-positive isolates from 23 of the 25 patients with LT-positive *E. coli* produced an active toxin detected in the bioassay. Seventy-three (84.8%) of 86 ST-positive isolates, obtained from 38 of 44 patients with ST-positive *E. coli*, gave positive results in the bioassay for ST. Of the enterotoxigenic isolates that gave positive results for both LT and ST by DNA hybridisation, 29 (82.8%) of 35 isolates from 12 of the 15 patients with LT + ST-positive *E. coli*, gave positive results for both toxins in the bioassay. The remaining 17.2% (six isolates from three patients) produced only LT in the bioassay (table). In the HeLa cell adherence assay, 94% of the LA probe-positive isolates (127 of 135 isolates from 69 of 72 patients) and 91.6% of the DA probe-positive isolates (66 of 72 isolates from 45 of 47 patients) gave positive results for LA and DA respectively. Fifty-nine (5.1%) isolates from 25 patients showed the AggA pattern. It was sometimes difficult to distinguish between the different adherence patterns. With 20 isolates, the opinion of two independent observers differed in the first instance, and the assay was repeated.

Initially, 28 isolates that gave negative results for ST in the bioassay gave positive results in the DNA hybridisation assay. However, 15 of these isolates hybridised with purified plasmid pBR325 DNA, the cloning vector for the ST probes. The second round of hybridisation of the 15 isolates (which hybridised with pBR325 DNA) with the ST probe in the presence of denatured unlabelled pBR325 DNA showed no hybridisation, leaving 13 probe-positive but bioassay-negative isolates (table). These 13 isolates came from six patients. All probe-negative isolates also gave negative results in the bioassays. None of the other probe-positive isolates hybridised with the corresponding cloning vectors.

Discussion

Three of 39 LT probe-positive and 13 of 86 ST-probe positive isolates gave negative results in bioassays (table). Of 29 isolates that gave positive results with both the LT and ST probes, six produced only LT in the bioassay. Another 15 isolates that hybridised with the ST probe but were bioassay negative were shown to be false positive, because they hybridised with the cloning vector. This could be due to the presence of vector DNA contaminating the probe. A similar problem has been described previously.¹⁸ The reason why the remaining 13 ST probe-positive isolates from six patients and three LT probe-positive isolates from two patients did not produce an active toxin in the bioassay is not clear. Possible explanations could be a lack of optimum expression of the toxin genes under the growth conditions or deletion of small regions of the LT or ST gene resulting in impairment of expression. The possible presence of cryptic sequences homologous to the LT or ST gene (unable

to produce an active toxin) in certain isolates may also contribute to this higher detection rate of ETEC by DNA hybridisation assay than in the bioassays. This level of disagreement between probe assay and suckling mouse assay for ST has been reported previously.²⁷ The finding that six isolates that gave positive results for both LT and ST in the probe assay gave positive results only for LT in the bioassay (table) indicated that expression of the LT and ST genes is independent of each other and that the absence of expression in one of these genes does not affect the expression of the other gene.

All probe-negative isolates also gave negative results in the bioassays; this indicated that the presence of DNA sequences homologous to the probe DNA in these isolates was consistent with the expression of the respective pathogenic trait. This also excluded the possibility of any high degree of genetic heterogeneity in these isolates for the expression of these toxins and adhesins. Furthermore, a negative result in the DNA hybridisation assay appeared to be more accurate than a positive result.

In agreement with a recent report,¹⁴ strains that gave positive results with the LA probe also gave positive bioassay results (with the exception of eight strains out of 135). Although the role of DA *E. coli* in the pathogenesis of diarrhoea is controversial,^{26,27} both the DNA probe assay and the HeLa cell adherence assay detected a significant number of DA-positive isolates and the two assay methods generally agreed. The reasons why eight LA probe-positive isolates (from three patients) and six DA probe-positive isolates (from two patients) gave negative results in the bioassays could be similar to those suggested for the enterotoxigenic isolates. Fifty-nine isolates showed the AggA pattern but it was sometimes difficult to distinguish between the true DA and AggA patterns. These AggA isolates need to be tested with the DNA probe for enteroaggregative *E. coli*.¹⁷

Because the HeLa cell adherence assay relies on observation of different patterns of adherence by microscopic examination, there is a considerable chance that an inexperienced observer may incorrectly identify the adherence pattern. The DNA hybridisation assay is usually free of such qualitative interpretation. There was, however, a problem of false positive results in this study with the plasmid-based DNA probe for ST, which was eliminated by supplementing the hybridisation solution with unlabelled denatured vector DNA.

From a practical point of view, bioassays are useful in obtaining rapid results while analysing a small number of isolates. There are different opinions regarding the sensitivity and specificity of the DNA hybridisation assay,^{11,18} but the major advantages of the assay are that a large number of isolates can be analysed quickly and it is more cost-effective than bioassays. Therefore, DNA probes are more useful than bioassays in screening large number of isolates in epidemiological investigations.

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