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Development of DNA probes for cytotoxin and enterotoxin genes in enteric bacteria

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Summary. DNA probes to identify the genes encoding toxins in enteric bacteria have been developed. Use of these probes reduces the number of animals required for toxicity testing, as suspect bacteria can be directly tested for the presence of toxin. We have augmented the gene probes available by developing probes against the *Escherichia coli* enterotoxin LTII and shiga toxin from *Shigella dysenteriae* 1.
The LTII gene from *E. coli* 357900 was identified and characterised and a suitable internal probe was obtained. The LTII gene was found not to be common among enterobacteriae from various geographical locations. Isolates predominately of animal origin from Nigeria and Thailand hybridized with the probe.
The shiga toxin gene was isolated from *S. dysenteriae* 1 by a combination of in vivo and in vitro methods. An internal probe was identified and used against different serogroups of *Shigella* and *E. coli* isolates. The probe was found to hybridize with *S. dysenteriae* 1 isolates and also some *S. flexneri* and *S. sonnei* strains. Representatives were tested for toxin production and found to produce toxin at low levels.
Key words. DNA probes; cytotoxin and enterotoxin genes; *Escherichia coli; Shigella* spp.

Introduction

Diarrhoea, the rapid loss of water and salts from body via the intestinal tract, is the most important infectious disease of our time: there are some 10^9 cases per year worldwide and several million deaths. Although the severe diarrhoea is mostly seen in developing countries, where most deaths due to diarrhoea occur in infants of two years and below, a high proportion of individuals in developed countries experience at least one episode of diarrhoea per year (often when travelling, hence 'travellers diarrhoea') and infants in day centres generally suffer multiple episodes per year. In addition to the human suffering caused by diarrhoea, large numbers of

newborn farm animals die from the disease each year. The immensity of diarrhoeal disease makes it not only a high public health priority but also a major human and economic burden.

There are many organisms that provoke diarrhoea and several different forms of the disease [20]. Some pathogens produce enterotoxins which cause a massive loss of fluids from the body and thus dehydration. The classical example of this is the watery diarrheoa caused by *Vibrio cholerae*. Other pathogens produce cytotoxins that cause bowel damage. Some species of *Shigella* produce high levels of a cytotoxin that also has enterotoxic activity [4], and an immunologically identical toxin, *slt*1 is produced by some diarrhoea-producing strains of enteropathogenic *Escherichia coli* (EPEC) and has been implicated in the pathology caused by some enterohaemorragic strains of *E. coli* (EHEC) [11, 12].

Despite the enormous progress in recent years in the diagnosis of diarrhoea and in the identification of diarrhoea-producing organisms, the causative agent in about 50% of cases cannot be identified. In recent years organisms which had previously been considered as part of the normal gut flora have been shown to be pathogenic e.g. enterohaemorragic *E. coli* (EHEC) [15]. Without being able to identify reliably the causative agent, it is difficult to develop effective vaccines to prevent diarrhoeal disease. Reliable tests are therefore needed to distinguish diarrhoea-producing organisms from the normal intestinal gut flora.

Until recently the only reliable test for enterotoxin was the rabbit ileal loop model, in which fluid accumulation in surgically exposed ligated segments of the small bowel is demonstrated. The animal model is ethically undesirable, expensive, time consuming, and requires a high level of expertise. It is inadequate for the testing of large numbers of organisms and thus for the type of testing that is currently needed.

In recent years, molecular genetic approaches have been used to identify pathogens [19]. A number of genes encoding toxins have now been isolated and characterised and used to construct DNA probes for the identification of bacteria producing such toxins. Probes are available for heat labile enterotoxin (LT) and heat stable enterotoxin (ST) from enterotoxigenic strains of *E. coli* (ETEC) and cholera toxin (CT) [8, 9, 13]. However CT, LT and ST probes do not detect all diarrhoea-producing organisms. To augment the available probes we decided to develop new cytotoxin and enterotoxin gene probes to detect additional diarrhoea-producing strains of *E. coli* and cytotoxin producing *Shigella* spp. Different approaches were used to identify and clone the genes responsible for toxin production, prior to probe construction.

Results

LTII from E. coli strain 357900, a new heat labile toxin

During an epidemiological screening of diarrhoea-causing *E. coli* strains isolated in India, one strain, 357900, showed no homology with DNA probes for *E. coli* LT and ST, even though it gave a positive result in the rabbit ileal loop test and the Chinese hamster ovary (CHO) tissue culture assay for enterotoxin production. Although the new enterotoxin is heat labile genetic analysis showed that unlike other heat labile toxins from *E. coli*, it was chromosomally determined and distinct from LT by various criteria (Clayton and Timmis, manuscript in prep.) This toxin was designated LT II.

A gene bank of total DNA from strain 357900 was constructed in cosmid pHC79 by established methods [7] and transformants containing recombinant cosmids were tested for production of the new LT toxin using the CHO tissue culture assay; one isolate containing the recombinant cosmid pEC167 produced enterotoxin with a titre of 1/320, a level 4–8-fold less than the titre obtained from the original strain. Subcloning of small DNA restriction fragments from the 40 kb insert in the hybrid cosmid localized the enterotoxin determinant on a 3.7 kb *Hin*dII-II-*Hin*cII fragment.

The enterotoxin gene was localized precisely by transposon mutagenesis with the insertion element Tn*1000*. Transposon insertion mutants were mapped by restriction endonuclease cleavage and SDS polyacrylamide gel electrophoresis of total protein was performed on selected subclones of pEC167 and transposon mutants; this allowed the localizaton of the genes encoding the two subunits of the toxin on a 2 kb fragment (fig. 1) (Clayton and Timmis). This in turn identified a suitable DNA fragment, a 0.5 kb *Pst*I-*Nco*I fragment, internal to the LT-like gene, as an appropriate DNA probe for homologous enterotoxin genes.

DNA hybridizations (colony blots) with a variety of *E. coli* strains reported to produce LT and ST were performed under high stringency conditions [7]; no homology was detected.

An epidemiological screening of large numbers of enterotoxigenic *E. coli* strains, of *Salmonella, Shigella, Yersinia, Aeromonas* and *Campylobacter* from various geographical locations was performed using the LT-like probe in order to ascertain the prevalence of the LT-like gene. The results are shown in table 1. 33/34 strains isolated in Thailand and Nigeria were found to hybridize with the probe. Pickett et al. [14] reported the cloning of an LTII enterotoxin gene from a different strain of *E. coli*. This probe was hybridized with representatives of our collection; all except two strains that hybridized with our LTII probe hybridized with this (data not shown) implying that the two genes are related but not identical.

As can be seen from table 1, the LTII gene is not widely distributed among diarrhoea-producing bacteria: 31/33 LTII positive strains were of animal origin.

Shiga toxin

Shiga toxin, the cytotoxin produced by *S. dysenteriae* serotype 1 is one of the most powerful cytotoxins

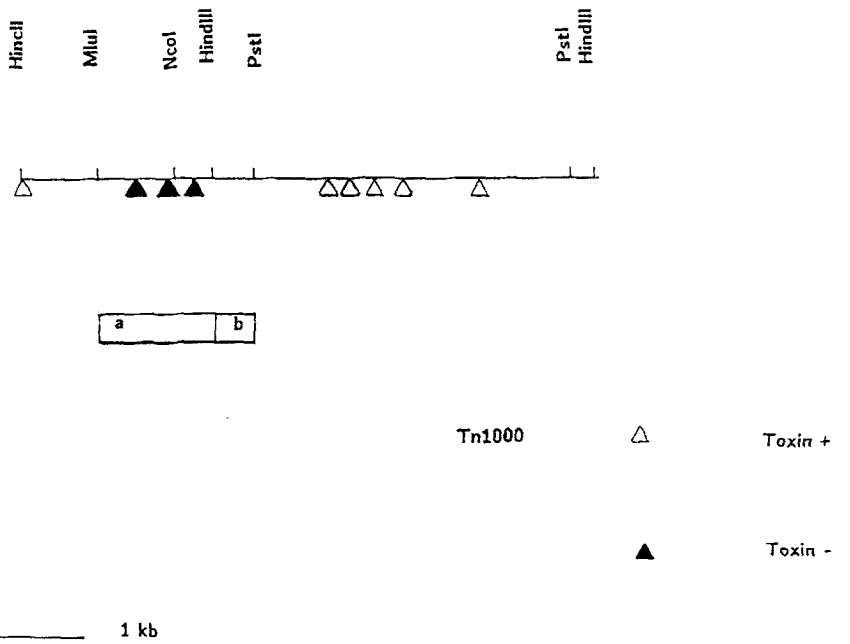

Figure 1. Restriction map of LT-like gene from *E. coli* 357900 showing Tn*1000* insertions in the cloned fragment. A and B denote toxin subunits identified by PAGE electrophoresis (Clayton and Timmis).

Table 1. Results of screening a series of diarrhoea-producing organisms with the 500 bp *Pst*I-*Nco*I probe for LT II

Species	Source	Number	Origin	Hybridization *Pst-Nco* probe
Escherichia coli	human LT II	1	Thailand	+
Escherichia coli	human LT II	1	Nigeria	+
Escherichia coli	human LT/ST	200	Japan (Osaka)	−
Escherichia coli	human LT	20	Africa (Rwanda)	−
Escherichia coli	human LT/ST	15	Australia	−
Escherichia coli	human EPEC	10	Peru	−
Escherichia coli	human LT/ST	15	Peru	−
Escherichia coli	human LT/ST	20	Bangladesh (Dhaka)	−
Escherichia coli	human LT/ST	6	India (Calcutta)	−
Escherichia coli	human LT/ST	35	Africa (Bangui)	−
Escherichia coli	animal bovine (LT II)	31	Thailand	+
Escherichia coli	animal porcine	21	England	−
Escherichia coli	animal bovine	1	England	−
Escherichia coli	animal porcine	12	Switzerland	−
Escherichia coli	animal bovine	4	Switzerland	−
Escherichia coli	animal bovine	58	Hungary	−
Escherichia coli	animal porcine	48	Hungary	−
Yersinia enterocolitica	human	34	England	−
Yersinia frederiksenii	human	16	England	−
Aeromonas spp.	human	64	England	−
Salmonella spp.	human	30	England	−
Shigella flexneri	human	7	England	−
Shigella boydii	human	4	England	−
Shigella sonnei	human	3	England	−
Shigella dysenteriae	human	1	England	−
Pleisiomonas shigelloides	human	3	England	−
Campylobacter spp.	human	35	England	−

known[10]. As molecular cloning of the shiga toxin determinant was prohibited by the N.I.H. Recombinant Advisory Committee, we decided to generate toxin negative mutants by in vivo methods prior to in vitro cloning. This, however, required the accurate localization of the toxin determinant on the *S. dysenteriae* 1 chromosome. To localize the shiga toxin determinant, we constructed high frequency transfer derivatives of *S. dysenteriae* 1 that could transfer their chromosomal genes from different points into a suitable recipient strain. The derivatives thereby generated, TS51, TS52, and TS59 were mated with *E. coli* TS50 and recipients having acquired individual chromosomal markers were selected (table 2).

Transconjugants were tested for cytotoxic activity against HeLa cells. Transconjugants having received *gal*, *trp* or *his* loci showed high levels of cytotoxin activity

($>10^7$ CD_{50}/ml of culture supernatant, table 3). The locus conferring high level toxin production was designated *stx*. Linkage analysis of these loci and the nearby *pyr*F, gave the gene order *trp*, *pyr*F, *stx*[18].

The levels of cytotoxin produced by *E. coli* transconjugants and transductants were as high as that of the parental *S. dysenteriae* 1 strain, and these activities could be neutralized by polyclonal anti-shiga toxin and monoclonal anti-*slt*1 antibodies, kindly provided by A. O'Brien[18].

An stx^+ $pyrF^+$ *E. coli* transconjugant was subjected to transposon mutagenesis. Over 10,000 independent transposon mutants were pooled and used propogate P1 phage. The phage lysate was then used to transduce $pyrF^+$ and a transposon marker into a $pyrF^-$, stx^+ *E. coli* strain. 86 independent $pyrF^+$ transductants containing the transposon marker were tested for cytotoxic activity and two transductants TS73 and TS74 were found to be negative. TS73 was used as a donor for transduction of the transposon into an stx^+ *E. coli* strain; all transductants were converted to stx^-. Thus TS73 and TS74 contain transposon mutant alleles of *stx*. The mutant alleles were designated *stx*1 (TS73) and *stx*2 (TS74). Transduction of the *stx*1 allele into *E. coli* Hfr strains KL99 and KL208 created TS76 and TS77, which could transfer the *stx*1 allele as an early marker into appropriate recipients. Upon transfer of the *stx*1 into *S. dysenteriae* 1 isolates, the ability to produce shiga toxin was lost. Hybridization of total DNA from *stx*1 and stx^+ bacteria with probes for the A and B subunits of the related toxin

Table 2. Characteristics of Hrf derivatives of *Shigella dysenteriae*. Derivatives of *Shigella dysenteriae* 1 in which the temperature-sensitive RP4-derived plasmid pMT999 had inserted into the chromosome were selected by growth of bacteria at 42 °C. The three derivatives TS51, TS52 and TS59 were mated with an appropriately-marked *E. coli* K-12 strain and transconjugants selected. The pattern of *Shigella* markers inherited by the *E. coli* recipient during these matings suggested the approximate sites of insertion of pMT999 in the chromosomes of TS51, TS52 and TS59, and thus their origins of chromosomal transfer.

	No of transconjugants obtained/10^6 donor cells							
Donor	$PurE^+$	Gal^+	Trp^+	His^+	$ArgG^+$	Ilv^+	Met^+	Leu^+
TS51	1	14	10	3	730	74.000	30	1
TS52	10	290	530	27	210	170	5	4
TS59	1300	650	8	3	8	3	4	0.1

Table 3. Mapping of *stx*: TS50 is the appropriately marked *E. coli* K-12 recipient strain used in the first matings.

Donor recipient	Selected phenotype	% co-inheritance							Recombinants examined
		$purE^+$	gal^+	fla^-	trp^+	stx^+	fla^-	his^+	
TS52	$PurE^+$	100	1.0	4.2	10.4	0	(4.2)	0	96
x	Gal^+	0	100	68.8	2.5	3.8	(68.8)	0	80
TS50	Trp^+	0	0	7.3	100	14.6	(7.3)	0	96
	His^+	0	0	(22.9)	1.0	1.0	22.9	100	96
TS59	Gal^+	0	100	11.3	0	ND	(11.3)	0	80
x	Trp^+	0	1.1	40.9	100	2.2	(40.9)	0	83
TS50	His^+	0	0	(28.0)	1.3	0	28.0	100	75

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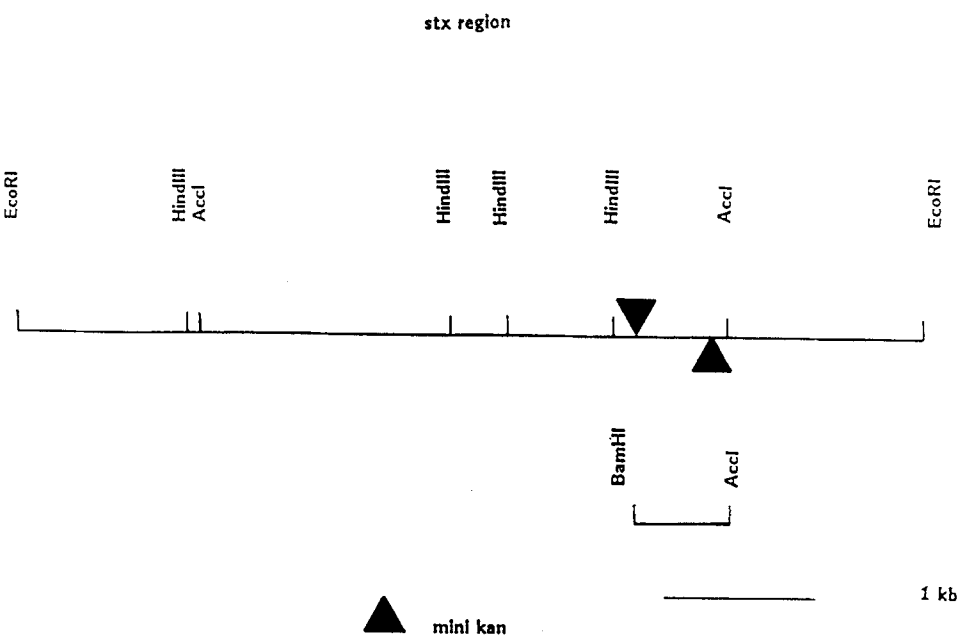

Figure 2. Restriction nuclease map of *stx* region from *S. dysenteriae* 1. Triangles denote transposon insertions. Each transposon was borderd by a *Bam*HI restriction site, the internal probe was constructed by isolating a 500 bp *Bam*Hi-*Acc*I from pDB74.

Table 4. Results of screening a collection of *Shigella* spp. and ETEC and EPEC *E. coli* with the 500 bp *Bam*HI-*Acc*I internal fragment from *S. dysenteriae* 1 *stx*. Numbers in brackets denote the number of strains tested.

Isolate	% isolates hybridizing
*S. dysenteriae*1	100 (30)
*S. dysenteriae*2	0 (4)
S. sonnei	82 (11)
S. boydii	0 (6)
S. flexneri	8 (12)
ETEC	30 (34)
EPEC	25 (8)

*slt*1, (kindly provided by A. O'Brien), indicated that the structural determinants of shiga toxin had been mutated in *stx*1 and *stx*2 mutant bacteria, as seen by a size difference in *Eco*RI fragments between mutant and wild type *stx* alleles corresponding to the size of the transposon (1.7 kb).

Having mapped and mutated the *stx* locus we were able to clone the region containing *stx*1 and *stx*2 alleles. Mapping of the cloned alleles with a series of restriction enzymes localized the transposons within a 500 bp region of the cloned fragments (fig. 2). Southern hybridizations with DNA probes isolate from the related *slt*1 gene localized the coding region for the A and B subunits of shiga toxin, and confirmed that the *stx*1 and *stx*2 mutations are located within an internal fragment that can be used as an *stx* gene probe.

This fragment was isolated and used to screen members of the genus *Shigella* and pathogenic *E. coli* strains for the presence of the toxin gene (table 4). Colony hybridizations were carried out by the steaming method of Maas[6].

All *Shigella* spp. isolates from different geographical locations hybridized with the *stx* probe. However, other serogroups of *S. dysenteriae* did not. No *S. boydii* isolates hybridized, but some isolates of *S. sonnei* and *S. flexneri* did. A series of pathogenic *E. coli* were screened with the probe and 9 LT-producing ETEC strains isolated from Central Africa and two EPEC strains were found to hybridize.

Representative hybridizing strains were then tested for cytotoxic activity against HeLa cells. The *S. sonnei, S. flexneri* and ETEC isolates tested showed low levels of cytotoxic activity 2.5×10^2 CD_{50}/ml compared to 10^7 CD_{50}/ml for wild type *S. dysenteriae* 1; cell destruction was not observed but rather a rounding of the cells and detachment from the monolayer occurred. The two EPEC *E. coli* did not show cytotoxic activity against HeLa cells.

Discussion

We have shown that the gene probes developed in this laboratory from LTII producing *E. coli* and *S. dysenteriae* 1 can detect cytotoxin and enterotoxin genes present in diarrhoea-producing bacteria.

All *S. dysenteriae* 1 isolates, which had been shown by the HeLa cell assay to be toxin positive hybridized with the gene probe. The *stx* probe could also identify other shigellae and some *E. coli*, which when assayed by the HeLa cell assay displayed weak cytotoxic activity. *S. sonnei* isolates hybridizing to the probe had all been isolated from patients which had returned to Britain from the Mediterranean area. It would be interesting to investigate the prevalence of *stx* positive *S. sonnei* on a worldwide basis as workers from the United States report that *S. sonnei* does not hybridize with the related *slt*1 probe[17]. Our results with *S. flexneri* confirm other reports that some strains do produce shiga toxin, albeit at a low level and hybridize with the related *slt*1 probe[5,17].

The results of probing LT-producing *E. coli* from Central Africa with *stx* suggested that these strains contain more than one toxin. This has been found to be the case with other *E. coli*, for example *E. coli* O157:H7 where both *slt*1 and *slt*2 are produced[16]. The identification of a strain which produces more than one toxin is an important factor in the diagnosis of diarrhoea produced by such organisms.

When other pathogenic *E. coli* were screened, two isolates were found to hybridize with the *stx* probe. However, these strains did not have cytotoxic activity against HeLa cells, suggesting either that the toxin is produced at low levels or that the gene is present but non-functional. The relevance of such a finding in vivo remains to be analysed.

The LTII toxin gene cloned from *E. coli* 357900 appears to be distinct from LT but similar to the LTII gene of Pickett (Clayton and Timmis). These toxins are not widely distributed, and are confined mainly to strains isolated from animal origin. Strains producing these toxins are not detected by current immunological tests using anti-LT antibodies and also give a negative result in the GMI-based enzyme-linked assay (Clayton and Timmis). However, colony hybridization with the new probe will unambiguously detect the toxin gene.

The hybridization method is extremely sensitive and is able to detect small numbers of bacteria in mixed cultures[8]; some workers have succeeded in detecting diarrhoea-producing bacteria in stool samples directly applied to filtres, thus saving considerable time and expense in stool culture[2]. New non-radioactive reagents are becoming available which allow hybridizations to be carried out in clinical laboratories without special apparatus and without risk from radiation[1,3]. This will greatly aid the utility and utilization of the technique.

As more toxin probes become available effective primary screening of diarrhoea-producing organisms will be possible. This will in turn reduce substantially the number of animal experiments and tests.

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Reduction of the number of mice used for potency testing of human and animal rabies vaccines

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Summary. Eight different rabies vaccines were tested for their potency in the standard mouse potency test using 3-, 5- and 7-week-old mice. 5-week-old mice seem to be best suited for this purpose, variability from test to test could be reduced considerably. An ELISA was used in parallel for the evaluation of the rabies glycoprotein content of rabies vaccines. Results of the mouse potency test correlated well with those of the ELISA if highly purified human vaccines were tested. Unspecific reactions in the ELISA caused by adjuvanted veterinary vaccines could not be blocked. Further experiments will be needed in order to evaluate the potency of inactivated veterinary rabies vaccines by a in vitro test.
Key words. ELISA; mouse test; potency; rabies; vaccine.

Introduction

The goal of any vaccine potency test is the evaluation of the protective activity of the vaccine. The most direct and reliable way to evaluate the potency of a vaccine is the demonstration of its capability to protect the species for which the vaccine is intended, e.g. a rabies vaccine must be able to protect humans or dogs against a rabies virus infection. In this test, which is not feasable in humans, vaccination is followed by experimental infection.

For many years laboratory animal testing has been used, as an alternative, to evaluate the potency of vaccines. Induced immunity may be checked by infection as in the target species. Laboratory animals, compared to the host species, have the advantage of easy handling and housing; they can be produced in great number, so that experiments can be conducted in a quantitative way, and finally, they are relatively cheap. Tests on laboratory animals