BACTERIAL PATHOGENICITY

Detection of *Vibrio cholerae* and *V. mimicus* heat-stable toxin gene sequence by PCR

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Previously the heat-stable enterotoxin in *Vibrio cholerae* and *V. mimicus* has been detected by suckling mouse assay, a non-specific approach, and by DNA probes, a time-consuming method. This report describes a polymerase chain reaction (PCR) procedure for the detection of the *stn* (NAG-ST) and *sto* (O1-ST) gene sequences that is rapid and specific, allowing toxin gene molecular characterisation. A total of 34 *V. cholerae* and *V. mimicus* isolates was examined for ST and CT genes. The NAG-ST gene sequence was amplified in 13 of 22 non-O1/non-O139 *V. cholerae* and three of five *V. mimicus* strains. A new enterotoxin gene sequence pattern was found with *Mse*I and *Taq*I restriction endonuclease PCR fragment digestion of two *V. cholerae* isolates, in addition to the pattern anticipated from the Genbank sequence, and found with the other ST⁺. These results show that ST-PCR detection is useful for the characterisation of *V. cholerae* and *V. mimicus*.

Introduction

With the spread of the Bengal strain in Asia in 1993 and the Latin American cholera epidemic in early 1992, the detection of virulence genes from clinical and environmental strains of *Vibrio cholerae* O1 and non-O1 has become an important issue. *V. cholerae* can harbour genes encoding two major enteric toxins: the cholera toxin (CT) present in all O1 and non-O1 epidemic strains [1] and the heat-stable enterotoxin (NAG-ST) encoded by the *stn* gene and found in some non-O1 strains associated with cases of gastroenteritis [2]. A very similar heat-stable toxin, O1-ST, is encoded by the *sto* gene of certain strains of *V. cholerae* O1 [3]. *V. mimicus* is considered as the reservoir of the heat-stable toxin gene among the species of the genus *Vibrio*, with 17% of strains tested hybridising to an *stn* gene probe [4]. In a recent report, Mallard and Desmarchelier [5] detected the presence of both CT and ST toxin genes in 6.5% of Australian *V. cholerae* O1 isolates.

Screening for ST toxin among vibrios has been based upon the suckling mouse assay and the molecular detection of the NAG-ST gene by hybridisation. An oligonucleotide based on the amino-acid sequence of NAG-ST toxin [6] or a longer DNA fragment [3] have

been used as probes. PCR amplification has been applied as part of the routine diagnostic method to detect CT gene positive *Vibrio* strains [7, 8], as well as for the detection of a large number of microbial pathogens. The DNA used in this reaction can be extracted from bacterial colonies or directly from food or stool swabs [9, 10].

Based on *stn* and *sto* nucleotide sequences [11, 12] – the *stn* gene derived from a non-O1 cholera vibrio which caused traveller's diarrhoea, and the *sto* gene from a CT-producing strain of *V. cholerae* O1 – a PCR protocol to detect heat-stable toxin genes was developed. This is a sensitive and specific method to identify the presence of genes. The development and application of this method in the detection and analysis of the ST gene sequence in *V. cholerae* and *V. mimicus* are described.

Materials and methods

Bacterial strains

Vibrio cholerae O1, non-O1 and *V. mimicus* strains from human or environmental origin, from our collection, were used in this work (Table 1).

PCR

Bacterial colonies or overnight liquid cultures were used. Samples of cultures (1 ml) were centrifuged and

the pellet was resuspended in 0.5 ml of sterile water, boiled in a water bath for 10 min and then frozen at -20°C . Three μl from a defrosted DNA preparation were used in the reaction. The PCR was carried out with a 50- μl standard reaction mixture with 3 mM MgCl_2 and 20 pmol of each primer. The cycling conditions were as follows: 25 cycles of 30 s at 94°C , 30 s at 50°C and 30 s at 72°C . The products were electrophoresed through an agarose 1.2% gel.

Primer design

A pair of oligonucleotide sequences for the amplification of heat-stable toxin gene was chosen based on the *stn* and *sto* sequences, Genbank accession numbers M36061 and L03220 respectively: ST-1: 5'-GAGAAACCTATTCATTGCA and ST-2: 5'-GCAAGCTGGATTGCAAC, which amplify a 216-bp fragment. CT was detected as described previously [8].

Restriction fragment length polymorphism (RFLP)

Restriction endonuclease digestion was performed on PCR products. The *stn* fragment was cleaved with *Mse*I (BioLab) and *Taq*I (BRL) under the standard conditions described by the manufacturers. The products were electrophoresed on polyacrylamide 5% (30:1) gels.

Zymovar analysis

The multilocus enzyme electrophoresis method was done as described by Salles and Momen [13].

Sequence comparison

The *sto*, *stn* and the heat-stable enterotoxin nucleotide sequence of *Escherichia coli* [14] were compared and

analysed with the use of the GCG (Genetic Computer Group, DNA Analysis Package of Programs, USA).

Results

Specificity of primers for vibrios heat-stable toxin genes

A comparison of the *sto* and *stn* nucleotide sequences revealed that they are identical in 97.3% of the sequence. The two primers, ST-1 and ST-2, were designed for *stn/sto* PCR amplification. In the ST-2 primer annealing region the identity between the sequences is 100% and a single mismatch is present in the ST-1 region. The same region from both genes can be amplified under high stringency conditions with this pair of primers. As shown in Fig. 1, a very strong and defined 216-bp PCR product was produced from each heat-stable toxin-producing strain of *V. cholerae* and *V. mimicus*. The ST *E. coli* gene showed 66% similarity with the *Vibrio* genes. An *E. coli* LT⁺/ST⁺ and an epidemic *V. cholerae* O1/CT⁺ (strain 254) from Brazil were used as negative controls and no PCR product corresponding to *stn* or *sto* genes was obtained. The positive controls were two ST⁺ *V. cholerae* non-O1 strains (686, 688) obtained from Dr G. Morris.

Heat-stable toxin and CT detection by PCR

As shown in Table 1, 25 non-O1 cholera vibrios, five *V. mimicus* and four *V. cholerae* O1 isolates from Dr Salles' collection were screened and the 216-bp ST-PCR product was obtained from 13 *V. cholerae* non-O1 and three *V. mimicus* strains. The CT gene was detected in two *V. cholerae* O1, all O139 and one non-O1/non-O139 (335) *V. cholerae* aldova strains [15].

Fig. 1. Agarose gel (1.2%) electrophoresis of products from PCR of heat-stable toxin gene (216 bp). Lane 1, 161; 2, 275; 3, 199; 4, 711; 5, 100 bp DNA ladder (BRL); 6, 263; 7, 686; 8, 724.

Table 1. *V. cholerae* and *V. mimicus* isolates used in this study

Serogroup	Strain	Source	Place	Date	Zvar*	CT/ST†
<i>V. cholerae</i> O1	38	Environment	UK	1977	12	— —
	121	Human	India	1970	14	+ —
	182	Environment	Australia	1983	11	— —
	254	Human	Brazil	1991	14	+ —
<i>V. cholerae</i> O139	172	Human	Bangladesh	1993	14	+ —
	179	Human	Bangladesh	1993	14	+ —
	197	Human	Bangladesh	1993	14	+ —
<i>V. cholerae</i> non-O1	147	Human	Brazil	1992	50	— —
	151	Environment	Mexico	1986	59	— —
	199	Human	Thailand	1990	30	— +
	208	Human	Thailand	1992	02	— —
	263	Human	Thailand	1991	30	— +
	265	Human	Australia	1986	48	— —
	271	Human	Thailand	1990	30	— +
	276	Human	Australia	1986	48	— —
	278	Human	Australia	1986	32	— —
	286	Human	Australia	1986	52	— —
	333	Human	Thailand	1990	70	— —
	335	Human	Czechoslovakia	1965	38	+ —
	686	Human	Japan	1985	30	— +
	688	Animal	Japan	1986	30	— +
	711	Human	Thailand	1982–85	30	— +
	712	Human	Japan	1985	30	— +
	716	Animal	Japan	1962	30	— +
	717	Human	Thailand	1982–85	30	— +
	718	Human	Thailand	1982–85	47	— +
	720	Human	USA	1988	61	— +
	723	Food	Thailand	1954	51	— +
	724	Human	Thailand	1982–85	30	— +
<i>V. mimicus</i>	160	ATCC	33653	...	20	— —
	161	ATCC	33654	...	21	— +
	162	ATCC	33655	...	19	— —
	275	Human	Australia	...	40	— +
	284	Human	Australia	1986	34	— +

*Zymovar.

†Results obtained by PCR analysis.

Zymovar analysis

The zymovars identified are shown in Table 1. The results reveal the effective distinction of some ST⁺ strains. Ten of 13 *V. cholerae* non-O1 strains analysed belong to zymovar 30, including strains 711 and 724, which are special in their RFLP.

RFLP

The amplified ST gene fragments obtained from *V. cholerae* and *V. mimicus* were digested with *Mse*I and *Taq*I restriction endonucleases. The predicted fragment sizes were 100, 97 and 19 bp, and 161 and 55 bp respectively. The digestions from the majority of the samples analysed gave the predicted pattern (Fig. 2a, b). Different products were obtained from two *V. cholerae* non-O1 strains 711 and 724, from Thailand. Both samples gave the same profile, revealing a polymorphism for these enzymes sites. The fragments found have an approximate size of 100, 50, 47 and 19 bp for *Mse*I (the 19-bp band was barely visible in the gel) and 161, 45 and 10 bp for *Taq*I (Fig. 2a, lanes 5 and 13; Fig. 2b, lanes 2 and 8).

Discussion

The identification of heat-stable toxin genes in *V. cholerae* and *V. mimicus* strains is an important step

when environmental or human isolates from cholera non-epidemic areas are under analysis. The demonstration that a NAG-ST-producing strain can cause diarrhoea of a severity comparable to cholera [16] increases the importance of developing a rapid and precise detection method, such as the PCR approach described here. The suckling mouse assay is not specific to this particular toxin, the El Tor haemolysin liberated by most strains of *V. cholerae* non-O1 is also detected by this approach [17]. As the *stn* and *sto* gene sequences are available, it was possible to design primers for PCR detection and analysis of the heat-stable toxin coding region. The results show that this reaction is specific to *V. cholerae* and *V. mimicus* ST genes, as no amplification product was observed in the case of the enterotoxigenic *E. coli* strain tested, harbouring a related ST gene.

In the present study there was a marked concentration of ST⁺ strains of zymovar 30. This may be due to a concentration of strains from Asia in the sample or it could reflect a correlation between this genetic background and the presence of the *stn* gene.

Distinct restriction patterns with the enzymes *Mse*I and *Taq*I were obtained with two of the non-O1 *V. cholerae* ST⁺ strains with an extra site for each of the enzymes. The positions where the sequence could

Fig. 2. Polyacrylamide gel (5%) electrophoresis of ST PCR fragments digested with restriction enzymes. **a**, *Mse*I: lane 1, 199; 2, 263; 3, 271; 4, 688; 5, 711; 6, 712; 7, 100 bp DNA ladder (BRL); 8, 161; 9, 686; 10, 275; 11, 716; 12, 723; 13, 724. **b**, *Taq*I: lane 1, 199; 2, 711; 3, 161; 4, 717; 5, 100 bp DNA ladder (BRL); 6, 275; 7, 723; 8, 724.

have changed to give these new sites were investigated with the three *V. cholerae* heat-stable toxin sequences in Genbank v. 96.0 and the size of the new restriction fragments. Counting from the beginning of the translated region, two transversions, an A → T in position 71 and a G → C in position 175, would generate an *Mse*I and a *Taq*I site, respectively. Both changes would produce an amino-acid change. These nucleotide substitutions lie outside the region of the mature toxin. As the polymorphisms detected would be caused by transversions, it is probable that other differences may exist, and hence these variant *stn* genes will be sequenced. Heterogeneity in the genome of some *stn*-positive strains has been detected

previously [18]; seven patterns were found by hybridising *Hind*III or *Eco*RI restriction fragments with a NAG-ST gene probe. Arita *et al.* [19] characterised a heat-stable enterotoxin (Vc-H-ST) in *V. cholerae* non-O1 serogroup Hakata, with an amino-acid sequence quite similar to that of the NAG-ST, except for the inclusion of a leucine residue at the N-terminus.

The polymorphism in the nucleotide sequence of the *stn* gene is now under investigation to establish whether it reflects changes in the biological activity of the toxin and to determine if it can be used as an epidemiological marker.

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