Rapid detection of *Vibrio* species using liquid microsphere arrays and real-time PCR targeting the *ftsZ* locus

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The development of rapid and sensitive molecular techniques for the detection of *Vibrio* species would be useful for the surveillance of sporadic infections and management of major outbreaks. Comparative sequence analysis of the *ftsZ* gene in the predominant *Vibrio* species that cause human disease revealed distinct alleles for each examined species, including *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Light Upon eXtension (LUX) real-time PCR assays were developed to target these species-specific polymorphisms, and were successful in rapidly differentiating the major pathogenic *Vibrio* species. Luminex liquid microsphere array technology was used to develop a comprehensive assay capable of simultaneously detecting *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. These assays permitted the identification of a presumptive *V. parahaemolyticus* isolate as *Vibrio alginolyticus*, which was verified using additional molecular characterization.

Received 1 June 2006
Accepted 2 October 2006

INTRODUCTION

Pathogenic species of the genus *Vibrio* pose a considerable public health threat as the causative agents of both sporadic and epidemic human infections. Cholera, caused by *Vibrio cholerae*, continues to spread globally in a seventh pandemic (O1 El Tor biotype), and the emergence of a non-O1 serogroup (O139 Bengal) has led to a new pandemic (Sack *et al.*, 2004; Faruque & Mekalanos, 2003). Unlike the watery diarrhoeal disease caused by *V. cholerae*, infection with *Vibrio parahaemolyticus* usually results in a self-limiting gastroenteritis with inflammatory diarrhoea and, in rare cases, septicaemia (Janda *et al.*, 1988). Opportunistic infections with *Vibrio vulnificus* can cause severe wound infections (Oliver, 2005) and fulminant septicaemia, with highly virulent strains causing extensive host-tissue damage and producing mortality rates of up to 60% (Linkous & Oliver, 1999). The threat posed by pathogenic *Vibrio* species has been highlighted by recent natural disasters, in which a number of survivors were infected in the aftermath of Hurricanes Katrina and Rita on the gulf coast of the USA (Centers for Disease Control, 2005, 2006), and the Indian Ocean tsunami (Lim, 2005). Fortunately, major outbreaks did not occur, but these disasters have served as an impetus

to develop rapid and sensitive molecular techniques that can be easily deployed to identify pathogenic *Vibrio* species during a public health emergency.

Traditionally, identification of *Vibrio* spp. has consisted of isolation on selective agar medium followed by biochemical and serological testing (Harwood *et al.*, 2004). The availability of genomic sequence data allows for *Vibrio* spp. comparative genomic studies, revealing not only a wealth of information on their evolution and pathogenesis, but potential targets for molecular typing and detection. Molecular techniques for *Vibrio* identification and subtyping have been developed, including oligonucleotide probes (Wright *et al.*, 1993) and DNA microarray technologies which target species-specific virulence determinants (Panicker *et al.*, 2004). PCR-based methods have been developed to identify and subtype *Vibrio* spp., including multiplex PCR primer sets for the amplification of *tlh/tdh/trh* (Bej *et al.*, 1999), *ompU/toxR/tcpI/hlyA* (Rivera *et al.*, 2001) and *vvh/viuB* (Panicker *et al.*, 2004). PCR-based molecular-typing studies have been performed in *V. cholerae* with amplified fragment length polymorphism (AFLP), BOX- and enterobacterial repetitive intergenic consensus (ERIC)-PCR (Singh *et al.*, 2001); in *V. parahaemolyticus* with multilocus sequence typing (MLST) (Chowdhury *et al.*, 2004); and in *V. vulnificus* with randomly amplified polymorphic DNA (RAPD) analysis (Parvathi *et al.*, 2005; Warner & Oliver, 1999). Recently, real-time PCR (RT-PCR) protocols have been developed for the identification of *V.*

Abbreviations: C_t, threshold cycle; LUX, Light Upon eXtension; RT-PCR, real-time PCR.

The GenBank/EMBL/DBJ accession numbers for the *ftsZ* sequences are DQ520257–DQ520276.

vulnificus, employing both TaqMAN (Campbell & Wright, 2003; Panicker & Bej, 2005) and SYBR Green technology, the latter targeting the *vwA* cytolysin gene alone (Panicker *et al.*, 2004) or in a multiplex real-time assay (Gubala, 2005). Alternatively, genetic variation at core genetic determinants can be exploited for speciation and disease surveillance.

We have previously utilized Light Upon eXtension (LUX) fluorogenic primers and liquid microsphere suspension arrays for the detection and typing of *Escherichia coli* (Gilmour *et al.*, 2006) and human-adapted *Salmonella enterica* serovars Typhi and Paratyphi A (Tracz *et al.*, 2006). In contrast to the 5' nuclease system which uses two unlabelled PCR primers and a fluorophore-labelled probe with a quencher, the LUX RT-PCR system requires a single pair of primers, a fluorogenic (that is self-quenching by the formation of a hairpin loop) primer and its unlabelled primer pair (Nazarenko *et al.*, 2002). Luminex microsphere suspension array technology utilizes allele-specific oligonucleotide probes conjugated to fluorescently coded microspheres which capture soluble DNA in a liquid phase and characterize hybridization partners by flow cytometry (Dunbar *et al.*, 2003). In this study, our goal was to apply LUX RT-PCR and Luminex microsphere technology to target species-specific polymorphisms at the *ftsZ* locus for the molecular typing of *Vibrio* species.

METHODS

Bacterial strains. Strains of *Vibrio* spp. (see Table 1) were obtained from the laboratories listed. Publicly available genomic sequence data were used to initiate comparative genomic studies at the *ftsZ* locus: *V. cholerae* O1 (El Tor biovar strain N16961, GenBank accession no. NC_002505); *V. parahaemolyticus* (RIMD 2210633, NC_004603); and *V. vulnificus* (CMCP6, NC_004459; YJ016, NC_005139) (Heidelberg *et al.*, 2000; Makino *et al.*, 2003; Chen *et al.*, 2003). Genomic sequence data from other Gram-negative bacteria were also used for assessing overall diversity at *ftsZ*: *E. coli* (EDL933, GenBank accession no. NC_002655; K12, NC_000913; CFT073, NC_004431); *Shigella flexneri* (2457T, NC_004741; 301, NC_004337); *Yersinia pestis* (Medievalis 91001, NC_005810; KIM, NC_004088; CO92, NC_003143); *Salmonella* serovar Typhimurium (LT2, NC_003197; DT104, Sanger Institute Microbial Pathogens Group, unpublished data); *Salmonella* serovar Typhi (CT18, NC_003198; Ty2, NC_004631); *Salmonella* serovar Paratyphi A (ATCC 9150, NC_006511); *Salmonella* serovar Enteritidis (LK5, University of Illinois at Urbana-Champaign, unpublished data; www.salmonella.org).

Bioinformatics. Initial screening of polymorphic loci was performed with BLASTN (www.ncbi.nlm.nih.gov/blast/) and multiple sequence alignments were completed using CLUSTALW (www.ebi.ac.uk/clustalw/) and Boxshade (www.ch.embnet.org). Neighbour-joining trees were constructed with Hasegawa-Kishino-Yano (HKY85) distance correction using SplitsTree4 (Huson, 1998), and genetic diversity statistics were calculated with DnaSP 4.10.3 (Rozas *et al.*, 2003), using only segments of *ftsZ* for which there was data for all examined strains (i.e. regions from complete genome data outside the amplified segments of *ftsZ* were not included). Split decomposition analysis was performed with SplitsTree4 using alignment inputs created by CLUSTALW and calculated using only parsimony-informative sites.

PCR and sequencing. Template DNA was prepared by centrifuging a loopful of bacterial culture grown on OMH agar (10% blood), resuspending the pellet in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and boiling for 10 min. Boiled cell debris was pelleted and the supernatant was removed and used as DNA template in real-time and standard PCR reactions.

Standard PCR was performed with Platinum High Fidelity Taq (Invitrogen), following the manufacturer's directions, with oligonucleotides described in Table 2, on an ABI GeneAmp 9700 thermocycler. PCR conditions were: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 68 °C for 45 s, with a final extension at 68 °C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced on an ABI 3730. A PCR method for detection of the *toxR* gene in *V. parahaemolyticus* was performed on isolate 97-1598 and selected control templates with primers *toxRF* and *toxRR* (368 bp PCR product; Table 2) as described elsewhere (Kim *et al.*, 1999).

LUX fluorogenic and unlabelled primer pairs (Table 2) were designed using the D-LUX designer software (Invitrogen) by targeting polymorphic regions in the *ftsZ* locus. For LUX RT-PCR, Platinum Quantitative PCR SuperMix-UDG (Invitrogen) was used for the amplification mixture with each LUX primer at a final concentration of 200 nM and 2.5 µl template for a total reaction volume of 25 µl. RT-PCR reactions were performed on the SmartCycler 2.0 (Cepheid). Samples were amplified by an initial denaturation at 95 °C for 3 min and 35 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s and an extension step at 72 °C for 15 s. Fluorescence was detected at the annealing step and the threshold level was set at 30 fluorescence units. Products for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* RT-PCR were 92, 91 and 64 bp, respectively. An RT-PCR reaction was considered positive at the point at which the fluorescent signal exceeded the background level [the cycle threshold (C_t) value], and each strain was tested a minimum of two times.

Liquid microsphere suspension arrays. Allelic discrimination of *ftsZ* was achieved after amplifying biotin-labelled target DNA from all *Vibrio* spp. in a PCR using *ftsZ*-F1 (common forward primer) and reverse primers *ftsZ*-R2-L (targeting *V. cholerae* only; 1215 bp product; Table 2) and *ftsZ*-R1-L (all other *Vibrio* species; ~1197 bp product; Table 2). PCR was performed as a 100 µl reaction with Platinum High Fidelity Taq (Invitrogen), following the manufacturer's directions with the following final primer concentrations: *ftsZ*-F1, 1 µM; *ftsZ*-R2-L and *ftsZ*-R1-L, 1.5 µM each. PCR reactions were purified as described above and eluted in 50 µl EB buffer (Qiagen), and T7 exonuclease was used to digest the unlabelled target DNA strand (Gilmour *et al.*, 2006; Nikiforov *et al.*, 1994). DNA digestion was performed by mixing 43 µl purified PCR product with 5 µl buffer 4 (USB) and 2 µl T7 exonuclease (20 U total), and incubating at 37 °C for 1 h. Enzymic digestion was inactivated by adding 2 µl 0.5 M EDTA. Oligonucleotide probes were designed matching the sense strand in regions characteristic of individual allele subtypes and screened using SBEprimer software (Kaderali *et al.*, 2003) for potential secondary structures or cross-hybridization between probes. xMAP carboxylated fluorescently coded microsphere sets 150, 155 and 160 (Luminex) were coupled to oligonucleotides VC-DOB98, VP-DOB99 and VV-DOB100, respectively (Table 2). Hybridization of biotin-labelled *ftsZ* target DNA strands to the capture probe-coupled microspheres and flow cytometry were performed in triplicate, as previously described (Gilmour *et al.*, 2006). The positive cut-off value for the *ftsZ* assay was chosen to be five times greater than the value for the negative control.

Table 1. Allelic discrimination of *ftsZ* in *Vibrio* spp. using liquid microsphere suspension and RT-PCR assaysAbbreviations: VC, *V. cholerae*; VP, *V. parahaemolyticus*; VV, *V. vulnificus*; +, positive; –, negative; ±, intermediate signal strength.

Species	ID*	Source†	LUX RT-PCR			Microsphere suspension array		
			VC	VP	VV	VC	VP	VV
<i>V. cholerae</i>	93-517 (O1)	NML	+	–	–	+	–	–
	ATCC 31503 (O1)	HCFD	+	–	–	±	–	–
	ATCC 35971 (non-O1/O139)	HCFD	+	–	–	+	–	–
	NIH-V-86 (O1)	NML	+	–	–	+	–	–
	93-302 (O139)	NML	+	–	–	+	–	–
	93-608 (O1)	NML	+	–	–	+	–	–
	569B (O1)	HCFD	+	–	–	+	–	–
	M010 (O139)	NML	+	–	–	+	–	–
	M04S (O139)	NML	+	–	–	+	–	–
<i>V. parahaemolyticus</i>	ATCC 14033 (O1)	HCFD	–	–	–	+	–	–
	04-1240	AB PLPH	–	+	–	–	+	–
	04-2192	SK HPL	–	+	–	–	+	–
	04-2548	OPHL	–	+	–	–	+	–
	04-2550	OPHL	–	+	–	–	+	–
	04-2551	OPHL	–	+	–	–	+	–
	04-2628	QPHL	–	+	–	–	+	–
	05-0998	ICDDRB	–	+	–	–	+	–
	05-0999	ICDDRB	–	+	–	–	+	–
	05-1000	ICDDRB	–	+	–	–	+	–
	05-1001	ICDDRB	–	+	–	–	+	–
	05-1002	ICDDRB	–	+	–	–	+	–
	05-3133	BCCDC	–	+	–	–	+	–
	ATCC 17802	HCFD	–	+	–	–	+	–
	JAPAN 2269	HCFD	–	+	–	–	+	–
<i>V. vulnificus</i>	02-5675	QPHL	–	–	+	–	–	+
	04-4159	MB CPL	–	–	+	–	–	+
	90-208	NML	–	–	+	–	–	–
	92-1751	NML	–	–	+	–	–	+
	ATCC 27562	HCFD	–	–	+	–	–	+
	C7184	HCFD	–	–	+	–	–	+
	FDA-QA1	HCFD	–	–	+	–	–	+
	LAM 624	HCFD	–	–	+	–	–	+
	W108	HCFD	–	–	+	–	–	+
<i>V. alginolyticus</i>	86-148	NML	–	–	–	–	+	–
	MWG656	NML	–	–	–	–	±	–
	S115-3	HCFD	–	–	–	–	±	–
	97-1598	NML	–	–	–	–	+	–
<i>V. mimicus</i>	86-852B	NML	–	–	–	–	±	–
	94-939	NML	–	–	–	–	–	–
	ATCC 33655	HCFD	–	–	–	–	–	–
<i>V. fluvialis</i>	86-108	NML	–	–	–	–	–	–
	89-751	NML	–	–	–	–	–	–
	ATCC 33809	HCFD	–	–	–	–	–	–
<i>V. furnissi</i>	96-0200	NML	–	–	–	–	–	–
	ATCC 35016	HCFD	–	–	–	–	–	–
<i>V. metschnikovii</i>	81-66	HCFD	–	–	–	–	–	–

*Serotypes for *V. cholerae* isolates given in parentheses.

†Sources: AB PLPH, Alberta Provincial Laboratory for Public Health; BCCDC, British Columbia Centre for Disease Control; HCFD, Health Canada Food Directorate; ICDDRB, International Centre for Diarrhoeal Disease Research, Bangladesh; MB CPL, Manitoba Cadham Provincial Laboratory; NB PHB, New Brunswick Public Health Branch; NML, National Microbiology Laboratory standard strain; OPHL, Ontario Public Health Laboratory; QPHL, Laboratoire de Santé Publique du Québec; SK HPL, Saskatchewan Health Provincial Laboratory.

Table 2. Oligonucleotide primers and probes used in this study

The species target is indicated in parentheses. Abbreviations: ALEXA 546, Alexa Fluor 546; HEX, 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein; JOE, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; Z, T-phosphorothioate; F, A-phosphorothioate; E, G-phosphorothioate; bases in lower-case type at the 5' end indicate those required for LUX primer hairpin formation and are not present in the target sequence; the penultimate 3' base in lower-case type was labelled with a fluorophore.

Target	Oligonucleotide*	Sequence (5'–3')	Platform
<i>ftsZ</i> (all <i>Vibrio</i>)	<i>ftsZF1</i>	CCGATGATGGAAATGTCTG	PCR/sequencing
<i>ftsZ</i> (all <i>Vibrio</i> except <i>V. cholerae</i>)	<i>ftsZR1</i>	TTAATCAGCCTGACGACGTA	PCR/sequencing
<i>ftsZ</i> (<i>V. cholerae</i>)	<i>ftsZR2</i>	TTAGTCAGCTTGACGTCTCA	PCR/sequencing
<i>ftsZ</i> (<i>V. cholerae</i>)	<i>ftsZcF1</i>	CTCAGGCACTGCGCAAAACG	Sequencing
<i>ftsZ</i> (<i>V. cholerae</i>)	<i>ftsZcR1</i>	GACCCATTTCCGACATCACAGTG	Sequencing
<i>ftsZ</i> (<i>V. cholerae</i>)	<i>ftsZcR2</i>	CATTTTCAGCAGCTTCTTCCGCG	Sequencing
<i>ftsZ</i> (<i>V. cholerae</i>)	<i>ftsZR2-L</i>	BIOTIN-zzfeTCAGCTTGACGTCTCA	PCR/Luminex
<i>ftsZ</i> (<i>V. cholerae</i>)	VC_ftsZ-RL	cggcTCCAGTCGCATATCTAGGCc[ALEXA 546]G	LUX RT-PCR
<i>ftsZ</i> (<i>V. cholerae</i>)	VC_ftsZ-FU	GCAATTTCTAGCCCGCTGCT	LUX RT-PCR
<i>ftsZ</i> (<i>V. cholerae</i>)	VC-DOB98	TCCGCGTAACTGTCGTGGCAACC	Luminex probe
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	<i>ftsZpF1</i>	GGCCGTGAGGCAGCTCTC	Sequencing
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	<i>ftsZpR1</i>	GCTTCTCGTTTGAATCGTAATTAAC	Sequencing
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	<i>ftsZpR2</i>	TCCAGACCAGCAGTGATGTTAAC	Sequencing
<i>ftsZ</i> (<i>V. parahaemolyticus</i> and <i>V. vulnificus</i>)	<i>ftsZR1-L</i>	BIOTIN-zzffTCAGCCTGACGACGTA	PCR/Luminex
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	VP_ftsZ-FL	cggcGAAAGGTGAAGACCGAGCc[HEX]G	LUX RT-PCR
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	VP_ftsZ-RU	AACAAGAACACCGCGAGCAC	LUX RT-PCR
<i>ftsZ</i> (<i>V. parahaemolyticus</i>)	VP-DOB99	AGTTGCGACAGGTATTGGCAATGAAAG	Luminex probe
<i>ftsZ</i> (<i>V. vulnificus</i>)	<i>ftsZvF1</i>	GTTGGTCGTGACGCAGCTCTT	Sequencing
<i>ftsZ</i> (<i>V. vulnificus</i>)	<i>ftsZvR1</i>	ATCTTCGCCTTTTGCCACACCG	Sequencing
<i>ftsZ</i> (<i>V. vulnificus</i>)	<i>ftsZvF2</i>	CAAGCCGTTTAGTTTCGAAGGAAAG	Sequencing
<i>ftsZ</i> (<i>V. vulnificus</i>)	<i>ftsZvF3</i>	GCAGGTGCTCGTGGCGTG	Sequencing
<i>ftsZ</i> (<i>V. vulnificus</i>)	<i>ftsZvF4</i>	TGGGTAGCGGTGTGGCAAAAGGC	Sequencing
<i>ftsZ</i> (<i>V. vulnificus</i>)	VV_ftsZ-RL	cggatTTACGACTGTTGCGTTATCc[JOE]G	LUX RT-PCR
<i>ftsZ</i> (<i>V. vulnificus</i>)	VV_ftsZ-FU	GATGAATTTGAAACGGTGGGTAA	LUX RT-PCR
<i>ftsZ</i> (<i>V. vulnificus</i>)	VV-DOB100	TTCGAGTGACGGTGGTTGCTACC	Luminex probe
<i>toxR</i> (<i>V. parahaemolyticus</i>)	<i>toxRF</i>	GTCTTCTGACGCAATCGTTG	PCR
<i>toxR</i> (<i>V. parahaemolyticus</i>)	<i>toxRR</i>	ATACGAGTGGTTGCTGTCATG	PCR

*All oligonucleotides were designed for this study except those for *tox* PCR, which was performed by the method of Kim *et al.* (1999).

RESULTS AND DISCUSSION

Genetic diversity at the *ftsZ* locus in the genus *Vibrio*

Using publicly available genomic sequences deposited in GenBank, including *V. cholerae* (strain N16961), *V. parahaemolyticus* (RIMD 2210633) and *V. vulnificus* (CMCP6, YJ016), we performed a comparative sequence analysis and identified *Vibrio* species-specific polymorphisms at the *ftsZ*, *trpS* and *dnaX* loci. These genes are core bacterial genetic determinants which serve as useful target loci in predicting overall interspecies genetic relatedness and do not regularly undergo mutation (Zeigler, 2003). Although analysis of the *trpS* and *dnaX* loci did not reveal targets that were appropriate for development of molecular assays, multiple sequence alignments of all available *ftsZ* sequences from the genus *Vibrio* and other major enteric pathogens suggested that the species-specific polymorphisms could be

exploited for the molecular identification of *Vibrio* species (Fig. 1).

Comparative genetic analysis of *ftsZ* between *Vibrio* species and other major Gram-negative enteric pathogens showed that the genus *Vibrio* has a clear phylogenetic separation at this locus from *S. enterica* serovars (Typhi, Enteritidis, Typhimurium, Paratyphi A), *E. coli*, *Sh. flexneri* and *Y. pestis* (Fig. 1a). Additionally, the three *Vibrio* species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) each encoded distinct *ftsZ* alleles, and amplification of a >1100 bp section with primers *ftsZF1/R2* (*V. cholerae*) and *ftsZF1/R1* (all other *Vibrio*) allowed for identification of species-specific variation. This genetically diverse region of *ftsZ* was sequenced for a panel of *Vibrio* spp., including *V. cholerae* (strain 93-0302), *V. parahaemolyticus* (04-1240), *V. vulnificus* (92-1751), *Vibrio alginolyticus* (86-148, 97-1598, MWG656), *Vibrio mimicus* (94-939, 86-852B), *Vibrio furnissi* (96-0200), *Vibrio fluvialis* (89-751) and *Vibrio metschnikovii* (81-66).

A total of 19 unique alleles of *ftsZ* were observed, and the genetic diversity and mutation types were calculated for a 785 bp region for which data were available for all strains. This segment represented 65 % of the complete *ftsZ* coding sequences (CDS) and encoded a total of 314 polymorphic sites (including 304 synonymous and 10 non-synonymous mutations). As an essential protein in cell division (Margolin, 2005; Errington *et al.*, 2003), adaptive mutation of *FtsZ* should be rare, and this was reflected in the low ratio of non-synonymous to synonymous mutations (dN/dS=0.03). Split decomposition analysis did not indicate recombination among *ftsZ* loci (data not shown). In comparison, a segment of the *hsp60* locus (556 bp) has previously been sequenced for 14 species (Kwok *et al.*, 2002), and in that dataset we calculated 180 polymorphic sites (of which 167 were synonymous).

RT-PCR identification of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*

Although the *V. cholerae* allele of *ftsZ* was similar to that of *V. mimicus*, and the *V. parahaemolyticus* allele was similar to that of *V. alginolyticus*, there was sufficient genetic diversity for the design of putatively species-specific primers. Targeting species-specific nucleotide polymorphisms in *ftsZ*, we developed RT-PCR assays employing LUX fluorogenic primer technology (Fig. 1c). RT-PCR was performed using template from a panel of *Vibrio* strains from Bangladesh and Canada, including representative type strains and clinical isolates from the major pathogenic species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*), as well as less common human *Vibrio* pathogens (*V. alginolyticus*, *V. fluvialis*, *V. furnissi*, *V. mimicus* and *V. metschnikovii*). The *V. cholerae* LUX primer set was successful in identifying all *V. cholerae* templates, with one exception (Table 1, Fig. 2a). All C_t values for the *V. cholerae* RT-PCR were consistently in the 16 to 19-cycle range (data not shown). *V. parahaemolyticus* and *V. vulnificus* LUX RT-PCR correctly identified all *V. parahaemolyticus* and *V. vulnificus* templates, respectively (Fig. 2b, c). Notably, there appeared to be two distinct groups of C_t values within the positive *V. vulnificus* RT-PCR results, one with C_t values of 18–19 and the other with C_t values of 25–28.

To confirm results from the LUX RT-PCR assay, we performed additional DNA sequencing of the *ftsZ* gene in selected *Vibrio* isolates. Only a single RT-PCR assay yielded an unexpected result, as *V. cholerae* ATCC 14033 was negative for *V. cholerae* RT-PCR. Sequencing of *V. cholerae* ATCC 14033 *ftsZ* found a 2 nt difference in the forward unlabelled primer sequence, which could prevent efficient primer binding and likely accounts for the negative result in *V. cholerae* RT-PCR. The two groups of C_t values observed in the *V. vulnificus* RT-PCR are likely due to the presence of nucleotide polymorphisms in the forward primer-binding region, as the sequenced templates from the late C_t value group had a 2 nt difference in the primer target sequence. Although there was a difference in C_t values among *V. vulnificus* templates, the *V. vulnificus* RT-PCR was still

effective in differentiating *V. vulnificus* from other *Vibrio* spp. Due to the requirements of LUX primer design, no other region in *ftsZ* offered an alternative target for *V. vulnificus* RT-PCR with this method. Intra-species allelic diversity has previously been observed for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Farfan *et al.*, 2002; Chowdhury *et al.*, 2004; Gutacker *et al.*, 2003), therefore it is not surprising that a single allele of *ftsZ* was not observed for all strains within each species in this study.

Liquid microsphere suspension arrays for *ftsZ*

Microsphere-based suspension arrays have previously been used for the molecular typing and detection of bacterial pathogens (Borucki *et al.*, 2005; Dunbar *et al.*, 2003; Gilmour *et al.*, 2006; Tracz *et al.*, 2006), and we investigated this technology as an additional technique for the allelic discrimination of *ftsZ* in *Vibrio* isolates. *Vibrio* probes were designed to target a region of *ftsZ* approximately 50 bp downstream of the LUX RT-PCR target sequences because of a species-specific polymorphism-rich region that could be covered by a single probe of ~20 oligonucleotides (Fig. 1c). The VC-DOB98 and VV-DOB100 probes were successful in hybridizing to all *V. cholerae* and *V. vulnificus* target DNA, respectively, with one exception (Table 1, Fig. 3). All *V. parahaemolyticus* isolates had positive hybridizations with the VP-DOB99 probe, which also produced positive or intermediate signals with all *V. alginolyticus* target DNA templates. Overall, excellent reproducibility of results with the microsphere suspension array was observed between repeat hybridizations on multiple days.

The Luminex microsphere assay produced several results that required sequence confirmation because of intermediate signals. The VV-DOB100 probe did not hybridize with *V. vulnificus* 90-208 on three separate occasions, and sequence analysis of the 90-208-encoded *ftsZ* allele showed an identical probe-binding region to that of other *V. vulnificus* templates that were positive in the microsphere assay. Additional factors must be influencing the VV-DOB100 probe–template hybridization, and may involve secondary structure or sequence outside the probe-binding region. Positive or intermediate signals were also produced with the VP-DOB99 probe from all *V. alginolyticus* templates, which were confirmed on multiple hybridization dates. Sequence analysis of these closely related *Vibrio* spp. revealed a 4–5 nt difference between *V. parahaemolyticus* and *V. alginolyticus* in the VP-DOB99 probe region. The presence of these polymorphisms would prevent optimal probe hybridization and likely accounts for the intermediate signal strength in these cases. Overall, positive hybridization results in the Luminex microsphere assay were clearly distinguishable from intermediate non-specific signals, and were useful in differentiating *Vibrio* spp.

Speciation of isolate 97-1598

Although they are distinct species of the genus *Vibrio*, it is not unusual for *V. parahaemolyticus* and *V. alginolyticus* to

Fig. 2. Allelic discrimination of species-specific polymorphisms in clinically important *Vibrio* species with *ftsZ* LUX RT-PCR targeting (a) *V. cholerae*, (b) *V. parahaemolyticus* and (c) *V. vulnificus*. Representative strains tested are indicated in the key; NTC, no template control. The horizontal line represents a threshold of 30 fluorescence units, the cut-off for positive signals (which are indicated with solid lines, whereas negative signals are indicated with dashed lines).

Fig. 3. Allelic discrimination of species-specific polymorphisms in selected isolates of clinically important *Vibrio* species using liquid microsphere suspension arrays. SEM is indicated by vertical lines on each bar. The ID of each strain is given in parentheses.

be incorrectly identified in the laboratory (Robert-Pillot *et al.*, 2002). Isolate 97-1598 was originally received by our laboratory as a *V. parahaemolyticus*; however, in the *V. parahaemolyticus* RT-PCR assay, this isolate was found to be negative, and sequencing of *ftsZ* determined that this allele was more similar to those encoded by *V. alginolyticus*. A PCR screen for the *toxR* gene (Kim *et al.*, 1999) was used to rule out isolate 97-1598 as a *V. parahaemolyticus*. Isolates 05-1000, 04-1240 and ATCC 17802 encoded the *V. parahaemolyticus toxR* determinant, whereas 97-1598 and two *V. alginolyticus* templates (86-148 and MWG656) were negative (data not shown). The results of this PCR test corroborate the results of the LUX primer assay and the sequence analysis for isolate 97-1598. Furthermore, our panel of *Vibrio* strains initially included an isolate of *Vibrio hollisae*, which was negative for all LUX RT-PCR and Luminex microsphere assays. However, this strain has been removed from the genus *Vibrio* and renamed *Grimontia hollisae* (Thompson *et al.*, 2003).

Advantages and disadvantages of assays targeting *ftsZ* polymorphisms

In this study, two novel assays were developed for the molecular typing of pathogenic *Vibrio*. The Luminex liquid microsphere assay involves a PCR that can amplify *ftsZ* from all clinically relevant *Vibrio* spp. and utilizes species-specific probes targeting the major *Vibrio* pathogens. Although the liquid microsphere assay did not have absolute sensitivity

and produced several intermediate signals, the results were useful in detection of *Vibrio* spp. Furthermore, the Luminex platform allows for the future design of additional probes to other *Vibrio* molecular targets and, in addition to speciation, may offer a powerful multiplex assay for identifying multiple species- and genus-specific virulence targets. The LUX RT-PCR assays were developed as singleplex reactions for each *Vibrio* sp., but offered many advantages, including speed, reproducibility and ease of primer design. The minimum time required to reach a positive signal in the RT-PCR method was 25 min from the point of template addition. All negative or late C_t signals in RT-PCR were accounted for by sequencing analysis of polymorphisms in the *ftsZ* primer-binding regions. The unique polymorphisms in *ftsZ* are excellent targets for molecular typing of clinically important *Vibrio* spp. and, overall, RT-PCR provided a more specific, rapid and cost-effective method for identifying *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.

ACKNOWLEDGEMENTS

Thank you to the DNA Core Facility at the National Microbiology Laboratory for DNA sequencing and oligonucleotide synthesis; to Connie Barton (NML) for thoughtful discussions; and to the Food Directorate, Health Canada (Ottawa), the International Centre for Diarrhoeal Disease Research (Bangladesh) and provincial laboratories of British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Quebec and New Brunswick for use of isolates. We would also like

to thank the genome sequencing centres that made their data publicly available, including the Sanger Institute and the University of Illinois at Urbana-Champaign.

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