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Evaluation of a highly discriminating multiplex multi-locus variable-number of tandem-repeats (MLVA) analysis for *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae is the etiological agent of cholera and may be used in bioterror actions due to the easiness of its dissemination, and the public fear for acquiring the cholera disease. A simple and highly discriminating method for connecting clinical and environmental isolates of *V. cholerae* is needed in microbial forensics. Twelve different loci containing variable numbers of tandem-repeats (VNTRs) were evaluated in which six loci were polymorphic. Two multiplex reactions containing PCR primers targeting these six VNTRs resulted in successful DNA amplification of 142 various environmental and clinical *V. cholerae* isolates. The genetic distribution inside the *V. cholerae* strain collection was used to evaluate the discriminating power (Simpsons Diversity Index = 0.99) of this new MLVA analysis, showing that the assay have a potential to differentiate between various strains, but also to identify those isolates which are collected from a common *V. cholerae* outbreak. This work has established a rapid and highly discriminating MLVA assay useful for track back analyses and/or forensic studies of *V. cholerae* infections.

1. Introduction

Vibrio cholerae is the etiological agent of cholera, a disease estimated to be responsible for over eleven million cases annually worldwide (Lanata et al., 2002). The latest large cholera outbreak, located in Zimbabwe, started in August 2008 and counted more than 73000 cases and 3500 deaths until February 2009 (WHO, 2009). More than 200 serogroups of *V. cholerae* are present, but only serogroup O1 and O139 are causing epidemic and pandemic cholera (Faruque et al., 1998). These serogroups generally harbour the genes encoding the cholerae toxin (CT) and toxin co-regulated pilus (TCP) which are considered the most important virulence factors. Strains of the O1 serogroup can be subdivided into serotypes Inaba, Ogawa or Hikojima. Each of these serotypes can be further categorized into a classical or an El Tor biotype based on biochemical properties. Seven large cholera pandemics have occurred since the 19th century (Sack et al., 2004), in which the seventh pandemic (El Tor biotype) started in 1961 in southern Asia and is still ongoing. The history of pandemic cholera, also in countries where cholera has disappeared, makes *V. cholerae* a potential agent for bioterrorism and biocrime.

Serological typing of *V. cholera* requires more than 200 different antisera for covering all known serogroups of this species (Salim et al., 2005). Since the 1980s molecular methods have been more common for identification and typing purposes of *V. cholerae* (Yam et al., 1989; Usera et al., 1994). Methods such as restriction fragment length polymorphism (RFLP) (Popovic et al., 1993), pulsed-field gel electrophoresis (PFGE) (Filetici et al., 1997), multi-locus sequence typing (MLST) (Byun et al., 1999; Kotetishvili et al., 2003), amplified fragment length polymorphism (AFLP) (Jiang et al., 2000), microarray (Dziejman et al., 2002; Vora et al., 2005; Pang et al., 2007) and repetitive-element PCR (rep-PCR) (Chokesajjawatee et al., 2008) have been used for genotyping purposes of *V. cholerae*. All of these methods have provided valuable contribution to the analysis of the genetic distribution of the *V. cholerae*. Nevertheless, some limitations exist when it comes to typing speed (MLST, rep-PCR, PFGE, microarray), portability (AFLP, PFGE) and high resolution (ribotyping) which are required in microbial investigations (forensics) related to bioterror actions.

Multi-locus variable-number of tandem-repeats analysis (MLVA) is a molecular method frequently applied for genotyping of prokaryotes, due to its simplicity and high discriminating power (Vergnaud and Pourcel, 2006; Lindstedt, 2005). Today, most medical relevant bacterial species, e.g. *Haemophilus influenzae*, *Staphylococcus aureus*, *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*, have been studied by this method (van Belkum 2007). MLVA is based on PCR analysis of tandemly repeated sequences in the bacterial genome, where specific primers are targeted the flanking sequences of several VNTR (variable-number of tandem-repeats) regions. Variations in

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number of repeats in each of the VNTRs generate PCR amplicons of varying fragment length. This size polymorphism is the basis for the generation of the various allele profiles. The MLVA results obtained in one laboratory may easily be compared with results obtained in other laboratories by exchange of allele profiles (string of figures) (Onteniente et al., 2003). Danin-Poleg et al. (2007) and Stine et al. (2008) have recently used MLVA to study the phylogeny and seasonal variation, respectively, in *V. cholerae*. However, in these studies the work was performed either on a limited number of strains or a geographically narrow collection of samples.

The aim of this study was to develop a multiplex high-resolution typing system for trace-back analyses of *V. cholerae* infections, based on polymorphism in six VNTR loci. The genetic distribution of 142 geographically diverse isolates of *V. cholerae* were analysed and evaluated by this MLVA scheme.

2. Materials and methods

2.1. Bacterial strains

Viable cells and DNA preparations of *V. cholerae* were kindly provided by Dr. Alessandra Caratolli (Department of Infectious Parasitic and Immuno-mediated Diseases, Istituto Superiore diSanita`, Rome, Italy), Dr. Herbert Tomaso (German Armed Forces, Institute of Microbiology, Munich, Germany), Dr. Gilles Vergnaud (Delegation Generale Pour L'armement, Centre d'ètudes du Bouchet, Paris, France), Cdt. Med. Carmen de YBARRA de Villavicencio (Unidad de Defensa Biológica Departamento NBQ Instituto Tecnológico la Marañosa, Madrid, Spain), Culture Collection, University of Göteborg (CCUG, Sweden), National Collection of Type Cultures (NCTC, United Kingdom), The Collection of Institut Pasteur (CIP, France) and Norwegian School of Veterinary Science (Fig. 1). Nucleotide sequence data were obtained from National Center for Biotechnology Information (NCBI) (Fig. 1).

Viable cells, DNA preparations and nucleotide sequence data were gathered to establish a strain collection of 142 various *V. cholerae* isolates (Fig. 1). The collection includes clinical and environmental strains isolated worldwide. In addition, twelve viable strains of other *Vibrio* species were included to check the *V. cholerae* primer specificity (5 *V. mimicus*, 3 *V. parahaemolyticus*, 2 *V. alginolyticus*, 1 *V. fluvialis* and 1 *V. metschnikovii*). Viable *Vibrio* cells were grown aerobically in tryptic soya broth (TSB) (Merck, Germany) at 37 °C overnight. Strain identification was verified using previously published *V. cholerae* specific primers (Fykse et al., 2007).

2.2. Extraction of DNA

A 10 ml *V. cholerae* culture was harvested by centrifugation at 5000 ×g for 10 min. Genomic DNA from the pellet was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the instructions given by the manufacturer. Extracted DNA was eluted in 250 µl TE buffer and stored at −20 °C until used as template in PCR. Samples were generally diluted 1:20 before used as template in PCR.

In order to further reduce the analytical time, the DNA extraction step was occasionally replaced by boiling a loopful of *V. cholerae* cells for 10 min followed by centrifugation (5000 ×*g* for 10 min) and use of the supernatant as PCR template.

2.3. DNA amplification

Multiplex PCR and real-time PCR reactions were performed in 15 and 20 µl, respectively, using 1.5 µl genomic DNA (10–20 ng of DNA) as template. The amplifications were carried out in the LightCycler® 480 System (Roche Diagnostics) using the LightCycler® 480 SYBR Green I Master kit (real-time PCR) and LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (multiplex PCR) according to recommendations given by the manufacturer. The optimal primer concentrations are stated in Table 1. The temperature program for the PCR reactions was as follows: 5 min initial denaturation at 95 °C followed by 35 or 25 cycles (real-time PCR or multiplex PCR, respectively) of denaturation at 95 °C for 10 sec, annealing at 58 °C for 10 s and primer extension at 72 °C for 30 s (25 s for real-time PCR). The amplifications were terminated after a final elongation of 5 min at 72 °C. The PCR fragments were verified by electrophoresis using the Bioanalyzer (Agilent Technologies, USA) prior to fragment analysis using capillary electrophoresis.

Real-time PCR screening was performed at DNA from all *V. cholerae* strains studied in order to evaluate the presence of genes encoding the cholerae toxin (*ctxA*) and the co-regulated pilus (*tcpA*) (Fykse et al., 2007).

2.4. In silico PCR

The allele profiles from twelve complete *V. cholerae* genome sequences were generated by performing *in silico* PCR (http://insilico.ehu.es/). Resulting *in silico* PCR fragments did not match our predefined allele- or size bins based on capillary electrophoresis (see section under) and therefore had to bee normalised. Thus, 3–6 different fragments from various VNTR loci were sequenced to reveal the real fragment length. The average numbers of basepair in difference between fragment lengths obtained by capillary electrophoresis and the real fragment length were calculated (sequencing and *in silico* PCR results in real size lengths) (see Supplementary data). These average numbers of basepair were subtracted from the *in silico* PCR fragment, and then adjusted to the closest size bin. To verify that the normalisation was correct, the number of repeats in the *in silico* fragments and corresponding size bins was counted and found to be identical (data not shown).

2.5. MLVA

In order to set up a 4MLVA application for *V. cholerae*, a search for potential VNTR candidates in the genomic sequence of *V. cholerae* O1 biotype El Tor, strain N-16961 was performed using bioinformatics resources (http://minisatellites.u-psud.fr/) with the following criteria: repeat length at least 6 bp and repeat sequences 100% conserved. Default settings were used for the remaining criteria. A total of 23 VNTR regions met the criteria. A one kb fragment covering the flanking sequences of 12 of these VNTR regions were applied for primer construction using the Primer3 software (Rozen and Skaletsky, 2000). Primer specificity and VNTR polymorphism were tested against twelve phylogenetic divers *V. cholerae* strains using real-time PCR. Six primers targeting polymorphic VNTRs were labelled in the 5'-end with the following fluorescent dyes and multiplexed into two solutions; multiplex 1: VC1-VIC, VC3-FAM and VC6-FAM and multiplex 2: VC4-NED, VC5-PET and VC9-FAM. One of each multiplex reaction mix was diluted 1:100 in sterile water. One µl of this dilution was further diluted 1:10 in HiDi formamide, containing the GeneScan™-500 LIZ size standard (Applied Biosystem), and analysed at the ABI prism® 310 Genetic Analyzer (Applied Biosystem). The samples were injected into the capillary at 15 kV voltages for 5 s and analysed for 28 min at 60 °C at 15 kV voltage using the POP4 separation polymer. The PCR amplicons were identified based on peak height and colour, and the fragment size was determined using GeneScan™-500 LIZ internal size standard (0.1 µl LiZ standard/12 µl HiDi formamide) and the GeneMapper v.3.0 software (Applied Biosystem). Different alleles or fragment sizes from each VNTR loci were identified and entered into the GeneMapper software (Applied Biosystem) as size bins. The size bins had an error range of +/−0.5 bp. If any product sizes were situated outside this interval, an error message was returned. Subsequent MLVA analysis of new samples was assigned allele profiles automatically using the GeneMapper software. The allele profiles were entered into BioNumerics version 5.1 software

Categorical
VNTR

Key	Code 1	Serotype	Genotype	Source	Location	Date/year	ctxA	tcpA	Allele size (bp)	Supplier
FFIVC039	024	non O1/O139	36	Uncooked scampi	Asia		-	-	197-115-214-179-201-152	5
FFIVC118	6/23	O1	37	Environment	Italy		-	-	220-115-214-170-201-152	1
FFIVC094	347	non O1/O139	38	Scampi	Bangladesh	2004-01-07	-	-	185-115-226-179-201-152	5
FFIVC098	359	non O1/O139	39	Mussel	Norway, Ryvika	2004-07-14	-	-	185-115-226-170-201-152	5
FFIVC097	358	non O1/O139	40	Mussels	Norway, Fedjane	2004-07-14	-	-	197-115-226-170-201-152	5
FFIVC109	503	non O1/O139	41	Water	Norway, Spirabukta	2004-08-18	-	-	175-115-197-179-201-152	5
FFIVC110	504	non O1/O139	41	Water	Norway, Spirabukta	2004-08-18	-	-	175-115-197-179-201-152	5
FFIVC084	246	non O1/O139	42	Mussel	Norway, Kue-Prestholmen	2003-03-31	+/-	+/-	175-115-274-170-201-152	5
FFIVC047	069	non O1/O139	43	Food	Asia		-	-	162-115-191-161-201-152	5
FFIVC103	5/31	non O1/O139	44	Environment	nd		-	-	162-115-209-170-201-152	1
NCTC8457	-	O1	45	Human	Saudi Arabia	1910	-	-	162-115-226-187-201-249	8
FFIVC055	198	non O1/O139	46	Mussel	Norway, Vallø	2001-08-28	-	-	180-115-220-179-201-152	5
FFIVC123	Vib2	O1	47	Human, faeces	Spain, Cordoba		+	+	180-115-250-179-201-152	4
FFIVC025	01/0177	O1	48	Human	Rwanda, Goma	1994	+	+	180-115-305-179-201-187	3
FFIVC011	B930	non O1/O139	49	Human, blood	Austria, Lake Neusiedl	2005-07-04	-	-	138-115-250-179-201-207	2
FFIVC036	018	non O1/O139	50	Shrimps	Thailand		-	-	169-115-232-205-201-201	5
FFIVC096	354	non O1/O139	51	Shrimps	Asia		-	-	169-115-263-170-201-194	5
FFIVC035	014	non O1/O139	52	Marinaramix	Thailand		-	-	169-115-274-148-201-152	5
FFIVC066	2/8	non O1/O139	53	Environment	Italy		-	-	169-115-232-152-201-152	1
FFIVC089	3/2	non O1/O139	54	Environment	nd		-	-	169-115-203-161-201-152	1
FFIVC072	232	non O1/O139	55	Human	nd		-	-	169-115-250-179-201-152	5
FFIVC074	234	non O1/O139	56	Human, faeces	nd		-	-	169-115-250-119-201-152	5
FFIVC016	NCTC8021	O1	91	Human	nd		+	+	175-115-293-152-201-159	7
FFIVC095	348	non O1/O139	92	Scampi	Bangladesh	2004-01-07	-	-	175-115-209-152-201-159	5
FFIVC121	99/0004	O395	93	Human	nd		+	+	169-115-274-152-201-159	3
O395	-	O1	93	Human	nd		+	+	169-115-274-152-201-159	8
FFIVC013	B932	non O1/O139	94	Human, stool	Turkey	2005-08-31	-	-	197-115-179-152-220-159	2
FFIVC076	236	non O1/O139	95	Human, faeces	nd		-	-	162-115-209-152-220-159	5
FFIVC053	161	non O1/O139	96	Mussel	Norway, Lennavika	2002-10-07	-	-	175-115-203-152-220-152	5
FFIVC101	4/76	O111	97	Human, septicemic	Italy		-	-	175-115-238-152-201-152	1
FFIVC021	002	non O1/O139	98	Food	Asia		-	-	151-115-209-152-198-152	5
FFIVC052	134	non O1/O139	99	Mussel	Norway		-	+/-	180-115-280-152-201-152	5
FFIVC106	5/47	O2	100	Human, septicemic	Italy		-	-	203-115-280-152-201-152	1
FFIVC009	B928	non O1/O139	15	Human, ear	Austria, Lake Neusiedl	2004-08-27			197-115-244-161-201-214	2
FFIVC054	177	non O1/O139	16	Mussel	Norway, Tømmervika	2002-11-18	-	-	203-115-244-161-201-173	5
FFIVC086	285	non O1/O139	17	Mussel	Norway, Ryvika	2003-07-28	-	-	203-115-293-161-201-152	5
FFIVC087	286	non O1/O139	17	Mussel	Norway, Ryvika	2003-07-28	-	-	203-115-293-161-201-152	5
FFIVC019	00/0065	non O1/O139	18	Fish	Germany, Elbe River		+	+	191-115-232-161-201-270	9
FFIVC078	238	non O1/O139	19	Human, faeces	nd		-	-	191-115-226-161-201-187	5
MZO-2	-	O12	20	Human	Bangladesh	2001	-	-	192-115-220-161-201-152	8
FFIVC070	230	non O1/O139	21	Human, faeces	nd		-	-	208-115-226-161-220-180	5
FFIVC093	344	non O1/O139	22	Mussel	Norway		-	-	208-115-280-161-201-201	5
FFIVC044	047	non O1/O139	23	Food	Asia		-	-	185-115-280-161-238-152	5
FFIVC137	NVH3858/92	non O1/O139	24	Human	Norway		-	-	185-115-232-161-220-152	5
FFIVC041	037	non O1/O139	25	Food	Asia		-	-	169-115-232-161-238-159	5
FFIVC071	231	non O1/O139	26	Human	nd		-	-	185-115-214-161-238-159	5
FFIVC049	086	non O1/O139	1	Shrimps	Asia		-	-	208-115-220-170-201-159	5
FFIVC100	4/57	O1	2	Environment	Italy		-	-	169-115-220-142-201-159	1
FFIVC004	B923	non O1/O139	3	Human, stool	Thailand	2001-03-07	-	-	203-115-220-179-201-187	2
FFIVC006	B925	non O1/O139	4	Human, ear	nd	2001-07-31	-	-	203-115-220-179-201-159	2
FFIVC037	021	non O1/O139	5	Uncooked scampi	Asia		-	-	175-115-250-161-201-159	5
FFIVC129	CCUG9120	O1	6	nd	Spain		+	+	180-115-168-161-201-159	6
FFIVC088	293	non O1/O139	7	Mussel	Norway, Vallø	2003-08-04	-	-	203-115-214-161-201-159	5
FFIVC080	240	non O1/O139	8	Human, faeces	nd		-	-	203-115-250-196-201-159	5
FFIVC077	237	non O1/O139	9	Human, faeces	nd		-	-	180-115-244-240-201-159	5
FFIVC048	084	non O1/O139	28	Scampi	Asia		-	-	203-115-209-170-201-166	5
RC385	-	O135	29	Environmental	USA, Chesapeake Bay		-	-	198-115-185-170-201-166	8
FFIVC014	B933	non O1/O139	30	nd	nd		-	-	232-115-209-196-201-166	2
FFIVC024	006	non O1/O139	31	Food	Asia		-		175-115-209-179-201-166	5
FFIVC040	027	non O1/O139	32	Scampi	Bangladesh		-	-	232-115-238-179-201-166	5
FFIVC028	01/0180	O1	33	Human	Rwanda, Goma	1994	+	+	191-115-214-142-220-166	3
FFIVC104	5/42	O158	34	Human, septicemic	Italy		-	-	180-115-299-161-220-166	1
FFIVC075	235	non O1/O139	35	Human, faeces	nd		-	-	156-115-197-196-220-166	5
2740-80	-	non O1/O139	64	Environmental	USA, Gulf coast		-	-	192-115-263-187-201-173	8
FFIVC018	00/0064	non O1/O139	65	nd	nd		+	+	191-115-203-187-220-173	9
FFIVC034	01/0188	O1	66	Human	Rwanda, Goma	1994	+	+	180-115-299-187-201-173	3
FFIVC099	4/53	O1	67	Human	Italy	1973	+	+	180-115-214-187-201-173	1
MAK757	-	O1	68	Human	nd		+	+	186-115-214-187-201-173	8
FFIVC069	229	non O1/O139	112	Human	nd		-	-	185-115-209-142-201-173	5
FFIVC136	NVH2276/92	non O1/O139	113	Human	Norway		-	-	185-115-256-205-201-173	5
FFIVC043	046	non O1/O139	69	Food	Asia		-	-	203-115-263-170-238-173	5
FFIVC045	048	non O1/O139	70	Shrimps	India		-	-	175-115-214-170-238-173	5
FFIVC032	01/0186	O1	71	Human	Rwanda, Goma	1994	+	+	180-115-293-187-201-187	3
FFIVC033	01/0187	O1	71	Human	Rwanda, Goma	1994	+	+	180-115-293-187-201-187	3
FFIVC029	01/0181	O1	72	Human	Rwanda, Goma	1994	+	+	180-115-250-187-201-187	3
FFIVC026	01/0178	O1	73	Human	Rwanda, Goma	1994	+	+	180-115-299-187-201-187	3
FFIVC030	01/0183	O1	73	Human	Rwanda, Goma	1994	+	+	180-115-299-187-201-187	3
FFIVC031	01/0185	O1	73	Human	Rwanda, Goma	1994	+	+	180-115-299-187-201-187	3
FFIVC015	CIP106855	O1	74	Human, stool	Bangladesh	1971	+	+	180-115-274-187-201-180	9
N16961	CIP106855	O1	74	Human	Bangladesh	1971	+	+	180-115-274-187-201-180	8
FFIVC107	5/64	non O1/O139	59	Environment	nd		-	-	180-115-203-142-201-173	1
FFIVC134	CCUG9122	O1	60	Human	Mecca, Saudi Arabia		+	+	180-115-203-142-201-152	6
FFIVC081	241	non O1/O139	57	Human, faeces	nd		-	-	180-115-250-142-201-180	5
FFIVC090	328	non O1/O139	61	Mussel	Norway, Hvaler	2003-08-31	-	-	180-115-197-142-201-152	5
FFIVC091	329	non O1/O139	61	Mussel	Norway, Hvaler	2003-08-31	-	-	180-115-197-142-201-152	5
FFIVC092	330	non O1/O139	61	Mussel	Norway, Hvaler	2003-08-31	-	-	180-115-197-142-201-152	5
FFIVC046	061	non O1/O139	63	Food	Asia		-	-	203-115-238-142-201-152	5
MZO-3	-	O37	58	Human	Bangladesh		-	-	180-115-238-142-201-201	8
FFIVC002	B921	non O1/O139	75	Human, ear	nd	2000-12-13	-	-	162-115-269-142-183-201	2
FFIVC068	216	non O1/O139	76	Barracuda	India		-	-	162-115-209-161-183-159	5
623-39	-	non O1/O139	77	Human			-	-	162-115-220-152-183-166	8
FFIVC042	045	non O1/O139	78	Food	Asia		-	-	162-115-220-152-201-180	5
FFIVC050	110	non O1/O139	79	Mussels	Norway		-	-	185-115-238-179-183-159	5
FFIVC051	117	non O1/O139	80	Mussel	Norway		-	-	175-115-263-179-183-159	5
FFIVC126	Vib8	non O1/O139	81	Human, faeces	Spain, Madrid		-	-	175-115-244-220-183-159	4
1587	-	O12	82	Human	Peru				185-115-274-205-201-180	8
FFIVC105	5/45	non O1/O139	83	Environment	nd		-	-	203-115-220-187-183-152	1
FFIVC005	B924	non O1/O139	84	Human, ear	nd	2001-05-11	-	-	180-115-256-158-183-152	2
FFIVC062	2/6	non O1/O139	85	Environment	nd		-	-	185-115-185-170-198-152	1
FFIVC079	239	non O1/O139	86	Human, faeces	nd		-	-	185-115-263-170-183-152	5
FFIVC022	004	non O1/O139	87	Food	Asia		-	-	169-115-203-196-183-152	5
FFIVC023	005	non O1/O139	88	Food	Asia		-	-	169-115-203-152-183-180	5
FFIVC102	5/10	non O1/O139	89	Environment	nd				169-115-185-179-183-152	1
FFIVC073	233	non O1/O139	90	Human, faeces	nd		-	-	169-115-214-170-183-249	5
FFIVC111	518	non O1/O139	115	Water	Norway, Holmenskjæret	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC113	552	non O1/O139	115	Water	Norway, Høvikodden	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC114	553	non O1/O139	115	Water	Norway, Høvikodden	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC115	570	non O1/O139	115	Water	Norway, Kalvøya	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC116	571	non O1/O139	115	Water	Norway, Kalvøya	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC117	572	non O1/O139	115	Water	Norway, Kalvøya	2004-08-11	-	-	180-115-203-170-198-166	5
FFIVC120	614	non O1/O139	115	Water	Norway, Fiskevollbukta	2004-08-10	-	-	180-115-203-170-198-166	5
FFIVC008	B927	non O1/O139	116	Human, stool	Tunisia	2002-10-11	-	-	180-115-203-187-198-214	2
FFIVC067	211	non O1/O139	117	Mussel	Norway, Vollen	2001-07-30	-	-	169-127-250-170-198-152	1
FFIVC085	250	non O1/O139	118	Oysters	Norway, Vågstrandpollen	2003-05-20	-	-	180-127-250-170-198-152	5
FFIVC122	Vib1	O1	106	Human, faeces	Guinea Ecuatorial, Bata		+	+	175-115-214-179-201-180	4
FFIVC124	Vib3	O1	106	Human, faeces	Guinea Ecuatorial, Bata		+	+	175-115-214-179-201-180	4
FFIVC125	Vib4	O1	106	Human, faeces	Guinea Ecuatorial, Bata		+	+	175-115-214-179-201-180	4
FFIVC119	6/31	O1	107	Human	Italy	2005	+	+	175-115-280-179-201-180	1
FFIVC020	00/0143	nd	110	Food	nd		+	+	185-115-232-179-201-180	3
FFIVC108	5/77	O1	111	Human	Peru	1991	+	+	185-115-226-179-201-180	1
FFIVC131	CCUG34708	O139	108	Human, faeces	USA, California		+	+	185-115-269-205-201-180	6
MO10	-	O139	108	Human	India /Bangladesh outbreak	1992	+	+	186-115-269-205-201-180	8
FFIVC001	-	O139	114	nd	nd		+	+	185-115-274-205-201-180	2
FFIVC128	Vib10	O139	114	Human	Spain		+	+	185-115-274-205-201-180	4
FFIVC130	CCUG34707	O139	114	Human	India, Madras		+	+	185-115-274-205-201-180	6
FFIVC132	CCUG47460	O139	114	nd	nd		+	+	185-115-274-205-201-180	6
FFIVC133	CCUG47461	O139	114	nd	nd		+	+	185-115-274-205-201-180	6
FFIVC003	B922	non O1/O139	10	Human, ear	Austria, Lake Neusiedl	2000-12-29	-	-	185-115-185-196-201-152	2
FFIVC012	B931	non O1/O139	11	Human, pharynx,	Austria, Lake Neusiedl	2005-08-04	-	-	185-115-197-196-201-207	2
V52	-	O37	12	Human	Sudan		+	+	198-115-293-196-201-187	8
FFIVC007	B926	non O1/O139	13	Human, stool	Tunisia	2002-08-29	-	-	180-115-226-196-198-159	2
FFIVC082	242	O1	14	Human, faeces	nd		+	+	180-115-226-196-201-180	5
FFIVC056	2/21	O1	101	Human	Italy	1994	+	+	175-115-209-196-201-180	1
FFIVC135	CCUG19445	O1	102	Human, faeces	Tunisia		+	+	175-115-274-196-201-180	6
FFIVC065	2/70	O1	103	Human	Albania	1994	+	+	175-115-226-196-201-180	1
FFIVC083	243	O1	104	Human, faeces	nd		+	+	175-115-250-196-201-180	5
FFIVC057	2/23	O1	105	Human	Italy	1994	+	+	175-115-203-196-201-180	1
FFIVC058	2/26	O1	105	Human	Italy	1994	+	+	175-115-203-196-201-180	1
FFIVC059	2/32	O1	105	Human	Italy	1994	+	+	175-115-203-196-201-180	1
FFIVC061	2/57	O1	105	Human	Albania	1994	+	+	175-115-203-196-201-180	1
FFIVC063	2/60	O1	105	Human	Albania	1994	+	+	175-115-203-196-201-180	1
FFIVC064	2/67	O1	105	Human	Albania	1994	+	+	175-115-203-196-201-180	1

Fig. 1. Genetic distribution of 142 strains of *V. cholerae* deduced from MLVA analysis. The dendrogram was constructed using categorical coefficients and the WARD algorithm. Code1: alias from other institutions, nd: not determined, +: PCR amplification, −: no PCR amplification, +/−: PCR amplification but weak band. Supplier: strains were obtained from; Department of Infectious Parasitic and Immuno-mediated Diseases, Istituto Superiore diSanita` (1), German Armed Forces, Institute of Microbiology (2), Delegation Generale Pour L'armement, Centre d'ètudes du Bouchet (3), Unidad de Defensa Biológica Departamento NBQ Instituto Tecnológico la Marañosa (4), Norwegian School of Veterinary Science (5), CCUG (6), NCTC (7), NCBI (8) and CIP (9).

Table 1
Primer sequence, repeat sequence, number of alleles, primer concentrations and diversity index at six polymorphic loci in 142 *V. cholerae* strains.

Primer	Primer sequence (5′→3′)	Tandem-repeat sequence	VNTR position[a]	Target gene or product	Primer concentration (μM)	Repeat variation	
						No. of alleles	DI[b]
VC1-f/r	f: CGGATACTCAAACGCAGGAT r: CTTTCGGTCGGTTTCTCTTG	AACAGA	137029	Cell division protein. *ftsY*	0.2	14	0.86
VC3-f/r	f: CATCAGCCCCAAATAACCAG r: GATGGCGTCAAGAGTGGAAT	TTTCTGCTCTTT	2162771	Hypothetical protein	0.2	2	0.03
VC4-f/r	f: TGTTTGAGAGCTCGCCTCTT r: TCATCAAGATGCACGACACA	TGCTGT	187661	Hypothetical protein	0.5	22	0.94
VC5-f/r	f: AGTGGGCACAGAGTGTCAAA r: AATTGGCCGCTAACTGAGTG	GATAATCCA	1778432	Collagenase	0.2	13	0.88
VC6-f/r	f: GGGAGCACCTTGTTATTTGG r: AACTGACCGACTTTGGGTTG	TCGAGTAGTAAAACAGACC	2492060	Intergenic regions	0.2	5	0.47
VC9-f/r	f: CGTTAGCATCGAAACTGCTG r: AGAAAACAATCGCCTGCTTG	GACCCTA	467017	Intergenic regions	0.4	12	0.83
ctxA-f/r	f:AGAAGGTGGGTGCAGTGGCTATAACA r: TGATCATGCAAGAGGAACTCA		–	*ctxA*	1.0	–	–
tcpA-f/r	f: CGCTGAGACCACACCCATA r:GAAGAAGTTTGTAAAAGAAGAACACG		–	*tcpA*	1.0	–	–
groEL-f/r	f: ATGATGTTGCCCACGCTAGA r: GGTTATCGCTGCGGTAGAAG		–	*groEL*	1.0	–	–

[a] Position refers to *V. cholerae* strain N-16961.
[b] DI, Simpsons Diversity Index.

(Applied-Maths, Belgium) as character values, and a dendrogram was constructed using the categorical coefficient and the WARD algorithm. A minimum spanning tree (MST) (BioNumerics software) was constructed using the highest number of single locus variants (SLVs) as priority rule (in case of two equal calculated trees in terms of distance, the tree with the highest number of branches connecting genotypes differing only in one locus should be selected). No creation of hypothetical types (missing links) was allowed.

The discrimination power of the MLVA assay was established calculating the Simpson's index of diversity (Simpson, 1949; Hunter and Gaston, 1998) for individual and a combined set of VNTR markers using the BioNumerics software. The script for calculating the diversity index for the combined set of VNTR loci was kindly provided by Dr. Gilles Vergnaud (the MLVA group, University of Paris South, Orsay, France).

2.6. Sequencing

PCR products for DNA sequencing were purified using the ExoSAP-IT® purification kit (USB, USA). Purified PCR products were eluted in 20 μl of sterile water and DNA concentrations were determined at 260 nm using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technology, USA). DNA sequencing was performed by using the BigDye® Terminator v.3.1 Matrix Standard Kit (Applied Biosystem, USA) in accordance with the manufacturer's manual, but with the following modifications; all reaction volumes were reduced by 50%, the samples were not vortexed during the washing step and the final single stranded (ss) DNA pellet was air dried for 45 min at room temperature. The DNA sequence of both ssDNA strands of the PCR product was determined. All sequences obtained in this study were edited by using the Staden package (Staden, 1996). Sequence alignments were performed by using clustalW as part of the MEGA4 software (Tamura et al., 2007).

3. Results

3.1. MLVA assay

In this study, a rapid and highly discriminating MLVA assay for genotyping of *V. cholerae* has been developed; primarily to enable investigation of cholera outbreaks related to future bioterror incidents or regular trace-back analyses of *V. cholerae* infections. A total of 142 *V. cholerae* isolates were examined for evaluation of this new typing scheme. Of twelve potential polymorphic VNTRs tested, six loci (VC1, VC3, VC4, VC5, VC6 and VC9) were identified to be polymorphic and used in our MLVA assay (Table 1). These loci were situated in four different genes and two intergenic regions in chromosome II of the *V. cholerae* genome (Table 1). Six primer pairs, flanking these VNTR regions, resulted in amplification of DNA from all tested *V. cholerae* strains, but resulted in equivocal PCR amplification of twelve closely related *Vibrio* species (data not shown).

The GeneScan-500 LIZ standard used generated peaks from 35 to 500 bp, which covered all fragment sizes resulting from the six VNTR loci. In each multiplexed PCR reaction, three amplicons were obtained, and these were easily differentiated based on colour and peak high in the electropherograms (Fig. 2). The size distribution of the PCR amplicons were 138–232 bp (VC1), 115–127 bp (VC3), 168–305 bp (VC4), 119–240 bp (VC5), 183–238 bp (VC6) and 152–270 bp (VC9). Nucleotide sequencing of the six amplified loci from various *V. cholerae* isolates confirmed that size polymorphisms were due to variation in repeat copy numbers (Fig. 3). However, a deviation was observed in isolates FFIVC005, 035 and 126 where an intermediate VC5 fragment size was obtained. This deviation can be explained by an insert of six nucleotides 16 bp upstream of the VNTR region (Fig. 3).

The time consumption of MLVA analysis on one *V. cholerae* isolate was estimated to be less than 5 h, and included DNA extraction 2.5 h (incubation time for protease K, 2 h), PCR reaction 60 min, fragment analysis 2×28 min, time for setting up the PCR reaction mix and preparation of the PCR products for fragment analysis. This indicates a fast assay for establishing a genetic fingerprint compared to methods such as MLST and PFGE (van Belkum et al., 1998).

A high level of polymorphism was observed among the 142 *V. cholerae* isolates using the novel MLVA assay (Table 1). In total, 115 distinct allele profiles or genotypes were identified with an allele variation from two (VC3) to 22 (VC4) (Table 1). Simpson's index of diversity of the various VNTR loci ranged from 0.47 to 0.94, except for 0.03 at VC3, indicating a high discriminating ability in individual loci. These figures were consistent or slightly higher than values reported by Danin-Poleg et al. (2007). Their work included 32 *V. cholerae* strains, and this limited number of isolates may have affected the individual diversity index values. The diversity index for a combined set of the VNTR locus was calculated to be 0.99, indicating that the MLVA method has a highly discriminating power. The diversity index of the MLVA scheme reported herein indicates a 99% probability for two randomly sampled *V. cholerae* isolates to be separated into two different genotypes.

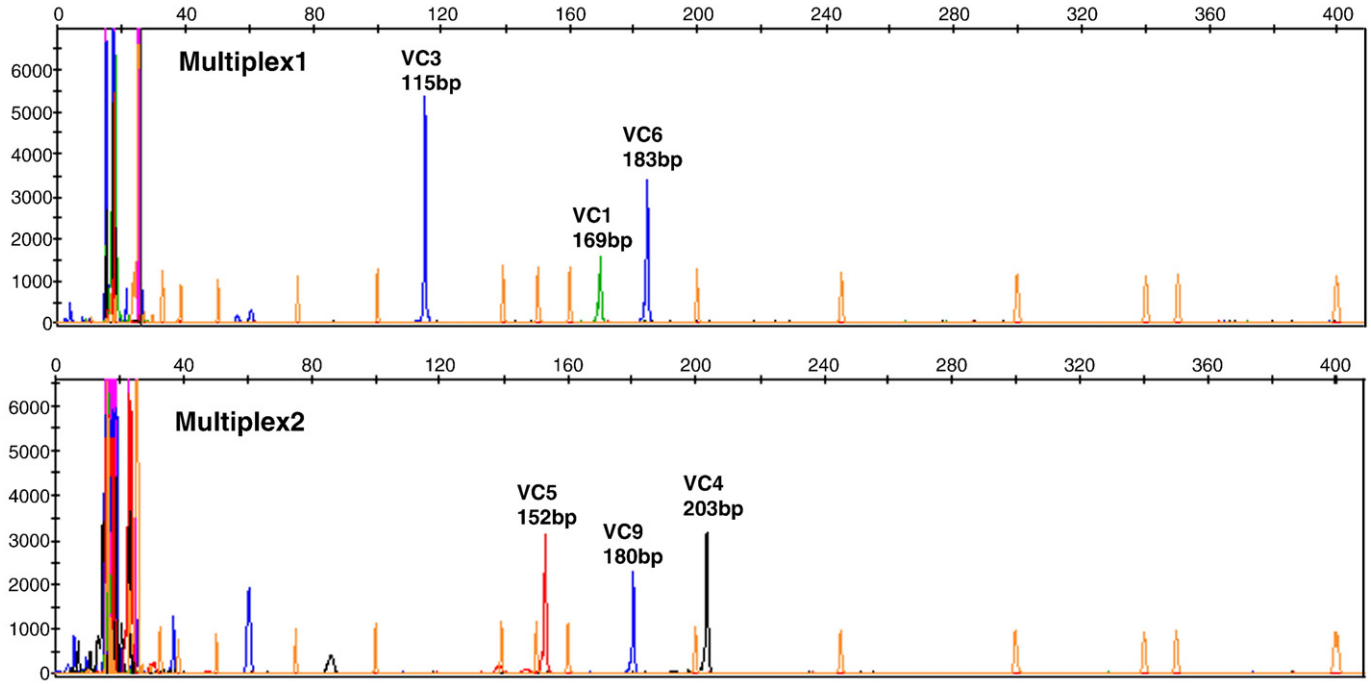

Fig. 2. Electropherogram showing separation of six PCR fragments from multiplex 1 and 2 defined by peak high and colour. Fragments are obtained from isolate FFIVC023. The lower peaks are from the GeneScan™-500 LIZ internal size standard.

3.2. In silico PCR

In order to include VNTR data available from complete *V. cholerae* genomes at NCBI, *in silico* generated PCR fragments were obtained for these strains. None of these fragments matched our pre-defined allele- or size bins derived from capillary electrophoresis. They were all a few basepair longer than the size bins assigned in the GeneMapper software. Deviations in fragment sizes obtained by using different methods (agarose gel, sequencing, capillary electrophoresis or use of various internal standards in the capillary electrophoresis) have previously been reported (Nederbragt et al., 2008). In order to avoid erroneous comparisons, caution should therefore be taken when PCR fragment determination is performed by various methods.

3.3. Phylogenetic analyses

A cluster analysis of the allele profiles from MLVA typing of 142 various *V. cholerae* isolates resulted in a dendrogram where the strains were divided into five larger clusters (Fig. 1). Cluster A consisted mainly of *V. cholerae* O1 and O139 strains, with five exceptions (FFIVC003, 007, 012, 020 and V52). Of these, only V52 is *ctxA* and *tcpA* positive (Fig. 1). Eight O1 strains from cluster A were isolated during an outbreak in Albania/Italia in 1994 (FFIVC056–059, 061 and 063–065), where six strains expressed identical allele profile. The O139 strains were differentiated in locus VC4 with only one repeat in difference. None of the non-O1/O139 strains in cluster A turned out to have more than three markers in common with the O1 or O139 strains. Cluster B3 is a cluster containing 32 different *V. cholerae* strains where 13 isolates are of serogroup O1. A small subcluster of eight O1 serogroup strain from an outbreak in Rwanda was differentiated only in the VC4 locus. This subcluster included the N16961 type strain, and differed only in VC4 and VC9 compared to the Rwanda strains.

Clusters B1, B2 and B4 contained mainly clinical and environmental non-O1/O139 isolates from different geographical location (Fig. 1). Only the B4 cluster included representatives of the serogroup O1 (FFIVC016, 025, 100, 118, 121, 123, 129 and NCTC8457). These three clusters represented a considerable number of different genotypes, which is not surprising since about 40% of the *V. cholerae* isolates included in the present study originated from the environment or marine animals, and thus reflect the environmental reservoir (Jiang et al., 2000). Surprisingly small genetic differences were observed between certain human O1 isolates and non-O1/O139 isolates with environmental origin. This can be exemplified by the finding of the difference in the VC4 locus (Fig. 4) of the FFIVC055 strain (genotype 46) isolated from mussels in Norway and the FFIVC123 strain (genotype 47), the latter an O1 Inaba strain isolated from human faces in Spain. However, the same genetic distance was identified between FFIVC090–091 and FFIVC107 (environmental strains) and the serogroup O1 isolate FFIVC134 (Fig. 4). These results support the paper by Karaolis et al. (1995) suggesting that toxigenic *V. cholerae* strains may be derived from environmental non-O1/O139 serogroup strains.

Twelve genotypes were repeatedly observed among the isolates analyzed. These genotypes represented clinical isolates sampled from

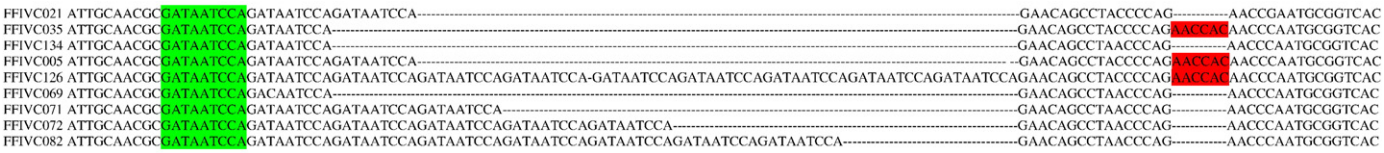

Fig. 3. Alignment of VNTR locus VC5 and respectively flanking sequence from nine various *V. cholerae* strains. The repeat motif GATAATCCA (green) constitute the size variation among the isolates. The intermediate fragment size observed in strain FFIVC005, 035 and 126 can be explained by an insert of 6 bp (marked in red) located 16 bp upstream of the VNTR region.

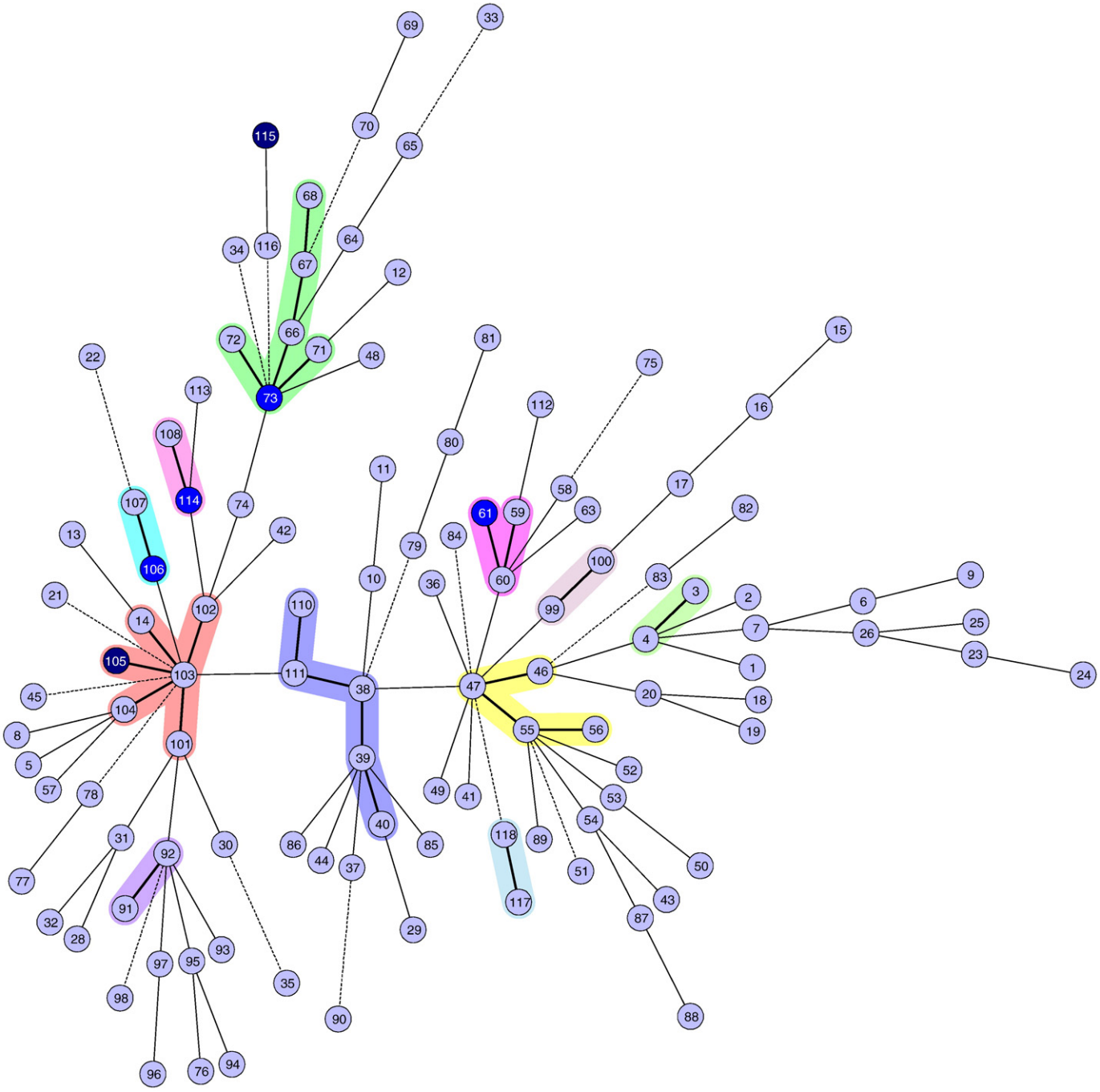

Fig. 4. The relationships between 115 MLVA genotypes identified among 142 isolates of *V. cholerae* using minimum spanning tree analysis. The numbers in the circles represent MLVA genotype. The thickness of the lines indicates the genetic distance; solid, thin and dotted lines represent single, double and triple or more locus variation, respectively.

various cholera outbreaks; Italia/Albania 1994 (strain FFIVC057–059, 061 and 063–064), Rwanda 1994 (FFIVC026 and 029–033), and Guinea Equatorial (FFIVC122 and 124–125) (Fig. 1), and environmental isolates from the same geographical location during the same time period (e.g. FFIVC111, 120 and 113–117) (Fig. 1). In contrast, FFIVC056 and FFIVC065 are not of the same genotype as the remaining isolates from the Italy/Albania outbreak but differ in one (FFIVC056) and four (FFIVC065) repeats in locus VC4 (Fig. 1). This is not consistent with previous RAPD or PFGE analyses, which showed that isolates from this particular outbreak all belonged to the same genotype (Pazzani et al., 2006). Similar results were observed for the Rwanda isolates (FFIVC029 and 032–33) and the O139 strains (FFIVC131 and MO10). By deleting the VC4 locus from the MLVA assay, only one genotype would be obtained from the Italian/Albania outbreak strains or strains of the O139 serogroup. These results showed that the VC4 locus is highly variable, and may have the ability to change the repeat copy number during the timescale of a cholera outbreak. This mechanism has earlier been identified among *Escherichia coli* isolates from epidemiologically well-characterized outbreaks in America (Noller et al., 2003).

As a complementary analysis to the clustering analysis (Fig. 1), a minimum spanning tree (MST) was constructed (Fig. 4). When a set of genetic distances is given for a number of samples, a MST analysis will generate a tree where all samples are connected in a way where the sum of distances from all branches is minimized. From a biological perspective, this signifies that evolution could be explained through as few events as possible. The lack of clonal complexes (few star-like

clusters of SLVs) in MST confirms the large genetic heterogeneity observed by cluster analysis. The toxigenic O1 and O139 strains in cluster A and B3 in Fig. 1 are organised to the left or upper left in the MSP tree. The more distantly related O1 isolates in cluster B4 are organised outside this area (genotype 2, 6, 37, 47, 91 and 93). There are two exceptions, NCTC8457 and FFIVC025, which clustered with genotype 103 (strain FFIVC065) and genotype 73 (three strains from Rwanda), respectively. The environmental O1 isolates from Italy and the O1 Hikojima strain clustered far from the serogroup O1 complex around serogroup 103. Interestingly, a close relationship was observed between the Norwegian clinical non-O1/O139 strain FFIVC136 (genotype 113) and the O139 isolates representing genotype 114 (Fig. 4), seeing that the *V. cholerae* O139 serogroup is mainly endemic in South-East Asia. This connection was not seen by cluster analysis.

4. Discussion

Fecal-contaminated food or water is the usual transmission route for classical cholera, an acute life-threatening diarrhoeal disease manifested by clear watery stools often referred to as rice water stool. Based on Centers for Disease Control and Prevention list of bioterrorism agents, *V. cholerae* is defined as a category B agent (Rotz et al., 2002), which implies a potential role of *V. cholerae* in biological terror scenarios. It is not necessarily a mass casualty agent, but rather a potential agent which can cause public fear and social disruption. In this context, it is important to be able to identify the source of infection as soon as possible in order to start adequate treatment of patients, preventive measures and initiation of outbreak investigation. The current work presents an MLVA assay for genotyping *V. cholerae* that will prove useful in epidemiological trace-back analysis or microbial forensic studies of this agent.

The MLVA protocol described herein was performed on a total of 142 clinical, environmental and geographically diverse isolates of *V. cholerae*. The assay included multiplex amplification of short specific products (115–305 bp) and unambiguous fragment size determination in an automatic sequencer. These features provide a highly discriminating MLVA method with a Simpson's index of diversity of 0.99. The current MLVA assay can be performed in less than 5 h. In order to further reduce the analytical time, the DNA extraction step was occasionally replaced by simply lysing the *V. cholerae* cells by boiling. This procedure was tested and resulted in reliable PCR amplification and a reduction of the analytical time to approximately 3 h (data not shown). Compared to more time demanding (MLST, PFGE, MLEE) or less inter-laboratory comparable methods (AFLP) (van Belkum et al., 1998; Chokesajjawatee et al., 2008: Lindstedt, 2005), we believe that this MLVA method is efficient, rapid, reliable and very convenient for laboratories performing epidemiological investigation of *V. cholerae* outbreaks. A disadvantage of the MLVA analysis is the high costs associated with the use of capillary electrophoresis and real-time PCR platforms, especially to laboratories with low economic budgets. Regardless, the MLVA method may be performed using cheaper standard endpoint PCR and agarose gel electrophoresis as long as a the PCR reactions are thoroughly examined, and a strictly evaluated allele standard is used in fragment size determination (Vergnaud and Pourcel, 2006, Chokesajjawatee et al., 2008).

A central part in microbial forensic is to perform a proper outbreak identification. A selection of the isolates in our strain collection was connected to known cholera outbreaks. This relationship was revealed by a common allele profile, or a highly related profile, for isolates from the Italia/Albania, Rwanda, and Guinea Equatorial outbreaks (Fig. 1). The Italia/Albania outbreak strains have previously been genotyped by PFGE and RAPD, resulting in no differentiation of 110 *V. cholerae* isolates (Pazzani et al., 2006). In contrast, the present results indicate that at least three different genotypes were present in this outbreak (Fig. 1). Danin-Poleg et al. (2007) demonstrated that the repeat copy number in two VNTR loci was stable over a 4-day period. However, our results indicate that the VNTRs in *V. cholerae* may evolve so rapidly that novel genotypes can emerge during the time of an outbreak, possibly caused by an initial single clone. These results showed that the present MLVA assay have a substantial higher discriminating power compared to PFGE and RAPD, and that the assay is suitable for outbreak investigations in which short-time genetic variations in the *V. cholerae* genome can be identified. Nevertheless, caution is warranted when using this method for evolutionary studies.

The cluster analysis performed on the allele profiles obtained, resulted in a phylogenetic tree divided into five larger clusters (Fig. 1). Toxigenic O1 and O139 strains of *V. cholerae* were located in cluster A or B3, however, some deviating O1 isolates were placed in cluster B4. This result is consistent with previous studies (Beltrán et al., 1999; Faruque et al., 2004; Keymer et al., 2007). In addition, the relatedness observed among the O1 and O139 strains of *V. cholerae* was expected since the O139 serogroup are considered to be a clone of the O1, El Tor biotype (Cholera Working Group, 1993; Comstock et al., 1996). It is difficult to explain the clustering of serogroup O1 outliers observed in this study, based on the present MLVA data, or available strain information. However, studies have shown that *ctx*/*tcp* negative environmental *V. cholerae* O1 isolates (i.e. FFIVC118 in cluster B4) are not necessarily closely related to toxigenic strains (Faruque et al., 2004). Strains of the Hikojima serotype (i.e. FFIVC129 in cluster B4) have previously been referred to as unstable (Chatterjeea and Chaudhuri, 2003) and may therefore deviate from other O1 strains.

In general the MST analysis was in agreement with cluster analysis. However, one interesting difference was the close genetic relationship found between the non-O1/O139 Norwegian strain FFIVC136 (*ctxA*$^-$ and *tcpA*$^-$) and the O139 strains (Fig. 4). The FFIVC136 strain was isolated in Norway from a patient suffering from severe watery diarrhoea (Henriksen et al., 1993). Considering the fact that *V. cholerae* O139 primarily is endemic to South-East Asia, finding strains highly related to this particular serogroup as far north as Norway is intriguing. In general, the marine background level of *V. cholerae* has been found to be low in Norwegian costal areas (Bauer et al., 2006). Our results illustrates that there is high genetic heterogeneity within the *V. cholerae* specie. On the other hand, non-O1/O139 isolates were found to be closely related to O139 isolates, which indicates that this serogroup may be present in non-tropical parts of the world. Eiler et al. (2006) indicated that *V. cholerae* may represent an overlooked health hazard in temperate and boreal regions of the world. Our results support this assumption.

In conclusion, a novel MLVA protocol for *V. cholerae* has been developed and evaluated. The assay is highly discriminating, fast (within 3–5 h) and easy to perform. This MLVA assay may be a useful tool in forensic studies of biological terror actions or in track back analyses of human *V. cholerae* infections connected to natural outbreaks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mimet.2009.06.011.

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