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Phenotypic and genetic analyses of 111 clinical and environmental O1, O139, and non-O1/O139 *Vibrio cholerae* strains from different geographical areas

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SUMMARY

A total of 111 clinical and environmental O1, O139 and non-O1/O139 *Vibrio cholerae* strains isolated between 1978 and 2008 from different geographical areas were typed using a combination of methods: antibiotic susceptibility, biochemical test, serogroup, serotype, biotype, sequences containing variable numbers of tandem repeats (VNTRs) and virulence genes *ctxA* and *tcpA* amplification. As a result of the performed typing work, the strains were organized into four clusters: cluster A1 included clinical O1 Ogawa and O139 serogroup strains ($ctxA^+$ and $tcpA^+$); cluster A2 included clinical non-O1/O139 strains ($ctxA^-$ and $tcpA^-$), as well as environmental O1 Inaba and non-O1/O139 strains ($ctxA^-$ and $tcpA^-/tcpA^+$); cluster B1 contained two clinical O1 strains and environmental non-O1/O139 strains ($ctxA^-$ and $tcpA^+/tcpA^-$); cluster B2 contained clinical O1 Inaba and Ogawa strains ($ctxA^+$ and $tcpA^+$). The results of this work illustrate the advantage of combining several typing methods to discriminate between clinical and environmental *V. cholerae* strains.

Key words: Bacterial typing, *Vibrio cholerae*.

INTRODUCTION

Vibrio cholerae is the causative agent of the severe dehydrating diarrhoeal disease cholera [1]. Traditionally, serological classification of *V. cholerae* is based on the somatic O antigens and requires about 206 antisera [2], whereas O139 serogroup is associated with cholera epidemics and O1 serogroup with cholera epidemics and pandemics [3, 4]. The current classification of *V. cholerae* distinguishes two O1 serotypes, Ogawa and Inaba. Apart from these serotypes, there is a third rare and unstable serotype (Hikojima), which agglutinates with both anti-Inaba and anti-Ogawa antisera [5]. Each serotype has been divided into classical and El Tor biotypes [1], although two additional variants have been proposed, i.e. hybrid and El Tor variant [6]. *V. cholerae* has been a part of human life for millennia. Throughout history, there have been seven pandemics caused by *V. cholerae* O1 serogroup strains where the human population has been decimated in the affected geographical areas.

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In 1992 a new serogroup named O139 appeared as a result of a lateral gene transfer that replaced a region encoding the O1 antigen of the seventh pandemic *V. cholerae* O1 El Tor strain [7]. Since then, both serogroups have co-existed. More than 600 outbreaks have been reported in recent years [8], of which about 83% occurred in Sub-Saharan Africa and South East Asia, whereas in Europe cholera arises mainly as sporadic cases. *V. cholerae* strains which cause cholera carry the cholera toxin (CT) and toxin co-regulated pilus (TCP), coded by the *ctxA* and *tcpA* genes, respectively [9, 10]. Non-O1/O139 serogroups may harbour virulence genes, indicating toxigenic potential from environmental sources [10, 11]. Given the fact that most virulence genes in *V. cholerae* are located in mobile elements, new pathogenical variants could emerge from the strains of these serogroups [12].

Recent economic, technological and social globalization has increased communications between countries throughout the world, and as a consequence easy dissemination of pathogenic agents is enabled. This fact, together with the possibility of using microorganisms in acts of bioterrorism, causes a real threat to public health. Due to the virulence and ease of dissemination of *V. cholerae*, it can be used as a biological weapon agent [13]. Therefore, it is crucial to have a deep knowledge about *V. cholerae* strains in order to perform epidemiological investigations and forensic studies. Several molecular methods have been used for identification and typing of *V. cholerae* strains: enterobacterial repetitive intergenic consensus (ERIC) sequence polymerase chain reaction (PCR), box elements PCR (BOX-PCR), amplified fragment-length polymorphism (AFLP) [10], single nucleotide polymorphism (SNP) [14], random amplified polymorphism DNA (RAPD) [15], pulsed-field gel electrophoresis (PFGE) [16–18], multi-locus sequence typing (MLST) [14, 19, 20] and variable number tandem repeat (VNTR) analysis (MLVA). The latter is a high-resolution method based on the tandem repeat analysis in multiple loci, used for genotyping and trace-back studies [21]. Clinical and environmental *V. cholerae* strains have been analysed by this method to study the relationship among isolates [11, 18, 22]. Phenotypic features (characteristics) of *V. cholerae* have also been established [15, 23, 24].

In this study, phenotypic and genetic analysis of 111 clinical and environmental O1, O139, and non-O1/O139 serogroup strains from different geographical areas was performed. Relationship among the strains was assessed by the combinations of obtained phenotypic and genetic data.

METHODS

Bacterial strains

The *V. cholerae* strains used in this work were isolated between 1978 and 2008 from different countries (see Supplementary Table S1, available online). Thirty-one strains were clinical isolates, 75 were environmental and five of unknown origin. Strains included O1 (28 Ogawa, 12 Inaba, 1 Hikojima), O139, O141 and non-O1/O139 serogroups. Seventeen strains of *V. cholerae* out of the 111 included in this study were previously characterized by means of phenotypic and genetic analysis [22, 25]. Viable bacteria and DNA preparations were obtained from the culture collections of the Biological Defence Unit, Instituto Tecnológico La Marañosa, San Martin de la Vega (Madrid, Spain) (ITM), Military Institute of Hygiene and Epidemiology (Pulawy, Poland) (MIHE) and Norwegian Defence Research Establishment (Kjeller, Norway) (FFI). DNA preparation and cell culture of *V. cholera* were performed by the above laboratories or kindly provided by Dr A. Echeita from the Institute of Health Carlos III (Madrid, Spain). Bacterial strains were streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar plates and incubated for 24 h at 37 °C (Oxoid, Spain), before being grown in tryptic soy broth (TSB; Oxoid, Spain).

Extraction of DNA

DNA was extracted using the QIAmp DNA Blood Mini kit (Qiagen GmbH, Germany) according to the manufacturer's protocols. Purified DNA was quantified using the NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., USA).

Phenotypic characterization

Biochemical identification

Strains were identified as *V. cholerae* using the microbiological culture analyser AutoSCAN®-4 (Siemens Healthcare Diagnostic S.L., Spain) or by classical biochemical reactions [1].

Serotyping

The serotypes were determined by slide agglutination with polyvalent O1 and O139 antiserums, and

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monospecific Inaba and Ogawa antisera (Oxoid, Spain).

Biotyping

Standard phenotypic tests were performed for biotype confirmation: susceptibility to polymyxin B (50 U) (Oxoid, Spain), chicken erythrocytes agglutination, haemolysis of sheep erythrocytes and Voges–Proskauer test [1].

Antibiotic susceptibility

In order to determine susceptibility of the strains to antimicrobial agents, the Kirby–Bauer diffusion method was performed [26] using commercial antibiotic disks (Oxoid, Spain): ampicillin (10 μg), tetracycline (30 μg) or doxycycline (30 μg), trimethoprim (25 μg), gentamicin (10 μg), nitrofurantoin (300 μg), streptomicyn (10 μg) and nalidixic acid (30 μg). The Control Laboratory Standards Institute (CLSI) has established interpretative criteria for *V. cholerae* for the following drugs: amplicillin, chloramphenicol, tetracycline group, and folate pathway inhibitors [27]. CLSI criteria for Enterobacteriaceae were used to interpret results of other antimicrobial susceptibility tests [28]. Antimicrobial susceptibility to colistin (4 μg/ml) was determined with the AutoSCAN®-4 sytem (Siemens Healthcare Diagnostic S.L.). A control strain of *Escherichia coli* (ATCC 25922) was used for these studies.

Amplification of *ctxA* and *tcpA* genes

The virulence genes *ctxA* and *tcpA* were amplified by polymerase chain reaction (PCR) as described previously [29, 30]. To verify the correct size, amplicons were electrophoresed in low electro-endo-osmosis 2% agarose gels stained with ethidium bromide, and visualized using UV light.

MLVA assay

The MLVA assay was performed by the ITM and MIHE laboratories according to FFI laboratory [22] procedures with the following modifications; the amplifications were performed by conventional PCR (final volume 15 μl). Primers targeting polymorphic VNTRs were labelled in the 5′-end with the following fluorescent dyes and multiplexed: VC4-NED, VC5-PET and VC9-FAM. The PCR mixture contained 10–20 ng purified DNA as template, 0·5 μM of each VC4-f/r primer, 0·2 μM of each VC5-f/r primer, 0·4 μM of each VC9-f/r primer, 1·5 mM $MgCl_2$, 2·5 U *Taq* DNA polymerase, and 0·2 mM of each dNTP in the buffer provided by the polymerase manufacturer (Bioline Inc., USA). The amplification was performed with one cycle at 95 °C for 5 min, 25 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and one cycle at 72 °C for 10 min using a Model 9700 thermal cycler (PE Applied Biosystems, USA). The multiplex reaction was diluted 1:100 in sterile water. Two μl of this dilution was diluted again 1:10 in HiDi formamide, containing the GeneScan™-500 LIZ size standard (0·16 μl LIZ standard/20 μl HiDi formamide) (Applied Biosystems). The samples were analysed with the ABI PRISM 310 genetic analyser (Applied Biosystems) by the corresponding genotyping laboratory. The samples were injected into the capillary at 15 kV voltages for 2 s or 5 s and analysed for 28 min at 60 °C using POP4 polymer. No variation in the sizing was observed using injection times of 2 s or 5 s with the same machine. Allele sizes were identified using GeneMapper v. 3.0 software (Applied Biosystems).

Analysis of the sequence data

In order to assign the correct allele number to the allele sizes obtained by capillary electrophoresis from the VC4, VC5 and VC9 loci (MLVA-3 assay), sequencing of several PCR fragments were performed. PCR products were purified using the QIAquick Gel Extraction kit (Qiagen Inc., Germany) and both strands of the PCR products were sequenced. Sequence reactions were performed using the Big-Dye™ Terminator Cycle Sequencing kit v. 1.1 on an ABI PRISM 310 (PE Applied Biosystems). Sequence alignments were performed using ClustalW, MEGA4 software [31].

Fragment size normalization and allele number assignment

In order to compare the fragment sizes obtained by capillary electrophoresis at different laboratories, it was necessary to normalize the above-mentioned fragment sizes. The MLVA-3 allele profiles from the *V. cholerae* strains FFIVC122, FFIVC123, FFIVC124, FFIVC125, FFIVC126 and FFIVC128 obtained at ITM and MIHE were compared with allele profiles previously obtained at FFI as reference data [22]. For each locus, the difference between the allele size obtained at ITM, MIHE and FFI (in base pairs) was calculated for each strain. From these

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values, the average number of base pairs was calculated and subtracted from the allele sizes obtained at ITM or MIHE (see Supplementary Table S2, available online).

In order to assign an allele number to a certain allele size, a marker-specific size ladder was constructed. The DNA fragment sizes obtained by capillary electrophoresis and sequencing, as well as the number of repetitions observed in the VNTR regions, were used to construct the ladder. The average number of base pairs to be subtracted from the allele size obtained by capillary electrophoresis (see above), and the standard deviation calculated from the corresponding data (see Supplementary Table S2) were used to establish the allele size range to which the allele sizes should be assigned.

Typing and clustering analysis

The genetic relationship among the *V. cholerae* strains were determined by clustering analyses using Bionumerics v. 6.5 software (Applied Maths, Belgium). Unweighted pair-group method with arithmetic averages (UPGMA) and minimum spanning tree (MST) analyses were performed based on antibiotic susceptibility, biochemical test, serogroup, serotype, biotype, VNTR analysis, and virulence genes *ctxA* and *tcpA* amplification. The UPGMA analysis was based on categorical coefficients and MST was performed as a complementary analysis to the UPGMA analysis, and was constructed using the highest number of single locus variants (SLVs) as priority rule, i.e. where types that differ by only one character are linked first. No hypothetical types (missing links) were introduced as branches of the MST.

The discrimination ability of individual or combined phenotypic and genetic analysis was calculated using the Hunter–Gaston diversity index (HGDI) [32].

RESULTS

Virulence factors

Thirty-three out of the 111 *V. cholerae* strains studied amplified the *ctxA* gene. Most of these were constituted by *V. cholerae* serotype O1 (29 O1, three O139, one non-O1/O139) (see Supplementary Fig. S1, available online). On the other hand, 86 *V. cholerae* strains amplified the *tcpA* gene, most of them being non-toxigenic of environmental origin (30 O1, three O139, 53 non-O1/O139).

Analysis of antibiotic resistance

The antimicrobial susceptibility tests performed in *V. cholera* strains revealed resistance (R) or intermediate resistance (I) to five or more of the eight antibiotics screened. The results observed for each antibiotic were as follows: ampicillin (R 39%, I 61%), tetracycline/doxycycline (R 3%, I 36%), trimethoprim (R 39%), gentamicin (R 8%, I 3%), nitrofurantoin (R 4%, I 60%), streptomycin (R 40%, I 62%), nalidixic acid (R 27%, I 73%), and colistin (R 27%, I 73%). Interestingly, nearly all human isolates expressed resistance to one or more antibiotics, including the isolates previously reported as susceptible [25].

MLVA

PCR amplicons from six *V. cholerae* strains analysed by capillary electrophoresis revealed up to 8 bp differences among laboratories (see Supplementary Table S2). The differences in average number of base pairs for each VNTR locus observed at MIHE and ITM compared to FFI were 7.00 and 4.17 for VC4, 0.50 and 1.83 for VC5 and 2.00 and 1.83 for VC9, respectively. Therefore, data normalization was performed based on the comparison of MLVA results in all three laboratories, in order to avoid errors in the assignment of allele numbers as recommended earlier [33]. This normalization is described in the 'Fragment size normalization and allele number assignment' section, and the results are shown in Table 1. The size distribution of the PCR amplicons observed were 155-317 (MIHE) and 182-303 (ITM) for VC4; 145-210 (MIHE) and 144-207 (ITM) for VC5, and 154-271 (MIHE) and 151-182 (ITM) for VC9. Deviation in several allele sizes was observed. In some cases, sequencing confirmed that these deviations were due to DNA fragments of intermediate size (Fig. 1).

Typing and clustering of *V. cholerae* strains by phenotypic and genetic analysis

Individual or combined phenotypic and genetic methods were used to analyse the relationship of 111 *V. cholerae* strains from different sources. The HGDI estimated for the phenotypic and genetic markers was analysed individually and combined (Table 2). The diversity index (DI) obtained in the MLVA-3 assay (DI = 0·985) was close to the DI of a six-loci scheme reported by FFI [21]. The antibiotic susceptibility assay yielded the lowest DI (0·610) of all performed

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Table 1. *Allele number, number of repeats and allele size obtained by capillary electrophoresis from indicated sources* (*in numbers of bases*) *for each VNTR locus*

VC4 (6 bp tandem-repeat sequence)					VC5 (9 bp tandem-repeat sequence)					VC9 (7 bp tandem-repeat sequence)				
		Size§					Size§					Size§		
Allele*	No. of repeats‡	FFI	MIHE	ITM	Allele*	No. of repeats‡	FFI§	MIHE	ITM	Allele*	No. of repeats‡	FFI§	MIHE	ITM
	1				1		119#				1			
23†	2		155			1					2			
	3				2	2	142¶	145	144	1	3	152	154	153
24†	4		169		3	2	148¶#	150#		2	4	159	161	161‖
1	5	168			4	3	152¶	154	154	3	5	166	167	
	6				14†	3			138‖#	4	6	173	175	
2	7	179	188	182	5	3	158¶#	160#		5	7	180	182	182‖
3	8	185	194		6	4	161¶	163		6	8	187	189	
4	9	191		194‖	7	5	170	172		7	9	194	196	
5	10	197			8	6	179¶	181	181	8	10	201		
6	11	203	207	206	9	7	187	190	190	9	11	207		
7	12	209	215	212	10	8	196¶	199	198‖	10	12	214		
8	13	214	221		11	9	205				13			
9	14	220	228		15†			210#			14			
10	15	226	235	230		10				13†	15		240#	
11	16	232	241		12	10	220¶#			14†	16		243	
12	17	238	247			11				11	17	249		
13	18	244	254	248‖		12					18			
14	19	250	260		13	13	240				19			
15	20	256	265							12	20	270	271	
16	21	263	270	266										
17	22	269	275											
18	23	274	283	279‖										
19	24	280	287	285‖										
25†	25		294	291										
20	26	293	299	297										
21	27	299		303										
22	28	305												
26†	29		317											

* Allele number described previously [22].
† New allele number observed in this study.
‡ Expected number of repeats.
§ Size obtained by capillary electrophoresis (in base pair) from indicated source.
¶ Fragment with known number of repeats by sequencing at FFI laboratory [22].
‖ Fragment with known number of repeats by sequencing in this study.
Suspected or observed intermediate size.

assays, followed by the biochemical test (DI = 0·790). Ninety-six different groups and a higher DI (0·997) were obtained when combining the MLVA-3 assay, the biochemical test and the antibiotic susceptibility test (Table 2). The UPGMA clustering analysis yielded a better clustering of strains according to source when combining these typing methods with *ctxA* and *tcpA* data.

Combined phenotypic and genetic methods used in this study allowed discrimination of 98 strains out of a total of 111 (DI = 0·997) (Table 2). The UPGMA clustering analysis resulted in a dendrogram where the strains were organized into four clusters (Supplementary Fig. S1). Cluster A1 contained 26 strains, where 24/26 were *ctxA*$^+$ and *tcpA*$^+$ human isolates from the following serogroups: O1 (n = 21), O139 (n = 3), non-O1/O139 (n = 1) and O141 (n = 1). O1 080025/FD strain (sewage water, Ceuta, Spain) was seen to be equal to O1 080025/EY and 080025/FC strains isolated from humans in Ceuta, Spain, which were associated with an outbreak in Morocco in 1990 [24]. The non-O1/O139 FFIVC084 (from mussels,

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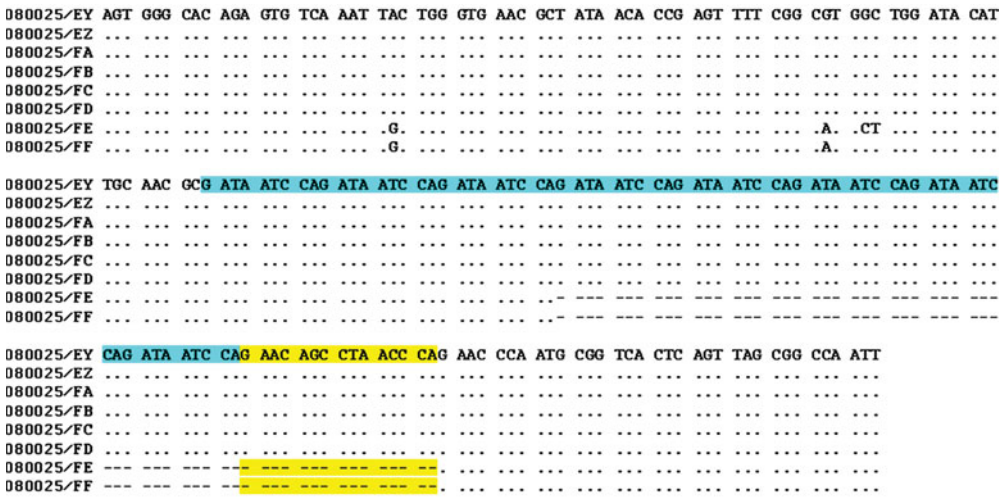

Fig. 1. Alignment of VNTR VC5 locus of eight *V. cholera* strains. The DNA fragments of 198 bp contain eight repeats of the motif GATAATCCA (in blue), while the smaller fragments of 138 bp have three repeats. These amplicons with deviation on the allele size have a 15-bp deletion (in yellow) below the VNTR.

Norway) *V. cholerae* strain (*ctxA*$^+$ and *tcpA*$^+$) was situated in the dendrogram close to the clinical O139 serogroup strains (*ctxA*$^+$ and *tcpA*$^+$). Cluster A2 included the environmental Norwegian FFIVC052 isolate (*ctxA*$^-$ and *tcpA*$^+$), environmental isolates from Ecuador, Spain and Norway (*ctxA*$^-$ and *tcpA*$^-$), and clinical Norwegian FFIVC136 and FFIVC137 strains (*ctxA*$^-$ and *tcpA*$^-$). FFIVC129 *V. cholera* strain of unknown origin isolated in Spain in 1979 established an 'outgroup' of clusters A1 and A2. This strain resulted in a unique MLVA-3 profile with atypical biochemical characteristics. Cluster B1 included 59 non-O1/O139 strains isolated from Poland and from Baltic water, O1 serogroup 13/154 strain (clinical, India) and O1 serogroup 21/635 strain (clinical, unknown geographical origin). Fifty-three out of 61 strains were *ctxA*$^-$ and *tpcA*$^+$, seven were *ctxA*$^-$ and *tcpA*$^+$ and one was *ctxA*$^+$ and *tcpA*$^+$. Cluster B2 contained six O1 serogroup strains (*ctxA*$^+$ and *tpcA*$^+$), one from an unknown source and five of human origin. O1 serogroup 14/2002/S *V. cholera* strain (Bug River, Poland), established an outgroup of clusters B1 and B2. This strain revealed a unique MLVA-3 profile.

The MST analysis resulted in a star-like organization of the environmental non-O1/O139 serogroup strains from Poland, indicating the existence of a clonal complex (Fig. 2). Clinical O1 13/154 (India), 1014 (Guinea) and 14/Jor (Jordan) strains (key nos. 018, 025, 016, respectively) were situated on the right-hand side of the MST tree, nearer to the Polish strains than the other strains included in this study. Norwegian clinical non-O1/O139 FFIVC136 strain (key no. 247) connects the Polish strains to other *V. cholerae* isolates by dotted lines, indicating the most probable connection between two types differing by more than two locus variants. The Spanish O1 serogroup strains were related to the *V. cholerae* strains isolated in an outbreak in Italy/Albania in 1994 (key nos. 172, 173, 179) through 080025/EZ and 080025/FB strains isolated in Ceuta, Spain in 1990 (key nos. 093, 095, respectively). Interestingly, strains imported from Ecuador to Spain (key nos. 101, 102, 103) were connected to two strains isolated from a marsh in Sevilla, Spain in 1991 (key nos. 099, 100). Norwegian environmental non-O1/O139 FFIVC084 strain (*ctxA*$^+$ and *tcpA*$^+$) (key no. 198) was connected to the Indian clinical O139 FFIVC130 strain (*ctxA*$^+$ and *tcpA*$^+$) (key no. 242). The latter and the clinical O139 FFIVC131 strain from California (key no. 261) were connected in the MST tree by a thin line, revealing a double character difference.

DISCUSION

The Centers for Disease Control and Prevention (CDC) has recently described a large cholera outbreak in Haiti (autumn 2010) [34]. During this outbreak, an increase of travel-associated cholera cases in neighbouring countries was reported, indicating that although cholera outbreaks occurred in areas with poor water and sanitation infrastructure, other countries are also at risk. In addition, CDC classified *V. cholerae* as a category B agent according to its

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Table 2. *Discrimination indices of individual or combined typing methods for* Vibrio cholerae

Typing method(s)*	No. of groups, size of largest group (%)	Diversity index (HGDI)
VC4	24 (11·71)	0·945
VC5	12 (21·62)	0·880
VC9	10 (32·43)	0·792
MLVA-3	67 (8·10)	0·984
AS	14 (58·55)	0·610
BT	17 (40·54)	0·790
BT/AS	36 (26·12)	0·897
MLVA-3/AS	75 (7·20)	0·988
MLV-3/BT	93 (3·60)	0·996
MLV-3/BT/AS	96 (2·70)	0·997
MLV-3/BT/ AS/*ctxA*/*tcpA*	96 (2·70)	0·997
MLV-3/BT/AS/*ctxA*/*tcpA*/ Serogroup/Serotype/Biotype	98 (2·70)	0·997

HGDI, Hunter–Gaston diversity index.
* MLVA-3, VC4, VC5 and VC9 combined markers; BT, biochemical test; AS, antibiotic susceptibility; BT/AS, MLVA-3/BT, MLVA-3/AS, MLVA-3/BT/AS, MLV-3/BT/AS/*ctxA*/*tcpA* and MLV-3/BT/AS/*ctxA*/*tcpA*/Serogroup/Serotype/Biotype combined markers; $n=111$.

potential use as a biological weapon [35]. Public health authorities are increasingly aware of the threat this agent may constitute. Identification and characterization of *V. cholerae* isolates is crucial for the control of the disease, and subsequent phylogenetic studies are useful for understanding the relationship between strains.

Environmental non-O1/O139 serogroup FFIVC084 strain (*ctxA*$^+$ and *tcpA*$^+$), clinical O141 serogroup 080025/FR strain (*ctxA*$^+$ and *tcpA*$^-$) and a large number of environmental non-O1/O139 serogroup strains from Poland (*ctxA*$^-$ and *tcpA*$^+$), which may constitute a potential danger to human health, have been included in this work. To illustrate this potential risk, two cases of septicaemia caused by *V. cholerae* non-O1/O139 serogroup, reported in Poland (summer 2006) can be mentioned. The first case was a 49-year-old man, who was found drowned in a lake. Microbiological examination of blood samples revealed a *V. cholera* infection of non-O1/O139 serogroup. Water samples collected from four different locations in the lake tested positive for a few *V. cholerae* non-O1/O139 serogroup strains. The second case of *V. cholerae* septicaemia occurred in a 79-year-old man. The patient was admitted to hospital in summer 2006 with symptoms of high fever, diarrhoea and severe abdominal cramps. Shortly after admission, he developed pneumonia and septicaemia. Laboratory investigation of two blood samples revealed the presence of *V. cholerae* [36].

Two separate serious cholera-like cases caused by non-O1/O139 strains (FFIV136 and FFIV137) were reported in Norway [37]. Furthermore, in our study the strains showed resistance to several antibiotics, which illustrates that environmental non-O1/O139 isolates are potentially dangerous, and supports the idea that new toxigenic strains could emerge through a lysogenic infection of non-toxigenic *V. cholerae* strains with the CTXΦ prophage harbouring virulence genes [38].

Several variants of CTXΦ prophage are in one or both *V. cholerae* chromosomes, as a single copy or in multiple tandemly arrayed copies, as a result of integration and excision mechanisms. These mechanisms of genetic exchange contribute to the considerable diversity of *V. cholerae* strains and, at the same time, they explain the origin of recent epidemic *V. cholerae* strains [39]. O139 isolates have been suggested to arise by genetic exchange with non-O1/O139 *V. cholerae* strains, as well as with clinical O1 strains [40]. The authors of that study pointed out that it seems possible that O139 strains derived from O1 progenitors could have epidemic potential, as opposed to O139 strains derived from non-O1/O139 progenitors. In accord with this study, there is a relationship between O139 FFIVC133 strain (*ctxA*$^+$ and *tcpA*$^+$) (key no. 244) and clinical O1 serogroup 080025/EZ and 080025/FB strains (*ctxA*$^+$ and *tcpA*$^+$) (key nos. 093, 095, respectively) (Fig. 2), supporting this hypothesis. The existence of environmental non-O1/O139 *V. cholerae* strains related to clinical O1 and O139 serogroup strains, as well as the fact that there are virulent clinical non-O1/O139 *V. cholerae* strains with resistance to multiple antimicrobial agents, highlights the need of continuous surveillance of this pathogen.

Thirteen O1 strains of different geographical origin resulted in an atypical biotype, herein referred to as 'intermediate'. No hybrid or El Tor variant strains were identified since the scheme proposed by Raychoudhuri *et al.* [6] was not used in our study. This information indicates that the hybrid or El Tor variant strains are more widespread than expected. Discrepancies between the biotypes previously published [25] and biotypes observed in the present study were detected. Five strains isolated in Spain previously biotyped as atypical by Usera *et al.* [25] have been assessed as El Tor biotype in our study. Additionally, one strain biotyped as El Tor was shown as

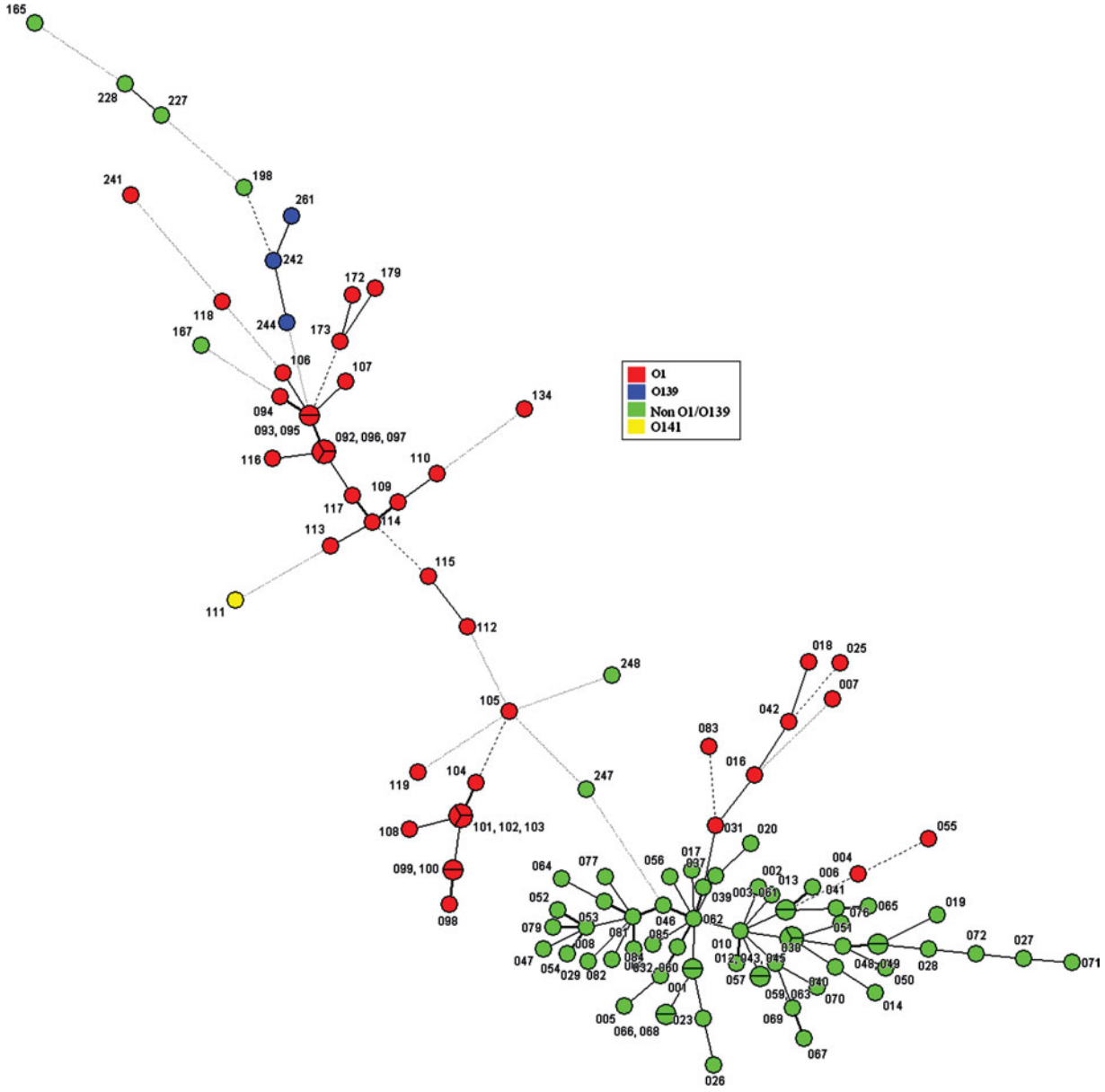

Fig. 2. Genetic relationship between 111 *V. cholerae* strains was analysed by creating a minimum spanning tree. The 111 strains were clustered based on the differences in the phenotypic and genetic markers. Each circle in the tree represents a different type. Circles are divided according the number of strains with a certain type. The species with different serogroups are represented by different colours, as indicated. The thickness of the lines indicate the genetic distance; solid and thin lines represent single and double locus variants, respectively; dotted lines indicate the most probable connection between two types differing by more than two locus variants; the length of the branches is proportional to the distance between the types.

hybrid biotype in our study. No reasons explaining the phenotypic variations identified through time can be given, although mutations in this strain can be speculated. The *V. cholerae* strains included in our study fell into four clusters, according to the phenotypic and genetic features analysed by the UPGMA method (Supplementary Fig. S1). The analysis revealed a clustering of *V. cholerae* strains by clinical or environmental source, and established a relationship among isolates from the same geographical area. Interestingly, our study differentiated the six Moroccan outbreak strains into three various genotypes, previously designated as one genotype by ribotyping, PFGE and multi-locus enzyme electrophoresis [25]. We observed two different genotypes for the three environmental *V. cholerae* strains isolated in Sevilla (Spain), which had previously been typed as one by ribotyping and PFGE [25]. On the other hand, three

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pairs of strains (FFIV130 and FFIVC133, FFIVC057 and FFIVC058, FFIVC114 and FFIVC115) had previously not been differentiated based on a six-loci MLVA scheme [22]. However, in our study, the UPGMA clustering analysis showed similarities of 90%, 93·7% and 90%, respectively, due to differences observed in the biochemical tests and the susceptibility to antimicrobial agents tests. These results indicate that, despite having an identical allelic profile, the strains are carriers of different phenotypic features.

Fifty-six out of 59 non-O1/O139 *V. cholera* strains isolated from water in Poland over a period of 10 years revealed a unique antimicrobial susceptibility pattern, diverse MLVA-3 profiles, and differences in biochemical pattern. The low level of phenotypic and genetic diversity in the isolates indicated that the Polish isolates may have originated from the same clone. The Polish 52/110/2006 and 8/110/2006 strains (key nos. 062, 010, respectively) (see Supplementary Table 1) isolated from Bug River in 1998 were centrally located in the lower part of the MST. They showed variation in VC5 and VC9 loci, and identical phenotypic profiles. Thus, it can be speculated that several SLV may have evolved from one of these two strains. More heterogeneity was observed in non-Polish strains, with types differing by more than two locus variants. In this case, significant variability in the antimicrobial susceptibility pattern, in the biochemical profile and in the allelic profile was observed by the combined tests performed.

In summary, we have studied different methods for typing *V. cholerae* strains, either individually or in combination, to obtain an optimal phylogenetic differentiation of this bacterium. The DI estimated from HGDI for individual phenotypic markers (antibiotic susceptibility and biochemical tests) were considerably low (DI = 0·610, DI = 0·790, respectively). However, when these data were combined with MLVA-3 data, a higher discriminatory level (DI = 0·997) was obtained, similar to the previously described six-loci MLVA scheme [22] or to the seven-loci scheme [11]. The 111 *V. cholerae* strains analysed were organized into 98 different groups based on phenotypic and genetic markers used in combination. Phylogenetic analysis of the combined tests showed a clear discrimination between clinical O1 and O139 serogroup strains and environmental isolates. We conclude that phenotypic tests in addition to genetic tests provide important information for characterization of *V. cholerae* strains isolated during epidemic and pandemic outbreaks, and are essential when performing phylogenetic studies or forensic trace-back studies of *V. cholera* strains.

NOTE

Supplementary material accompanies this paper on the Journal's website (http://journal.cambridge.org/hyg).

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DECLARATION OF INTEREST

None.

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