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Clonal relationship among *Vibrio cholerae* O1 El Tor strains isolated in Somalia

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Abstract

One hundred and three *Vibrio cholerae* O1 strains, selected to represent the cholera outbreaks which occurred in Somalia in 1998–1999, were characterized by random amplified polymorphic DNA patterns, ribotyping, and antimicrobial susceptibility. All strains showed a unique amplified DNA pattern and 2 closely related ribotypes (B5a and B8a), among which B5a was the more frequently identified. Ninety-one strains were resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfamethoxazole, and trimethoprim, conferred, except for spectinomycin, by a conjugative plasmid IncC. These findings indicated that the group of strains active in Somalia in the late 1990s had a clonal origin.

Keywords: Cholera; Clonal analysis; Antimicrobial resistance; Somalia

Introduction

Cholera still remains a serious infectious disease in many developing countries, most often centred in tropical areas (Lipp et al., 2002). The ongoing 7th pandemic was caused by *Vibrio cholerae* O1 biotype El Tor and began in Indonesia in 1961 (Faruque et al., 1998). *V. cholerae* O1 El Tor spread rapidly through Asia and the Middle East reaching the African continent in 1970.

Within the 7th pandemic, the history of cholera in Somalia has been documented for the large epidemics which occurred in 1971 and 1985 (Swerdlow and Isaäcson, 1994; Maimone et al., 1986). In 1994, cholera re-emerged with 27,904 clinical cases notified to WHO. In the following decade, cholera established permanently with a major peak of cases notified in 1999 (17,757 cases).

In this paper, we present phenotypic and genotypic features of 103 *V. cholerae* O1 strains representative of the 1998–1999 period of epidemic in Somalia. Strains were characterized by Random Amplified Polymorphic DNA (RAPD) assay, BglI ribotyping, antimicrobial susceptibility tests, and by PCR for genes encoding the cholera toxin subunit A (*ctx*A), zonula occludens toxin

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(*zot*), accessory cholera enterotoxin (*ace*), and toxin coregulated pilus (*tcp*A).

Materials and methods

Bacteria strains, growth conditions, and antibiotic susceptibility testing

A total of 103 *V. cholerae* O1 strains were retrospectively analysed in this study (Table 1). All *V. cholerae* O1 El Tor strains were isolated from clinical cases representative of 11 outbreaks which occurred in Somalia in 1998–1999. The strains were isolated from rectal swabs in Cary-Blair transport medium. Original stock cultures of isolates were kept in 20% glycerol Luria-Bertani (LB) broth at −70 °C. Phenotypic strain characterization was performed as described previously (Scrascia et al., 2003). Antimicrobial susceptibility was determined by the disc diffusion technique and interpretative criteria of the National Committee for Clinical Laboratory Standards (NCCLS, 2004). The antimicrobial disc contents were ampicillin (AMP; 10 μg), chloramphenicol (CHL; 30 μg), doxycycline (DOX; 30 μg), kanamycin (KAN; 30 μg), streptomycin (STR; 10 μg), spectinomycin (SPT; 10 μg), tetracycline (TET; 30 μg), sulfamethoxazole (SMX; 25 μg), and trimethoprim (TMP; 5 μg) (Oxoid, Milan, Italy). *Escherichia coli* ATCC 25922 was used as a quality control strain.

RAPD assay

Genomic DNA extraction and PCR reactions were performed as described previously (Pazzani et al., 2006). All primers (ERIC1, ERIC2, VCR1, VCR2, ATX1, and ATX2) were synthesized commercially and purified through a reverse phase chromatography by Invitrogen Life Technologies. PCR reactions were performed in duplicate, and results were found to be reproducible. PCR products were separated in 2% (w/v) agarose gel, 45 mM Tris-Borate, 1 mM EDTA buffer, at 60 V and at 14 °C. Agarose gels were stained with ethidium bromide at the final concentration of 0.5 μg/ml. Images from gels were converted into digital form by the Gel-Doc 2000 photo documentation system (Bio-Rad, Milan, Italy).

Ribotyping

Molecular characterization by BglI restriction patterns of 16S and 23S rRNA was performed as described previously (Pourshafie et al., 2000). Digitisation and interpretation of patterns were done with programs in the Taxotron package (Taxolab, Institut Pasteur, Paris). The membranes were first scanned and the images searched for bands by RestrictoScan. The fragment sizes were interpolated from migration data by RestrictoTyper (Damian et al., 1998; Machado et al., 1998). *Citrobacter koseri* strain CIP 105177 (Collection de l'Institut Pasteur) DNA was cleaved by *Mlu*I restriction endonuclease (Amersham Pharmacia Biotech), and the fragments were then used as the molecular size standards.

Gene detection by PCR

Detection of genes encoding cholera toxin subunit A (*ctxA*), zonula occludens toxin (*zot*), accessory

Table 1. Geographical distribution, RAPD cluster type, ribotypes, and resistance patterns of 103 *V. cholerae* O1 strains isolated in Somalia in 1998 and 1999

Province of isolation (no. of strains)	RAPD cluster type (no. of strains)	Ribotype (no. of strains)	Resistance pattern[a] (no. of strains)
Saanag (2)	VIII (2)	B5a (2)	AMP CHL SMX SPT STR TMP (2)
Bari (1)	VIII (1)	B5a (1)	AMP CHL SMX SPT STR TMP (1)
Mudug (8)	VIII (8)	B5a (8)	AMP CHL SMX SPT STR TMP (8)
Galguduud (2)	VIII (2)	B5a (1); B8a (1)	SPT (2)
Hiraan (7)	VIII (7)	B5a (7)	AMP CHL SMX SPT STR TMP (7)
Shabeellaha Dhexe (5)	VIII (5)	B5a (5)	AMP CHL SMX SPT STR TMP (4)
			SPT (1)
Baanadir (46)	VIII (46)	B5a (33); B8a (9)	AMP CHL SMX SPT STR TMP (42)
		B5a (4)	SPT (4)
Bay (6)	VIII (6)	B5a (6)	AMP CHL SMX SPT STR TMP (6)
Shabeellaha Hoose (11)	VIII (11)	B5a (5); B8a (4)	AMP CHL SMX SPT STR TMP (9)
		B5a (2)	SPT (2)
Gedo (10)	VIII (10)	B5a (10)	AMP CHL SMX SPT STR TMP (10)
Jubbada Hoose (5)	VIII (5)	B5a (2)	AMP CHL SMX SPT STR TMP (2)
		B5a (3)	SPT (3)

[a]AMP, ampicillin; CHL, chloramphenicol; SMX, sulfamethoxazole; SPT, spectinomycin; STR, streptomycin; TMP, trimethoprim.

cholera enterotoxin (*ace*), and toxin-coregulated pilus for intestinal colonization (*tcpA*) was performed by PCR as described previously (Pourshafie et al., 2000; Tamayo et al., 1997). The amplicons were electrophoresed through agarose 0.8% gel (Appligene, Illkirch, France) and then stained with ethidium bromide. A negative control (reaction mixture without template) and a toxin-positive control (*V. cholerae* O1 strain O395) were included in each run.

DNA sequencing

Amplified DNA products of interest were cloned into pGEM-T Easy vector (Promega, Milan, Italy), according to the manufacturer's instructions. *E. coli* JM83 was used as a recipient strain. The cloned products were purified before sequencing by using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Milan, Italy) and sequenced by the Big Dye Terminator method (BMR Genomics, Padova, Italy). The resulting DNA sequences were analysed for similarity by using the BLAST program available at the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997).

Nucleotide sequence accession numbers

The sequence of the 1150-bp DNA amplicon from the identified IncC plasmid (GenBank accession no. EU011830) has been submitted to GenBank.

Results

Genomic typing of 103 *V. cholerae* O1 strains isolated in Somalia in 1998–1999 (Table 1) was first performed by RAPD fingerprinting. Each strain was characterized by an individual RAPD cluster type represented by the combination of the 6 single RAPD patterns (Fig. 1A). Combinations with 6 identical amplified DNA patterns or with one different amplicon in only one pattern out of the 6 were classified in the same specific RAPD cluster type. Ninety-one strains exhibited the RAPD pattern E2/E14/V2/V5/A2/A8, while 12 isolates exhibited the RAPD pattern E1/E14/V2/V5/A2/A8. RAPD pattern E2 differed from E1 for the presence of an additional DNA amplicon of 1150 bp. All *V. cholerae* O1 strains isolated in Somalia in 1998–1999 were then classified within a single RAPD cluster type designated VIII.

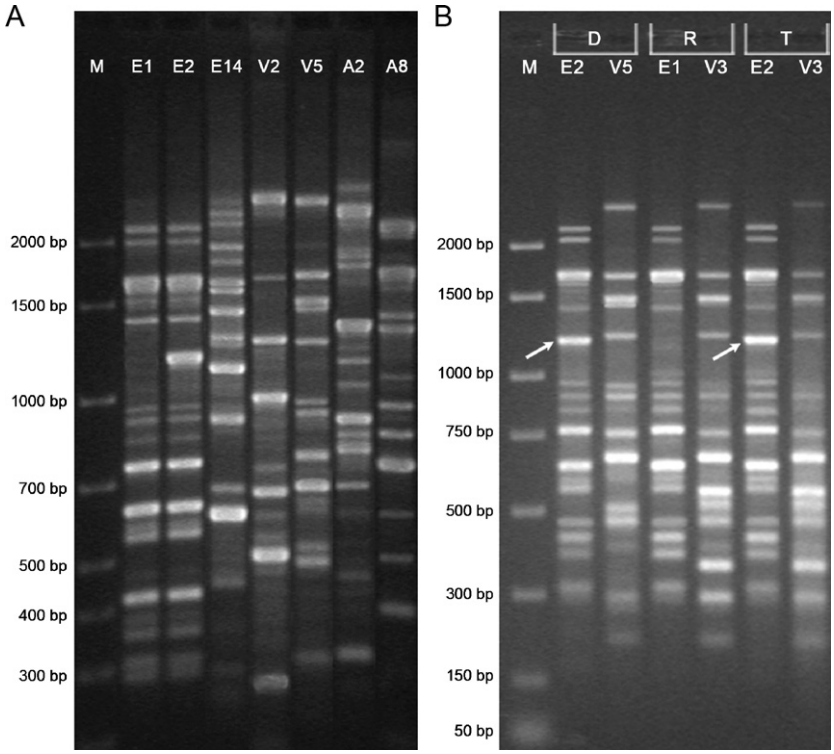

Fig. 1. (A) RAPD patterns of genomic DNA of *V. cholerae* O1 strains generated by 6 primers. Each specific pattern is designated by a letter with a serial number. Molecular size markers are given on the left. (B) RAPD patterns of genomic DNA of *V. cholerae* O1 donors (D), recipients (R), and transconjugants (T). White arrows indicate the 1150-bp DNA amplicon linked to the identified IncC plasmid. Molecular size markers are given on the left.

Table 2. Analysis for the presence of *ctxA*, *zot*, *ace*, and *tcpA* genes in 103 *V. cholerae* O1 strains isolated in Somalia in 1998 and 1999

Province of isolation (no. of strains)	No. of strains	PCR detection for genes			
		ctxA	*zot*	*ace*	*tcpA*
Saanag (2)	2	+	+	+	+
Bari (1)	1	+	+	+	+
Mudug (8)	7	+	+	+	+
	1	–	+	–	–
Galguduud (2)	1	+	+	+	+
	1	+	–	+	+
Hiraan (7)	7	+	+	+	+
Shabeellaha Dhexe (5)	4	+	+	+	+
	1	+	–	+	+
Baanadir (46)	45	+	+	+	+
	1	+	+	+	–
Bay (6)	6	+	+	+	+
Shabeellaha Hoose (11)	11	+	+	+	+
Gedo (10)	10	+	+	+	+
Jubbada Hoose (5)	5	+	+	+	+

Strains were further characterized by *Bgl*I ribotyping (Table 1). Two closely related ribotypes were detected, 89 isolates were ribotype B5a and 14 ribotype B8a (Damian et al., 1998).

Antimicrobial susceptibility testing identified 2 distinct patterns of antimicrobial resistance (Table 1). Ninety-one strains were resistant to AMP, CHL, SMX, SPT, STR, and TMP, while 12 strains exhibited resistance only to SPT.

The resistance markers to AMP, CHL, SMX, STR, and TMP could be transferred as a linkage group in conjugation experiments with *V. cholerae* O1 recipient strains (average frequency of transfer 3.5×10^{-6}). Incompatibility tests and physical analysis of plasmid DNA in donors and transconjugants identified a conjugative plasmid of incompatibility class C (IncC) of the same molecular size (unpublished results).

Interestingly, all *V. cholerae* O1 strains which exhibited the RAPD pattern E2 were resistant to AMP, CHL, SMX, SPT, STR, and TMP, whereas the 12 isolates which exhibited the RAPD pattern E1 were resistant only to SPT. This prompted us to investigate if the 1150-bp DNA amplicon, which distinguished pattern E2 from E1, was linked to the IncC plasmid encoding multiple resistance. Multiple resistant *V. cholerae* O1 strains, selected from the single provinces of isolation, were used as donors in conjugation experiments with *V. cholerae* O1 strain CIRPS1305 as recipient (characterized by RAPD patterns E1 and V3). *V. cholerae* O1 transconjugants while retaining the pattern V3, which distinguished donors from recipient, shifted the pattern from E1 to E2 following acquisition of the IncC plasmid AMP–CHL–SMX–STR–TMP (Fig. 1B). These data demonstrated the linkage of the 1150-bp DNA amplicon to the IncC plasmid encoding multiple resistance.

The DNA sequence of the 1150-bp DNA amplicon was determined for both donors and transconjugants. The sequence showed 99% identity, over 1167 bp, with the *tra*D gene, type IV conjugative transfer system, identified in the IncA/C plasmid pIP1202 harboured by *Yersinia pestis* strain IP275 biovar orientalis (GenBank accession no. CP000602).

PCR detection for *ctxA*, *zot*, *ace*, and *tcpA* was positive for most of the strains. Only, 2 isolates were negative for *zot*, 1 for *tcpA*, and 1 for *ctxA*, *ace*, and *tcpA* (Table 2).

Discussion

Two major routes for the introduction of cholera to Africa have been described (Lan and Reeves, 2002). One originates in the Western countries of Guinea, Sierra Leone, Liberia, Nigeria, and other coastal countries expanding inland along rivers and trade routes. The second is thought to originate in the Middle East extending to the Eastern African countries of Djibouti, Ethiopia, and Somalia.

Since 1970, when the 7th pandemic reached the African continent, Somalia has been suffering an increased number of cholera epidemics. In 1985–1986, major cholera outbreaks accounted for more than 30,000 clinical cases notified to WHO. The re-emergence of cholera in Somalia in 1994 can probably be traced back to the spreading of *V. cholerae* O1 strains responsible for the large cholera outbreak which hit Djibouti in 1993 (10,055 cases), after 7 years of absence. In 1994, cholera extended to Somalia spreading from north throughout the whole country and, over the following decade, cholera established itself permanently with an average of 9000 annually clinical cases.

This study was carried out to investigate the extent of the clonal relationship among 103 *V. cholerae* O1 strains representative of the 1998–1999 period of epidemic in Somalia. Results obtained by RAPD assay and *Bgl*I ribotyping highlighted the presence of a group of closely related strains. Apart from a unique RAPD cluster type, most of the isolates were characterized by B5a, the predominant ribotype identified among *V. cholerae* O1 strains of the 7th cholera pandemic (Aidara et al., 1998).

The upsurge of cholera outbreaks in the late 1990s was also characterized by the appearance of an increased number of multi-resistant *V. cholerae* O1 strains (resistance

to $\geqslant$3 antimicrobial agents). Ninety-one strains exhibited resistance to AMP, CHL, SMX, SPT, STR, and TMP conferred, except for SPT, by a conjugative plasmid of incompatibility class C (IncC). Resistance to AMP, CHL, SMX, and TMP increased constantly in Somalia from 1994 to 1996 (Materu et al., 1997). Thus, acquisition of the IncC plasmid AMP–CHL–SMX–STR–TMP was the most simple explanation for the appearance of the multidrug-resistant strains which spread in Somalia in 1998–1999. *V. cholerae* O1 strains with indistinguishable molecular and antimicrobial resistance features were also found along the eastern Kenyan border with Somalia (Scrascia et al., 2006), demonstrating the spread of the Somalian strains to nearby Kenya as well.

Overall, the data reported in this study indicated that the group of strains active in Somalia in the late 1990s had a clonal origin.

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