Journal of Antimicrobial Chemotherapy (2009) **63**, 438–442
doi:10.1093/jac/dkn542
Advance Access publication 20 January 2009

JAC

SXT-related integrating conjugative element and IncC plasmids in *Vibrio cholerae* O1 strains in Eastern Africa

N. Pugliese[1], F. Maimone[2], M. Scrascia[1], S. F. Materu[3] and Carlo Pazzani[1]*

[1]Dipartimento di Genetica e Microbiologia, Università di Bari, Via G. Amendola 165/A 70126, Bari, Italy; [2]Interuniversity Research Center for Sustainable Development, Università 'La Sapienza', Palazzo Doria Pamphilj 00038, Valmontone, Roma, Italy; [3]African Medical and Research Foundation (AMREF), PO Box 30125, Nairobi, Kenya

Received 10 September 2008; returned 31 October 2008; revised 25 November 2008; accepted 16 December 2008

***Objectives*: The objective of this study was to investigate the extent of resistance patterns and associated mobile genetic elements in epidemic *V. cholerae* O1 El Tor strains isolated from Eastern Africa in the late 1990s.**

***Methods*: Self-transmissible genetic elements and associated clusters of genes encoding resistance were detected by conjugation experiments. Detection of SXT-related integrating conjugative elements (ICEs) and associated antibiotic resistance genes was performed by PCR to amplify the SXT element-integrase gene (*int*), right SXT element-chromosome junction (*attP-prfC*) and genes conferring resistance to chloramphenicol (*floR*), sulfamethoxazole (*sulII*), streptomycin (*strA*) and trimethoprim (*dfrA1*). Genomic relatedness was established by random amplified polymorphic DNA patterns.**

***Results*: Of 224 strains analysed, 200 isolates exhibited resistance to four or more antimicrobials. An IncC plasmid, encoding resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim, conferred multidrug resistance to 113 strains isolated from Somalia and Ethiopia, whereas an SXT-related ICE, encoding resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim, conferred multidrug resistance to 74 strains isolated from Sudan, Kenya and Tanzania.**

***Conclusions*: This study has shown the spread of SXT-related ICEs among *V. cholerae* O1 African isolates. It has also highlighted the role of two distinct genetic elements in conferring multiple resistance to the two distinct groups of *V. cholerae* O1 strains that, in the late 1990s, spread through Eastern Africa, a critical geographic region for the persistence and transmission of cholera to the entire continent.**

Keywords: conjugation, *V. cholerae*, antimicrobial resistance, mobile elements

Introduction

Cholera is a severe diarrhoeic disease caused by the Gram-negative bacterium *Vibrio cholerae*. The seventh and most recent pandemic originated on the island of Sulawesi in Indonesia in 1961, reaching Africa in 1970.[1] Over the following years, cholera spread throughout this continent and, since the late 1990s, more than 80% of the cholera cases notified annually to the WHO have been in Africa.

Apart from rehydration and electrolyte replacement, antimicrobial therapy may considerably reduce the severity of diarrhoea and the duration of vibrio excretion. However, over the past few decades, an increased number of multidrug-resistant *V. cholerae* epidemic strains have been isolated, raising concerns on the limitation of available antimicrobial options.

This study investigated the extent and genetic nature of the different components of resistance patterns in epidemic *V. cholerae* O1 El Tor from Eastern Africa in the late 1990s.

Materials and methods

Bacterial strains, growth conditions and antibiotic susceptibility testing

In this study, 224 *V. cholerae* O1 strains were retrospectively analysed to determine the relatedness between antimicrobial resistance patterns and self-transmissible genetic elements. Phenotypic strain characterization and antimicrobial susceptibility tests (disc diffusion method) were performed as described previously.[2] MICs were

*Corresponding author. Tel: +39-080-5443379; Fax: +39-080-5443386; E-mail: pazzani@biologia.uniba.it

determined by the broth dilution method. *Escherichia coli* ATCC 25922 was used as a quality control strain.

Bacterial conjugation and plasmid analysis

V. cholerae O1 strain CIRPS1305, a rifampicin-resistant mutant of CIRPS1006, was used as recipient. Conjugation experiments, incompatibility tests and plasmid detection were performed as described previously.[3] The frequency of transfer of a genetic marker was expressed as the number of transconjugants per recipient cell.

Random amplified polymorphic DNA (RAPD) assay

Genomic DNA extraction and PCR reactions for RAPD assay were performed as described previously.[4] Combinations with six identical amplified DNA patterns or with one different amplicon in only one of the six patterns were classified in the same specific RAPD cluster type. *V. cholerae* O1 classical strains 569B and O395 (RAPD cluster type III—reference pattern E1/E11/V13/V19/A9/A5), and El Tor strains E7946 (RAPD cluster type II—reference pattern E1/E14/V2/V3/A2/A8) were included for comparison.

Detection of SXT-related integrating conjugative elements (ICEs) and associated antibiotic resistance genes

Paired primers int1-F and int1-B were employed to detect the SXT element-integrase gene (*int*). Chromosomal integration was detected by amplification of the right SXT element-chromosome junction (*attP-prfC* gene sequence). Original primers SXT-R1F (5′-CAA GCGGAAAAAAATCCATA-3′) and SXT-R1R (5′-AGAGTCAA CTGCGGTCAGAG-3′), corresponding to the SXT sequence of *V. cholerae* O139 strain MO10 (nt 98 916–98 933) and the *prfC* gene sequence of *V. cholerae* O1 El Tor strain N16961 (nt 704213–704232), respectively, were used.

PCR detection of genes conferring resistance to chloramphenicol (*floR*), sulfamethoxazole (*sulII*), streptomycin (*strA*) and trimethoprim (*dfrA1*) was performed by the primer pairs FLOR-F/FLOR-2, SUL2-F/SUL2-B, STRA-F/STRA-B, and DFRA-F (5′-TCGAAG AATGGAGTTATCGG-3′)/DFRA-R (5′-TTAACCCTTTTGCCAGA TTT-3′), respectively.[5,6]

PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 10 min. *V. cholerae* O139 strain MO10 and *V. cholerae* O1 El Tor strain E7946 were used as PCR positive and negative controls, respectively.

Nucleotide sequence accession numbers

The internal DNA sequences of *dfrA1*, *floR*, *strA*, *sulII* and *int* genes and the right SXT element-chromosome junction were assigned GenBank accession numbers FJ442932, FJ442933, FJ442934, FJ442935, EU011828 and EU011829, respectively.

Results

Four distinct patterns of antimicrobial resistance were identified in 224 *V. cholerae* O1 strains representative of the large epidemics in Eastern Africa in the late 1990s (Table 1). The corresponding MIC ranges for all isolates are shown in Table S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Genomic typing was performed by RAPD fingerprinting. Two major RAPD cluster types designated as VIII (reference pattern E2/E14/V2/V5/A2/A8) and IV (reference pattern E5/E12/V2/V5/A1/A8) were identified (Figure 1).

To ascertain whether resistance genes were located on self-transmissible genetic elements, 48 *V. cholerae* O1 strains selected for their resistance pattern, place and year of isolation, and RAPD cluster type were used as donors in conjugation experiments with *V. cholerae* O1 strain CIRPS1305 as recipient.

In matings between El Tor strains with resistance pattern A and CIRPS1305, the resistance markers were transferred into recipient cells as a linkage group at an average frequency of 3.5×10^{-6}.[3,7]

For all the strains tested, incompatibility tests and plasmid DNA detection in donors and transconjugants identified a conjugative plasmid of incompatibility class C (IncC) encoding resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim.

Matings of El Tor strains with resistance pattern B and CIRPS1305 revealed transfer of the resistance determinants to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim as a linkage group at an average frequency of 1.5×10^{-6}. As no plasmid was detectable by gel electrophoresis in donors or transconjugants from any of the conjugation experiments, the results suggested that the resistance determinants could be located on an ICE.

To identify this possible ICE-mediated resistance, the El Tor donors and resistant El Tor transconjugants were tested for molecular features typical of SXT-related elements. The PCRs for detection of the SXT element-integrase gene (*int*) yielded an amplicon of the same size of 592 bp. The DNA sequence of the product was determined, showing 96% identity with the SXT element *int* sequence in *V. cholerae* O139 strain MO10 (GenBank accession no. AYO55428).

The amplification experiments to investigate the right SXT element-chromosome junction yielded a PCR product of the same size of 825 bp. The DNA sequence of the product showed 99% identity with the right SXT element-*prfC* junction in *V. cholerae* O139 strain MO10.

PCR assays designated for the amplification of the internal sequences of *floR*, *sulII*, *strA* and *dfrA1* were performed in El Tor donors, recipient CIRPS1305 and resistant El Tor transconjugants. All, except for the recipient, yielded DNA amplicons of the same size. The DNA sequence of the products showed more than 99% identity with the *floR*, *sulII*, *strA* and *dfrA1* resistance genes harboured by SXT elements identified in *V. cholerae* O1 El Tor strains. This indicated that the cluster of genes encoding resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim in the El Tor strains with pattern B were located on an SXT-related ICE.

Of the Kenyan El Tor isolates with pattern C, three strains were mated with *V. cholerae* O1 CIRPS1305 as recipient. The resistance patterns of transconjugants and the frequencies of transfer revealed two clusters of resistance genes: one of genes encoding resistance to sulfamethoxazole and tetracycline, which was transferred at an average frequency of 1.5×10^{-2}, and the other encoding resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim, which was transferred at an average frequency of 1.5×10^{-6}. As was done for the characterization of resistance elements in El Tor strains with patterns A and B, incompatibility tests, plasmid DNA detection and PCR

Table 1. Drug resistance patterns, RAPD cluster types and resistance-associated genetic elements in 224 *V. cholerae* O1 strains isolated in 1998–99 from Eastern Africa

Resistance pattern (number of strains)	Pattern designation	RAPD cluster type	Country(ies) of isolation (number of strains or year)	Number of strains tested as donors	Conjugative genetic element(s)	Frequency of transfer in matings *V. cholerae*× *V. cholerae*
AMP CHL STR[a] SXT TMP (120)	A	VIII	Ethiopia (22)	5	IncC plasmid encoding resistance to AMP, CHL, STR, SXT and TMP	3.5×10^{-6}
			Somalia (91)	15		
			Kenya (7)	5		
CHL STR[a] SXT TMP (74)	B	IV	Sudan (16)	5	SXT-related ICE encoding resistance to CHL, STR, SXT and TMP	1.5×10^{-6}
			Kenya (55)	8		
			Tanzania (3)	2		
CHL STR[a] SXT TET TMP (6)	C	IV	Kenya (6)	3	SXT-related ICE encoding resistance to CHL, STR, SXT and TMP	1.5×10^{-6}
					IncC plasmid encoding resistance to SXT and TET	1.5×10^{-2}
STR (24)	D	VIII	Somalia (12)	3	not detected	
		VII	Kenya (12)	2		
Reference strains						
El Tor/E7946	—	II	Bahrain (1978)	—	—	—
classical/569B	—	III	India (1948)	—	—	—
classical/O395	—	III	India (1948)	—	—	—

AMP, ampicillin; CHL, chloramphenicol; STR, streptomycin; SXT, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.
[a]High levels of resistance (>256 mg/L).

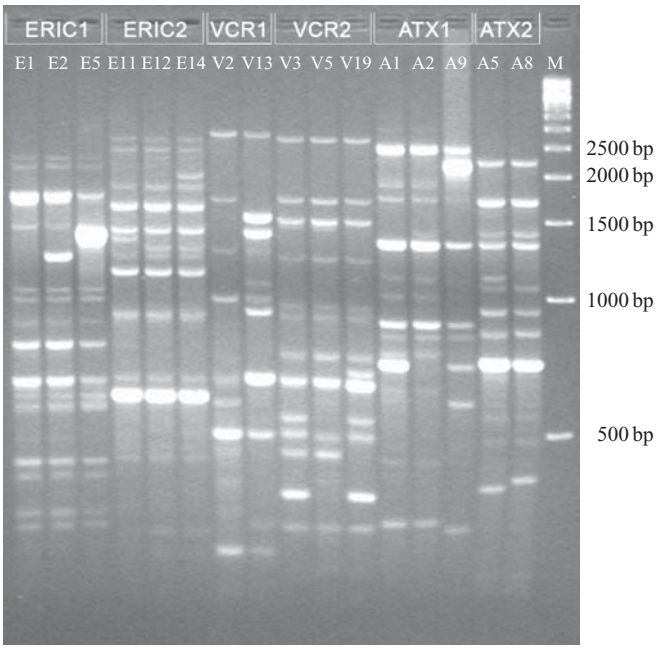

Figure 1. RAPD patterns of genomic DNA of *V. cholerae* O1 strains generated by six primers. All representative variants of single RAPD patterns are included. Each specific pattern is designated by a letter with a serial number (E1, V2, A1, etc.) and the corresponding primer is reported at the top of the gel (ERIC1, VCR1, ATX1, etc.). Molecular size markers are given on the right-hand side.

analysis were also performed on donors, recipient and resistant transconjugants. The Lake Victoria strains harboured two distinct genetic resistance elements: an IncC conjugative plasmid encoding resistance to sulfamethoxazole and tetracycline and an SXT-related ICE indistinguishable from that found in El Tor strains with pattern B. Five El Tor strains with pattern D were examined in detail. No plasmid DNA, SXT-related elements or transfer of streptomycin resistance determinant(s) were detected.

Discussion

Since 1970, Eastern Africa has been suffering an increased number of cholera epidemics; in 1997, it accounted for 56% of the annual clinical cases notified by the entire continent to WHO, remaining high (20%) in 1998–99. The upsurge of cholera outbreaks in the late 1990s was also characterized by a high number of multidrug-resistant *V. cholerae* O1 strains: of 224 strains analysed, 200 isolates were multidrug-resistant (resistance to three or more antimicrobials) and clustered in three resistance patterns encoded by two distinct types of conjugative genetic elements.

An IncC plasmid encoding resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim was identified in most strains active in Ethiopia, Somalia and along the South-Eastern border of Kenya with Somalia. In contrast, a different conjugative genetic element, an SXT-related ICE, encoding resistance to chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim, characterized most multidrug-resistant *V. cholerae* O1 strains (patterns B and C) active in Sudan, Kenya and Tanzania. This class of ICEs was first identified in MO10, and subsequently in *V. cholerae* O1 El Tor strains isolated in the Indian subcontinent in 1994. Since the early 1990s, SXT-related ICEs have spread widely among *V. cholerae* epidemic strains in Asia where, nowadays, most isolates contain these ICEs.[8] In the past decade, SXT-related ICEs have also been detected in *V. cholerae* O1 strains in Southern Africa.[9,10] Data produced in this study show that SXT-related ICEs were also to be found in *V. cholerae* O1 strains in Eastern Africa, thus highlighting their role in conferring the multiple resistance shown by isolates that spread throughout this area in the late 1990s.

The identification of two separate groups of *V. cholerae* O1 isolates, each with their own peculiar resistance pattern, conjugative genetic element, geographic areas of isolation and genomic relatedness, may be consistent with the hypothesis of independent introductions of these strains to Africa, which then gradually spread coming face to face with each other in Eastern Africa in the late 1990s.

Funding

This study was supported by grant IC18-CT97-0231 (F. M.) from the European Commission under the INCO-DC programme and, in part, by research funds of the Faculty of Sciences, University of Bari, and the PhD course in Genetics and Molecular Evolution at the University of Bari.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1. Kaper JB, Morris JG, Levine MM. Cholera. *Clin Microbiol Rev* 1995; **8**: 48–6.

2. Scrascia M, Maimone F, Mohamud KA *et al.* Clonal relationship among *Vibrio cholerae* O1 El Tor strains causing the largest cholera epidemic in Kenya in the late 1990s. *J Clin Microbiol* 2006; **44**: 3401–4.

3. Scrascia M, Pugliese N, Maimone F *et al.* Clonal relationship among *Vibrio cholerae* O1 El tor strains isolated in Somalia. *Int J Med Microbiol* 2008; doi:10.1016/j.ijmm.2008.07.003.

4. Pazzani C, Scrascia M, Dionisi AM *et al.* Molecular epidemiology and origin of cholera reemergence in Italy and Albania in the 1990s. *Res Microbiol* 2006; **157**: 508–12.

5. Hochhut B, Lofti Y, Mazel D *et al.* Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob Agents Chemother* 2001; **45**: 2991–3000.

6. Iwanaga M, Toma C, Miyazato T *et al.* Antibiotic resistance conferred by a class I integron and SXT constin in *Vibrio cholerae* O1 strains isolated in Laos. *Antimicrob Agents Chemother* 2004; **48**: 2364–9.

7. Scrascia M, Pugliese N, Maimone F *et al.* Cholera in Ethiopia in the 1990s: epidemiologic patterns, clonal analysis and antimicrobial resistance. *Int J Med Microbiol* 2008; doi:10.1016/j.ijmm.2008.10.004.

8. Burrus V, Marrero J, Waldor MK. The current ICE age: biology and evolution of SXT-related integrating conjugative elements. *Plasmid* 2006; **55**: 173–83.

9. Ceccarelli D, Bani S, Cappuccinelli P *et al*. Prevalence of *aadA1* and *dfrA15* class 1 integron cassettes and SXT circulation in *Vibrio cholerae* O1 isolates from Africa. *J Antimicrob Chemother* 2006; **58**: 1095–7.

10. Mwansa JCL, Mwaba J, Lukwesa C *et al*. Multiply antibiotic-resistant *Vibrio cholerae* O1 biotype El Tor strains emerge during cholera outbreaks in Zambia. *Epidemiol Infect* 2007; **135**: 847–53.