Jpn. J. Med. Sci. Biol., 50, 227 - 232, 1997.

Short Communication

ISOLATION OF A NEW VARIANT OF *VIBRIO CHOLERA* O1: *V. CHOLERA* O1 RIBOTYPE B27 TOXINOGENOTYPE TB31 DURING THE LAST CHOLERA EPIDEMIC IN SENEGAL

Awa AIDARA-KANE, Cheikh. S. B. BOYE¹, Stephania KOBLAVI²,
Francine GRIMONT and Patrick GRIMONT²

Institut Pasteur, BP 220, Dakar, Senegal, ¹C. H. U. Aristide Le Dantec, Dakar, Senegal and ²Institut Pasteur Paris, France

(Received October 15, 1997. Accepted March 25, 1998)

SUMMARY: A total of 205 *Vibrio cholerae* O1 isolates from recent cholera epidemic in Senegal were analyzed by conventional methods, polymerase chain reaction (PCR) for genes encoding cholera toxin (*ctx A*), zonula occludens toxin (*zot*) and accessory cholera enterotoxin (*ace*), ribotyping and toxinogenotyping. Ribotyping after *Bgl* I digestion of total DNA revealed that ribotype B5a, the predominant ribotype of the seventh pandemic in Africa and Asia, was not isolated. A new ribotype designated B27 in our database is predominant and was associated with a new toxinogenotype designated TB31.

Cholera remains an important public health problem in most developing countries and cholera epidemics continue to cause emotionally charged disasters (1).

The pathogenicity of *Vibrio cholerae* is related to the presence of a set of virulence genes implicated in the intestinal colonization and in the induction of severe diarrhea in patients (2). The major virulence factor is cholera toxin (CT), but two other toxins were described: The zonula occludens toxin (ZOT) and the accessory cholera enterotoxin (ACE), the *ctx* gene encoding CT, the *ace* gene encoding ACE and the *zot* gene encoding ZOT are located on a chromosome segment flanked by two or more

Table I. Characteristics of oligonucleotides used for PCR amplification of *ctxA*, *ace* and *zot* genes

Gene	Oligonucleotide	Sequence	Position
<i>ctxA</i>	<i>ctx</i> 1	5'-CGG GCA GAT TCT AGA CCT CCT G-3'	564
	<i>ctx</i> 2	5'-CGA TGA TCT TGG AGC ATT CCC AC-3'	
	<i>ctx</i> probe	5'-CGT TAA TGA TGT ATT AGG GGC ATA-3'	
<i>ace</i>	<i>ace</i> 1	5'-TAA GGA TGT GCT TAT GAT GGA CAC CC-3'	314
	<i>ace</i> 2	5'-CGT GAT GAA TAA AGA TAC TCA TAG-3'	
	<i>ace</i> probe	5'-CCG CTT ATC CAA CAG GCT AT-3'	
<i>zot</i>	<i>zot</i> 1	5'-TGG CTT CGT CTG CTG CCG GCG ATT-3'	1083
	<i>zot</i> 2	5'-CAC TTC TAC CGA CAG CGC TTG CG-3'	
	<i>zot</i> probe	5'-GCC ACT TTA ACC GCG CCA C-3'	

copies of a repeated sequence called RS1 (3). The distribution of these different genes forming the "virulence cassette" has been investigated (4,5).

A collection of 205 strains of *V. cholerae* O1 strains isolated in Senegal between August 1995 and March 1996 were first characterized by conventional methods using biochemical, serological and antibiotic susceptibility tests.

These methods were not able to discriminate among strains: the etiologic agent of all cases of cholera recorded in Senegal was *V. cholerae* O1 biotype El Tor serotype Ogawa and all the strains were resistant to the vibriostatic agent O129 (2,4 diamino 6,7 diisopropyl pteridine phosphate), cotrimoxazole and chloramphenicol.

PCR analysis of total DNA was performed by using primers described in Table I. An amount of 500 ng of DNA was used as target in the presence of 50 pmol of primers, 200 nmol of DNTP and 2.5 IU of Taq DNA polymerase. The samples were subjected to 25 rounds of amplification steps consisting of 1 min denaturation at 94 C, 1 min annealing at 65 C and 2 min extension at 72 C. The amplification program was preceded by denaturation for 5 min at 94 C and ended with 5 min of extension at 72 C. After PCR, the amplicon was transferred to a nylon membrane N+ and hybridized in the same conditions as for ribotyping with a specific probe (Table I) nonradioactive labeled with digoxigenin-11dUTP (Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate) following the manufacturer's instructions. PCR analysis showed that all *V. cholerae* O1 strains isolated during this Senegales outbreak have the complete virulence cassette.

Fig. 1. Dendrogram obtained by comparison of *Bgl* I ribotyping patterns from the more frequently isolated ribotypes of *V. cholerae* after 1937.

Newer methods of molecular typing including arbitrary PCR (6), pulsed field gel electrophoresis (7) and ribotyping (8,9) are considered as important tools for epidemiological and taxonomical purposes. In this study ribotyping and toxinogenotyping were used as molecular methods typing *V. cholerae* O1 strains. Ribotyping and toxinogenotyping were performed as follows: approximately 2 µg of extracted DNA was cleaved for at least 3 hr at 37 C with *Bgl*I according to the suppliers instructions (Boehringer Mannheim, France). Restriction fragments were separated in 0.8% (w/v) agarose gel (Appligene Illkirch, France) in Tris borate buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) for 16 hr at 1.5 V/cm. Restriction fragments of *Citrobacter koseri* strain 32 DNA cleaved by *Mlu*I (Pharmacia-Biotech, Saint-Quentin, France) were used as fragment-size markers and were loaded in four

Bgl I

Fig. 2. Ribotyping and toxinogenotyping pattern of Senegalese *Vibrio cholerae* O1 strains isolated after *Bgl*I digestion in 1995/1996.

lanes of a 20-lane gel prior to migration. Gels were stained with ethidium bromide (Sigma) 2 µg/ml for 20 min and photographed at 365 nm UV transillumination. DNA fragments were depurinated with 0.25 M HCl and transferred to a nylon membrane (Hybond N+, Amersham International, Amersham, UK) by the method of Southern using an alkali blotting procedure and a vacuum blotter (Vacugene system, Pharmacia-Biotech). After transfer, DNA was fixed in an oven at 80 C for 30 min. Prehybridization and hybridization were carried out at 68 C for an hour and 58 C for 16 hr, respectively.

The probe used for hybridization was a set of five synthetic nucleotides corresponding to conserved sequences of RNA 16S and 23S labelled with digoxigenin (Boehringer Mannheim, Germany). For more accurate subtyping of *V. cholerae* strains, the study of the toxin gene pattern (toxinogenotyping) was associated to the RNA gene restriction pattern (ribotyping). For toxinogenotyping, the probe for hybridization was plasmid PJM17 (3) in which was inserted a fragment of *V. cholerae*

Fig. 3. Dendrogram obtained by comparison of *Bgl* I ribotyping patterns from the more frequently isolated toxinogenotypes of *V. cholerae* after 1937.

carrying genes *ctx*, *zot* and *ace* (10,11). Fragments were detected colorimetrically as previously described (12).

Digitalization and interpretation of ribotypes were done by using the programs of the Taxotron package (Taxolab, Institut Pasteur, Paris, France) and the profiles were compared with those of the 53 ribotypes in our data bank obtained from 605 strains of *V. cholerae* isolated from all continents (data not shown, see ref. 12) over a period of 58 years (1937-1995). Otherwise, 98 toxinogenotypes were identified (data not shown, see ref. 12), by using the modified plasmid PJM 17 probe.

The major pattern encountered during this seventh pandemic is ribotype B5a of Koblavi, corresponding to ribotype 5 of Popovic (8) which represents more than 50% of the strains of our collection (308/605). This ribotype is often associated with four major toxinogenotypes: TB 10 (30%), TB 12a (13%), TB 30 (11%) and TB42 (7.5%). Ribotype B5a was isolated during the Senegalese epidemic in 1978 and it was associated with ribotype B9 during the Senegalese outbreak in 1988 (Fig. 1); none of these two ribotypes was isolated during this outbreak.

All the strains isolated during this Senegalese outbreak had the same pattern characterized by three bands around 10 kb and one band around 4 kb. This ribotype has never been identified before and it was designated B27 in our data bank. This ribotype was associated with a new toxinogenotype never identified before that we designated TB31 (Figs. 2 and 3).

This finding emphasises that epidemic waves of biotype El Tor could be due to the emergence of new clones.

REFERENCES

1. Siddique, A. K., Salam, A., Islam, M. S., Akram, K., Majundar, R. N. and Zaman, K. (1995): Why treatment centres failed to prevent cholera deaths among Rwandan refugees in Goma, Zaire. *Lancet*, 345, 359-361.
2. Kaper, J. B., Morris, J. G. and Levine, M. M. (1995): Cholera. *Clin. Microbiol. Rev.*, 8, 48-86.
3. Mekalanos, J. J. (1983): Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell*, 35, 253-263.
4. Karasawa, T., Mihara, T., Kurazono, H., Nair, G. B., Garg, S., Ramamurthy, T. and Takeda, Y. (1993): Distribution of the zot (zonula occludens toxin) gene among strains of *Vibrio cholerae* O1. *FEMS Microbiol. Lett.*, 106, 143-146.
5. Kurazono, H., Pal, A., Bag, P., Nair, G. B., Karasawa, T., Mihara, T. and Takeda, Y. (1995): Distribution of genes encoding cholera toxin, zonula occludens toxin, accessory cholera enterotoxin and El Tor hemolysin in *Vibrio cholerae* of diverse origins. *Microb. Pathog.*, 18, 231-235.
6. Coelho, A., Andrade, R. C., Vicente, A. C. P. and Salles, C. A. (1995): New variant of *Vibrio cholerae* O1 from clinical isolates in Amozonia. *J. Clin. Microbiol.*, 33, 114-118.
7. Cameron, D. N., Khambaty, F. M., Washmuth, I. K., Tauxe, R. V. and Barrett, T. J. (1994): Molecular characterisation of *Vibrio cholerae* O1 strains by pulsed field gel electrophoresis. *J. Clin. Microbiol.*, 32, 1685-1690.
8. Popovic, T., Bopp, C., Olsvik, O. and Wachsmuth, K. (1993): Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. *J. Clin. Microbiol.*, 31, 2474-2482.
9. Koblavi, S., Grimont, F. and Grimont, P. A. D. (1990): Clonal diversity of *Vibrio cholerae* O1 evidenced by rRNA gene restriction patterns. *Res. Microbiol.*, 141, 645-657.
10. Baudry, B., Fasano, A., Ketley, J. and Kaper, J. B. (1992): Cloning of a gene (zot) encoding a new toxin produced by *Vibrio cholerae*. *Infect. Immun.*, 60, 428-434.
11. Trucksis, M., Galen, J. E., Michalski, J., Fasano, A. and Kaper, J. B. (1993): Accessory cholera enterotoxin (Ace), the third toxin of *Vibrio cholerae* virulence cassette. *Proc. Natl. Acad. Sci. USA*, 90, 5267-5271.
12. Koblavi, S. (1996): Identification et typage moleculaire des vibronacees, these de doctorat de l'Univeriste paris VII, specialite Microbiologie.