Volume 315, number 1, 74–76 FEBS 11942 January 1993

Identification of toxigenic *Vibrio cholerae* from the Argentine outbreak by PCR for *ctx* A1 and *ctx* A2-B

Pablo Varela[a], Marta Rivas[b], Norma Binsztein[b], Maria Laura Cremona[a], Patricio Herrmann[c], Oscar Burrone[d], Rodolfo A. Ugalde[e] and Alberto C.C. Frasch[e]

[a]*Tecnologia Genetica, Buenos Aires, Argentina,* [b]*Instituto Nacional de Microbiologia 'Carlos G. Malbran', Avenida Velez Sarsfield 563, (1281) Buenos Aires, Argentina,* [c]*Laboratorios BioCiencia, Buenos Aires, Argentina,* [d]*International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy* and [e]*Instituto de Investigaciones Bioquimicas Fundacion Campomar, Antonio Machado 151, (1404) Buenos Aires, Argentina*

Received 21 September 1992; revised version received 16 November 1992

A polymerase chain reaction (PCR) to detect a region of the A1 cholera toxin gene was applied to the identification of 43 *Vibrio cholerae* strains isolated from the recent outbreak in Argentina. A good correlation was observed between the GM1-enzyme-linked immunosorbent assay (GM1-ELISA) to detect the B subunit of the enterotoxin and PCR. However, a *V. cholerae* non-01 strain that was negative by the ELISA test, was positive by the PCR assay for the A1 region. A second PCR test to detect the A2-B coding region was developed to solve this case. We propose that routine detection of toxigenic *V. cholerae* by PCR should include analysis of A2-B coding region or the whole cholera toxin operon.

Vibrio cholerae; Toxin gene; Polymerase chain reaction

1. INTRODUCTION

Infection by *Vibrio cholerae* cause alterations in the digestive tract when the enterotoxin gene is present and expressed [1]. Some strains of *V. cholerae* 01 may not produce the enterotoxin (*CT*) [2] while other *V. cholerae* non-01 may be enterotoxigenic [3]. The locus for the cholerae toxin is a single transcription unit made up of sequences encoding the toxin subunits A (*ctx*A) and B (*ctx*B) [4]. The single polypeptide A is usually proteolytically nicked to form two disulphide-linked polypeptides named A1 and A2. The A subunit is responsible for the toxic activity and B subunit is involved in the binding of the toxin to the ganglioside GM1 of the target cell [4].

In 1991, cholera made a dramatic reappearance in the Americas [5]. From February to July 1992, 450 cases of cholera were detected in Argentina. As expected, several non-toxigenic *V. cholerae* have been identified after the cholera outbreak. Thus, detection of *CT* (or *ctx*) has become essential to assess the possible involvement of a given strain in pathology. The polymerase chain reaction (PCR) could be a good alternative for this purpose [6,7]. In this paper, we show that it is necessary to amplify by PCR both regions, *ctx*A1 and *ctx*A2-B, in order to obtain a perfect correlation with GM1-enzyme linked immunosorbent assay (GM1-ELISA) for CT detection. This approach was applied to strains isolated from the recent Argentine outbreak.

Correspondence address: A.C.C. Frasch, Instituto de Investigaciones Bioquimicas Fundation Campomar, Antonio Machado 151, (1404) Buenos Aires, Argentina.

2. MATERIALS AND METHODS

Forty-three isolates of *V. cholerae* were obtained from the recent outbreak in Argentina. They were collected from human infections and from water or fishes taken from the Pilcomayo River in the North of Argentina (Table I). *V. cholerae* and *Escherichia coli* strains were identified by standard techniques [8]. Well characterized strains and indigenous isolates of *V. cholerae* were cultured in AKI medium at 37°C for 18 h in a stationary test tube [9]. Their serological characterization was performed by slide agglutination using polyvalent and monovalent 0-antisera produced at the 'Instituto Nacional de Microbiologia'. For toxin production tests, all *V. cholerae* strains were subjected to GM1-ELISA as described [10]. *E. coli* strains were grown in Evans medium [11]. Enterotoxins produced by *E. coli*, were characterized by GM1-ELISA with monoclonal antibodies [12,13]. PCR assays were carried out with purified DNA [14] or with 1 μl of boiled bacterial cultures with identical results. Oligonucleotides homologous to a region of *ctx*A1 were designed, which differ in sequence from those of the heat-labile toxin of enterotoxigenic *E. coli* [15–17]. The sequence used was 5′-AGACGGGATTTGTTAGGCACGAT-3′ (forward primer, positions 715–737) and 5′-AGAACCTCGTAAGGGTGTTGGGC-3′ (reverse primer, positions 1123–1145). A second set of primers to detect the region encoding the A2 and B subunits was constructed. The sequence of these primers were 5′-TAGAGCTTGGAGGGAAGAGCCGT-3′ (forward primer, positions (1082–1104) and 5′-ATTGCGGCAATCGCATGAGGCGT-3′ (reverse primer, positions 1647–1625). PCR reactions were performed in 50 μl of a final mix containing 50 mM KCl, 10 mM Tris, pH 8, 5 mM $MgCl_2$, 0.01% gelatin, 1 μM of primers, 200 μM of each dNTP (deoxynucleotides), 100 ng of DNA or the equivalent of 1 μl of boiled bacterial cultures and 2 units of Taq polymerase (Bethesda Research Laboratories, USA). After 5 min at 100°C, DNA was amplified through 30 cycles of 2 min at 94°C, 1 min at 55°C and 1 min of 72°C. Amplified DNA fragments were subjected to 2% agarose gel electrophoresis.

 Published by Elsevier Science Publishers B.V.

Table I

Results of GM1-ELISA and PCR to detect a region encoding *ctx*A1 in *V. cholerae* strains isolated in Argentina

Serogroup	Serotype	No. of isolates	Origin[a]			Number of isolates positive by	
			H	W	F	GM1-ELISA	PCR (A1)
01	Ogawa	26	19	5	2	24	24
01	Inaba	10	7	3	0	8	8
Non-01		7	4	2	1	0	1

[a]H = human; W = water; F = fish.

3. RESULTS AND DISCUSSION

Forty-three *V. cholerae* strains isolated from human infections, water and fishes in Argentina during the recent cholera outbreak were obtained. All of them were tested for the presence of enterotoxin by GM1-ELISA and for the presence of the gene encoding part of the A1 subunit by PCR. The results are summarized in Table I and examples of the amplified products obtained in *ctx*A1 PCR are shown in Fig. 1. PCR reactions were considered positive when an amplified fragment of about 431 bp was obtained (Fig. 1A) and when digestion of this PCR product with the enzymes *Taq*I and *Hpa*II generated fragments of 239 bp and 192 bp, or 356 bp and 75 bp, respectively (Fig. 1B, and data not shown). *V. cholerae* isolates were considered *ctx*A1 PCR negative when they did not show an amplified product of the expected size and failed to hybridize with a probe specific for *ctx*A (Fig. 1A, and data not shown). Weak and unspecific amplified products were observed in some reactions. These products can be eliminated by increasing the temperature of annealing during PCR. Four toxigenic *V. cholerae* 01, El Tor biotype, three non-toxigenic *V. cholerae* as well as six toxigenic and one non-toxigenic *E. coli* reference strains showed a perfect correlation between GM1-ELISA and PCR for *ctx*A1 (results not shown).

Thirty-six out of the 43 Argentine isolates were identified as *V. cholerae* 01, El Tor biotype (26 Ogawa and 10 Inaba serotypes) (Table I). Twenty-four out of 26 Ogawa and 8 out of 10 Inaba isolates were positive by both, GM1-ELISA and PCR methods, for *ctx*A1. Two Ogawa and two Inaba strains were negative by both methods. Thus, results from all 36 *V. cholera* 01 isolates showed a perfect correlation between GM1-ELISA and PCR for *ctx*A1. Seven of the 43 *V. cholerae* strains tested were non-01 and they were, as expected, negative by GM1-ELISA. However, one out of the seven non-01 isolates was positive for *ctx*A1 (Fig. 1C, and Table I). This isolate was named S563. Since this result was rather unexpected, two independent cultures of S563 were prepared and tested by GM1-ELISA and by *ctx*A1 PCR. The same results were obtained. The identify of the S563 PCR product was confirmed by digestion with *Taq*I, which generated the same bands as shown in Fig. 1B for GM1-ELISA positive strains (not shown). To know whether S563 had a mutation in a region other than *ctx*A1, primers to detect the A2-B region were

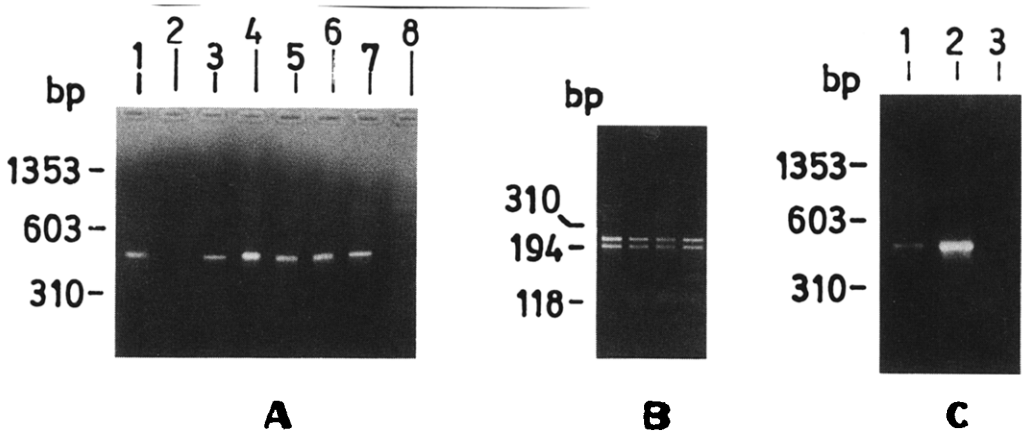

Fig. 1. Identification of *ctx*A1 PCR products in Argentine isolates of *V. cholerae*. (A) PCR amplified fragments from toxigenic and non-toxigenic Argentine isolates. Lanes 1 and 3–7, *V. cholerae* 01 El Tor biotype from human infections, GM1-ELISA positive; lanes 2 and 8, *V. cholerae* 01 El Tor biotype isolated from a fish and from water, GM1-ELISA negative. Molecular weight marker was φX174 phage DNA digested with the restriction enzyme *Hae*III. (B) Digestion with the restriction enzyme *Taq*I of PCR amplified products from four *V. cholerae* 01 El Tor biotype from human infections, GM1-ELISA positive. (C) Lane 1, amplified products in *ctx*A1 PCR from the non-01 *V. cholerae* isolate S563. Lane 2 is a positive control (*V. cholerae* 01 El Tor biotype from a human infection, GM1-ELISA positive), and lane 3 is a negative control (*V. cholerae* non-01, GM1-ELISA negative).

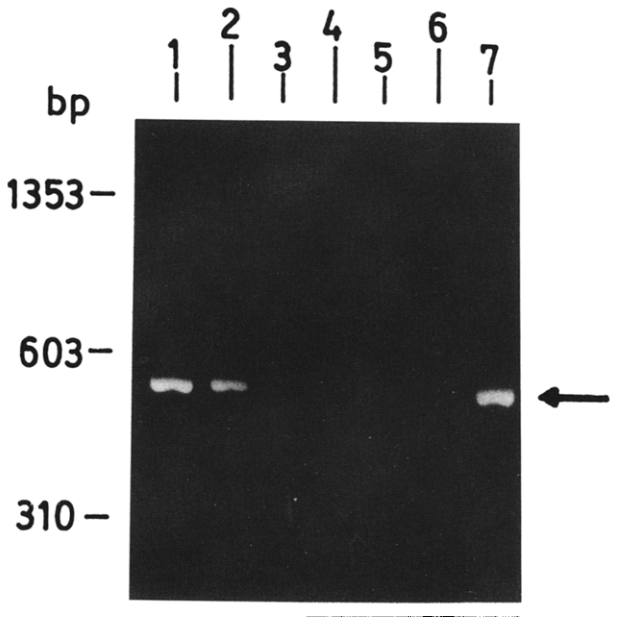

Fig. 2. Identification of PCR products in *V. cholerae* strains isolated in Argentina using primers for *ctx*A2-B. Arrow indicates the position corresponding to the 566 bp A2-B amplified product. Lanes 1, 2 and 7, *V. cholerae* 01 GM1-ELISA positive isolates; lanes 3 and 4, *V. cholerae* non-01 GM1-ELISA negative isolates; 5, *V. cholerae* 01 strain GM1-ELISA negative; 6, *V. cholerae* non-01 S563 strain, GM1-ELISA negative.

constructed and used in PCR assays (Fig. 2). Non-01 *V. cholera* S563 was negative in *ctx*A2-B PCR (Fig. 2, lane 6). *V. cholerae* isolates that were negative by GM1-ELISA and in *ctx*A1 PCR, failed to generate any amplified product by *ctx*A2-B PCR (Fig. 2, lanes 3–5). Positive controls were GM1-ELISA and *ctx*A1 PCR positive *V. cholera*, which generated the expected amplified product of 566 bp (Fig. 2, lanes 1, 2 and 7). Further digestion of these products with restriction enzymes *Alu*I, *Mbo*II and *Hinc*II confirmed that these DNA bands were amplified from *ctx*A2-B (not shown). We conclude that the mutation present in S563 strain is different to those present in other non-toxigenic *V. cholera* isolates. When this work was completed, two related papers were published. Both used PCR for *ctx*A and found a perfect correlation with toxin production as detected by GM1-ELISA [6,7]. We found, however, that in some cases it might be necessary to use a second set of primers to avoid false positive results. Given the possible wide utilization of PCR in samples like food and water, we suggest that amplification of *ctx*A2-B, which appears to perfectly correlate with the GM1-ELISA procedure or, alternatively, the whole cholera toxin operon, should be analyzed in routinely tests for toxigenic *V. cholerae* detection by PCR.

Acknowledgements: This work was supported by grants from Swedish Agency for Research Cooperation with Developing Countries (SAREC), Pan American Health Organization/World Health Organization and Fundacion Life, Buenos Aires, Argentina. The technical assistance of Marcelo Lifschitz is acknowledged.

REFERENCES

[1] Finkelstein, R.A. (1973) Crit. Rev. Microbiol. 2, 553–623.
[2] Kaper, J.B., Moseley, S.L. and Falkow, S. (1981) Infect. Immun. 32, 661–667.
[3] Craig, J.P., Yamamoto, K., Takeda, Y. and Miwatni, T (1981) Infect. Immun. 34, 90–97.
[4] Guidolin, A. and Manning, P.A. (1987) Microbiol. Rev. 51, 285–298.
[5] Glass, R.I., Libel, M. and Brandling-Bennett, A.D. (1992) Science 256, 1524–1525.
[6] Shirai, H., Nishibuchi, M., Ramamurthy, T., Bhattacharya, S.K., Pal, S.C. and Takeda, Y. (1991) J. Clin. Microbiol. 29, 2517–2521.
[7] Fields, P.J., Popowic, T., Wachsmuth, K. and Olsvik, O. (1992) J. Clin. Microbiol. 30, 2118–2121.
[8] Balows, A., Hausler, W.J., Herrmann, K.L., Isenberg, H.D. and Shadomy, H.J. (eds.) (1991) Manual of Clinical Microbiology, 5th edn., American Society for Microbiology, Washington, DC, pp. 360–395.
[9] Iwanaga, M. and Yamamoto, K. (1985) J. Clin. Microbiol. 22, 405–408.
[10] Rivas, M., Binsztein, N., Lopez Moral, L. and Cinto, R. (1987) Rev. Arg. Microbiol. 19, 91–100.
[11] Evans, D.J.J., Evans, D.G., Richardson, S.H. and Gorbach, S.L. (1976) J. Infect. Dis. 133 (Suppl.), 5097–5102.
[12] Svennerholm, A.M. and Wiklund, G. (1983) J. Clin. Microbiol. 17, 596–600.
[13] Svennerholm, A.M., Wikstrom, M., Lindblad, M. and Holmgren, J. (1986) J. Clin. Microbiol. 24, 585–590.
[14] Maniatis, T., Fritsch, E.F. and J. Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.
[15] Mekalanos, J.J., Swartz, D.J. and Pearson, G.D.N. (1983) Nature 306, 551–557.
[16] Yamamoto, T., Gojobori, T. and Yokota, T. (1987) J. Bacteriol. 169, 1352–1357.
[17] Yamamoto, T., Tamura, J. and Tokota, T. (1984) J. Biol. Chem. 259, 5037–5044.