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Research Report

New Biotype of *Vibrio cholerae* O1 from Clinical Isolates in Surabaya

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ABSTRACT

A surveillance of new pathogenic variants of Vibrio cholerae O1 strains was initiated to identify the emerge and spread throughout Surabaya. Findings from seven years (1994–2000) and from years 2008 until now by using a two-fold surveillance strategy was pursued involving 1) hospital-based case recognition, and 2) environment samples. Rectal swabs and environment samples were transported to ITD-UNAIR, Surabaya for culture and isolates were characterized by serotypic identification and arbitrarily primed PCR fingerprints revealed a group of strains with similar fingerprint patterns that are distinct from those of the current El Tor epidemic strain. These strains have been analyzed by in vitro technique and the group has been denominated the Surabaya-Indonesian variant of V. cholerae O1.

Key words: *Vibrio cholerae O1, Cholera toxin, AP-PCR finger print*

INTRODUCTION

Cholera toxin (CT) is a major virulence determinant of *Vibrio cholerae*. *V. cholerae* is indigenous to fresh and blackish water environments in worldwide especially in tropical areas primarily to developing countries with warm climates. *V. cholerae* causes seafood borne infection, water-borne outbreaks and epidemics in terrestrial environments (Barua, D. 1974 and, Bauer *et al*., 1966).

Most *V. cholerae* isolates from the environment do not produce Cholera Toxin (CT), nor do they possess the genetic potential to produce Cholera Toxin. *V. cholerae* O1 and O139 are the major seroytpes associated with illness, and some *V. cholerae* non-O1 and non-O139 isolates produce CT.

Detection of CT-producing *V. cholerae* using conventional culture-, biochemical- and immunological-based assays is time-consuming and laborious. A rapid, reliable and practical assay for the detection of CT-producing *V. cholerae* has been used as like as PCR assays which offer a more sophisticated approach to the identification of *Vibrio cholerae* (Brosius *et al*., 1981).

Although PCR assays provide more rapid identification of *Vibrio cholerae* than conventional assays, they require the use of electrophoresis to detect amplified products, which is time-consuming and tedious.

The worldwide epidemiological situation in cholera El Tor at the beginning of this century is presented; among its characteristic features are continued extensive epidemics and outbreaks in African and Asian countries with cases of import of this infection to other continents. Outbreaks caused by a new variant of the infective agent of cholera, Vibrio cholerae O139, are still registered at limited territories in the countries of South-East Asia (Oneshchenko et al., 2005).

The emergence of Vibrio cholerae O139 Bengal during 1992–1993 was associated with large epidemics of cholera in India and Bangladesh and, initially, with a total displacement of the existing V. cholerae O1 strains. However, the O1 strains reemerged in 1994 and initiated a series of disappearance and reemergence of either of the two serogroups that was associated with temporal genetic and phenotypic changes sustained by the strains. Since the initial emergence of the O139 vibrios, new variants of the pathogen derived from multiple progenitors have been isolated and characterized. The clinical and epidemiological characteristics of these strains have been studied. Rapid genetic reassortment in O139 strains appears to be a response to the changing epidemiology of V. cholerae O1 and also a strategy for persistence in competition with strains of the O1 serogroup. The emergence of V. cholerae O139 has provided a unique opportunity to witness

genetic changes in V. cholerae that may be associated with displacement of an existing serogroup by a newly emerging one and, thus, provide new insights into the epidemiology of cholera (Faruque *et al*., 2003).

Vibrio cholerae is causing a severe epidemic in east Java after being absent from the region for about 10 years (Tauxe *et al*., 1994). Vibrio cholerae typically contains a prophage that carries the genes encoding the cholera toxin, which is responsible for the major clinical symptoms of the disease (Safa *et al.*, 2010). The taxonomy of this species has been the object of our interest, and we recently developed a method for distinguishing pathogenic groups by using arbitrarily primed PCR (APPCR) fingerprints (Coelho *et al.*, 1993 and Coelho *et al.*, 1995) on the basis of the general methodology of AP-PCR (Welsh *et al*., 1990 and Williams *et al.*, 1990). By this technique a single oligonucleotide with an arbitrary sequence is used in a PCR with the DNA of the strain under analysis. Low-stringency conditions for hybridization are used, and the oligonucleotide can find regions of pairing, leading to the amplification of various genome fragments. Our study (6) involved four groups of pathogenic *V. cholerae*: classical, El Tor, Ogawa and Hikojima, all of which are distinguishable with the fingerprints.

When applied to strains isolated from environment the results were similar to those from clinical isolated strains. However, when this method was used to study a group of strains from patients with diarrheal disease in the north part of Surabaya, a quite distinct fingerprint pattern emerged for some of these strains. In the work described here we further extended this observation by using other in vitro techniques to evaluate the degree of relatedness between this group and the other pathogenic strains.

MATERIALS AND METHODS

Isolation and Identification of Vibrio cholerae Serogroups O1 and O139

Enrichment in alkaline peptone water

Inoculated APW with liquid stool or a rectal swab. Incubate the tube with the cap loosened at 37°C for 8 hours. Then subculture to TCBS with two loopfuls of APW from the surface of the broth. If the broth cannot be plated after 8 hours of incubation, subculture a loopful at 18 hours to a fresh tube of APW. Subculture the second tube to TCBS agar after 8 hours of incubation.

Inoculation of TCBS

Inoculate the TCBS plate after 24 hours incubation at 37° C. Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2 to 4 mm in diameter. The yellow color is caused by the fermentation of sucrose in the medium. Sucrose-nonfermenting organisms, such as *V. parahaemolyticus,* produce green to blue-green colonies.

Isolation of suspected *V. cholerae*

One of each type of sucrose-fermenting colony was selected from the TCBS plate to inoculate a heart infusion agar (HIA) slant. Incubate the HIA slants at 37° C for up to 24 hours; for serologic testing. Slide serology with polyvalent O1 and O139 antisera is sufficient for a presumptive identification.

Screening tests for suspected *V. cholerae* isolates

Oxidase test

Conduct the oxidase test with fresh growth from an HIA slant medium. Place 2 to 3 drops of oxidase reagent (1% *N,N,N*′*N*′-tetramethyl-*p*-phenylenediamine) on a piece of filter paper in a petri dish. In a positive reaction, the bacterial growth becomes dark purple immediately. Oxidase-negative organisms will remain colorless or will turn purple after 10 seconds. Positive and negative controls should be tested at the same time. Organisms of the *Vibrio cholerae* is oxidase positive.

Reactions of V. cholerae in screening tests

Oxidase test Positive

Kligler iron agar (KIA) alkaline/acid, no gas produced (red slant/yellow butt)

Triple sugar iron agar (TSI) acid/acid, no gas produced (yellow slant/yellow butt)

Lysine iron agar (LIA) alkaline/alkaline, no gas produced (purple slant/purple butt)

Gram-negative curved rods

Serologic Identification of V. cholerae O1 and O139

Presumptive identification using O1 antisera

Slide agglutination testing with polyvalent O1 antisera, fresh growth of suspected *V. cholerae* from a nonselective agar medium HIA should be used. Using growth from TCBS agar may result in false-negative reactions. Presumptive *V. cholerae* O1 isolates should be tested in monovalent Ogawa, Inaba antisera and Hikojima.

Confirmation of *V. cholerae* O1 using Inaba and Ogawa antisera

The O1 serogroup of *V. cholerae* has been further divided into three serotypes, Inaba, Ogawa, or Hikojima. A positive reaction in either Inaba, Ogawa or both Hikojima antiserum is sufficient to confirm the identification of a *V. cholerae* O1 isolate. Isolates that agglutinate weakly or slowly with serogroup O1 antiserum but do not agglutinate with either Inaba or Ogawa antiserum are not considered to be serogroup O1. Agglutination reactions with Inaba and Ogawa antisera should be examined simultaneously, and the strongest and most rapid reaction should be used to identify the serotype. Strains that agglutinate very strongly and equally with both the Ogawa and Inaba antisera are suspected may be referred to as "possible serotype Hikojima."

Strains of *V. cholerae.*

The strains used in the present study are listed in Table 1.

AP-PCR.

The AP-PCR mixtures consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 100 mM (each) deoxynucleoside triphosphate (dNTP), 30 pmol of one of the oligonucleotides, and 100 ng of DNA in a total volume of 25 ml. The mixture was overlaid with mineral oil, and 1.5 U of *Taq* DNA polymerase was added. The program consisted of 45 cycles, and an annealing temperature of 32° C was used (Coelho *et al*., 1995). Two sets of fingerprints were done, one of them with oligonucleotide 1 (5'-GGTGCGGGAA) and the other with oligonucleotide 3 (5'-CCAGATGCAC) (Coelho *et al*., 1995). Analysis of the amplified fragments was done on 1.4% agarose gels (GIBCO-Bethesda Research Laboratories) in Tris-borate buffer (TBE) (Sambrook *et al*., 1989) running at 100 V for 3 h.

Analysis of presence of virulence genes by PCR.

The basic program for the PCRs for the specific genes included 1 min initial denaturation step at 94° C, followed by 35 three-step cycles at 94° C (45 sec), annealing 55°–60° C for (45 sec) and extension at 72° C (1 min). A final extension at 72° C for 5 min was included in all reactions. A total of 100 ng of DNA, 20 pmol of each primer, 0.25 mM dNTPs, and 1.5 U of *Taq* DNA polymerase were used, with a 1.5 mM $MgCl_2$ buffer, in a total volume of 50 ml. The oligonucleotides used for *ctxA1* amplification were 5'_ CGG GCA GAT TCT AGA CCT CCT G _3' (sense) and 5'_ CGA TGA TCT TGG AGC ATT CCC AC_ 3' (antisense), which were designed

Table 1. Strains of *V. cholerae* analyzed in the study

Strain[a]	Place[b]	Date		Serotype	Type
VF-57	Surabaya	February	1995	Ogawa	El tor
VF-58	Surabaya	February	1995	Ogawa	El tor
VF-59	Surabaya	February	1995	Ogawa	El tor
VF-60	Surabaya	October	1995	Ogawa	-
VF-61	Surabaya	February	1995	Ogawa	El tor
VF-62	Surabaya	February	1995	Ogawa	El tor
VF-64	Surabaya	March	1995	Ogawa	Classic
VF-65	Surabaya	January	1995	Ogawa	El tor
VF-66	Surabaya	March	1995	Ogawa	El tor
VF-67	Surabaya	February	1995	Ogawa	El tor
VF68	Surabaya	June	1995	Ogawa	El tor
VF 69	Surabaya	June	1995	Ogawa	Classic
VF70	Surabaya	June	1995	Ogawa	El tor
VF-71	Surabaya	February	1995	Ogawa	El tor
VF-72	Surabaya	February	1995	Ogawa	El tor
VF-73	Surabaya	February	1995	Ogawa	El tor
VF-74	Surabaya	June	1995	Ogawa	El tor
VF-75	Surabaya	February	1995	Ogawa	El tor
VF-76	Surabaya	February	1995	Ogawa	El tor
VF-77	Surabaya	February	1995	Ogawa	El tor
VF-80	Surabaya	February	1998	Ogawa	-
VF-81	Surabaya	February	1998	Ogawa	-
VF-82	Surabaya	March	1998	Ogawa	-
VF-83	Surabaya	March	1998	Ogawa	-
VF-84	Surabaya	March	1998	Ogawa	-
VF-85	Surabaya	March	1998	Ogawa	-
VF-86	Surabaya	March	1998	Ogawa	-
VF-87	Surabaya	March	1998	Ogawa	-
VF-88	Surabaya	September	1998	Ogawa	-
VF-89	Surabaya	September	1999	Ogawa	-
VF-90	Surabaya	September	1998	Ogawa	-
VF-91	Surabaya	September	1998	Ogawa	-
VF-92	Surabaya	April	1997	Ogawa	El tor
VF-94	Surabaya	March	1998	Ogawa	-
VF-95	Surabaya	June	1998	Ogawa	-
VF-96	Surabaya	September	2008	Ogawa	-
VF-192	Surabaya	June	2009	Hikojima	
VF-193	Surabaya	July	2009	Ogawa	-
VF-194	Surabaya	July	2009	Hikojima	

Strain[a]	Place[b]	Date		Serotype	Type
VF-195	Surabaya	September	2009	Hikojima	
VF-196	Surabaya	September	2009	Ogawa	-
VF-200	Surabaya	December	2009	Hikojima	
VF-217	Surabaya	January	2010	?	

[a] The 36 first strains correspond to the original group of strains analyzed. The seven El Tor strains included in this group were used in the tests described in the text. The last four Hikojima strains were identified later. All isolates came from patients; isolates VF-96 were obtained from environment.

[b] All locations are in Surabaya and adjacent region.

for classical strains. The oligonucleotides used for *ctxA2* amplification were 5'_ ACA GAG TGA GTA CTT TGA CC_ 3' (sense) and 5'_ ATA CCA TCC ATA TAT TTG GGA G_ 3' (antisense), which were designed for classical strains (Lipp *et al*., 2003).

Biochemical identification.

The biochemical characterization of Surabaya-Indonesian strains was done by a battery of standard tests (Farmer *et al*., 1985.) including oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, requirement for Na^+ (growth in nutrient broth with 0, 1, and 3% NaCl), motility, indole, gas from glucose, susceptibility to O/129 (150-mg discs), and acid production from D-glucose, L-arabinose, cellobiose, lactose, maltose, D-mannitol, salicin, and sucrose. Identification of biotypes was performed by detection of acetylmethylcarbinol (Voges-Proskauer test) and determination of susceptibility to polymyxin B (Oxoid) by spot inoculation onto Mueller-Hinton agar (Difco) containing 15mg of polymyxin B per ml (Roy et al., 1965), hemolysis of sheep erythrocytes, and hemagglutination activity for human (O group) and chicken erythrocytes.

O1 somatic antigen characterization.

Expression of O1 antigen by Ogawa-El Tor strains was further evaluated by tube agglutination tests against polyvalent O1 antisera prepared by immunizing rabbits with heat-killed cells of Inaba or Ogawa and then absorption of the antisera with the heat-killed cells of the heterologous serotype. Polyvalent O1 antiserum was obtained by mixing the two monospecific antisera. Tests were performed with live cultures grown for 3 h (Barua, 1974.).

Antimicrobial susceptibility tests.

Antimicrobial susceptibility testing was carried out on Mueller-Hinton agar (Difco) by the disc diffusion method (Clinical and Laboratory Standards Institute, 2008) for levofloxacin, nalidixic acid, ampicillin, chloramphenicol, imipenem and ceftriaxone.

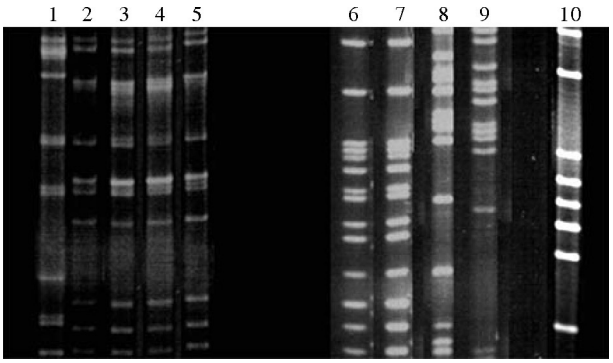

Figure 1. Pathogenic *V. cholerae* O1 fingerprints obtained by AP-PCR with oligonucleotide *ctx*A1 and *ctx*A2. Lanes 1 to 5, Surabaya-Indonesian variant strains (biotype Ogawa-El Tor VF-69, VF-59, VF-76, VF-77 and VF-92) respectively; lanes 6 to 9, biotype Hikojima strains VF192, VF-200, biotype Ogawa VF-193 and VF-196, respectively; lane 10 1-kb ladder size marker (GIBCO Bethesda Research Laboratories).

RESULTS AND DISCUSSION

We studied 43 *V. cholerae* O1 strains (Table 1) isolated in 1995, 1997, 1998, 2008 and 2009 in east and south Surabaya and, in particular, from the villages adjacent region north of Surabaya. Most of them came from patients with diarrhea, and their co-cultures did not show other enteropathogenic bacteria. A screening was done with these strains by using biochemical identification and AP-PCR fingerprints. Two oligonucleotides, oligonucleotides *ctx*A1 and *ctx*A2, were used in separate reactions. Each of the oligonucleotides showed that there were two markedly different groups of strains in the sample. One of these groups yielded the fingerprints found with other El Tor strains (Coelho *et al*., 1995 and Lipp *et al*., 2003), and the other group, comprising five strains Hokojima and denominated in Surabaya-Indonesian variant in 2009–2010, produced fingerprints different from those of the other pathogenic groups studied

previously. The fingerprints of the strains within the group were identical. The results with oligonucleotide *ctx*A1 (Fig. 1) showed a completely different pattern for the Surabaya-Indonesian strains, in which a 1.3-kb band seemed to be the only band common to the bands for the El Tor strains. In the case of oligonucleotide *ctx*A2, a prominent 0.55-kb El Tor band was absent from the fingerprints of the Surabaya-Indonesian strains; the other bands were the same (data not shown). These same oligonucleotides have been used against representative El Tor, classical and Inaba strains (Coelho *et al*., 1995). A serotype difference is not detected with these oligonucleotides. All of the Surabaya-Indonesian strains tested belonged to the Ogawa serotype, but other Ogawa strains in the sample were normal El Tor isolates, producing their characteristic AP-PCR fingerprints. The Surabaya-Indonesian variant strains came from the small town (villages) bordering of Surabaya¸ and a few other villages 50 km adjacent Surabaya. A further search of our collection revealed five other Surabaya-Indonesian strains among the Ogawa strains from the same region.

The AP-PCR fingerprints used in the present study are tuned at a less discriminative level that groups together the El Tor strains (except the environment strains that form a separate group). This grouping occurs because of the choice of oligonucleotides, which were selected to distinguish between broad pathogenic groups and not strains within a group (Coelho *et al*., 1995). A more conspicuous distinction between the Surabaya-Indonesian variant and the El Tor strains was produced with AP-PCR. The Surabaya-Indonesian strains behaved in the biochemical tests as typical representatives of *V. cholerae*. Biological markers such as O-antigen specificity and antimicrobial susceptibility were also evaluated. It was shown that all Surabaya-Indonesian strains but one exhibited O1 agglutination titers of 1,024 (five strains) or 2,048 (three strains). The homologous titer for the classical Ogawa strain was 4,096. Surabaya-Indonesian strain VF-217 was autoagglutinable, preventing its testing. Further testing with monospecific antiserum showed that Surabaya-Indonesian strains reacted only with Ogawa antiserum. Antimicrobial susceptibility tests showed that the Surabaya-Indonesian and El Tor strains isolated from the same geographical area were equally susceptible to all antimicrobial agents tested. Biotyping showed that these strains were Voges-Proskauer test positive and susceptible to polymyxin. Taken together these results support the previous findings of AP-PCR analyses that Surabaya-Indonesian strains are a separate group distinct from the El Tor (polymyxin resistant, Voges Proskauer test positive) and the classical (polymyxin susceptible, Voges-Proskauer test negative) biotypes of *V. cholerae* O1. The presence of *ctx*A gene was investigated in various ways in the Surabaya-Indonesian strains. PCR amplifications were done with positive results for all Hikojima strain and 2 Ogawa El Tor strains. The restriction fragment length polymorphisms of the *ctx* genes were tested. A cholera toxin DNA fragment of 982 bp was used as a probe. This DNA fragment was produced by PCR amplification from an El Tor strain, and it includes most of the *ctx*A1 and *ctx*A2 genes. This indicates that the toxin genes, if present, have a very divergent sequence. Only seven strains (VF-92, VF-192, VF-193, VF-194, VF-195, Vf-196 and VF-200) produced cholera toxin.

The presence of other *V. cholerae* virulence genes was investigated by PCR. Oligonucleotides specific for *ctxB*. All of these amplifications gave negative results. Positive controls with an El Tor strain were included in all of the experiments.

Preliminary phenetic analysis places the Surabaya-Indonesian clone at a considerable distance from other pathogenic O1 clones (ElTor, Classical, and Hikojima). The Surabaya-Indonesian variant seems to be restricted, for the time being, to a small area of the Surabaya and adjacent region and is probably unable to compete with the invading El Tor strains. A parallel may be traced with the early isolates of El Tor from the hospitals. Their epidemiological relevance is, at present, negligible, but as in the latter case, future developments of cholera in Indonesia may outline its importance.

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