Original Article

Phenotypic and Genotypic Characterization of Vibrio cholerae Clinically Isolated in Surabaya, Indonesia

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SUMMARY: The phenotypic and genotypic characteristics of 6 clinical strains of Vibrio cholerae isolated in Surabaya, Indonesia in 2009 were examined. The DNA fingerprints obtained suggested that these isolates were not from a single clone. Furthermore, all isolates produced cholera toxin and possessed the classical type of toxin B subunit gene, thus meaning that this is the first report of the occurrence of El Tor variants of V. cholerae in Indonesia. Although all isolates were sensitive to almost all antibiotics tested, including ampicillin, chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, kanamycin, nalidixic acid, norfloxacin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline, and had no mutation in the gyrA and parC genes, they nevertheless possessed the class I integron that is a molecular vehicle for the acquisition of antibiotic resistance genes, suggesting that they have the potential to acquire the genetic element for drug resistance.

INTRODUCTION

Vibrio cholerae is a gastrointestinal pathogen that causes cholera, one of the most notorious enteric diseases with serious morbidity and mortality worldwide. It is estimated that cholera kills more than 100,000 people worldwide every year, mainly infecting children between 1 and 5 years old (1). However, only V. cholerae serogroups O1 and O139 produce the enterotoxin, cholera toxin (CT), that is reported to cause the disease (2). To the best of our knowledge there have been 7 major cholera pandemics since the early 19th century. The first 6 pandemics were caused by toxigenic strains belonging to the classical serogroup O1 biotype, whereas the 7th, which technically began in the Celebes Islands of Indonesia in 1961, was caused by the El Tor biotype (3). This latter pandemic was initially limited to Asian countries in the 1960s, but soon spread to African countries in the 1970s and further to countries of both American continents from the 1990s onward (1,3).

New pathogenic V. cholerae variants have emerged and spread throughout many Asian and African countries over the past decade. These variants display a mixture of phenotypic and genotypic traits from the two main biotypes ("Classical" and "El Tor"), thus suggesting that they are genetic hybrids (4). Indeed, cholera cases caused by an El Tor variant with the classical-type CT B subunit gene (ctxB) were reported in Mozambique (5), Bangladesh (6), and several other countries in Asia and Africa (7–9). Meanwhile, V. cholerae is increasingly developing resistance towards many antimicrobials used to treat diarrhea (1). For example, the recently isolated V. cholerae strains in India were found to be resistant to multiple drugs, including ampicillin, streptomycin, tetracycline, ciprofloxacin and nalidixic acid, chloramphenicol, sulfamethoxazole, and trimethoprim (10,11). Likewise, most Ethiopian V. cholerae clinical isolates showed resistance to chloramphenicol and ampicillin (12), whereas those isolated in Iran were resistant to nalidixic acid, trimethoprim, and sulfamethoxazole (13). Furthermore, increased fluoroquinolone or tetracycline resistance was reported in V. cholerae clinical isolates in India (14,15). There is increasing evidence to suggest that the genetic elements associated with virulence and drug resistance in V. cholerae are diverse. It is therefore of paramount importance to identify both phenotypic and genetic variations in V. cholerae, which is currently endemic to different regions of the world. In this context, there have been very few reports regarding the presence of El Tor variants and multidrug resistant strains of V. cholerae in Indonesia, other than those inferred to be of Indonesian origin as a result of their isolation from people who have traveled to Indonesia (16).
or from food materials imported from Indonesia (17). Herein we describe the phenotypic and genotypic characteristics of 6 V. cholerae strains isolated clinically from patients in Surabaya, Indonesia’s second-largest city and the capital of the province of East Java, in 2009.

**MATERIALS AND METHODS**

**Bacterial strains:** Fresh stool specimens were obtained from 6 pediatric patients aged between 5 and 14 years with typical cholera diarrhea who attended the Dr. Soetomo’s General Hospital or local clinics in Surabaya, Indonesia in 2009. None had received antibiotic treatment prior to collection of the stool specimens (Table 1). All specimens were stored in Cary-Blair transport medium, transported at ambient temperature to the laboratory within 2 h, and then plated directly on thiosulfate citrate bile salt sucrose (TCBS) agar (Oxoid, Basingstoke, UK). Six strains (VF-192, VF-193, VF-194, VF-195, VF-196, and VF-200 [Table 1]) with sucrose-fermenting yellow colonies on TCBS agar, all of which were oxidase-positive and grew in nutrient broth without added NaCl, were tentatively identified as V. cholerae and were stored in heat infusion agar slants with 2% salt until further phenotypic and genotypic characterization. A further 2 strains of CT-producing V. cholerae O1 El Tor (5H332 and 18H24 [Table 1]) were obtained from Osaka Prefectural Institute of Public Health in Japan for comparison of the serological and genotypic characteristics and antibiotic resistance profiles. Strains 5H332 and 18H24 were isolated clinically from Japanese patients who traveled to Indonesia and India in 1994 and 2006, respectively.

**Phenotypic characterization:** Strains identified as V. cholerae were confirmed by standard biochemistry (18). The O serogroup of these strains was determined by agglutination tests with a commercial polyclonal antisera specific to the V. cholerae O1 polysaccharide (Denka Seiken, Tokyo, Japan). The Inaba and Ogawa serotypes of the O1 strain were differentiated using a commercial latex agglutination kit (V. cholerae AD; Denka Seiken). CT production was determined using a latex agglutination kit (VET-RPLA; Denka Seiken) according to the manufacturer’s instructions. Susceptibility testing was performed using the disk diffusion method with antibiotic disks (BBL Sensi-Disk; Becton Dickinson and Company, Sparks, Md., USA) containing ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, levofloxacin, nalidixic acid, norfloxacin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline. Briefly, the strains were subcultured in Tryptic soy broth and plated on Mueller-Hinton agar with the disks before incubation for 24 h at 37°C. After incubation, antibiotic susceptibility or resistance was determined based on the size of the inhibition zones around each antibiotic disk according to the interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI) (19).

**Genotypic characterization:** A PCR assay targeting 16S-23S rRNA intergenic spacer regions (ITS) specific to V. cholerae (20) was performed to confirm their taxo-

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Phenotypic identity1)</th>
<th>Serogroup, serotype2)</th>
<th>Biotype3)</th>
<th>CT production3)</th>
<th>Month, Year isolated</th>
<th>Place isolated</th>
<th>Source</th>
<th>Patient status</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF-192</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>June, 2009</td>
<td>Local clinic in Surabaya, Indonesia</td>
<td>Stool sample</td>
<td>7-year-old Local resident</td>
</tr>
<tr>
<td>VF-193</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>July, 2009</td>
<td>as above</td>
<td>as above</td>
<td>5-year-old Local resident</td>
</tr>
<tr>
<td>VF-194</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>as above</td>
<td>as above</td>
<td>as above</td>
<td>7-year-old Local resident</td>
</tr>
<tr>
<td>VF-195</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>September, 2009</td>
<td>as above</td>
<td>as above</td>
<td>7-year-old Local resident</td>
</tr>
<tr>
<td>VF-196</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>as above</td>
<td>as above</td>
<td>as above</td>
<td>13-year-old Local resident</td>
</tr>
<tr>
<td>VF-200</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>November, 2009</td>
<td>Dr. Soetomo’s General Hospital, Surabaya, Indonesia</td>
<td>as above</td>
<td>14-year-old Local resident</td>
</tr>
<tr>
<td>5H332</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>1994</td>
<td>Osaka Prefectural Institute of Public Health, Osaka, Japan</td>
<td>as above</td>
<td>Japanese resident who traveled to Bali, Indonesia prior to clinical manifestation</td>
</tr>
<tr>
<td>18H24</td>
<td>V. cholerae</td>
<td>O1 Ogawa</td>
<td>El Tor</td>
<td>+</td>
<td>2006</td>
<td>as above</td>
<td>as above</td>
<td>Japanese resident who traveled to India prior to clinical manifestation</td>
</tr>
</tbody>
</table>

1): determined by standard biochemical tests (18).
2): determined by commercial serotyping kits of slide and latex agglutination test using polyclonal and monoclonal antibodies, respectively.
3): determined by commercial cholera toxin detection kit using a monoclonal antibody.
nomic identities as *V. cholerae*. A “*V. cholerae*” repeat
targeted PCR assay (VCR-PCR) (21) was performed to
assess whether the Surabaya isolates had a novel geno
type of integron island by comparing their VCR-PCR
band patterns with those reported previously (21). Fur
thermore, 3 different DNA fingerprinting methods,
namely the random amplified polymorphic DNA
method (RAPD) (22), enterobacterial repetitive inter
genic consensus-PCR (ERIC-PCR) (23), and BOX ele
ment PCR (BOX-PCR) (23), were performed in order to
determine the degree of inter-strain heterogeneity or
homogeneity. A further set of PCR assays using
primers specific to O1 antigen biosynthesis genes and
virulence-related genes, including *ctxA-1*, *ctxA-2*, *tcpA*,
toxT, *ompW*, tcpB, zot, *rtxA*, *hlyA*, *ompU*, and
toxR, was also performed (20). The presence of class 1
integron and SXT constin (a conjugative, self-transmis
sible integrating element) was subsequently determined
using primers specifically chosen to amplify within these
two elements (24). PCR assays targeting several drug
resistance-associated genes within the class 1 integron or
SXT constin were performed. These genes included
*aadA* (encoding resistance to streptomycin and spec
tinomycin) and *blaP1* (resistance to *β*-lactams) in the
class 1 integron, and *strA* (encoding for streptomycin
resistance), *floR* (encoding for chloramphenicol
resistance), *dfrA1* (encoding O1-specific trimethoprim
resistance), and *sulII* (encoding sulfamethoxazole
resistance) in the SXT constin (25–27). The above PCR
assays were performed essentially following the
methodology (e.g., primer design, PCR, and ele
ctrophoresis conditions) described for each target gene
elsewhere (11,20,22–28). Finally, the presence of point
mutations in the quinolone resistance-determining
regions (QRDR) of *gyrA* and *parC* were determined by
dNA sequencing of the genes described by Baranwal et
al. (29). In addition, a mismatch amplification mutation
assay (MAMA)-PCR, as described by Morita et al. (28),
was performed to differentiate classical and El Tor
tcxB-type strains.

**Ethics clearance:** Approval for this study was ob
tained from the ethics committees of the Institute of
Tropical Disease, Airlangga University. Verbal in-
formed consent regarding use of the clinical samples for
research purposes was obtained from all participants be
fore sample collection.

**RESULTS**

The phenotypic and genotypic characterization
results for all strains, except for the antibiotic resistance
profile, are presented in Tables 1 and 2, respectively.
All strains were identified as CT-producing *V. cholerae*
O1 El Tor based on their phenotypic and genotypic
characteristics. The VCR-PCR band patterns of the 6
Surabaya isolates was identical to the band pattern of *V.
cholerae* O1 El Tor Ogawa strains isolated in many
Asian countries, including Japan, China, Thailand, and
Indonesia, in the 1990s, as reported by Tokunaga et al.
(21; data not shown). The Surabaya isolates were
grouped into 4 genotypes on the basis of the combined
results of the 3 DNA fingerprinting patterns (Fig. 1),
and all possessed the virulence-associated genes tested
for in the present study, therefore their CT genes were
identified as the classical type.

The antibiogram and the antibiotic resistance-associ
ated gene profiles of the strains are presented in Table
3. The Surabaya strains were susceptible to all antibiot
ics tested, except for erythromycin, and did not possess
any of the antibiotic resistance-associated genes tested.
There was no mutation in the *gyrA* and *parC* genes of
QRDR.

**DISCUSSION**

Although a number of researchers have reported the
genetic characteristics of *V. cholerae* strains isolated in
several Asian countries over the past decade (as re
viewed by Safa et al. [4]), there have been very few
reports regarding Indonesian isolates to date. This is
therefore the first report to document the distribution of
virulence-associated genes in Indonesian isolates. The
results of our DNA fingerprinting studies suggest that
the Surabaya isolates did not arise from a single clone.
The distribution of virulence-associated genes in the
Surabaya isolates was comparable to that in the refer-

![Fig. 1. Electrophoretic patterns of (A) RAPD, (B) ERIC-PCR, and (C) BOX-PCR on DNA preparations of 6 Surabaya isolates and 2 reference strains of *V. cholerae* O1 El Tor. Lanes 1, 2, 3, 4, 5, 6, 7, and 8 were electrophoretic patterns observed for strain nos. VF-192, VF-193, VF-194, VF-195, VF-196, VF-200, 5H332, and 18H24. Lane M, 1 kb Plus DNA ladder (Invitrogen, Carlsbad, Calif., USA).]
Table 2. Genotypic characteristics other than antibiotic resistance-associated gene profile of *V. cholerae* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genetic identity</th>
<th>Serogroup genetically determined</th>
<th>Genotype as determined by RAPD</th>
<th>Genotype as determined by ERIC-PCR</th>
<th>Genotype combined</th>
<th>Virulence-associated genes</th>
<th>CT genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF-192</td>
<td><em>V. cholerae</em> O1</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VF-193</td>
<td><em>V. cholerae</em> O1</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>II +</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>VF-194</td>
<td><em>V. cholerae</em> O1</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>VF-195</td>
<td><em>V. cholerae</em> O1</td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>III +</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>VF-196</td>
<td><em>V. cholerae</em> O1</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>I</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>VF-200</td>
<td><em>V. cholerae</em> O1</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>IV +</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>5H332</td>
<td><em>V. cholerae</em> O1</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>V</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>18h24</td>
<td><em>V. cholerae</em> O1</td>
<td>C</td>
<td>D</td>
<td>A</td>
<td>VI +</td>
<td>+ + + + + + + + + + + + + +</td>
<td>+</td>
</tr>
</tbody>
</table>

1) determined by PCR assay targeting *V. cholerae*-specific DNA sequence of 16S–23S rDNA intergenic spacer region (15).
2) determined by PCR assay targeting *V. cholerae* O1 serogroup-specific gene (15).
3) genotyped based on combined profiles of RAPD (5), ERIC-PCR (30) and BOX-PCR (30).
4) determined by MAMA PCR assay (16).

Table 3. Antibiogram and antibiotic resistance-associated gene profiles of *V. cholerae* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Antibiotic resistance and susceptibility</th>
<th>Antibiotic resistance-associated region and gene</th>
<th>Mutation of amino acid in QRDR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
<td>CHL</td>
<td>CIP</td>
</tr>
<tr>
<td>VF-192</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>VF-193</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td>VF-194</td>
<td>S</td>
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<td>VF-195</td>
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<tr>
<td>VF-196</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>VF-200</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>5H332</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>18h24</td>
<td>S</td>
<td>I</td>
<td>I</td>
</tr>
</tbody>
</table>

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; LEV, levofloxacin; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; S, susceptible; I, intermediate; R, resistance.
ence strains and those reported elsewhere (4). Nevertheless, the fact that all Surabaya strains possessed the classical type of ctxB is quite a novel finding. The ctxB is known to have shifted from the classical-specific type in India and Bangladesh around 1993, and this type became globally predominant soon afterwards (5–8,30). As the reference strain, 5H332, used in the present study, which was isolated from a Japanese patient who had traveled to Bali, Indonesia in 1994, possessed the El Tor type of ctxB, it is suggested that the El Tor variant emerged in India or Bangladesh and spread to Indonesia thereafter. Alternatively, phage-mediated lateral classical CT gene transfer, as suggested by Udden et al. (31), might have occurred in the Indonesian El Tor strains. Further extensive phenotypic and genotypic analyses of clinical and environmental V. cholerae strains, including these toxigenic non-O1 strains in different areas of Indonesia, will, however, be required to distinguish between these two possibilities.

Cholera endemics in Indonesia have been reported sporadically in previous publications over the past 20 years (32–38), with some of these studies noting the drug resistance of V. cholerae isolates during this period. For example, a cholera-specific surveillance study undertaken in Indonesia between 1993 and 1999 (34) reported that V. cholerae O1 isolates were susceptible to chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, ceptriaxone, ciprofloxacin, norfloxacin, and nalidixic acid. Likewise, Tjanadi et al. (38) reported that V. cholerae O1 isolated clinically between 1995 and 2001 was sensitive to ceptriaxone, norfloxacin, and ciprofloxacin, with a few isolates being resistant to ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline. More recently, Agtini et al. (32) reported that a majority of V. cholerae O1 El Tor strains isolated clinically in North Jakarta, Indonesia from August 2001 to July 2003 were susceptible to trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, ciprofloxacin, and chloramphenicol. An antibiogram of the 6 Surabaya isolates showed a similar spectrum of drug susceptibility. Correspondingly, they did not seem to possess any gene or genetic mutation, if not all, associated with drug resistance, including quinolone, thus suggesting that antibiotic treatments remain effective for most cholera cases in Indonesia. However, the presence of a class I integron in the Surabaya V. cholerae genome is a warning sign as such integrons have been reported to be a vehicle for the acquisition of antibiotic resistance genes (39). Antibiotic resistance in the Surabaya strains may therefore evolve in the future through integron-mediated acquisition of further drug resistance-associated gene cassettes.

In conclusion, this paper documents the phenotypic and genotypic profiles of V. cholerae strains isolated clinically in 2009 in Surabaya, Indonesia. The findings suggest that multidrug resistance is not prevalent, at least in the strains in Surabaya, although periodic surveillance of the drug resistance of V. cholerae in Indonesia is necessary since all strains possessed a class I integron, a necessary vehicle for the acquisition of antibiotic resistance genes, thus suggesting that these strains may acquire resistance in the near future. Furthermore, this is the first report to document the occurrence of the El Tor variant in Indonesia, which is potentially more virulent than the conventional V. cholerae O1 El Tor (40). It must, however, be noted that the present study reports the genotypic and phenotypic characteristics of a relatively small number of V. cholerae strains isolated clinically in an Indonesian city over a limited period of time. Further phenotypic and genotypic characterization of a larger number of V. cholerae isolates in more extensive areas of Indonesia will therefore be required to assess the current pathogenic status of V. cholerae in Indonesia.

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During the preparation of this paper, Dr. Gary Cores de Vries, a co-investigator, passed away, and we extend our sincere condolences to his family, friends, and colleagues. We very much appreciated his dedication to our entire research activities. The two reference strains of V. cholerae, 5H332 and 18H24, used in the present study were generously provided by Osaka Prefectural Institute of Public Health, Osaka, Japan.

Conflict of interest None to declare.

REFERENCES