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Molecular characterization of recent *Vibrio cholerae* O1, El Tor, Inaba strains isolated from hospitalized patients in Kolkata, India

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Summary *Objectives:* To study the phenotypic and genotypic characterization of newly emerged *V. cholerae* O1, Inaba strains isolated from patients with diarrhoea.
Methods: Bacterial characterization was made using polymerase chain reaction, ribotyping, PFGE and RFLP.
Results: After its first appearance in July 2004, O1 Inaba became the dominant serotype by March 2005 and totally replaced the former dominant serotype, Ogawa from May 2005. Most of the Inaba isolates belong to a new ribotype RIV. Ogawa and also some Inaba strains isolated during the same period were identified as RIII. Similarly, the majority of the Inaba isolates belong to 'H1' pulsotype and one isolate is type 'H', while the Ogawa isolates were mostly 'H' pulsotype. Presence of CTX prophage was detected in a single site of the chromosome with at least two RS elements.
Conclusions: There has been a switch of dominant serotype from Ogawa to Inaba in the Kolkata region. This is not necessarily due to emergence of a new clone but does serve as an epidemiological marker. Further analysis at the molecular level will be required to define this trend and to monitor future spread to other regions.

Introduction

The past decade has witnessed a resurgence in the global incidence of cholera and it continues to be a growing concern in most of the developing countries.[1] The toxigenic *Vibrio cholerae* belonging to O1 and O139 serogroups are the causative agents of cholera. *V. cholerae* strains belonging to

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O1 serogroup are further classified into two biotypes, classical and El Tor and into two major serotypes — Inaba and Ogawa. Since its emergence in 1961 as a causative agent of seventh pandemic, the El Tor vibrios has spread to more than 75 countries in Asia, Africa, and South America.[2] The Latin American episode of cholera and the unprecedented genesis of *V. cholerae* O139 in 1992 are outstanding examples of the unpredictable nature of cholera.[3–5] The extent and rapidity of the spread of the O139 serogroup led to conclude that this event was the beginning of the eighth pandemic of cholera. In fact, *V. cholerae* O1 was virtually extinct among the cholera patients for a period of 6 months after the appearance of O139 serogroup in Kolkata.[6] However, the scenario changed quickly with a decline in O139 cases and interestingly, *V. cholerae* O1 strains replaced the O139 strains since 2003 in Kolkata. At present, *V. cholerae* O1 was the causative agent of cholera in most parts of the globe, while the serogroup O139 was confined mostly in China with almost 59% of cholera cases.[2] After the emergence of *V. cholerae* O139, incidence pattern of serogroups and clones are constantly changing in many cholera endemic regions. In this context, some of the important epidemiological events recorded in the Indian subcontinent include: (i) shift in the incidence between O1 and O139 serogroups[6,7]; (ii) emergence of new clones with multidrug resistance strains of *V. cholerae* O1 Ogawa and Inaba[8–10]; (iii) identification of hybrids of classical and El Tor biotypes based on the *rstR* genes[11,12]; and (iv) increase in the prevalence of O1 Inaba serotypes in India.[13,14]

In this study, we report the findings of a surveillance, which has enabled us to detect subtle changes in traits of *V. cholerae* isolates. The subsequent molecular analysis revealed the presence of newly emerged O1, Inaba strains with distinct ribotype and pulsotype, which displaced the previously prevalent O1, Ogawa strains.

Materials and methods

Hospital surveillance

The present study is a part of the active surveillance program of the National Institute of Cholera and Enteric Diseases (NICED) on cholera and was approved by the scientific advisory committee (SAC) of this Institute. Analysis for this study was made for a period of 24 months from January 2004 to December 2005. Stool specimens were obtained from patients admitted to the Infectious Diseases Hospital (IDH), Kolkata, in McCartney bottles using sterile catheters. Rectal swabs were taken from patients from whom stool specimens could not be obtained. Stool specimens and rectal swabs in Cary-Blair medium were transported within 1 h of collection and were processed within 1 h of arrival at the laboratory for the isolation of *V. cholerae* strains.

Bacteriology and serogrouping

After initial enrichment in alkaline peptone water (pH 8.5), thiosulphate citrate bile salts sucrose agar (TCBS) (Eiken Chemical Co. Ltd., Tokyo, Japan) was used as the selective medium for the isolation of *V. cholerae*. A multitest medium was used for presumptive identification of *V. cholerae*.[15] All the typical strains were subsequently examined for the oxidase reaction and the identity of *V. cholerae* O1 was confirmed by serogrouping with polyvalent O1 and monospecific Inaba and Ogawa antisera. *V. cholerae* strains which did not agglutinate with the O1 antiserum were checked with monoclonal O139 antibody developed at our Institute. *V. cholerae* strains, which were biochemically tested but did not agglutinate with either O1 or O139 antisera were assumed to belong to the non-O1 non-O139 serogroups. The representative *V. cholerae* strains covering the whole study period was used for further characterization.

Polymerase chain reaction (PCR) assays

A multiplex PCR-based assay was used to detect the toxigenic trait and to biotype strains of *V. cholerae* by determining the presence of *ctxA* (encoding the enzymatic subunit of cholera toxin) and *tcpA* (encoding the major structural subunit of the toxin coregulated pilus), respectively.[16,17] Three pairs of primers were used to detect the following: a 301-bp amplicon of *ctxA*, a 472-bp fragment of the El Tor variant of the *tcpA* sequence, and a 618-bp sequence of the classical variant of *tcpA*. In separate assays, specific *rstR* primers were used for the detection of allelic types of *rstR*[11] among different strains with appropriate volumes of 10× amplification buffer (500 mM KCl, 100 mM Tris—HCl, 15 mM $MgCl_2$), [pH 8.3]), 2.5 mM each of dNTP mixture, 10 pmole each of the primer, 1.25 unit of rTaq DNA polymerase (Takara Shuzo, Otsu, Japan) and 5 μl of template. The reaction volume was adjusted to 25 μl using sterile triple distilled water. Uniplex and multiplex PCRs were performed in an automated thermocycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA, USA) for 30 cycles using previously described PCR conditions.[17] The primer sequences used in this study are shown in Table 1.

Pulsed-field gel electrophoresis (PFGE)

PFGE of *V. cholerae* was performed as described previously.[9] *Not*I (Takara) digested inserts of *V. cholerae* were applied to contour-clamped homogenous electric fields in a CHEF Mapper system (Bio-Rad, Hercules, CA, USA) using 1% PFGE grade agarose in 0.5× TBE (44.5 mM Tris—HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) for 40 h 24 min at 14 °C. Run conditions were generated by the auto-algorithm mode of the CHEF Mapper, PFGE system using a size ranges of 20—300-kb. After electrophoresis, the gels were stained with ethidium bromide (1.0 μg per ml) in distilled water for 30 min, destained for 15 min and photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad). A DNA size standard (lambda ladder; New England Biolabs, Beverly, MA, USA) was used as the molecular mass standard.

Southern blotting and DNA hybridization

A 7.5-kb *Bam*HI fragment of plasmid pKK3535 containing 16S and 23S *rRNA* genes of *Escherichia coli* was used as the *rRNA* probe and the *ctxA* probe consisted of a 540-bp *Xba*I-*Cla*I