Introduction

The etiological agent of cholera, *Vibrio cholerae*, is a serologically diverse organism comprising of more than two hundred ‘O’ antigen based serogroups, three serotypes namely Ogawa, Inaba, Hikojima. The O1 serogroup strains have two bio types – Classical and El Tor. The O1 and O139 serogroup strains of *V. cholerae* are known to cause epidemics and hence are known as epidemic strains. All the other serogroup strains are known as non epidemic strains [1].

The pathogenesis of cholera is a complex process and involves a number of factors which help the pathogen to reach and colonize the epithelium of the small intestine and produce the cholera enterotoxin (CT) which leads to rapid dehydration, and death occurs in 50 to 70% of untreated cases. The cholera enterotoxin (CT), encoded by the gene ctxAB is directly responsible for severity of the disease. *V. cholerae* also possess a variety of virulence factors which contribute to the pathogenesis of the disease and not all of the strains possess every one of them, but still most of them remain virulent. This study was carried out to find the presence of the cholera toxin gene and other virulence associated genes in *V. cholerae* strains isolated from Chennai, South India. Genotyping was done based on the presence and absence of these genes and seven genotypes were identified.

Materials and Methods

*V. cholerae* strains

A total of two hundred and fifty eight clinical isolates of *V. cholerae*, isolated from patients admitted to the Communicable Disease Hospital (CDH), Tondiarpet, Chennai, with Acute Diarrhoeal Disease (ADD) during a five year period were collected and identified by performing a set of standard biochemical tests including Oxidase, Indole, Lysine decarboxylase, Arginine dihydrolase, Citrate utilization, Methyl Red test and Voges Proskauer test [6]. Organisms, identified as *V. cholerae* were serogrouped and serotyped by performing agglutination test with O1- Inaba, Ogawa and O139 type specific anti-serum.

**PCR Assay**

PCR amplification of the target DNA was carried out by multiplex PCR assay, in a thermal cycler (Eppendorf Mastercycler Gradient, Germany); with 200 µL PCR tubes with a reaction mixture volume of 50 µL. Previously described methods and primers were used to amplify part of the ompW, ctxA, tcpA, ace, zot, toxR and nanH genes [7, 8, 9]. *V. cholerae* cells were grown overnight at 37°C on Luria Bertani broth (LB broth). Bacterial cells were pelleted by centrifugation, suspended in 100µl of sterile nuclease free water and boiled for 10 minutes. Cell debris were removed
by centrifugation at 12,000 rpm for 10 min and the super-
natant containing the template DNA was used for PCR 
assay. Primers and master mix (2X) were obtained from 
Sigma-Aldrich, U.S.A. The amplification consisted of initial 
denaturation at 94°C for 2 minutes, followed by 30 cycles 
consisting of 94°C for 1 minute, 62°C for 1 minute and 
72°C for 1 minute and a final extension at 72°C for 10 min-
utes. PCR products thus obtained, were electrophoresed 
through 1.5% (wt/vol) agarose gel with ethidium bromide 
(final concentration 0.5 μg/mL), to resolve the amplified 
products and visualized. A 100 base pair (bp) molecular 
weight marker was run along with the PCR products to de-
termine the molecular weight of the PCR amplicons. The 
results were recorded on a BioRad UV gel documentation 
system (BIO-RAD, Hercules, CA, USA). The genes present 
in each of the isolates were noted and tabulated. Table 1 
shows the primers used in the PCR assay.

### Table 1 - Primers used for PCR assay

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Nucleotide Sequence (5’-3’)</th>
<th>Amplification Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompW - F</td>
<td>5’- GCCAGAGCTGTACCTTTGTT-3’</td>
<td>588</td>
</tr>
<tr>
<td>ompW - B</td>
<td>5’- GCAGAGCTTGTTACCTTTGTT-3’</td>
<td>564</td>
</tr>
<tr>
<td>ctxA - F</td>
<td>5’- AACCTTACACCACCTGGAAG-3’</td>
<td>316</td>
</tr>
<tr>
<td>ctxA - B</td>
<td>5’- AACCTTACACCACCTGGAAG-3’</td>
<td>316</td>
</tr>
<tr>
<td>acet - A</td>
<td>5’- GTGGATGCTTACCTTTGTTG-3’</td>
<td>947</td>
</tr>
<tr>
<td>acet - B</td>
<td>5’- GCCAGAGCTTGTTACCTTTGTT-3’</td>
<td>588</td>
</tr>
<tr>
<td>zot - A</td>
<td>5’- GGCTTACGTTAGCGGGG-3’</td>
<td>620</td>
</tr>
<tr>
<td>zot - B</td>
<td>5’- GCCAGAGCTTGTTACCTTTGTT-3’</td>
<td>588</td>
</tr>
<tr>
<td>tcpA - F</td>
<td>5’- GCCAATGAGGCGGCGGGG-3’</td>
<td>453</td>
</tr>
<tr>
<td>tcpA - B/Cls</td>
<td>5’- GCCAATGAGGCGGCGGGG-3’</td>
<td>453</td>
</tr>
<tr>
<td>tcpA - El</td>
<td>5’- GCCAATGAGGCGGCGGGG-3’</td>
<td>453</td>
</tr>
<tr>
<td>toxR - F</td>
<td>5’- GGTGTTACAGCGGGG-3’</td>
<td>779</td>
</tr>
<tr>
<td>toxR - B</td>
<td>5’- GGTGTTACAGCGGGG-3’</td>
<td>779</td>
</tr>
<tr>
<td>nanH1 - F</td>
<td>5’- GGTTGTTACAGCGGGG-3’</td>
<td>639</td>
</tr>
<tr>
<td>nanH1 - B</td>
<td>5’- GGTTGTTACAGCGGGG-3’</td>
<td>639</td>
</tr>
</tbody>
</table>

### Results

Serotyping, serotyping and biotyping of the V. cholerae isolates revealed that, 199 isolates were O1 Ogawa, 25 
were O1 Inaba, 24 were O1 Hikojima, 5 were O139 and 
5 were non epidemic strains. One of the non epidemic 
strains was identified as V. cholerae O81. All the isolates 
were phenotypically confirmed to be biotype El Tor. All the 
258 biochemically confirmed V. cholerae strains were posi-
tive for the species specific gene ompW. The cholera toxin 
gene ctxA was found in 253 (98%) of the isolates. Four out 
of the 258 strains which tested negative for the ctxA were 
non epidemic strains of V. cholerae. One of the non epi-
demic V. cholerae strain (O81) had the ctxA gene. All the 5 
V. cholerae O139 isolates did not possess the nanH gene. 
Based on the presence and absence of the cholera enterotoxin 
and the other virulence associated genes, seven geno-
types of V. cholerae were identified, which are tabulate-
below. The results of the PCR assay are tabulated in Table 2.

### Table 2 – Result of PCR assay of V. cholerae Isolates

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. Tested</th>
<th>No. Positive</th>
<th>Positive %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompW</td>
<td>258</td>
<td>258</td>
<td>100</td>
</tr>
<tr>
<td>ctxA</td>
<td>258</td>
<td>253</td>
<td>98</td>
</tr>
<tr>
<td>toxR</td>
<td>258</td>
<td>232</td>
<td>90</td>
</tr>
<tr>
<td>ace</td>
<td>258</td>
<td>229</td>
<td>89</td>
</tr>
<tr>
<td>zot</td>
<td>258</td>
<td>226</td>
<td>88</td>
</tr>
<tr>
<td>tcpA</td>
<td>258</td>
<td>251</td>
<td>97</td>
</tr>
<tr>
<td>nanH1</td>
<td>258</td>
<td>253</td>
<td>98</td>
</tr>
</tbody>
</table>

### Genotypes of the Isolates

ctxA+, toxR+, ace+, zot+, tcpA+, nanH+ = 212 isolates. 
ctxA+, toxR-, ace+, zot-, tcpA-, nanH+ = 7 isolates.

### Discussion

All the strains tested, were positive for the species specif-
cic gene ompW, thus confirming that all the isolates were 
V. cholerae. Among all the isolates, 212 (82%) of the V. 
cholerae isolates had the complete cassette of virulence 
genes and formed the genotype 1. Apart from this group, 
second highest number of isolates (19) was found in geno-
type 6. 98% of the isolates carried the cholera toxin gene 
ctxA. All 4 strains which tested negative for the ctxA were 
non epidemic strains. However, these cholera toxin nega-
tive strains were able to induce cholera like diarrhoea in 
the patients, which establishes the importance of the other 
toxins that the V. cholerae possesses. All the ctxA negative 
isolates were positive for the accessory cholera enterotoxin 
gene ace and the zonula occludens toxin gene zot. One 
of the non epidemic strain isolated (V. cholerae O81) pos-
sessed the ctxA gene, which is unusual and indicates the 
event of genetic transfer between the epidemic and the non 
epidemic strains.

Toxigenic coliforms gupus gene (tcpA) was present in 97% 
of the isolates, this may be considered important because 
the expression of TCP is correlated with the expression of 
CT [3]. The regulatory gene toxR was found in 92% of the 
isolates. The toxR gene has been shown to be involved in 
the regulation and expression of several genes of V. cholo-
rae [1]. All the V. cholerae O139 isolates, did not have the 
nanH gene, since in general the V. cholerae O139 have a 
truncated pathogenicity island 2, which does not include the 
nanH gene. All the V. cholerae strains were clinical 
specimens, isolated from patients suffering from cholera, 
pertaining to the same geographical location, but screen-
ning for the virulence genes revealed seven different pat-
tern or genotypes based on the presence or absence of 
the virulence genes, thus revealing the diversity among the 
organism.

### Conclusion

Despite being an ancient disease and after more than a 
century of research, cholera still presents many surprises 
and challenges to us. One of the major problems in chol-
era research is the emergence of new clones of the same 
serotypes and biotypes and identification of these strains 
and establishment of clonal relationship are of great im-
portance. Cholera remains a fascinating and deadly dis-

---

**Note:** The content above is a transcription of the document, and the numbered list and tables have been represented in a structured format. The document discusses the PCR assay and genotyping of V. cholerae isolates, focusing on the presence and absence of virulence genes, with specific emphasis on the role of ctxA and other key genes in the diversity among the isolates. The results are presented in a tabular format for clarity.
ease about which microbiologists and physicians should be knowledgeable. Constant monitoring, and in-depth genomic and proteomic research is necessary to track the emergence of new pathogenic strains.

Acknowledgement
The authors wish to thank Mr. Theodore James and Dr. Janarthanan of Communicable Diseases Hospital (CDH), Tondiarpet, Chennai, South India, for all the help rendered.

REFERENCE