RESEARCH PAPER | Medical Sciences | Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Genotyping of *Vibrio cholerae* strains based on the cholera toxin and virulence associated genes.

KEYWORDS: Cholera, *Vibrio cholerae*, Genotyping, Virulence factors

P. Vivian Joseph Ratnam	T. Sundararaj	S. Rajkumar,
Assistant Professor, Pondicherry Institute of Medical Sciences, Kalapet, Puducherry, India (Corresponding author)	Director, JASMN Education and Research Foundation, Perungudi, Chennai, India.	Director, Hubert Enviro Care Systems, Ashok Nagar, Chennai, India

Anjali Anup Anand	Shabana Banu
JASMN Education and Research Foundation, Perungudi, Chennai, India	JASMN Education and Research Foundation, Perungudi, Chennai, India

ABSTRACT *Cholera is a serious public health hazard in many developing countries and Vibrio cholerae, the causative agent of cholera remains a major cause of morbidity and mortality throughout the world. Cholera is characterized by devastating watery diarrhoea 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.*

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) that disrupts ion transport by intestinal epithelial cells. All pandemic *V. cholerae* O1 and O139 strains possess the *ctxAB* gene on the 6.9-kb CTX prophage integrated within chromosome 1. The *ctxAB* gene encodes for the cholera enterotoxin, which is responsible for the clinical severity of cholera [2]. Although production of CT, is directly responsible for the manifestation of diarrhoea, cholera pathogenesis relies on the synergistic action of a number of other genes like *tcp*, *nanH* coding for colonization factors, toxin co-regulated pilus and neuraminidase, *ace* coding for accessory cholera enterotoxin, *zot* coding for zonula occludens toxin and *toxR* the regulatory gene [3, 4].

The detection of these genes can help to understand and characterize the organism completely and among the genotype based detection systems; the Polymerase Chain Reaction (PCR) is widely accepted and used. In PCR, a specifically defined DNA segment is amplified through repeated synthesis. The ends of the amplified DNA are determined by the location of two short, specific oligonucleotides that are used as primers for DNA synthesis [5]. In this study, we used PCR to screen clinical isolates of *V. cholerae* for the presence of *ompW, ctxA, tcp, toxR, ace, zot* and *nanH* genes and based on the presence or absence of these genes, the isolates were grouped into genotypes.

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. *V. cholerae* O1 biotype El Tor strains were identified by performing, test for haemolysis, Polymyxin B (50 U) sensitivity, Voges Proskauer test and chick cell agglutination (CCA) test [6]. All the *V. cholerae* isolates were tested for the species specific gene *ompW* and other virulence genes *ctxA, tcpA, ace, zot, toxR* and *nanH*.

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^{0}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

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

by centrifugation at 12,000 rpm for 10 min and the supernatant 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^{0}C for 2 minutes, followed by 30 cycles consisting of 94^{0}C for 1 minute, 62^{0}C for 1 minute and 72^{0}C for 1 minute and a final extension at 72^{0}C for 10 minutes. 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 determine 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

Target Gene	Nucleotide Sequence (5'-3')	Amplicon Size (bp)
ompW - F	5'- CACCAAGAAGGTGACTTTATTGTG-3'	588
ompW - B	5'- GAACTTATAACCACCCGCG-3'	
ctxA - F	5'- CGGGCAGATTCTAGACCTCCTG-3'	564
ctxA – B	5'- CGATGATCTTGGAGCATTCCCAC-3'	
ace - F	5'- TAAGGATGTGCTTATGATGGACACCC-3'	316
ace – B	5'- CGTGATGAATAAAGATACTCATAGG-3'	
zot – A	5'- TCGCTTAACGATGGCGCGTTTT-3'	947
zot - B	5'- AACCCCGTTTCACTTCTACCCA-3'	
tcpA -F	5'- CACGATAAGAAAACCGGTCAAGAG-3'	620 453
tcpA- B/Clas	5'- TTACCAAATGCAACGCCGAATG-3'	
tcpA- B/El Tor	5'- CGAAAGCACCTTCTTTCACACGTTG-3'	
toxR - F	5'- CCTTCGATCCCCTAAGCAATAC-3'	779
toxR – B	5'- AGGGTTAGCAACGATGCGTAAG-3'	
nanH1 - F	5'- GGTTGGTGCAAGGTATTGG-3'	639
nanH2 – B	5'- TGAGGTTGAGCTCGGTATCC-3'	

Results

Serogrouping, 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 positive 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 epidemic *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 genotypes of *V. cholerae* were identified, which are tabulated below. The results of the PCR assay are tabulated in Table 2.

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

Genes	No. Tested	No. Positive	Positive %
ompW	258	258	100
ctxA	258	253	98
tox R	258	232	90
ace	258	229	89
zot	258	226	88
tcpA	258	251	97
nanH	258	253	98

Genotypes of the Isolates

ctxA+, toxR+, ace+, zot+, tcpA+, nanH+ = 212 isolates.
ctxA+, toxR+, ace+, zot+, tcpA+, nanH- = 5 isolates.
ctxA+, toxR+, ace-, zot-, tcpA+, nanH+ = 6 isolates.
ctxA-, toxR+, ace+, zot+, tcpA+, nanH+ = 5 isolates.
ctxA+, toxR+, ace-, zot+, tcpA+, nanH+ = 4 isolates.
ctxA+, toxR-, ace-, zot-, tcpA+, nanH+ = 19 isolates.
ctxA+, toxR-, ace+, zot-, tcpA-, nanH+ = 7 isolates.

Discussion

All the strains tested, were positive for the species specific 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 genotype 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 negative 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) possessed the *ctxA* gene, which is unusual and indicates the event of genetic transfer between the epidemic and the non epidemic strains.

Toxin coregulated pilus 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 .cholerae* [7]. 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 [10, 11]. All the *V. cholerae* strains were clinical isolates, isolated from patients suffering from cholera, pertaining to the same geographical location, but screening for the virulence genes revealed seven different pattern 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 cholera 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 importance. Cholera remains a fascinating and deadly dis-

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

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 the all the help rendered.

REFERENCE
1. Samuel Rajkumar, Vivian P. Joseph Ratnam, N. Narmada, Eiji Arakawa and T. Sundararaj. 2011. Enterotoxigenicity screening of viable environmental Vibrio cholerae strains from rainwater pools in a university campus in Chennai, South India. Scandinavian Journal of Infectious Diseases. 43(5):325-28. | 2. Muhammad Ali Shah, Ankur Mutreja, Nicholas Thomson, Stephen Baker, Julian Parkhill, Gordon Dougan, et al. 2014. Genomic Epidemiology of Vibrio cholerae O1 Associated with Floods, Pakistan, 2010. Emerging Infectious Diseases. 20(1): 13 – 20. | 3. Kaper, J. B., Morris, Jr. J. G., Levine, M. M. 1995. Cholera. Clin. Microbiol. Rev. 8: 48-86. | 4. Faruque, S. M., M. J. Albert and J. J. Mekalanos. 1998. Epidemiology, genetics and ecology of toxigenic Vibrio cholerae. Microbiol. Mol. Biol. Rev. 62:1-14. | 5. Tanja Popovic, Patricia I. Fields and Orjan Olsvic. 1994. Detection of Cholera toxin genes. Vibrio cholerae and Cholera: Molecular to Global Perspectives. Edited by I. Kaye Wachsmuth, Paul A. Blake and Orjan Olsvic. American Society for Microbiology, Washington DC. 41-52. | 6. Kelly, M. T., F. W. Hickman – Brenner, and J.J. Farmer, III. 1991. Vibrio, In A. Balows, W.J. Hausler, Jr., K.O. Herrmann, H.D. Isenberg, and H.J. Shadomy (ed.), Manual of Clinical Microbiology, 5th ed. ASM Press, Washington, D.C. 384 – 395. | 7. Nandi, B., Ranjan K. Nandy, Sarmishtha Mukhopadhyay, G. Balakrish Nair, Toshio Shimada and Asoke C. Ghose. 2000. Rapid method for species- specific identification of Vibrio cholerae using primers targeted to the gene of outer membrane protein. J. Clin. Microbiol. 38 : 11:4145-4151. | 8. Singh, D.V., Sree Renjini Isac and R.R. Colwell. 2002. Development of a hexaplex PCR assay for the rapid detection of virulence genes in Vibrio cholerae and Vibrio mimicus. J. Clin. Microbiol. 40(11):4321-24. | 9. Folgosa, E., S. Mastrandrea, P. Cappuccinelli, S. Uzzau, P. Rappelli, Brian, M. J., et al. 2001. Molecular identification of pathogenicity genes and ERIC types in Vibrio cholerae O1 epidemic strains from Mozambique. Epidemiol. Infect. 127:17-25. | 10. Figueiredo, S. C. A., Anna Cristina Neves-Borges, Ana Coelho. 2005. The neuraminidase gene is present in the non-toxigenic Vibrio cholerae Amazonia strain: a different allele in comparison to the pandemic strains. Mem. Inst. Oswaldo. Cruz. Rio de Janeiro, 100(6):563-569. | 11. Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S. and Kaper, J. B. 1992. Role of Vibrio cholerae neuraminidase in the function of cholera toxin. Infect Immun. 60:406-415. |