Abundance of hybrid genomes among *Vibrio cholerae* in the aquatic environment determined by fluorescence labelled gene cassette PCR and pulsed field gel electrophoresis: An Australian perspective.

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Introduction:

Vibrio cholerae is the causative agent of cholera, a life threatening and watery diarrhoea that causes an estimated 3–5 million cholera cases and100000–120000 deaths per year (Zuckerman *et al.*, 2007). The current pandemic of cholera is caused by El Tor biotype of the O1 serotype distinct from the classical biotype that is responsible for the previous pandemics. Recently, *V. cholerae* strains isolated from Asia and Africa have been reported to harbour phenotypic and genotypic characteristics (Nair *et al.*,2002 ; Safa *et al.*, 2005; Safa *et al.*, 2009) from both the classical and El Tor (ET) biotypes suggesting that they are genetic hybrids (Safa *et al.*, 2009). These hybrid strains possess all the necessary virulence genes and genetic traits leading to fears that these strains may initiate a new pandemic. The fact that the *V. cholerae* hybrid strains possess characteristics related to the classical biotype is noteworthy since the classical biotype was believed to be extinct (Safa *et al.*, 2009). The types of *V. cholerae* residing in the aquatic environment of Sydney, Australia and its relatedness to known strains remains unknown due to the lack of bacteriological and molecular investigations. Using a new technique profiling integron-associated gene cassettes PCR (Labbate *et al.*, 2007) and Pulse Field Gel electrophoresis (PFGE), we provide evidence for the environmental presence of *V. cholerae* in Sydney with relatedness to characterized hybrid strains.

Materials and Methods:

Collection of and transportation of environmental samples: Water samples were collected weekly between August and September 2009 from four rivers in Sydney and June-September 2010 monthly from only one river. All samples were collected with aseptic technique using sterile dark Nalgene falcon tubes (Nalgene Nunc International, St. Louis, Mo.) and placed in an insulated plastic box. Samples were immediately transported at ambient air temperature from the site of collection (Alam *et al.*, 2006) to the laboratory at the University of Technology Sydney, Australia.

Plating and Storing:
100 µl of unprocessed samples were plated, using a glass spreader, onto thiosulfate–citrate-bile-salts-sucrose (TCBS) agar and incubated at 37°C for 18 to 24 h. Colonies with the characteristic appearance of *V. cholerae* were selected and subcultured on nutrient agar (NA) media. Long term storage of isolates was at -80°C after resuspending cells in tryptone soy (TS) broth containing 10% (v/v) glycerol

DNA techniques:
Genomic DNA (gDNA) was extracted from overnight cultures using the XS buffer protocol (Tillett & Neilan, 2000). Standard PCR was performed using PCR master mix (Promega) containing 25 U Taq DNA polymerase/ml, 800 mM dNTPs and 1.5 mM MgCl2. Primers were used at a final concentration of 0.5 mM each.

***V. cholerae* mobile gene cassettes (MGCs) size profiling:**
The integron arrays of *V. cholerae* strains were profiled according to the presence or absence of mobile gene cassette (MGC) size classes. MGCs from the *V. cholerae* arrays were amplified using 6-carboxyfluorescein (6-FAM)-labelled degenerate primers (HS1201 and HS1202) that anneal to the *attC* sites of MGCs (Labbate *et al.*, 2007). Samples were run in triplicate and the fluorescently tagged PCR products (containing the MGCs) were analysed on an Applied Biosystems (3130xl) genetic analyzer to detect the different size classes of MGCs and their relative abundance. The ABI 3130xl instrument utilized a sophisticated automation capabilities allowing increased productivity. The data obtained by the ABI 3130xl instrument were analysed by Genemapper (Applied Biosystems, version 4.1) software.

Pulsed-Field Gel Electrophoresis (PFGE):
PFGE was performed following the instructions of Pulse Net Asia Pacific. The data were analysed using BioNumerics (Applied Maths, Belgium) software. After background subtraction and gel normalization, the fingerprint patterns were subjected to typing based on banding similarity and dissimilarity. Dendrograms were computed using the Bionumeric Software Package (Applied Maths, Belgium), Dice similarity coefficient, and UPGMA clustering methods, as recommended by the manufacturer, and these were represented by Multi-Dimensional Scaling (MDS) (Fig 2C).

Results:

A. Isolation of *V. cholera* strains from the aquatic Environment of Sydney:
In the present study we analysed 53 environmental samples and selected 23 isolates to be positive for *V. cholerae* based on phenotypic properties. Of the 23 isolates, nine showed a positive amplicon with *V. cholerae* species specific *ompW* primers. Of those, seven were further confirmed to be *V. cholerae* by sequencing the 16S rRNA gene. However, only one of the strains (ES-17) possessed the *wbe*O1 pandemic marker gene. None of the strains had *ctxA*, the main virulence gene of pathogenic *V. cholerae*.

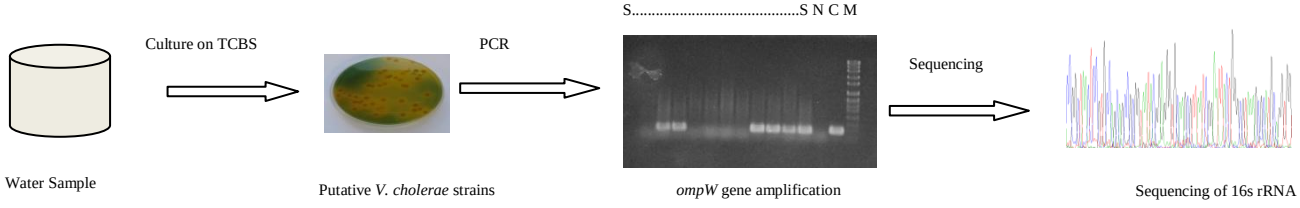


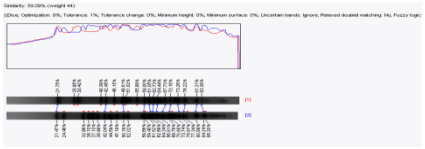
Fig.1: Steps involved in the isolation of *V. cholerae* from the aquatic environment of Sydney, Australia. In the Fig S=Sample, N=Negative control, C= Positive Control and M=Molecular weight marker.

B. Genome analysis based on PFGE:

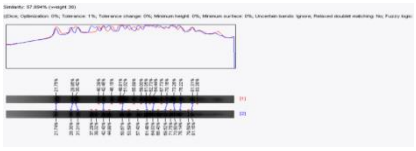
We analysed all (n=7) *V. cholerae* strains from Sydney employing PFGE and compared them with *V. cholerae* strains belonging to serogroup O1, O139, non-O1/non-O139 and recently emerged hybrid strains from Bangladesh. One strain (ES-17) yielded an identical PFGE pulsotype to the recently emerged hybrid strain (Fig.2A). We found that one Sydney isolate (ES-17) exhibited genomic similarity with the hybrid strains. Another Sydney isolate (ES-12) showed more similarity (59%) with classical strain (Fig.2B upper panel) than the ET reference strain, which exhibited 57% similarity (Fig.2B lower panel). Moreover, the Multi-Dimensional Scaling (MDS) showed unique pattern of Australian strains in most of the cases and some showed relatedness with hybrid strains (Fig. 2C).

A

B

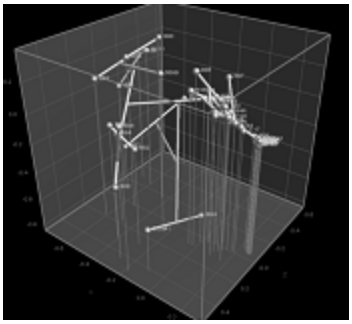


V. cholerae (Australia) VS *V. cholerae* CL



V. cholerae (Australia) VS *V. cholerae* ET

C



MDS of *V. cholerae*

Fig.2: PFGE pulsotype of Australian strain. Pulsotype of Hybrid strain and ES-17 (A) . Pair wise comparision of ES-12 (non O1/non O139 strain) and classical reference strain 569B and Pair wise comparision of ES-12 (non O1/non O139 strain) and El-Tor reference strain N16961v(B). MDS of *V. cholerae* isolated from Bangladesh and Australia (C).

C. Mobile Gene Cassette size profiling using Fluorescence labelled Gene Cassette PCR:

We employed previously developed (Labbate *et al*., 2007) and recently refined (Islam *et al*. 2010) novel genotyping technique based on fragment analysis of mobile gene cassettes to compare the fragment of the same strain (ES-17) that yielded identical pulsotypes to hybrid strain. The strain (ES-17) showed nearly identical fragment with the hybrid strain (Fig: 3). Moreover the fragment of MGCs of strain ES-17 were compared with reference EL and classical strain (Data not shown here). We found that some of the *V. cholerae* strains showed more similarity with the classical biotype. The results obtained from this novel technique is consistent with the pulsotype obtained by PFGE. Moreover, this technique could compare *V. cholera*e strains in more detail (Labbate *et al*., 2007, Islam *et al*., 2010)

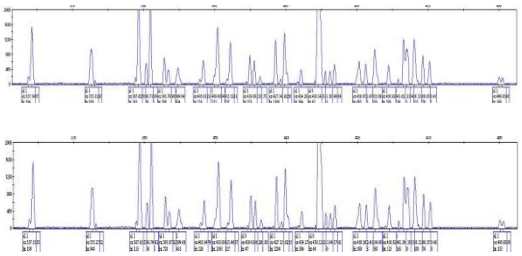


Fig.3: Fragment analysis of *V. cholerae* strains using cassette PCR. Similar fingerprints were observed for the hybrid strain (upper) and Australian isolate ES-17 (lower).

Discussion:

Genomic plasticity among *Vibrio cholerae* (*V. cholerae*) is of immense interest to researchers working with this deadly pathogen. The recent emergence of hybrid strains and their genomic relatedness with classical strains have been reported from Bangladesh (Nair *et al.*, 2002; Safa *et al.*, 2005; Safa *et al.*, 2009). The present study was designed to overcome from the paucity of knowledge in the environmental strains of *V. cholerae* in Sydney, Australia and to determine their relatedness to *V. cholerae* from Bangladesh. In November 2006, NSW health authorities were notified of three cases of El Tor cholera in patients with no recent travel history (Forssman *et al.*, 2007), but to date there is no record of isolating *V. cholerae* from the water reservoir of Sydney. We for the first time isolated six *V. cholerae* isolates in 2009 and one in 2010 from aquatic environment of Sydney, Australia. The environmental presence of this pathogen in Sydney warrants for long-term environmental surveillance.

The presence of *Vibrio cholerae* belonging to serogroup non O1/Non O139 and *ctx* negative O1 strains in the aquatic environment of Sydney pose a public health significance because lateral transfer of virulence genes in *Vibrio cholerae* can potentially lead to virulent strains (Waldor and Mekalanos *et al.*, 1996; Grim *et al.*, 2010). As those rivers are connected with Botany Bay, potential risks are associated with sea food consumption and use of the water of those rivers in recreational activities. Moreover, non-culturable cells of *V. cholerae* can produce clinical symptoms of cholera (Colwell *et al.*, 1994). The result of this study is consistent with previous other studies (Kirschner *et al.*, 2008; Grim *et al.*, 2010).

In this study we used the cassette PCR, a novel method for strain comparisons (Labbate *et al.*, 2007, Islam *et al.*, 2010) which can show insertion or excision of integron associated gene cassettes in the small chromosome of *V. cholerae* genome. We found significant genomic relatedness of Australian native strains with recently emerged hybrid strains. Both PFGE and cassette PCR showed similarity with hybrid strains which suggest the presence of hybrid strains in Australia. The MDS data obtained by PFGE suggest that most of the strains of Australian origin do have unique genomic structure. However, one of the isolates was identical to the hybrid strain and the other was more closely related to classical. These findings suggest that those strains have regional signature and they are also following evolutionary trends as have been observed in Asia and Africa (Nair *et al.*,2002 ; Safa *et al.*, 2005; Safa *et al.*, 2009,). Recently we used a retrospective microbiological, molecular and phylogenetic study to show cholera infection associated with classical, El Tor and El Tor variants of *V. cholerae* in Mexico between 1991 and 1997 (Alam *et al.*, 2010). Earlier macro-evolution were reported among *V. cholerae* from Asia and Africa (Nair *et al.*,2002 ; Safa *et al.*, 2005; Safa *et al.*,

2009). This study is a growing list of investigations demonstrating not only environmental survival but presence of hybrid genomes among *V. cholerae* in the aquatic environment of Australia. In our present study we are investigating micro-evolution among pandemic and non pandemic strains using cassette PCR.

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