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Galleria mellonella is low cost and suitable surrogate host for studying virulence of human pathogenic *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae causes a severe diarrheal disease affecting millions of people worldwide, particularly in low income countries. *V. cholerae* successfully persist in aquatic environment and its pathogenic strains results in sever enteric disease in humans. This dual life style contributes towards its better survival and persistence inside host gut and in the environment. Alternative animal replacement models are of great value in studying host-pathogen interaction and for quick screening of various pathogenic strains. One such model is *Galleria mellonella*, a wax moth which has a complex innate immune system and here we investigate its suitability as a model for clinical human isolates of O1 El TOR, Ogawa serotype belonging to two genetically distinct subclades found in Pakistan (PSC-1 and PSC-2). We demonstrate that the PSC-2 strain D59 frequently isolated from inland areas, was more virulent than PSC-1 strain K7 mainly isolated from coastal areas ($p = 0.0001$). In addition, we compared the relative biofilm capability of the representative strains as indicators of their survival and persistence in the environment and K7 showed enhanced biofilm forming capabilities ($p = 0.004$). Finally we present the annotated genomes of the strains D59 and K7, and compared them with the reference strain N16961.

1. Introduction

Infectious diseases are responsible for approximately 25% of human deaths annually (WHO Communicable Diseases, 2002 Progress Report, http://www.who.int/infectious-disease-news/). Diarrheal diseases are leading causes of morbidity and mortality among children under 5 years of age in developing countries e.g. enteric pathogens alone results in approximately 750,000 deaths each year (Vinekar et al., 2015; Carter et al., 2015). Among such enteric pathogens, *Vibrio cholerae* the cause of cholera has been classified as one of the "emerging and re-emerging infections" (Satcher, 1995) and has become a threat for many low-income countries due to its ability to cause large outbreaks with high death rates (Faruque et al., 1998; Campbell-Lendrum and Rosalie Woodruf, 2006; Rose et al., 2001; Ryan et al., 2006). The spectrum of disease ranges from asymptomatic carriage to profuse life-threatening diarrhea due to rapid electrolyte loss from the gut resulting in severe dehydration. More than 200 serogroups of *V. cholerae* have been identified, however, all major epidemics are mainly caused by serogroups O1 and O139 whereas the remaining serogroups, collectively referred as non-O1/non-O139, generally cause mild diarrhea due to absence toxin co-regulated protein (TCP: receptor for entry of CTXϕ) and cholera toxin (CT). (Heidelberg et al., 2000; Yamai et al., 1997; Vanden Broeck et al., 2007). Recent trends show that *V. cholerae* is rapidly becoming endemic globally mainly due to unhygienic and sanitary conditions and the estimate for only one year (2009 to 2010) suggested that the disease incidence increased to 50% (Page et al., 2012). The recent case of Haiti testifies this where the time between appearance of typical symptoms to death was only 12 h suggesting the need for rapid and specific detection tests and in particular for better outbreak management (Anon, 2010; Keddy et al., 2013).

V. cholerae has developed multiple mechanisms to survive in the environment and interact with invertebrate and non-invertebrate hosts. For a holistic understanding of the determinants important in survival and virulence, there is a need for simple and low cost infection models to better understand the outcome of infection cycle as well as long term persistence in environment (Waldor and Mekalanos, 1996; Pukatzki et al., 2006) Many in vitro or in vivo models have been developed to identify the virulence factors, which help in establishing the infection

Abbreviation: CT, cholera toxin; TCP, toxin-coregulated pili; PSC-1, Pakistan subclade 1; PSC-2, Pakistan subclade 1; *deoC*, deoxyribose-phosphate aldolase; VPI-1, *Vibrio cholerae* pathogenicity Island; VPI-2, *Vibrio cholerae* pathogenicity Island; *acfC*, accessory colonizing factor; MSHA, mannose-sensitive hemagglutinin ()

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process of various important pathogens. For example, yeast, flies, fish and mice have been extensively used as 'surrogate hosts' due to the reasonable proportion of common gene pool with human and hence to understand the disease process. In particular, due to the similar susceptibility range of microbial infections as that of humans, mice have long been used as models for infection studies (Pradel and Ewbank, 2004; Bruno and Ueli, 2010). However, the only established mouse model of cholera is by oral inoculation which lacks manifestation of diarrheal pathology as a result of *V. cholerae* challenge (Klose, 2000). Moreover, the study of *V. cholerae* infection in vivo presents a challenge by employing complicated suckling mouse and infant rabbit infection models (Ritchie and Waldor, 2009; Faruque et al., 2003) for comparing frequently seen genetically diverse epidemic strains for their virulence potential. It is believed that a rabbit infant model mimics the pathology of cholera in humans (exhibit massive diarrhea) when inoculated with live *V. cholerae* orally or intestinally (Ritchie et al., 2010), however for quick screening of epidemic strains for their virulence potential it may not be the appropriate model.

Therefore, a simple robust alternative non-mammalian model is an attractive option for studying infection to establish virulence before embarking upon existing routinely used animal models (Klose, 2000; Ritchie and Waldor, 2009). The *Galleria mellonella* (wax moth), mounts an innate immune response, has been extensively used as a surrogate model for the study of many human microbial pathogens i.e., *Burkholderia* spp., Enteropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria* spp., *Acinetobacter baumannii*, *Campylobacter jejuni*, *Francisella tularensis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Legionella pneumophila*, *Shigella*, Enteroaggregative *Escherichia coli* and hence is considered least complex and useful model organism (Seed and Dennis, 2008; Wand et al., 2011; Kavanagh and Reeves, 2004; Scully and Bidochka, 2006; Leuko and Raivio, 2012; Miyata et al., 2003; Mukherjee et al., 2010; Peleg et al., 2009a, 2009b; Senior et al., 2011; Aperis et al., 2007; Ayres et al., 2008; Jander et al., 2000; Park et al., 2007; Schell et al., 2008; Seed and Dennis, 2009; Harding et al., 2012; Barnoy et al., 2017; Khalil et al., 2016). Moreover, the ability of pathogens to kill *G. mellonella* larvae correlates with the extent of their pathogenic potential, just as the outcome expected with mammalian model organisms (Leuko and Raivio, 2012; Harding et al., 2012; Barnoy et al., 2017; Gundogdu et al., 2015).

In this study, we isolated and compared the differently evolving El Tor *V. cholerae* subclades (PSC-1 and PSC-2) by using two representative strains K7 and D59 for their ability to form biofilms and to infect *G. mellonella*. Furthermore, K7 and D59 were fully sequenced and compared with the *V. cholerae* N16961 (toxigenic and the etiological agent of 7th cholera pandemic and falls within the biotype O1 and belongs to serogroup EI Tor) genome.

2. Material and methods

2.1. Ethical statement

The ethical approval was obtained from the Departmental Ethical Review Board. Bacterial representative isolates used in this study were selected from already existing collection of our previous study (Shah et al., 2014).

2.2. Bacterial strains

Bacterial representative epidemic isolates belonging to distinct subclades PSC-1 (K7) and PSC-2 (D59) isolated from South (Province Sindh: coastal region) and North (Province KPK: inland) from children (age < 5 years) were used in this study.

2.3. Genome sequencing of strains and analysis

The two strains K7 and D59 were completely sequenced (using Illumina, San Diego, CA, USA) as reported previously (Shah et al., 2014) and were mapped to the reference genome of *V. cholerae* O1 El Tor strain N16961 (isolated from Bangladesh, 1975, accession number AE003852-3). The detailed genomic comparison was initiated using *V. cholerae* O1 El Tor pandemic strain N16961 as a reference for ordering the draft genome of *V. cholerae* O1 El Tor strains K7 and D59 for further comparative analysis. The complete sequence of both chromosomes of *V. cholerae* N16961 (Accession No. NC_002505 and NC_002506) was retrieved from National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/). The raw genomic sequences were subjected to annotation using PROKKA, a software tool for prokaryotic genomes annotations (Seemann, 2014). The genomes were then structurally compared with the reference genome N16961 utilizing Artemis Comparative Tool (ACT) (Gogoulou et al., 2005). For exploring large-scale evolutionary events such as rearrangement and inversion the whole genomes were aligned using software Mauve version 2.4.0

2.4. G. mellonella infection assay for V. cholerae

G. mellonella were purchased from Live Foods UK of uniform age, weight and free from antibiotic treatment, were used in infection assays as described previously (Senior et al., 2011). Briefly, after surface disinfection using 70% (v/v) ethanol, 10 µl volumes containing bacterial suspension (10^2 to 10^8 CFU/ml: harvested at OD_{600} = 0.5) were injected into the first right proleg of larvae (10 per group) by using a Hamilton syringe with a 30-gauge needle and kept at 30 °C (60% relative humidity) in 90 mm petri plates containing a 90 mm diameter Whatman filter paper. *G. mellonella* larvae were scored at 24 and 48 h for survival. Death of larvae was distinguished by melanisation of the larvae and lack of movement. Control larvae were injected with PBS diluent and 10 larvae were left untreated. Data from three independent experiments were combined and percentage of killing for different infective doses i.e. 10^8 CFU to 10^2 CFU was calculated. Significant differences were compared using a Student's *t*-test.

2.5. Biofilm assays

The assay was performed as described previously (Kierek and Watnick, 2003). Briefly, borosilicate glass tubes (VWR) were filled with 300 µl of the growth medium (AKI: 1.5% Bacto peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% $NaHCO_3$) inoculated with the strain under study. For reproducibility, experiments were initiated at an optical density at 600 nm (OD_{600}) of 0.02, cells were allowed to adhere to the surface during 24 h of incubation at 28 °C. Crystal violet-stained (1-mg/ml) borosilicate tubes were visualized for biofilm formation. In parallel, surface-adherent cells were quantified after removing the planktonic cells and tubes were rinsed twice with the media and filled with 300 µl of fresh medium. An equivalent volume of 1-mm diameter borosilicate glass beads (Biospec) was added to each tube. Biofilm associated cells were mechanically removed by vortexing in the presence of these beads for 10 s. Finally, the OD_{600} of the biofilm cell suspensions were measured. All assays were performed in triplicate to quantify biofilm-associated cells and the results were reproducible. Student's *t*-test was used to compare the results.

3. Results

3.1. Comparative genome analysis

The assembling result indicated that the genome size of K7 is 67,359 bps, D59 is 67,068 bps and reference genome (*V. cholerae* N16961) is 94,702 bps. Annotation of these genomes revealed 3564 proteins in D59, 3598 proteins in K7 and 5453 proteins in the reference genome N16961. Genome comparison was carried out using Artemis Comparison Tool (ACT) as shown in Fig. 1. Regions of sequence similarity are linked by blocks, which are colored red (same orientation) or