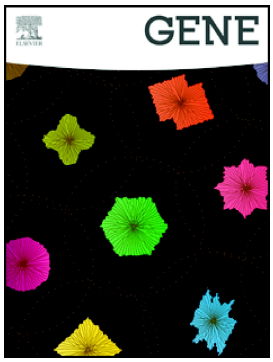

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Galleria mellonella is low cost and suitable surrogate host for studying virulence of human pathogenic *Vibrio cholerae*

Short Title: **Virulence Investigation of unique *V. cholerae* subclades in *G. mellonella* model**

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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 worm 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.

Keywords: *V. cholerae*, *Galleria mellonella*, Surrogate host model, Virulence studies

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 *et al.,* 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 *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 hours suggesting the need for rapid and specific detection tests and in particular for better outbreak management (Update, 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 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) 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.,* 2009; Senior *et al.,* 2011; Aperis *et al.,* 2007; Ayres *et al.,* 2008; Jander *et al.,* 2000; Park *et al.,* 2007; Peleg *et al.,* 2009; 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.,* 2010) .

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.,* 2010) 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, 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 hours 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 sec. 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 3,564 proteins in D59, 3,598 proteins in K7 and 5,453 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 blue (reverse orientation), with saturation indicating the relative degree of identity (dark=high similarity, to light=low similarity). Comparative analysis shows that K7 and reference strain N16961 has an extra region at 3' end when compared with D59 strain which may be due to deletion of genomic

blocks in D59 or insertion of new blocks in K7 as extended region of K7 do not show similarity even with reference genome N16961. Aligning genomes using mauve gave us a clear indication of inserted or deleted regions shown in Fig. 2.

As shown in Fig. 2, a large deletion (appx. of 27,343 bps) can be observed in D59 and K7 as compared to reference genome (N16961) at 3' end. There was another small genomic block observed deleted at the 3' end of D59 resulted in the truncation of 291 bps. This deleted block was found to have five missing genes in D59 (*deoC* encoding Deoxyribose-phosphate aldolase, *deoA* encoding Thymidine phosphorylase, *deoB* encoding Phosphopentomutase, *deoD*: encoding Purine nucleoside phosphorylase and *sbmA* encoding a peptide antibiotic transporter. Other regions of D59 show the same synteny as K7 and there is a highly conserved block in both the strains when compared with reference genome N16961 (shown in Figure 2) which may have the essential and evolutionary conserved genes. Genes found deleted in D59 and K7 as compared to N16961 have been listed in Supplementary Table 1. Genome comparison also revealed that both strains have 1,158 genes in common with the reference genome, appoximately 2300 genes common in both (K7 and D59) whereas 29, 56 and 3,974 unique genes in D59, K7 and N16961 respectively (Fig. 3).

3.2 Signature Genomic Alterations in PSC-1 and PSC-2 Isolates

The study suggests that not only the positioning of genes in the genomes but also the locations of the genes are analogous among Pakistan strains (PSC-1: K7 and PSC-2: D59) and Bengal strain N16961 (Fig. 1). The Vibrio seventh pandemic (VSP-2) island genes VC0496 and VC0497 are deleted in PSC-1and PSC-2 but present in Bengal strain. Members of PSC-1 exhibit a unique pattern with respect to the deletion of three genes of *Vibrio* pathogenicity island-1 (VPI-1) namely VC0819 (1385 bps), VC0820 (3042 bps) and VC0821 (1140 bps) encoding aldehyde dehydrogenase (AldA), ToxR regulated gene A, a secreted mucinase/protease, (TagA) and a

hypothetical protein. The other sub-clade specific deletion included four genes (VC_0495-0498) of VSP-2 and was also reported among Bangladesh cholera outbreak strains in 2004 (Hendriksen *et al.*, 2011).

The major duplication events which occurred in the regulatory genes, in all members of PSC-1 and PSC-2, include the duplication of the gene *smcR* (virulence regulatory gene) which has its 2 copies in reference strain as well. Moreover, VC0142 which is a C4-dicarboxylate transport regulator is duplicated in PSC-1. Some genes of the pathogenic island of *V. cholerae* VPI-1 and VPI-2 seem to show variation in their gene dosage due to perhaps duplication events. VC0847 a VPI-1 gene has its three copies in PSC-2 and a single copy in reference strains whereas VC1788 which belongs to VPI-2 is duplicated in PSC-2 and Bengal strains. However, VC1789 and VC1790 of VPI-2 are duplicated equally with its two copies in PSC-1 and PSC-2 whereas six copies in the reference strain. Moreover, VC0166 which is a transcriptional regulator of the gene *cspA* (stress response) is duplicated in PSC-2 compared to the PSC-1 and reference strain.

3.3 *G. mellonella* Infection Model

The virulence potential of clinical *V. cholerae* isolates belonging to PSC-1 (K7) and PSC-2 (D59) were compared using *G. mellonella* infection model. (Supplementary Fig. 1). Results suggested that K7 and D59 isolates caused time-dependent death of at least 40% and 60% of the *G. mellonella* larvae when 1000 CFU injected after 24 hours' post infection respectively. No mortality was observed in the control PBS injected *G. mellonella* larvae. These results demonstrate that the susceptibility of *G. mellonella* to K7 and D59 varies to some extent.

Furthermore, the results suggest that the mortality caused by K7 (PSC-1) and D59 (PSC-2) was dependent on dose (the number of injected bacteria) and exposure time. *G. mellonella* larvae

were injected ranging from 10^2 to 10^8 CFU/larvae and monitored for 24-48 hours (Fig. 4A; Fig. 4B). Results have shown that infection with 10^8 to 10^6 CFU of D59 resulted in 100% to 95% *G. mellonella* mortality within 48 h of infection. Furthermore, for lower dose of bacteria injected ranging from 10^5 to 10^2, the mortality was reduced from 95% to 84%. However, the results due to infection of *G. mellonella* with 10^8 to 10^6 CFU of K7 show 93% to 80% mortality whereas at dose 10^5 to 10^2 the mortality was reduced from 80% to 67% (Fig. 4). LD50 of K7 was found to be $3.17x10^6$ CFU which is significantly higher than that of D59 i.e., $1.3x10^2$ CFU suggesting that D59 is more lethal even at low dose (p=0.0001) (Table 1)

3.4 Biofilm Forming Potential of *V. cholerae* O-1 (PSC-1 & PSC-2)

The biofilm forming potential of both types were investigated and results indicate that the K7 (PSC-1) was found to be more efficient to form biofilms compared to D59 (PSC-2) (p=0.004) (Supplementary Fig. 2).

4 Discussion

The animal models (mouse, rat, rabbit, fish etc.) for infection studies has been used for decades and are important source of knowledge (Wiles *et al.,* 2006) but when used unnecessarily in large numbers this has ethical and cost implications. The only established *V. cholerae* infection model that manifests severe watery diarrhea is the infant rabbit model (Ritchie and Waldor, 2009). This model is costly, difficult to use and can have inconsistent results..

G. mellonella model have been recently used to investigate the pathogenic potential of range of human pathogens including diarrheal pathogen enteropathogenic *E. coli* (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.,* 2009; Senior *et al.,* 2011; Aperis *et al.,* 2007; Ayres *et al.,* 2008; Jander *et al.,* 2000; Park *et al.,* 2007; Peleg *et al.,* 2009; Schell *et al.,* 2008; Seed and Dennis, 2009; Harding *et al.,* 2012; Barnoy *et al.,* 2017; Khalil *et al.,* 2016) and fish pathogen, *Vibrio anguillarum* (normally causes skin disease and inflammation) (McMillan *et al.,* 2015) . *G. mellonella* has also been used for comparing the virulence potential of different natural variants , however, the comparative analysis of the pathogenic potentials of *V. cholerae* O-1 isolates from diarrheagenic clinical human cases has not been previously exhibited in this model. The current study was undertaken to test the virulence potential of two such natural genetic variants *V. cholerae* O-1 EL TOR strains K7 and D59 (belonging to two distinct subclades) using *G. mellonella* model.

We have shown that this simple invertebrate model was able to demonstrate the pathogenic potential of *V. cholerae* O-1 subclades reported to cause cholera in different geographical locations of Pakistan during 2010 floods . Furthermore, results suggest that K7 (PSC-1) and D59 (PSC-2) virulence in *G. mellonella* is influenced by the relative injected dose. At high concentrations ranging up to 10^6 CFU/larva both subclades (K7 and D59) of *V. cholerae* O-1 induces 100% killing or septic death in *G. mellonella*. However, at lower infectious doses the trends indicate that PSC-1 (K7) was less virulent compared to PSC-2 (D59).

Some genes of the pathogenic island of *V. cholerae* VPI-1 and VPI-1I seem to show variation in their gene dosage due to perhaps duplication events and may influence pathogenic capabilities such as relative transmissibility, virulence and better adaptation to environment (Pallen and Wren, 2007) . Similarly, variation in killing ability of K7 and D59 can be due to range of virulence factors including their genome content which may have impact on the relative transmissibility and virulence of the respective subclades. We used *G. mellonela* to differentiate virulence potential of two

subclades of V. cholerae i.e., PSC1 and PSC2. For instance, K7 (PSC-1) isolates has a unique 3-gene deletion in the VPI-1 including *aldA* (aldehyde dehydrogenase), *tagA* (a mucinase: plays a role in host cell surface modification), and a predicted coding sequence encoding a hypothetical protein and a 4-gene deletion within the VSP-2, also previously identified in outbreak El Tor strains in Bangladesh in 2008 (Pang et al., 2007). Relatively low virulence of K7 could be linked to the lack of *tagA* gene (ToxR-activated gene A) which has been shown to contribute significantly to bacterial virulence (*Aeromonas hydrophila*) in a mouse model (Pillai *et a.*, 2006). For PSC-2 isolates, 18-gene deletion (VC_0495-0512) in VSP-2 is similar to deletions reported in *V. cholerae* El Tor isolates from cholera outbreaks in Haiti 2010 and southeastern China in 2005 (Hendriksen *et al.*, 2011; Pang et al., 2007) and an important gene located in this deletion encodes a putative type IV pilin (VC0502, which may affect the colonization and virulence potential of these strains. Moreover, D59 also possesses frame shift mutation in the *acfC*, accessory colonizing factor (VC_0841) (Shah *et al.*, 2010) .

Comparative analysis of K7 and D59, revealed a large homologous block however deletion of a block at 3' end of D59 was also observed which might be due to environmental stress or through course of evolution. This conserved block harbors some important or essential genes including enzymes, virulence genes, antimicrobial resistant genes and many others genes. Deletion of five genes in D59 may not have much influence on the overall behaviour of the organism however *deoA, deoB, deoC* and *deoD* are the major enzymes involved in the catabolic pathways usually found in the form of operon *deoCABD* in the pathogens (Christensen *et al.*, 2003) . Deletion of this operon might have influenced the long-term persistence in environment but nevertheless need to be further investigated.

This is the first report using the simple and inexpensive *G. mellonella* as a surrogate host to study the relative virulence of two distinct clinical sublineages of *V. cholerae* O1 El TOR. Our study suggests that *G. mellonella* is a simple, cheap and useful model system for assessing virulence of different clonal lineages of *V. cholerae* O-1. Although the K7 strain appears to be less pathogenic in *G. mellonella* compared to D59, however this variant more readily formed biofilms and such ecology-evolution perspective may account for the varied transmission behavior and pathogenic potential of *V. cholerae* in different infection models (Pallen and Wren, 2007) .

V. cholerae is widely distributed in aquatic environments of cholera endemic regions and the ability of biofilm formation is important for their long-term persistence and environmental survival. In our study no association between biofilm forming ability and virulence against *G. mellonela* was observed as our results suggest that K7 was better biofilm former compared to D59 (PSC-2: lacking a putative type IV pilin). Similar results have been reported previous study in *Acinetobacter baumannii* where the ability of strains to form biofilms does not necessarily correlate with increased virulence and only biofilm adapted cells showed enhanced virulence in *G. mellonella* (Wand *et al.*, 2012), On the other hand, the previous study has shown that the presence of the mannose-sensitive hemagglutinin (MSHA) type IV pilus enhance interaction between bacterial cell to host surface (Watnick *et al.,* 1999) resulting in formation of micro-colonies and hence biofilms by synthesizing an exopolysaccharide (EPS) and hence better survival in environment (Watnick *et al.,* 1999; Yildiz and Schoolnik ,1999; Yildiz *et al.,* 2001; ; Wai *et al.,* 1998). However, D59 (PSC2) seemed more virulent compared to K7 (PSC-1) in *G. mellolnela* infection model, perhaps due to the differential expression of other virulent genes due to SNPs or natural deletions as explained above.

The role of VSP-2 in pathogenesis or survival has not been explored in detail and evolutionary changes in this pathogenic island manifest as deletion of genes VC0496 and VC0497 in

K7 (PSC-1) as seen in the Haitian isolates as well as other unique genes deletions (e.g. VC095-VC098 in VSP-2 and VC0819-VC0821 in VP-1) may perhaps not be required for its superior environmental survival. It can be hypothesized that if there is no selective advantage in keeping the gene it is usually converted to a pseudogene and sometimes disappears/deleted from the genome (Mutreja *et al.,* 2011; Hirotsune *et al.,* 2003; Kiyoi *et al.,* 1998) suggesting that VC0496 and VC0497 also have gone through reductive evolution. On the contrary, double dosage of stress response gene VC0166 **(***cspA***)** in D59 may likely enhance its virulence potential as reflected in previous study where its attenuation led to decreased virulence (Leuko and Raivio, 2012). Our results suggest that selection could be a compelling driver of gene loss and selective reductive genome evolution in K7.

It is now revealing from the genomic data that gene loss as a universal source of genetic variation that can cause adaptive phenotypic diversity, however, it is still largely unknown that what are actual mechanisms and evolutionary driving forces involved in genome reduction and loss of gene functions. In most of the cases these deletions are linked with a negative effect on fitness, it is also believed that these deletions accumulate by chance through non-adaptive genetic drift together with fundamental mutational deletion bias (Koskiniemi *et al.,* 2012; Albalat and Cañestro).We can speculate from this study that deletions and variations in the copy number of genes in K7 and D59 which may be correlated with the difference in their killing potential of *G. mellonella* but also in outcome of their biofilm forming phenotype and hence better environmental persistence of one strain over other. Furthermore, the deletion of important set of genes involved in metabolic pathway i.e. *deoCABD*, in D59 strain may also play contributory role in reduced biofilm phenotype and hence its long term environmental persistence. However, more detailed studies are required for definitive answer.

5. Conclusion

Comparative genome analysis of two distinct subclades of *V. cholerae* found in Pakistan (PSC-1 and PSC-2) showed significant difference at genomic level. *Galleria mellonela* was used as an infection model to evaluate the virulence potential of both subclades, which showed that PSC-2 strain D59 was more virulent than PSC-1 strain K7. In contrast K7 (PSC1) showed enhanced biofilm potential indicating better survival and persistence in the environment as compared to K7.

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T**able 1: LD50 values for *Vibrio cholerae* subclade PSC1 (k-7) and PSC2 (D-59).** Lethal dose of both strains were determined using Probit analysis based on three independent *Galleria mellonella* infections at T = 24 h. Y=Yintercept + slope*log(X)

Table 1:

Strain	LD_{50} Average	SD	p-value
K-7	3.17E+06	2.24E+06	0.0001
D-59	1.3E+02	2.255	

Figure Legends

Fig. 1. Comparative analysis of K7 and D59 with reference genome N16961: The conserved regions are shown in the form of red and blue bars. Red bars point towards the similarly oriented regions and blue bars show regions that are conversely oriented.

Fig. 2. Whole genome alignment of D59, K7 with reference genome (N16961): The genomes were compared with each other using progressive MAUVE with default parameters. The colinearity of the genomes and the two deletions (regions) between them have been labelled.

Fig. 3. Venn diagram showing the number of conserved, accessory and unique genes among D59, K7 and reference strain N16961.

Fig. 4. Comparison of killing potential of D-59 and K-7 against *G. mellonella* at different doses and time. A range of 10^2-10^8 CFU of bacterial cells (D-59 and K-7) were injected to *G. mellonella* and % killing was recorded after 24 (4A) and 48 (4B) hours. Results are the means of three independent experiments.

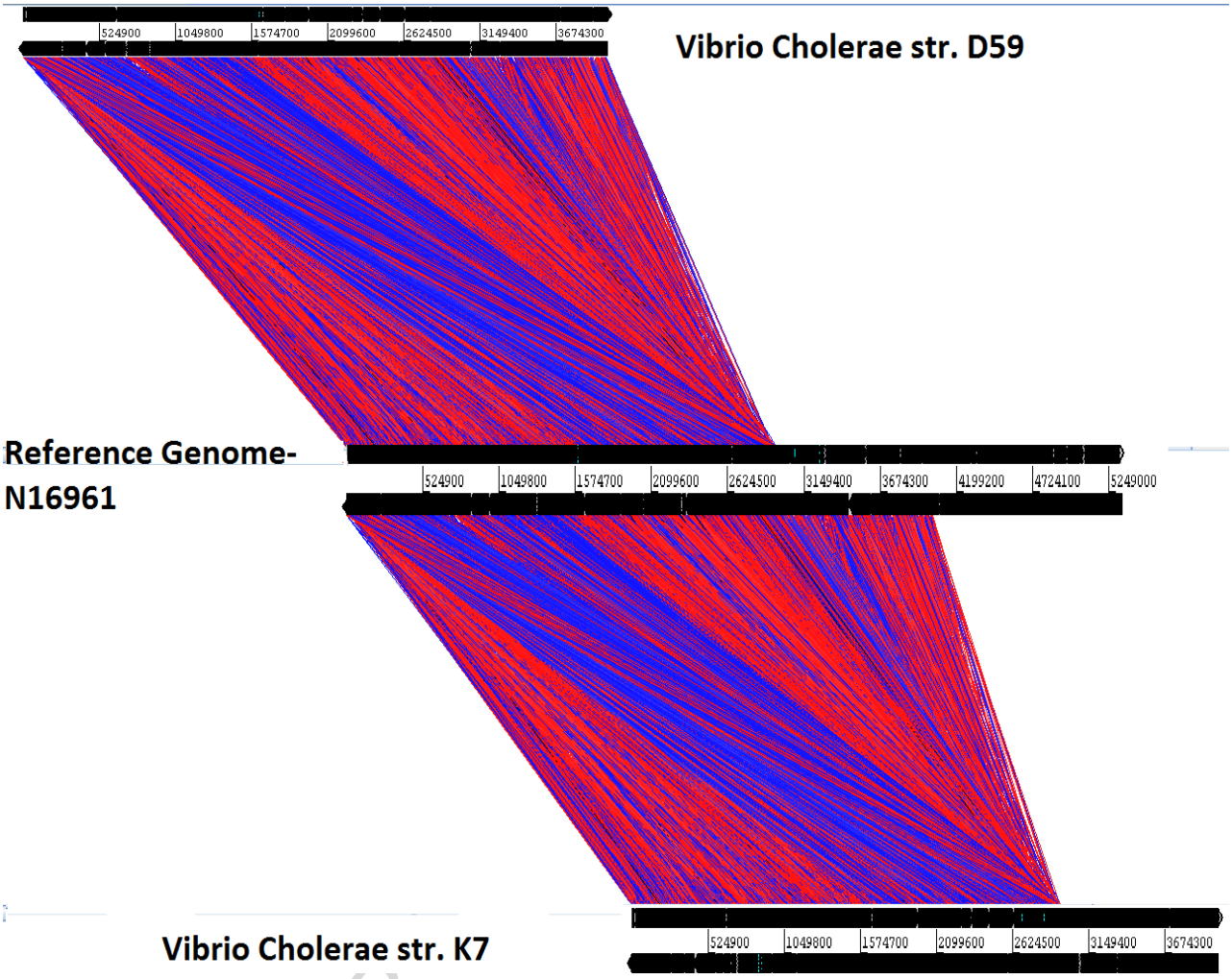

Fig 1

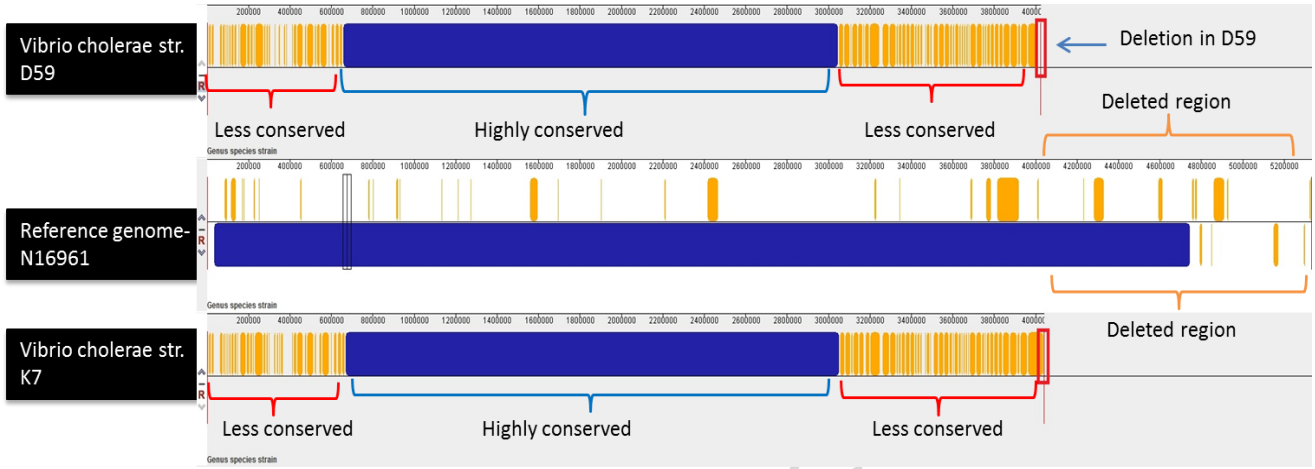

Fig 2

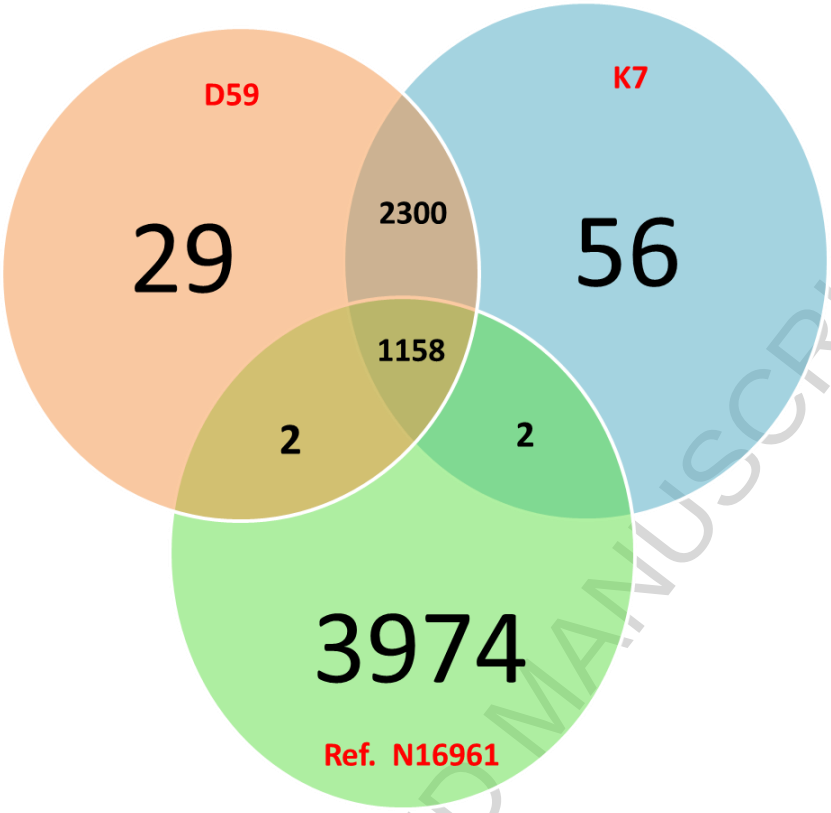

Fig 3

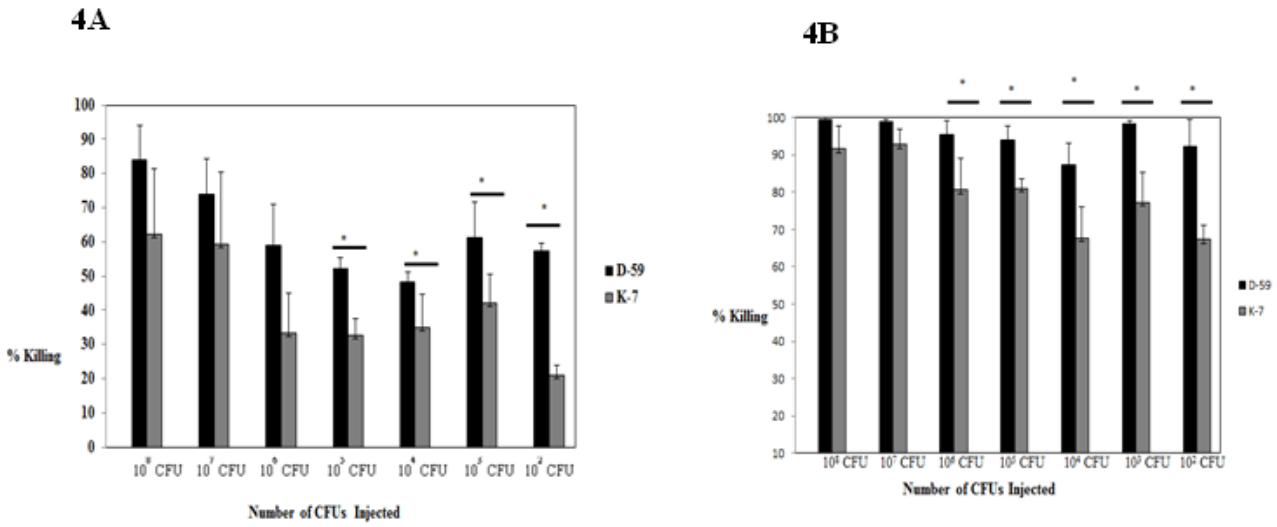

Fig 4

Table Legends

Fig 4

Highlights

- *Galleria mellonela* was used as an efficient in-vivo model against *Vibrio cholerae*.
- D59 (PSC2) showed more virulence potential towards *Galleria mellonela*
- K7 (PSC-1) exhibited enhanced biofilm forming capabilities as compared to D59.

List of 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 ()