Type III secretion system confers enhanced virulence in clinical non-O1/non-O139 *Vibrio cholerae*

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Abstract

Vibrio cholerae O1 infections mainly are responsible for significant mortality and morbidity amongst children, however, non-O1/non-O139 *V. cholerae* have also been reported to cause mild to severe infections because of their virulence potential. The pathogenic mechanisms of non-O1, non-O139 isolates are not as clearly understood as for that of O1 and O139 isolates. Type three secretion system (TTSS) is also considered one of the important virulent factors and during the current study, we investigated the role of TTSS in association with non-O1/non-O139 clinical isolates. We report that the presence of TTSS in non-O1/non-O139 *V. cholerae* clinical isolate (D13) from a child confers more virulence compared to the one lacking it (D15) in another clinical case during the small cholera epidemic. Moreover, the antibiotic susceptibility profiles of D13 and D15 indicate that they are multiple drug resistance (MDR) isolates. The sequence analysis for TTSS cluster was carried out for D13 and compared with the TTSS positive

reference *Vibrio parahaemolyticus* RIMD2210633 and *V. cholerae* AM19226 non-O1/non-O139. Furthermore, the pathogenic potential of D13 & D15 was also explored in simple and economical invertebrate host model, *Galleria mellonella* and the results revealed that $TTSS^{+ve}$ isolate (D13) was more virulent compared to $TTSS^{-ve}$ isolate (D15). We suggest that this distinct genetic difference, seen in natural variants D13 and D15, is also reflected by the clinical picture of the former in contributing towards the severity of disease symptoms and this finding was further validated by assessing virulence potential of both isolates using inexpensive *G. mellonella* infection model.

1. Introduction

Vibrio cholerae, a gram-negative bacterium, belonging to the ɣ-subdivision of the family proteobacteriaceae causes acute watery diarrheal disease known as cholera [1]. Cholera has claimed millions of lives in the past, but today cholera is still endemic in Africa and South Asia where it has been reported for at least 1,000 years [2]. Cholera is mostly a disease of the tropics, it is often associated with low quality sanitation management where the people had limited access to microbiologically safe drinking water. Natural calamities such as floods, earthquakes, and political disturbances resulting in wars, also increase the likelihood of a cholera epidemic. *V. cholerae* is a highly heterogenous species, based on somatic O antigen >200 serogroups exists, the majority of which are non-pathogenic and only O1 and O139 out of 200 serogroups are known to cause widespread and repeated epidemics. The O1 and O139 can be further subdivided into two biotypes, El Tor and classical [3-4].

Seven cholera pandemics have been recorded since 1817. It is widely accepted that the first six epidemics are caused by biotype: classical, while the current seventh pandemic was due to E1 Tor. Interestingly, recent studies indicate that non-O1/non-O139 *V. cholerae* are

increasingly recognized as a cause of sporadic cases of gastroenteritis usually resulting in severe bloody diarrhea and are also associated with extra-intestinal infections including bacteremia, skin and wound infections in immuno-compressed individuals [5-6].

V. cholerae O1, O139, and non-O1/non-O139 serogroups differ in their clinical outcome because of the number of virulence factors ranging from array of toxins, colonization factors, antibiotic resistance, resistance to disinfectant provided by capsular polysaccharides, as well as unique surface antigens (O139 lipopolysaccharide and O antigen capsule) [7-10].

In different parts of Africa, USA, Asia, and Europe cholera outbreaks were recorded and their studies [11-12] revealed that *V. cholerae* non-O1/non-O139 strains are the causative agent of these outbreaks. Some of these isolates although non-pandemic serogroups possess known genes/systems associated with virulence including the cholera toxin phage (CTX), genomic islands VSP-1 and 2, and VPI-1 and 2. However, even without typically associated virulence factors with O1 and O139 serogroup isolates such as cholera toxin (CT), carried by CTX and TCP genes required for human intestinal colonization, additional virulence factors comprising heat-stable enterotoxin (ST), haemolysin (HlyA), mannose-sensitive hemagglutinin (MSHA) pilus, repeats in toxin (RTX), and the type three secretion system (TTSS) enhance virulence of non-agglutinating vibrios (NAGs) i.e. non-O1/non-O139 isolates [13-17].

Previous studies revealed that the non-O1/non-O139 *V. cholerae* strain AM-19226 successfully colonized the infant mouse intestine as well as induced a cholera-like disease condition in adult rabbits [17-18]. The genome analysis of AM-19226 further revealed that it lacked CT and TCP suggesting that perhaps other virulence factors including type III secretion system (TTSS) may have compensated for the lack of CT and TCP in infant mouse and rabbit models [17]. TTSS has been reported from a wide spectrum of gram-negative bacteria including

non-O1/non-O139 *V. cholerae* isolates of both clinical and environmental origins [19-20, 23-24]. They facilitate direct translocation of TTSS secreted effectors proteins through a syringelike a needle into the host cytosol [19-20]. The translocated effector proteins are known to interfere cellular processes including depolymerization of cytoskeleton components [21] and more specifically the products of factors such as *op*F, *vop*E, stimulates actin nucleation and promotes actin depolymerization respectively [22].

The genome analysis of non-O1/non-O139 TTSS shows strong homology to *V. parahaemolyticus* TTSS gene clusters [17] and have been proposed to play a role in virulence and environmental adaptation of these isolates [17]. However, the significance of TTSS particularly in association with human non-O1/non-O139 *V. cholerae* and other less characterized effectors in the pathogenesis of the diarrheal disease is not well understood.

We identified two non-O1/non-O139 *V. cholerae* isolates from children under 10 years of age showing cholera-like symptoms and, investigated their susceptibility profiles as well the virulence potential of the isolates with a focus on TTSS. This is the first report where TTSS in association with non-O1/non-O139 *V. cholerae* clinical cases was investigated from Pakistan and we believe a greater understanding of the differences between pandemic and non-pandemic serogroups will have implications for our better monitoring and surveillance of *V. cholerae* in the future.

2. Material and methods

2.1 Ethical statement

This study was first approved by an ethical board of the Department of Biosciences, CUI, Islamabad.

2.2 Sample collection and identification:

Suspected cholera stool samples were collected in Cary-Blair transport medium within 24 hours of the patient being admitted and transported to Microbiology & Public Health Laboratory at Department of Biosciences, COMSATS University – Islamabad. For enrichment of samples, 1x alkaline peptone water was used after 6-8 hrs incubation at 37°C. After enrichment, the inoculum was taken and smeared on solid thiosulfate citrate bile salts (TCBS) sucrose medium and then it was placed at 37°C for 16-20 hrs and yellowish-green colonies, characteristic of *Vibrio species*, were selected and biochemical and molecular tests were performed as described previously [21-22]. Isolates were preserved in BHI broth at -8°C containing 20% glycerol.

2.3 Serotyping determination

Slide agglutination test was carried out to confirm if the isolates belonged to O1 and O139 serogroups or non-O1/non-O139. Monovalent Inaba and Ogawa antisera and Polyvalent Inaba and Ogawa antisera (Denka Seiken, Japan) were used to determining serogroup O1. The serogroup specific encoding regions were amplified by multiplex PCR [25].

2.4 Antimicrobial susceptibility test

According to CLSI guideline (CLSI, 2009) [26], antimicrobial susceptibility to different antibiotics was checked by Kirby Bauer disc diffusion tests on Muller-Hinton Agar (Oxoid). The antibiotics used were: trimethoprim (25 µg), tetracycline (30 µg), ampicillin (10 µg), sulfamethoxazole/trimethoprim (23.71/1.25 µg), erythromycin (15 µg), streptomycin (10 µg), ofloxacin (5 µg), nalidixic acid (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), and chloramphenicol (30 µg). As a susceptible control, *Escherichia coli* ATCC 25922 were used.

2.5 DNA extraction and genomic characterization of D13 and D15

Overnight culture of *V. cholerae* D13 (isolate from 9 year old child) and D15 (isolate from 10 year old child) isolates in nutrient broth was used to extract DNA by ArchiPure DNA extraction Kit (Prime 5, UK). PCR was performed to screen for *rtxA* and *ompW* [27, 28]. Further, screening through PCR using various gene specific primers was carried out for detecting virulence factors i.e. *tcpA*, *ctxA*, TTSS (*vcsC*, *vspD*, *vcsV*) (Table 1).

2.6 Genetic characterization of antibiotic resistance

The isolated strains were analyzed by PCR to detect conjugative and integrative element SXT [29], integrons [30], and genetic basis for resistance to sulfamethoxoles [31-32], fluoroquinolones [33-34], streptomycin [35-36], chloramphenicol [36] and trimethoprim.

Table 1: Primers used in the study

Gene	**Primer sequence (5' to 3')**	**Annealing Temp. (°C)**	**Product (bps.)**	**References**
rtxA	F: AGCAAGAGCATTGTTGTTCCTACC R: ACTTCCCTGTACCGCACTTAGAC	61	120	[27]
ompW	F: CACCAAGAAGGTGACTTTATTGTG R: GAACTTATAACCACCCGCG	61	588	[28]
ctxA	F: CTCAGACGGGATTTGTTAGGCACG R: TCTATCTCTGTAGCCCCTATTACG	60	301	[37]
O1 *rfb*	F: GTTTCACTGAACAGATGGG R: GGTCATCTGTAAGTACAAC	55	192	[25]
O139 *rfb*	F: AGCCTCTTTATTACGGGTGG R: GTCAAACCCGATCGTAAAGG	55	449	[25]
$tcpA^{Cl}$	F: CACGATAAGAAAACCGGTCAAGAG R: ACCAAATGCAACGCCGAATGGAGC	60	472	[37]
$tcpA^{ET}$	F: GAAGAAGTTTGTAAAAGAAGAACAC R: GAAAGGACCTTCTTTCACGTTG	60	672	[37]
*vcs*C2	F:CGTCGACGTTACCGATGCTATGGGT R:GCTCTAGAAGTCGGTTGTTTCGGTAA	60	535	[42]
*vcp*D	F:CGTCGACAGTTGAGCCAATTCCATT R:AGACGACCAAACGAGATAATG	55	422	[17]
*vcs*V2	F:ATGCAGATCTTTTGGCTCACTTGATGG R:ATGCGTCGACGCCACATCATTGCTTGC	60	535	[17]

2.7 Genome sequencing of D13 and D15

D13 and D15 isolates were sequenced by Illumina HiSeq platform. Initially by creating multiplex sequencing libraries were created each with 250 bps capacity. To get paired end reads of 72 bases of 96 libraries, libraries were loaded on the sequencing platform cell. Unique index tagging was used to labeled each library. For both samples 200X- average coverage was achieved and using a *de novo* genome assembly program Velvet version 0.7 [44], the 72 bps paired end reads were assembled. For best k-mer size and at least 20×k-mer coverage, parameters were set to appropriate values. By using SMALT software, genome of *V. cholerae* O1 El Tor strain N16961 is mapped to the 72-base paired end read data of both isolates and whole genome alignment of both isolates was obtained. The data was used to predict and compare TTSS cluster with reference sequences (RIMD2210633; AM19226).

2.8 Type three secretion system (TTSS) prediction and comparison

The TTSS gene cluster in *V. cholerae* D13 and D15 genome sequences were predicted using BLAST [45]. *V. cholerae* AM19226 was taken as the reference sequence against which *V. cholerae* D13 and D15 genomes were queried. BLAST comparisons among the multiple genomic regions of interest were further visualized using Easyfig [46].

2.9. Genome Comparison of D13 & D15

To do the annotation of both D13 and D15, the genome sequences of both strains had been submitted through the SEED-RAST server.

2.10 Galleria mellonella infection model for clinical non-O1/non-O139 V. Cholera

To investigate the virulence potential of TTSS positive and negative clinical non-O1/non-O139 *V. cholerae* isolates, infection assays were performed by using an invertebrate model system, *Galleria mellonella* as described previously [47, 48]. Briefly, 10 µl taken from overnight (O/N) culture, grown at 30°C carrying 10^4 CFU to 10^8 CFU were injected into first right proleg of *G. melonella* larvae. 10 larvae were injected in each case. 70% (vol/vol) ethanol was used for surface disinfection, and experimental models were scored at 24 and 48 hours for phenotypes such as survival, movement and change in color. In parallel, PBS diluent was injected to control larvae and the experiment considered successful when no control larvae died. Larvae which show no movement/response when touched were considered dead. Data from at least three replicates was analyzed and for each strain, 50% lethal doses were calculated.

3. Results

3.1 Clinical manifestations of TTSS$^+$ and TTSS$^-$ non-O1/non-O139 isolates

This study is based on two child patients presenting cholera-like illness. On the basis of standard biochemical tests and PCR, stool sample of both patients were considered positive for presence of *V. cholerae* [28, 49]. Briefly, cholera isolate-D13 was isolated from a 9 year old male patient admitted to pediatric ward of civil hospital, Dera Ismail Khan (Khyber Pukhtoon Khwa province) with symptoms of watery stool accompanied with fever (102°F), concomitant with episodes of vomiting, severe dehydration and frequent passage of watery stools (Isolate-D13). The second isolate (Isolate-D15) was taken from a 10 year old male patient with profuse watery stool and other gastroenteritis/diarrhea like symptoms(Isolate-D15).

Serogroup determination by using slide agglutination test revealed that *V. cholerae* isolates (D13 and D15) belonged to non-O1/non-O139 serogroups as they lack O1*rfb*/O139*rfb* or *tcp*A^{Cl}/*tcp*A^{El} the latter was confirmed by separate multiplex PCRs. PCR also showed both isolates lacked the cholera toxin and the cholera prophage encompassing genes *ctxA*, *zot*, *ace* and RS1. These isolates also lacked *tcpA,* an important virulence factor located on vibrio pathogenicity island -1. However, using TTSS gene -specific primers (Table 1), D13 was found positive for TTSS (Figure 1.)

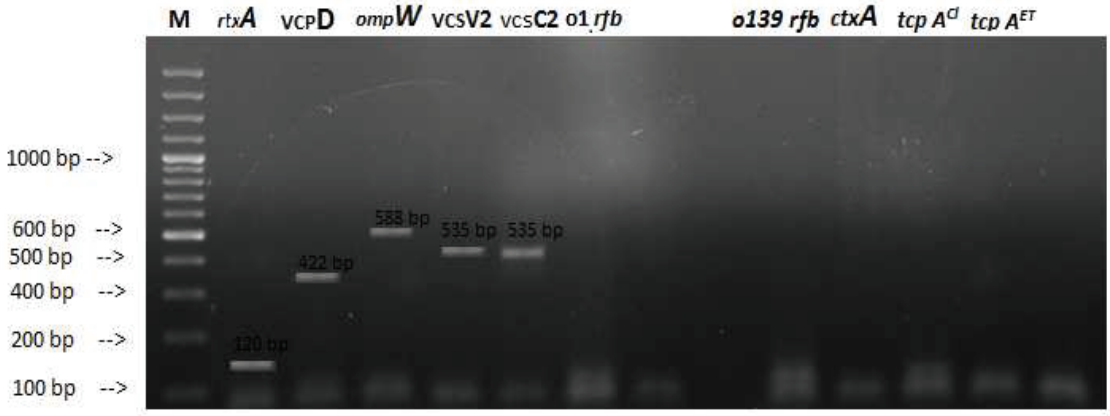

Figure 1: Detection of TTSS in D-13**.**

3.2 Genotypic and Phenotypic characterisation of antimicrobial resistance in D13 and D15

D13 and D15 were multiple drug resistant as they exhibited resistance phenotypes for streptomycin, co-trimoxazole, nalidixic acid and ciprofloxacin on Mueller-Hinton (MH) agar. However, resistance to ceftazidime and ofloxacin varied as D13 was resistant to ceftazidime whereas D15 to ofloxacin. Response to tetracycline, erythromycin and ceftaxime were intermediate or sensitive in both isolates.

Integrons of all three classes were absent in D13 and D15 as consensus sequences of their integrases were not amplified by PCR. Integrative and conjugative element, SXT was not found in these isolates. *strA* and *strB* (encoding streptomycin resistance) were present as the PCR revealed amplification of 284 and 248 bps fragments respectively whereas in case of sulfamethoxazole resistance encoding genes, *sul2* was present and *sul1* and *sul3* were absent. Furthermore, *qnrA*, *qnrB* and *qnrS* (fluoroquinoles resistance) were not present in D13 and D15. Among, other antibiotic resistance encoding genes, *florR* (chloramphenicol resistance) was only present in D15. (Table 2)

Table 2: Antibiotic resistance characterization

Non-O1/non-O139 *V. cholera*	**Antimicrobial resistance encoding genes present**	**Antimicrobial resistance encoding elements not detected**
D13	*sul2*, *strA*, *strB*,	*SXT,* dfrA1, *dfrA18*, *tetA,* , *qnrA*, *qnrB*, *qnrS* , *sul1*, *sul3, intI*, *int2*, *int3*, *florR*
D15	*sul2*, *strA*, *strB*, *flo*rR	*SXT, dfr*A1, *dfrA18*, *tetA* , *qnrA*, *qnrB*, *qnrS*, *sul1*, *sul3*, *intI*, *int2*, *int3*.

3.3 TTSS comparative sequence analysis

The conserved TTSS gene cluster in the assembled completed genomes of *V. cholerae* D13 and D15 was identified based on similarity searches through BLAST. The alignment results confirmed the existence of TTSS cassette in D13 isolate while it was not found in the genome D15 isolate. Comparative analysis results of the conserved TTSS genes cluster of *V. parahaemolyticus* RIMD2210633, *V. cholerae* AM19226, and *V. cholerae* D13 were visualized and shown in Figure 2. The result from TTSS gene cluster genome alignment has also shown the difference in gene organization of conserved TTSS gene cluster in all these strains.

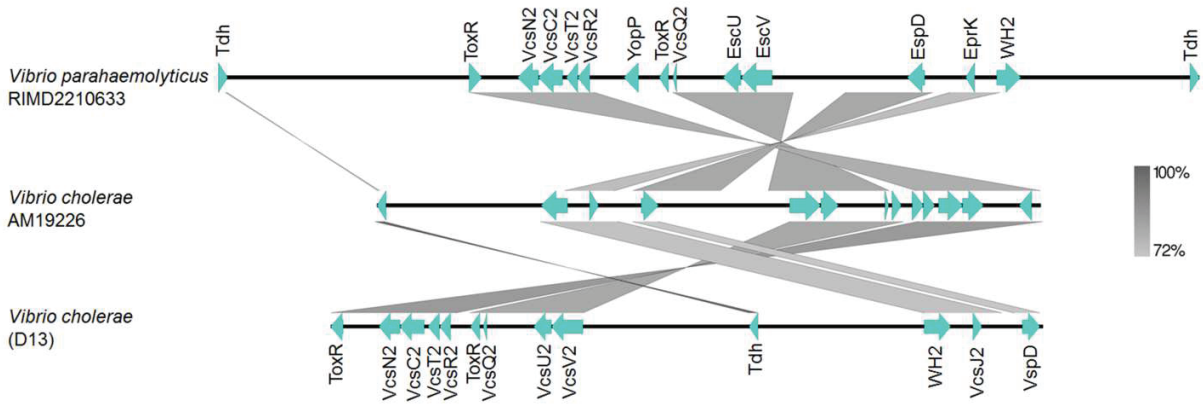

Figure 2. Comparison of TTSSs gene cluster of genomes of *V. parahaemolyticus* RIMD2210633, *V. cholerae* AM19226, and *V. cholerae* D13. Arrows represent TTSS CDSs which comes through BLAST hits and annotation. Vertical lines between sequences show regions having shared homology .

3.4. Genome Comparison of D13 & D15

In general, both genomes (D13 and D15) showed the similar number of genes that have been annotated. The genome statistic also showed the similar characters. The results of analysis are shown as in Appendix A (Figures A1 & A2).

3.5 Mortality in G. mellonella infected with V. cholerae TTSS^{+ve} isolate and TTSS^{-ve} isolate

G. *mellonella* is injected with a range of 10^4 to 10^8 CFU of bacterial cells (D-15 and D-13) and number of surviving insects were recorded after 24 hrs (A) and 48 hrs (B), and are shown in tabular form under the graph. The numbers (CFUs) shown in these results are the mean values of three independent experiments.

Keeping in consideration the different clinical manifestations shown by TTSS^{+ve} (D13) and TTSS^{-ve} (D15) isolates**,** we investigated their pathogenicity in *G. mellonella* larvae. In the first right proleg of larvae, 10^4 to 10^8 CFUs of bacterial cells (D15, D13) were injected, and their survival was monitored for 24 hours and 48 hours. The virulence potential of D13 and D15 was

compared with PBS injected control group. No mortality of *G. mellonella* larvae was observed in control group. Death of at least 90% larvae was observed due to presence of TTSS^{+ve} strain. On the other hand, larvae infected with TTSS^{-ve} strain showed 60% deaths at 10^4 CFUs, 24 hours post injection (Figure 3A). As the dose increased, the number of larval deaths increased, and 100% larval deaths were recorded at 10^7 CFUs (Figure 3A) and 10^6 CFUs (Figure 3B), at 24 hr and 48 hr respectively. The number of surviving larvae, injected with TTSS^{-ve} strain, remained higher as compared to larvae injected with TTSS^{+ve} strain, regardless of time and dose. (Figure 3). The comparative percentage of survival results indicates the *G. mellonella* is more susceptible to TTSS^{+ve} non-O1/non-139 *V. cholerae*.

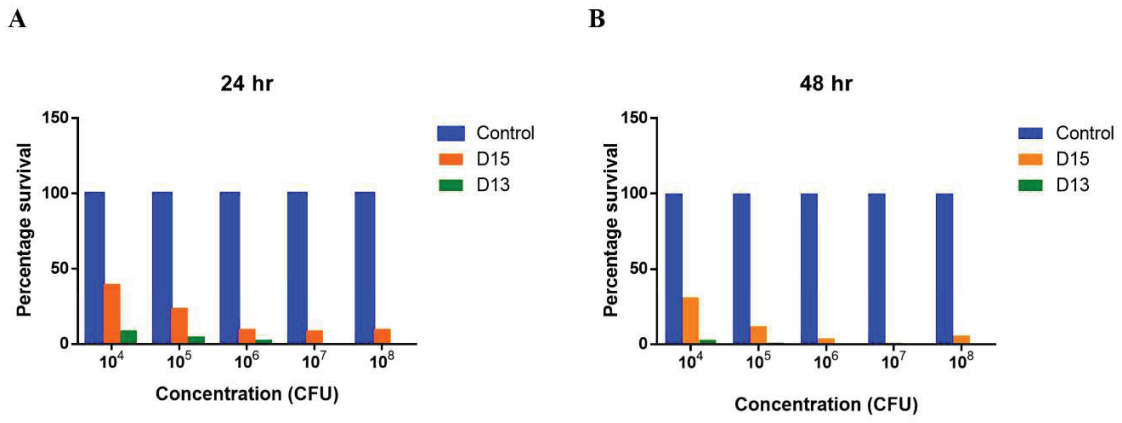

Figure 3: Comparison of killing potential of D-15 and D-13 against *G. mellonella* at different doses and time. CFUs, colony forming units.

4. Discussion

Vibrio cholerae associated with acute watery diarrhea has a substantial prevalence in Pakistan, since the disease is endemic in South Asia and some African and South American countries [4]. Cholera results in higher morbidity and mortality in children and the elderly population. Since *V. cholerae* is transmitted through fecal oral route so, increase in cases is

observed during monsoon seasons often resulting in floods contaminating water reservoirs with cargo pathogens including *V. cholerae. V. cholerae* has a great variety of serogroups, among them only O1 and O139 cause epidemics because these strains encode the cholera toxin and other virulent arsenals which are responsible for characteristic cholera symptoms [24]. However, the non-O1/non-O139 strains also caused local outbreaks which are increasing day by day due to consumption of raw sea food or extra-intestinal infections from recreational waters [50]. Furthermore, these strains are acquiring more virulent characteristics which are helpful in evolving more pathogenic non-O1/non-O139 isolates posing higher levels of threats.

In this study, two non-O1/non-O139 *V. cholerae* associated diarrheal cases with distinct clinical picture were analyzed to better understand the pathogenicity based on phenotypic and genomic characteristics. The study revealed that both non-O1/non-O139 isolates (D13 and D15) lacked the cholera toxin as well as toxin co-regulated pili which are the unique feature of toxigenic *V. cholerae* of O1 and O139 serogroups. However, D13 caused severe acute diarrheal disease whereas D15 caused mild diarrhea. Both the isolates possessed *rtx*A and hemolysin (*hly*A) as a majority of the non-agglutinating vibrios (nag) from other regions also shown to possess hemolysin [38, 39, 41, 42, 51]. Furthermore, both isolates possessed *dth* (gamma-thermostable hemolysisn), the product of which is similar to Tdh hemolysin of *V. parahemolyticus*, however *dth* role in disease has not been clearly elucidated among non-O1/non-O139 *V. cholerae* [52]. Moreover, key virulence players such as TcpA from vibrio pathogenicity island-1 (VPI-1) as well as other important members of VPI-1 such as *toxT*, *toxR*, *acf*, *tagA* and *aldA* were not found. Partial or intact VPI-1 were found in some non-O1/non-O139 *V. cholerae* from some parts were isolated but this is usually not common. It has also been seen

that presence of *tcp*A or VPI-1 in NAG vibrio may be associated with acquisition of *ctx* phage in shaping up highly virulent non-O1/non-o139 in the environment [53].

VSP-1 and VSP-II found in the contemporary toxigenic *V. cholerae* O1 El Tor, usually absent in the six pandemic O1 *V. cholerae* and majority of the non-O1/non-o139 vibrio or environmental vibrios [41]. VSP-1 and VSP-2 are rarely present in non-O1/non-O139 isolates as majority of the studies did not find these islands in these vibrios or only encode some open reading frames in case of their existence [41, 43] and that was the case for D13 and D15 isolates as well for current study.

Both (D13 and D15) the strains showed a multiple antibiotic resistance phenotype with resistance to streptomycin, co-trimoxazole, nalidixic acid, ciprofloxacin, ceftaxime and ofloxacin compared to some recent studies where non-O1/non-O139 were susceptible to antibiotics [23, 54]. However, enhanced level of resistance to ampicillin, chloramphenicol, fluoroquinolones, co-trimoxazole have been reported by many studies [55-57] like the current study. However, the resistance pattern of these non-O1/non-O139 isolates was different from O1 isolates of clinical origin in Pakistan [58]. Genotypically, integrative and conjugative element (ICE-SXT) was absent in these NAG isolates which is now consistently encoded by O1 El tor. In addition, mobile genetic elements such as integrons encoding resistance to various antibiotics were non-existent. However, for chloramphenicol resistance encoding *florR* was present similarly, sulfamethoxazole resistance encoding *sul2* was also detected by PCR. The different drug profile from those of O1 *V. cholerae* suggested that empirical antibiotic cholera therapy is relevant for non-O1 infections and it must be revised. Moreover, there is also a threat of an increase in antibiotic resistance as, like other reservoirs, the marine and aquatic habitats are also under risk due to increased anthropogenic activities [59].

On the other hand, *ctxA*, *ace*, *zot*, *mshA*, were absent in both isolates investigated during the current study. Previous studies also show that non-O1/non-O139 isolates usually do not produce cholera toxin unlike O1 and O139 [60]. However, these non-O1/non-O139 isolates may acquire cholera toxin carrying CTX phage and transfer of CTX phage from *ctx* positive to *ctx* negative non-O1/non-O139 isolates [61].

The genome analysis in the TTSS gene cluster support the similarity of non-O1/non-O139 TTSS *V. cholerae* D13 with *V. parahaemolyticus* TTSS gene cluster [17]. The high similarity of TTSS gene cluster and organizations were found among *V. cholerae* D13 and *V. parahaemolyticus* in comparison with *V. cholerae* AM 19226. However, some genes are present only in *V. parahaemolyticus* while missing in *V. cholerae* D13, on the other hand, some genes are present only in *V. cholerae* D13 while missing in *V. parahaemolyticus*. *Galleria melonella* infection model analysis indicated that the presence of TTSS (D13) has contributed in enhancing its virulence compared to another non-O1 strain (D15) lacking TTSS and which somehow has been corroborated with the clinical picture of the patient as well.

At this time, we cannot say exactly what molecular mechanism of pathogenesis lead to frequent death of the *G. mellonella* larvae after infection with $TTSS^{+ve}$ nonO1/nonO139 but it may have contributed either directly or indirectly by allowing the production of other virulence factors or toxins such as RTX.

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Appendix A

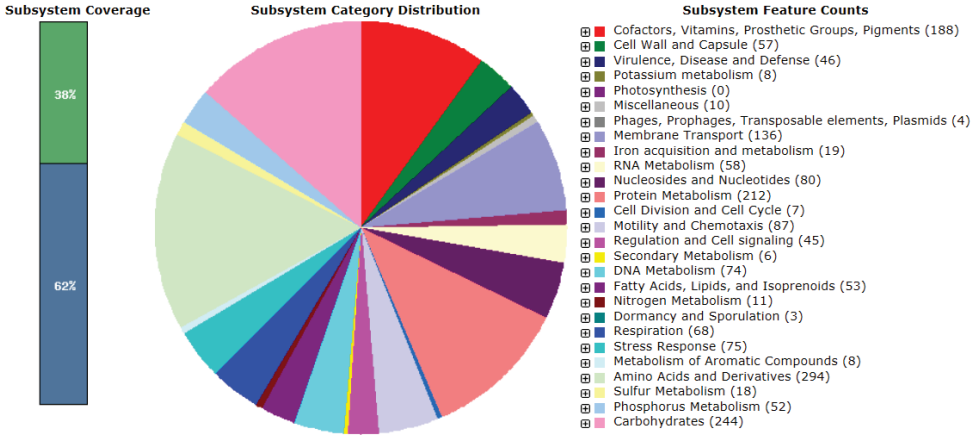

Figure A1: The Diagram of V. cholerae D13 based on Subsystem Category

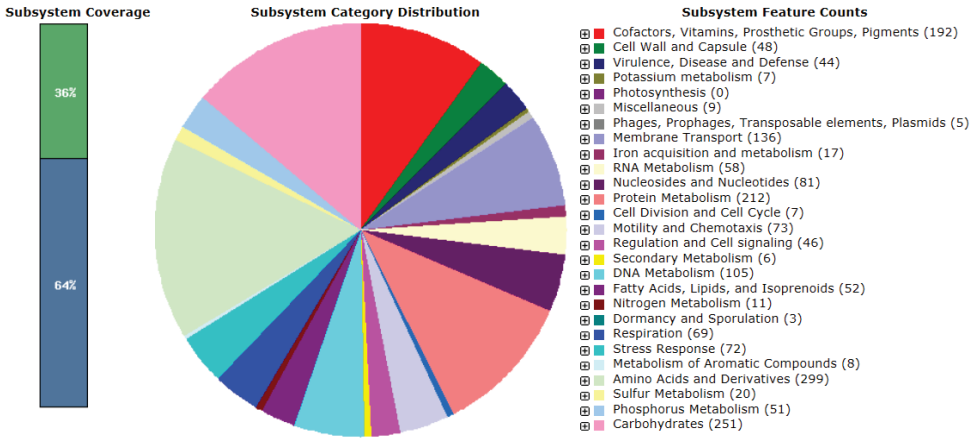

Figure A2: The Diagram of V. cholerae D15 based on Subsystem Category

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Type III secretion system confers enhanced virulence in clinical non-O1/non-O139 *Vibrio cholerae*

Highlights:

- Type three secretion system (TTSS) is also considered one of the important virulent factors and during the current study we investigated the role of TTSS in association with non-O1/non-O139 clinical isolates.
- This article report that the presence of TTSS in non-O1/non-O139 *V. cholerae* clinical isolate (D13) from a child confers more virulence compared to the one lacking it (D15) in another clinical case during the small cholera epidemic.
- The antibiotic susceptibility profiles of D13 and D15 indicate that they are multiple drug resistance (MDR) isolates.
- The results revealed that that $TTSS^{+ve}$ isolate (D13) was more virulent compared to $TTSS^{-ve}$ isolate (D15).
- These finding was further validated by assessing virulence potential of both isolates using inexpensive *G. mellonella* infection model.