Ranjana Tripathi (2011). ***Exploration of type III secretion system in non-01, non-0139 strains of vibrio cholerae.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

The dreaded disease 'cholera' that afflicts millions every year all over the world, is caused by *Vibrio cholerae* strains belonging to O1 and O139 serogroups (Kaper *et al.*, 1995). More than 200 seroroups of *Vibrio cholerae* have been reported till now. Strains belonging to serogroup other than O1 and O139 collectively known as non-O1, non-O139 and were considered to cause localized outbreaks and extra-intestinal infections in man. However, in recent past, an unusual upsurge in the clinical isolation of the non-O1, non-O139 strains were associated with cholerae patients (Sharma *et al.*, 1998b). In fact, the relative preponderance of *V. cholerae* non-O1, non-O139 strains as compared to epidemic causing counterparts of O1 and O139 strains from diarrhoeal patients admitted to Infectious Diseases Hospital, Kolkata ranged between 33 to 36% in the last three years.

The genome of *V. cholerae* consists of two chromosomes and complete nucleotide sequences of both the chromosomes are available for O1 El Tor strain N16961. Available data suggest that genome of *V. cholerae* is very dynamic in nature with respect to acquisition of potential virulence factors through horizontal gene transfer and generation of new clones (Heidelberg *et al.*, 2000). Although much attention was attributed towards the biology and pathogenesis of O1 and O139 strains, very little or no information is available on the nature of pathogenesis of non-O1, non-O139 strains which cause diarrhoea clinically indistinguishable from cholera. Interestingly, recent genome sequencing and comparative genome analysis of non-O1, non- O139 strains revealed that these strains harbour genes of putative exotoxins and type III secretory system (Chen *et al.*, 2007).

Moreover, sequence data mining also reveals that the genes encoding type III secretion system (T3SS) in these strains are further related to T3SS2 gene cluster of *Vibrio parahemolyticus*. Since non-O1, non-O139 strains are by and large devoid of cholera toxin and toxin co-regulated pili, it is therefore, conceivable that the type III system could contribute towards the pathogenesis of these strains. Further, Mekalanos and coworkers have characterized a T3SS effector molecule VopF (NT01VC2350, WH2 motif domain protein) which has been posited to play a key role in enhancing the intestinal colonization of non-O1, non-O139 strains. T3SS has been reported to be involved in the environmental fitness of those strains (Dziejman *et al.*, 2005). T3SS is

sophisticated bacterial machinery, assembled in a needle like apparatus, which bacteria employed to translocate its effector molecules directly into host cells.

In 1968, an outbreak had occurred by a strain belonging to serogroup O37 in Sudan. In 1969, a strain had been isolated from a patient in India which also belonged to same clone of O37 serogroup which was earlier isolated from Sudan. Strain from O37 serogroup was closely related to *V. cholerae* ElTor (Beltran *et al.*, 1999). T3SS is present in all strains of O141 serogroup and interestingly these strains are distributed throughout the world and known to cause sporadic diarrhoea disease (Dziejman *et al.*, 2005). Unlike their pathogenic counterpart O1 and O139, acquisition of type III secretion system as well as effector molecules that are being delivered by such systems has been considered as a major contributing factor in the pathogenesis of non-O1, non–O139 strains. Though significant information has been gained, little is known regarding the T3SS system in the clinical strains of Indian origin which further motivated us to embark on this study.

In this piece of this work, we screened the distribution of T3SS genes among clinical isolates of Indian origin. Southern blot analysis revealed the presence of five major T3SS genes in seventeen clinical isolates out of fifty. We have indentified VopF, a key effector molecule in all the strains positive for other T3SS genes. VopF harbours three WH2 domains, one FH1 domain and one PRM domain. WH2 domain and FH1 domain are actin binding motifs, present in actin binding proteins, which modulate actin dynamics. These proteins include β thymosins, profilin which maintain actin in its monomeric form (G actin) and others include WASP, WAVE, Spir etc. which activate cellular ARP2/3 complex through their WH2 domains. Activated ARP2/3 complex initiates actin polymerization inside cell during cellular processes like endocytosis, vesicular trafficking, and cell cycle events. Spir contains four WH2 domains through which it nucleates actin monomers. Some bacterial pathogens secrete T3SS effectors directly inside host, subvert cellular signalling cascades and hijack host systems to get own benefits. They hijack cellular machinery by mimicking some host cell signalling proteins or directly activate ARP2/3 complex which leads to actin polymerization on the bacterial surface, supporting their movement inside host from cell to cell. Besides this, some pathogens translocate

effector molecules into host cells and directly mediate actin polymerization without using host cellular proteins *e.g.* VopF, VopL, VopN and TARP.

Further, we conversed our attention on VopF; the T3SS effector of *V. cholerae* that has all the domains necessary for actin polymerization thus, promotes actin polymerization in host cell. Mechanistically, VopF exerts differential influence on the architecture of cytoskeleton, thus promoting colonization of pathogens in gut epithelia. So far, detailed molecular analysis of WH2 and PRM domains as well as their contributions in the functionality of these proteins has not been illustrated, which motivated us to develop a yeast model to study functional behaviour of each individual WH2 domain along with wild type VopF.

Simultaneously, our results demonstrated that VopF confers toxicity when expressed ectopically in yeast. Furthermore, to get deep insight, we deleted WH2 domains of VopF to explore individual domain function. Domain deletion studies revealed that all WH2 domains and FH1 domain contribute toxicity to VopF in a cumulative manner. Interestingly, the results obtained by domain deletion studies also suggested that Wh2 domain 3 has maximum contribution to toxicity of VopF. Now the question arises here, why does domain 3 confer maximum contribution to toxicity of VopF. The importance of WH2 domain 3 regarding VopF toxicity can be justified by our ClustalW data analysis which reveals that Wh2 domain 3 is completely conserved among various non-O1, non-O139 strains and has maximum numbers of conserved residues.

In an effort to dissect the mechanism by which VopF exerts its toxic effect on yeast, we performed Flow Cytometry and stained yeast cellular DNA with propidium iodide. FACS analysis indicated that VopF expression leads to cell cycle arrest resulting in loss of viability of yeast cells. Interestingly, it is known that cell cycle arrest and or actin perturbation trigger a morphogenesis check point which is sensed by yeast cells that further leads to growth defect phenotype. Possibly, VopF exerts its toxic effect by exploiting the morphogenesis checkpoint mediated pathway.

Precisely, WH2 domain containing cellular protein WASP, N-WASP, WAVE plays an important role in the regulation of actin dynamics. Therefore, VopF has been identified as an actin nucleator protein; this gives advantage to bacterium for intestinal

colonization. To localize VopF in yeast cell, we tagged this with mCherry (giving red **fluorescence)** at its C-terminus. Cell cytoskeletal actin was stained with FITC-**phalloidin** to see the localization pattern of VopF in context of actin. We reported that **VopF** has been co-localized with actin network and disrupt actin cytoskeleton which led to diffuse pattern of actin in the cell.

After getting insight into individual WH2 domain activity parameter, our next objective of this study was to investigate particular amino acid, responsible for toxicity of individual WH2 domain. To, achieve this objective, we constructed various alanine mutants for each individual WH2 domain by site directed mutagenesis using Gene tailor kit. Alanine scanning is quite appropriate approach to identify the role of particular amino acid in a stretch of amino acids. Results were quite interesting and we have characterized the role of each amino acid in WH2 domain. Amino acids which have the role in the toxicity of WH2 individual domain 1 and 3 are mostly hydrophobic. Alanine substitutions at residues **L6A, M7A, I10A, V14A and L16A** rescued yeast growth suggesting their maximum role in the toxicity of WH2 domain 1. In a similar manner, we have replaced each amino acid of WH2 domain 3 with alanine by site directed mutagenesis to find out their role in WH2 domain 3 toxicity. Results suggested that alanine substitutions at **L6A, I10A, F13A, K15A, L18A and R19A** were able to restore maximum growth, implying that they have crucial role in WH2 domain 3 toxicity.

Concisely, distribution of T3SS genes helps us to explore the pathogenesis of *V. cholerae* non-O1, non-O139 strains as many of them are devoid of CT and TCP, and are associated with sporadic cholera like disease. Futher, we developed yeast model to study the functions of VopF, a key effector molecule of *V. cholerae*. This model will be a valuable tool to expedite the identification of intracellular target of VopF and also useful to screen small molecule inhibitors against this effector molecule. Additional studies are necessary to address these issues.