Effect of Type Three Secretion System and Phospholipases on Virulence of *Harveyi Clade* Vibrios (*Vibrio harveyi* and *Vibrio campbellii*)

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Abstract

Vibrios belonging to *Harveyi clade* (*V. harveyi* and *V. campbellii*) are pathogenic marine bacteria in shrimp causing devastating vibriosis in aquaculture industry. Several virulence factors are involved in the virulence including type three secretion system (TTSS) and phospholipases. The TTSS are specialized secretion apparatus used by pathogens to inject virulence factors directly into host cells. To facilitate the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes such as phospholipases. But there is a knowledge gap on expression of virulence genes and it's relation to the virulence. Therefore this study aimed at detecting the connection between the expression of virulence factors and the virulence of *Harveyi clade* vibrios. The level of expression of *genes of type three secretion system such as, vopD* (gene for outer protein), *vcrD* (gene for calcium response protein), *vscP* (gene for secretion protein) and five genes responsible for phospholipase activity (*pl1, pl2, pl3*: phospholiphase genes; *omplA1, omp1A2*: outer membrane phospholiphase A genes) *were quantified.* Genes of type three secretion system (TTSS) showed clear differences in expression levels between virulent and avirulent strains with significantly high expression in avirulent isolates (Fold-expression 27.4, 19.7 and 13.4 for *vopD, vcrD* and *vcsP* respectively) and lower in virulent isolates (Fold-expression 1 for all three genes). Two out of the five phospholipases genes showed significant differences between the different strains having relatively higher expression in low virulence strains and having relatively low expression in high virulence strains. The TTSS genes and some of the phospholipases genes expression is inversely proportionate to the virulence. Findings of this research can have significant consequences on the implementation of control measures for the devastating luminescent Vibriosis problem in the aquaculture industry.

Key words: Gene expression, Virulence, Type three secretion system, Phopholipases, Vibriosis

Introduction

During the last fifty years, the world aquaculture has grown very rapidly and owing to that the farming methods have been shifted from extensive to intensive to achieve maximum profits. Due to these changes aquaculture industry is threatened by many diseases caused by bacteria and viruses. *Harveyi clade vibrios have* been recognized as one of the most significant pathogens in aquaculture of marine fish, crustaceans and mollusks (Zhang and Austin 2000). Many virulence factors have been identified in *Harveyi clade* vibrios, including type three secretion system genes, proteases, phospholipases, hemolysins, cysteine protease, metalloprotease, serine protease and chitinase (Zhang and Austin 2000). However, it is not yet clear which virulence factors are most important in the progression of the disease caused by *vibrios belonging to the Harveyi clade.* Some studies revealed that the presence of virulence genes within the genome of these bacteria is not immediately linked to virulence, with differences in virulence not being correlated to presence or absence of a specific virulence factor (Ruwandeepika et al. 2010). The quantity of the virulence products produced by these bacteria might be a critical factor and the virulence of the bacteria might be more closely linked to the expression level of the virulence genes. The aim of the present study was to determine the expression level of the virulence genes of type three secretion system and some phospholipases in virulent, non virulent and moderately virulent isolates belonging to the Harveyi *clade*, and to study the relation between virulence

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towards gnotobiotic brine shrimp (*Artemia franciscana)* and virulence gene expression.

Materials and Methods

The bacterial strains used in the study included virulent, moderately virulent, avirulent strains (Table 1). Bacteria were obtained from Laboratory of Aquaculture and Artemia Reference Centre, Ghent University, Belgium. In order to check the virulence of bacterial strains used, the challenge experiments were performed with high quality cysts of *Artemia franciscana* (INVE Aquaculture, Baasrode, Belgium), according to Ruwandeepika *et al.*, (2011). *Artemia* were challenged with bacteria at the dose of 10^5 CFU per ml of *Artemia* culture water. The survival of *Artemia* was counted 48 h after the challenge. Each treatment was done in triplicate and the sterility of the control treatments was checked at the end of the challenge and the relative percentage of survival (RPS) was calculated (Ruwandeepika *et al.*, 2011). The strains were grown to late exponential phase, and virulence gene expression was measured by reverse transcriptase real-time PCR as described before (Ruwandeepika *et al.*, 2011) and expressed relative to the expression in virulent strain. Gene specific primers were designed based on the consensus region for respective gene sequences deposited in GenBank using Primer3 software (http: // frodo. wi. mit.edu/primer3). RNA extraction, reverse transcription and real time PCR was adopted from Ruwandeepika *et al.* (2011). The level of expression of *genes of type three secretion system such as, vopD* (gene for *V. harveyi* outer protein), *vcrD* (gene for *V. harveyi* calcium response protein), *vscP* (gene for *V. harveyi* secretion protein) and five genes responsible for phospholipase activity (*pl1*, *pl2*, *pl3*: phospholiphase genes; *omplA1*, *omp1A2*: outer membrane phospholiphase A genes) *were quantified by using* ABI PRISM 7300 Fast Real Time System thermal cycler (Applied Biosystems) as described by Ruwandeepika *et al.* (2011) using gene specific primers. Real-time PCR data was analysed using the $2^{-\Delta\Delta CT}$ method. *rpoA* was used as the house keeping gene and virulent strain was used as the calibrator for calculating the gene expression level. The analysis of data was carried out using the SPSS statistical software (version 19, SPSS Inc. IBM, 2010). Gene expression (ΔCt) and survival data were compared with one way ANOVA, followed by a Tukey's post-hoc test. For all statistical analyses, 5% significance level was used.

Results and Discussion

This study quantified the expression levels of type three secretion system (TTSS) genes and five phospholipase. These genes are important in the process of invasion of organisms to the host and establishing the infection in the host. In this study, genes of type three secretion system (TTSS) showed clear differences in expression levels between virulent (causing 29% Relative Percentage Survival/RPS) and avirulent strains (81-84% RPS), with significantly high expression in avirulent isolates (Fold-expression 27.4, 19.7 and 13.4 for *vopD, vcrD* and *vcsP* respectively) and lower in virulent isolates (Fold-expression 1 for all three genes) (Table 1). Invasion of host tissues and colonization in host tissues are the important events of pathogenesis of bacterial disease. Organisms will start expressing genes which are important for survival as individuals which helps in invasion and colonization of host tissues. The expression levels of these genes are higher in avirulent or low virulent organisms as they need to overcome the host defense than the virulent organisms. Defoirdt *et al.* (2010) found that some virulent genes such as chitinase which is important for the attachment and invasion, showed the higher expression in lower virulent organisms and vice versa. The TTSS are specialized secretion apparatus used by pathogens to inject virulence factors directly into host cells. Bacteria will start expressing genes which are important for survival as individuals to thrive the host defense mechanisms and to localize the infection. Our identification of the virulence and TTSS gene expression can have significant consequences for the implementation of control measures for the devastating luminescent Vibriosis problem in the aquaculture industry. This is the first study showing the relationship between virulence and TTSS gene

expression in *V. harveyi* and in *V. campbellii.*

This study quantified the expression of five phospholipase genes including *pl1, pl2, pl3*: phospholiphase genes; *omplA1, omp1A2*: outer membrane phospholiphase A genes. The bacterial phospholipases comprise a diverse group of proteins that are considered to be virulence factors for bacterial species that cause serious diseases the virulence factors that damage host cells. To facilitate the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes such as phospholipases that destroy or derange constituents of host cell membranes, leading to membrane dysfunction and/or physical disruption. Two out of the five phospholipases genes showed significant differences ($p < 0.05$) between the different strains having higher expression in low virulence strains and having low expression in high virulence strains (Table 1). *pl1* and *pl3* showed higher expression in low virulent strains while *pl2*, *omp1* and *omp2* did not show statistical significant between virulent and low virulent strains of *Harveyi clade* vibrios. To aid in the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes that destroy or derange constituents of host cell membranes, leading to membrane dysfunction and/or physical disruption. By cleaving phospholipids, phospholipases destabilize the membrane resulting in cell lysis. Evidence implicating phospholipase in host cell penetration, injury, and lysis by microorganisms has been reported in various types of bacteria and fungi (Songer, 1997).

This study concluded that the TTSS genes and some of the phospholipases genes expression is inversely proportionate to the virulence. Findings of this research can have significant consequences for the implementation of control measures for the devastating luminescent vibriosis problem in the aquaculture industry.

Table 1. Expression of five phospholipase genes (*pl1, pl2, pl3*: phospholiphase genes; *omplA1, omp1A2*: outer membrane phospholiphase A genes) and type three secretion system genes *vopD* (gene for *V. harveyi* outer protein), *vcrD* (gene for *V. harveyi* calcium response protein) and *vscP* (gene for *V. harveyi* secretion protein) (TTSS) relative to *rpoA* mRNA in the pathogenic Harveyi clade isolate (LMG21363).

Bacterial isolates	Virulence	*pl2*	*omp1*	*omp2*	*pl1*	*pl3*	*vopD*	*vcrD*	*vsP*	survival (RPS)
LMG21363	High	1^{l}	1^{s}	1^{t}	1^{a}	1^{d}	1^{x}	1^{l}	1^{m}	29^{p}
JAF548	Low	$1,70^{l}$	$1,00^{s}$	$1,01^{t}$	$2,42^{b}$	$3,68^{e}$	$27,41^{y}$	$19,65^{j}$	$13,39^{n}$	53^{q}
BB120	Moderate	$0,93^{i}$	$1,04^{s}$	$0,94^{t}$	$0,92^{a}$	$0,95^{d}$	$7,36^{y}$	$4,66^{k}$	$2,06^{m}$	82^{r}

[a]: Values in the same column showing the same superscript letter are not significantly different ($P>0.05$).

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