Comparison of Virulence between Luminescent and Non-Luminescent *Vibrio harveyi* and *Vibrio campbellii in vivo*

HAD Ruwandeepika[1,2,3], TS Prasad Jayaweera[3], I Karunasagar[2] and P Bossier[1]

[1]Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium.

[2]UNESCO MIRCEN for Marine Biotechnology, Karnataka Veterinary, Animal and Fisheries Sciences University, College of Fisheries, Mangalore, Karnataka, India

[3]Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka

Abstract

Luminescent Vibriosis caused by *Vibrio harveyi* and *Vibrio campbelliis* a devastating disease in aquatic spp including finfish and shell fish. These vibrios are luminous Gram-negative bacteria widely distributed in the marine environment. Luminescence is an outcome of quorum sensing (bacterial cell to cell communication). Since luminous vibrios are serious pathogens causing severe losses in aquaculture, gnotobiotic *Artemia* (Brine shrimp) was used as the host animal (*in vivo* model) to examine the virulence of luminous vibrios in a challenged experiment. In this study wild-type *V. harveyi* BB120, *V. campbellii* LMG 21363, quorum sensing mutants of *V. harveyi* BB120 and their previously reported non-luminescent isogenic counterparts were used. In addition quorum sensing maximally active mutant JAF483 (QS+) which is a constitutively luminescence and quorum sensing inactive mutant JAF548 (QS-) which is a dark (non-luminescent) mutant were also used. This study investigated the expression levels of the some virulence gene regulator (*luxR*) and virulence factors (metalloprotease *vhp* and hemolysin *vhh*) by reverse transcriptase real time PCR too. The challenge test revealed that all the non-luminescent strains were less virulent. The most virulent strain of luminescent LMG21363 had a relative percentage survival (RPS) value of 25 and its non-luminescent counterpart had only 49 RPS which is statistically significant ($p<0.05$). Furthermore, the non-luminescent variants produced lower levels of the quorum sensing master regulator *luxR* and *vhp* ($p<0.01$). Moreover the QS- mutant (non-luminescent) showed significantly higher RPS and lower gene expression (*luxR* and *vhp*) than the QS+ mutant (luminescent). This study concludes that the non-luminescent variants of *Vibrio harveyi* and *Vibrio campbellii* were less virulent than luminescent counterparts under *in vivo* conditions and produced lower levels of some virulence genes.

Key words: *Artemia*, Gene expression, Luminescence, Non-luminescence, Virulence

Introduction

Vibrio harveyi and *Vibrio campbellii* are ubiquitous, bioluminescent bacteria causing life threatening vibriosis in marine vertebrates and invertebrates, leading to severe losses in the aquaculture industry. These two species are closely related showing 61-74% DNA-DNA similarity and over 97% 16S rDNA similarity. The pathogenicity mechanisms of these bacteria are imprecisely understood and it is suggested that the ability to attach and to form biofilms, the secretion of various extracellular products including proteases and hemolysin, lipopolysaccharide and bacteriocin like substances and the presence bacteriophages are playing a major role.

Luminescence is one of the phenotypes that are regulated by quorum sensing in vibrios. Bioluminescence consumes approximately 20% of the cellular energy and consequently, it must have a positive selective value because otherwise it would have been lost during evolution. One of the hypotheses is that *V. harveyi* emits light to stimulate DNA repair. Interestingly, *V. harveyi* bioluminescence was also shown to be involved in the detoxification of H_2O_2, thus playing a role in the protection against oxidative stress (Czyz and Wegrezyn 2001). As H_2O_2 is an important part of the defense of eukaryotic hosts against infections, bioluminescence can be considered as a virulence factor.

Virulence factor expression of vibrios has been reported to be controlled by different regulatory systems, including quorum sensing. Quorum sensing, bacterial cell-to cell communication, involves the production, secretion, and detection of extracellular signal molecules (called autoinducers) to collectively regulate gene expression. Quorum sensing is studied widely in *V. harveyi* and it uses three channels that are mediated by the signal molecules Harveyi Autoinducer1 (HAI-1), autoinducer 2 (AI-2) and Cholerae Autoinducer 1 (CAI-1). The signals are detected at the cell surface by two-component receptor proteins which feed a common signal transduction cascade which controls the production of the quorum sensing master regulator LuxR and directly or indirectly controls many other quorum-sensing target genes (Waters and Bassler 2006). In *V. harveyi,* quorum sensing controls bioluminescence, biofilm formation, type III secretion (TTS), extracellular toxin, siderophore and metalloprotease production. It has been shown that the quorum sensing process regulates the virulence of luminescent vibrios towards different hosts *in vivo* (Defoirdt *et al.* 2008).

This study was designed to check the virulence of luminescent and non luminescent *Vibrio harveyi* and *Vibrio campbellii* using an *Artemia* (Brine shrimp) as the *in vivo* host model. Further, some virulence genes expression such as *luxR*, metalloprotease (*vhp*) and hemolysin (*vhh*) was quantified.

Materials and Methods

Wild-type *V. harveyi* BB120, *V. campbellii* and quorum sensing mutants of *V. harveyi* and non luminescent strains of isogenic strains were used in the study (Table 1). Bacteria were obtained from the 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 Colony Forming Units (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 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 respective luminescent strain. RNA extraction, reverse transcription and real time PCR was adopted from Ruwandeepika et al., 2011. Virulence genes (*vhh* hemolysins, *vhp* metalloproteases) and regulator (*luxR* quorum sensing master regulator) were quantified by using ABI PRISM 7300 Fast Real Time System thermal cycler (Applied Biosystems) as described by Ruwandeepika

Table 1. Relative percentage of survival of *Artemia* nauplii (mean ± standard error of three replicates)

		Artemia survival (relative percentage survival)[a]		Gene expressions		
Strain	Mutation in	Non-luminescent	Luminescent	*luxR*	*vhp*	*vhh*
Control		100±0	100±0			
BB120	wild type	78±1	53±7 (0.008)	0,6 (0.017)	0,3 (0.002)	0,6 (0.028)
BB152	Mutation in *luxM* (HAI1 - AHL synthase)	65±4	43±10 (0.036)	0,8 (0.192)	0,4 (0.051)	0,9 (0.875)
BB170	Mutation in *luxN* (HAI1 - AHL receptor)	69±8	49±7 (0.025)	0,9 (0.565)	0,6 (0.055)	0,9 (0.861)
BB886	Mutation in *luxP* (AI-2 receptor)	96±8	91±7 (0.468)	0,6 (0.049)	0,5 (0.159)	0,9 (0.606)
MM30	Mutation in *luxS* (AI-2 synthase)	96±8	85±8 (0.184)	0,6 (0.064)	0,5 (0.001)	0,9 (0.741)
LMG21363	wild type	49±11	25±8 (0.031)	0,5 (0.005)	0,5 (0.151)	0,7 (0.403)
JAF483*	*luxO* (QS maximally active)	40±2	-	3.3 (0.004)	6.8 (0.003)	1.1(0.3)
JAF548*	*luxO* (QS inactive)	-	82±2 (0,01)	1	1	1

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 housekeeping gene and respective luminescent strain was used as the calibrator for calculating the gene expression level. The analysis of data was carried out using the SAS® statistical software (9.1). Survival data of *Artemia* was analyzed by an independent sample t-test. The gene expression levels were analyzed by an independent sample t-test using ΔCt data. Significant level was chosen to be 5% and 1% for survival and gene expression, respectively.

Results and Discussion

The survival of *Artemia* larvae challenged to non-luminescent variants of the wild type strains BB120 and LMG21363, and the HAI-1 (AHL synthase and AHL receptor) mutants BB152 and BB170 was significantly higher than survival of larvae challenged to the corresponding luminescent strains ($P < 0.05$) (Table 1). QS+ luminescent mutant had lower survival than the QS- dark mutant, which indicates that the luminescence has a relation with virulence. This is consistent with the findings of Phuoc et al. (2009), who also found lower mortality of *Artemia* when challenged with non-luminescent strains.

The reverse transcriptase realtime PCR results indicate that the expression of the quorum sensing master regulator gene *luxR* was significantly affected in the non-luminescent wild type strains BB120 (*V. harveyi*) and LMG21363 (*V. campbellii*). Moreover the QS+ luminescent mutant had significantly higher luxR expression than the QS- dark mutant (Table 1).Also for the AI-2 mutants, *luxR* mRNA levels were lower in the non-luminescent variants, although only significant in the AI-2 receptor mutant BB886. In contrast, there were no differences in *luxR* mRNA levels in the HAI-1 mutants. Apparently, the non-luminescent variation has a more significant effect on *luxR* mRNA levels in wild type and AI-2 mutants than it has on *luxR* mRNA in the HAI-1 mutants.

At this moment, the cause of the non-luminescent phenotype still is unclear. Phuoc et al. (2009) reported the presence of quorum sensing molecules in the non-luminescent strains and thus the non-luminescent phenotype was not caused by inability of the strains to produce signals. Findings of the study indicate that it might be a mutation or rearrangement in the quorum sensing system or in another regulatory protein that is integrated in the quorum sensing response. Although similar effects were observed for the different strains, it is not possible to exclude the possibility that the non-luminescent phenotype is caused by different mutations in the different strains. Interestingly, Nelson et al. (2007) recently reported that all of the bioluminescence structural genes (*luxABCDE*) were present in cryptically bioluminescent *Vibrio salmonicida* strains, whereas the strains showed a novel arrangement and copy number of the homologues of the *Vibrio fischeri* quorum sensing regulatory genes $luxI_{vf}$ and $luxR_{vf}$ (which is not a homolog of the *V. harveyi* quorum sensing master regulator *luxR*). The authors argued that the novel arrangement of these quorum sensing regulatory genes generate antisense transcripts that might be responsible for the reduced luminescence.

after 48 h challenge with isogenic luminescent and non-luminescent *Vibrio campbellii* and *Vibrio harveyi* strains and the expression of the quorum sensing master regulator *luxR*, metalloprotease (*vhp*) and hemolysin (*vhh*) [a] For each experiment, the survival in the uninfected control was set at 100% and all other survival data were normalized accordingly.

Survival in challenge with luminescent and corresponding non-luminescent strain was compared using an independent samples t-test. Significant differences ($P < 0.05$) are in bold and italics.

JAF483is QS maximum mutant (QS+) which has the highest luminescence and JAF548 is QS inactive mutant (QS-) which has dark phenotype.Survival and the gene expression of JAF483 were compared relative to the JAF 548 which is a non-luminescent mutant ($p<0.01$). (P values are within the brackets).

Proteases are known to be putative virulent factors and in this study the metalloprotease gene expression was showing approximately twofold lower expression levels in the non-luminescent variants of all tested strains, although the difference was only significant for the wild type BB120 and the AI-2 synthase mutant

MM30 (Table 1). In addition the QS+ luminescent mutant had higher metalloprotease gene expression than the QS- dark mutant. According to the previous findings of Mok et al. (2003), the expression of metalloprotease is positively regulated by quorum sensing. Hence, the non-luminescent variants producing lower levels of metalloprotease mRNA is consistent with our observation that *luxR* mRNA levels were lower in non-luminescent variants when compared to their luminescent counterparts. The difference in metalloprotease expression might explain the lower virulence of the non-luminescent variants in the challenge tests. Moreover, the level of the quorum sensing master regulator luxR was also lower in the non-luminescent strains. Quorum sensing has been shown before to regulate virulence towards brine shrimp larvae (Defoirdt et al. 2008) and in addition to metalloprotease, quorum sensing has been shown to regulate the expression of a type III secretion system, extracellular toxin and a siderophore *in vitro*. Hence, a lower activity of this regulatory mechanism might also be an explanation for the reduced virulence of the non-luminescent variants, not only the decreasing metalloprotease levels, but also the expression levels of other virulence factors that were not investigated in this study. Apart from that, hemolysin is also one of the vital virulence products in many vibrios having cytotoxic and hemolytic activity in the host tissues causing severe damages to the host. However, in the current study we could not find significant difference in expression level of this putative gene in non-luminescent strains compared with the luminescent strains (Table 1).

in non-luminescent *V. harveyi* strains grown to log phase relative to their isogenic luminescent counterparts.

Conclusions

This study conclude that the non-luminescent variants of *Vibrio harveyi* and *Vibrio campbellii* variants were less virulent under in *vivo* conditions and produced lower levels of some virulence genes (*luxR, vhp*).

References

Czyz A and We grzyn G 2001 On the function and evolution of bacterial luminescence. In: Case JF, Herring PJ, Robinson BH, Haddock SHD. Kricka LJ and Stanley PE (eds) Bioluminescence and chemiluminescence. Singapore: World Scientific Publishing Co., pp 31-34

Defoirdt T, Verstraete W and Bossier P 2008 Luminescence, virulence and quorum sensing signal production by pathogenic *Vibrio campbellii* and *Vibrio harveyi* isolates. *J Appl Microbiol* 104: 1480-1487.

Mok KC, Wingreen NS and Bassler BL 2003 *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* 22: 870-881.

Nelson EJ, Tunsjo HS, Fidopiastis PM, Sorum H and Ruby EG 2007 A novel *lux* operon in the cryptically bioluminescent fish pathogen *Vibrio salmonicida* is associated with virulence. *Appl Environ Microbiol*73:1825-1833.

Phuoc LH, Defoirdt T, Sorgeloos P, and Bossier P 2009 Virulence of luminescent and non-luminescent isogenic vibrios towards gnotobiotic *Artemia franciscana* larvae and specific pathogen-free *Litopenaeus vannamei* shrimp. *J Appl Microbiol* 106(4):1388-96.

Ruwandeepika HAD, Bhowmick PP, Karunasagar I, Bossier P and Defoirdt T 2011 Quorum sensing regulation of virulence gene expression in *Vibrio harveyiin vitro* and *in vivo* during infection of brine shrimp (*Artemia franciscana*). Environmental Microbiology Reports 3(5); 597-602

Waters CM and Bassler BL 2006 The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* 20:2754-2767.