# A comparison of virulence levels between *Vibrio harveyi* strains 639 and 1114 infecting black tiger shrimp in Thailand

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## Abstract

Pathogenic *Vibrio harveyi* remains a major cause of vibriosis in shrimp, resulting in serious production losses. In Thailand, highly virulent *V. harveyi* 1114 and 639 have often been used for vibriosis studies. The virulence of 1114 is enhanced by bacteriophages with well-defined phenotypes, but phenotypes of 639 are poorly understood. Here, we characterized phenotypes of both strains and compared their virulence degree to *Penaeus monodon* under the same experimental conditions. In bacterial immersion challenge tests, 639 and the lysogenized form of 1114 showed a similar virulence level, and they caused a higher pathogenicity than the non-lysogenized form of 1114. In addition, the plaque assay results confirmed the existence and production of bacteriophages in the lysogenized form of 1114, whereas there was no interaction between 639 and the phages of 1114. *V. harveyi* 639 grew slightly faster than 1114 did, and bacterial cell aggregation, a feature of biofilm formation, was observed in 639 but not in 1114. Therefore, the high virulence of 639 may be associated with a non-bacteriophage-mediated mechanism, e.g. biofilm formation, but the existence of different phages for 639 cannot be ruled out.

**Keywords:** *Vibrio harveyi*, bacteriophage, virulence, *Penaeus monodon*, phenotypic characterization

## Introduction

*Vibrio harveyi*, a major vibriosis pathogen in shrimp, can cause a high mortality in two high-economic-value farmed shrimp, the black tiger shrimp *Penaeus monodon* and the pacific white shrimp *P. vannamei* (Intaraprasong et al., 2009; Soto-Rodriguez et al., 2012). A high mortality rate (up to 100%) can be induced by either bathing the *P. monodon* larvae with a highly virulent strain of *V. harveyi* isolated in the Philippines at concentration of $10^2$ CFU/ml (Lavilla-Pitogo et al., 1990, Harris 1999) or injecting juvenile *P. vannamei* with $10^3$ CFU/g shrimp of *V. harveyi* CAIM 1792 isolated in Mexico (Soto-Rodriguez et al., 2012). On the contrary, low virulent strains of *V. harveyi* required a higher dose of $10^6$ CFU/ml to induce mortality in shrimp laevae (Pizzutto and Hirst, 1995). Taken together, these results suggest the virulence variation among *V. harveyi* strains and different virulence mechanisms in *V. harveyi* pathogenic strains. Possible pathogenicity mechanisms in *V. harveyi* include the secretion of various extracellular products (ECPs), quorum sensing, biofilm-forming ability, iron-binding ability and the association with bacteriophage (Austin and Zhang, 2006).
Isolated in Thailand *V. harveyi* strains 1114 and 639 are highly virulent to *P. monodon*. Strain 1114 is susceptible to the bacteriophage *Vibrio harveyi* siphophage 1 (VHS1), and the

lysogenized form (VH1) shows ~100 times higher virulence level to *P. monodon* than the non-lysogenized form (VH0) (Khemayan et al., 2006). Interestingly, such bacteriophage-enhanced virulence of 1114 was not observed in *P. vannamei* (Intaraprasong et al., 2009). Although 639 also causes a high mortality in *P. monodon* (Ponprateep et al., 2009), information on its phenotypes has been limited. Importantly, there was no report on bacteriophage involvement for 639. In addition, degrees of virulence in 639 and 1114 have never been compared under the same experimental conditions. Therefore, bacterial challenge test experiments with these two virulent strains 1114 and 639 to *P. monodon* were conducted to compare their virulence levels. Furthermore, phenotypes of both strains were characterized in terms of bacteriophage interaction using dot plaque assays and investigated their growth curves.

**Methodology**

Media and artificial seawater

All Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) used in this study were supplemented with 3% artificial sea salt (ASS) (Marinum). Artificial seawater at 16 ppt of artificial sea salt in water was used for shrimp stocking and immersion challenge tests.

Preparation for shrimp stock

*P. monodon* (~10 g each) were obtained from Shrimp Genetic Improvement Center, Surat Thani, Thailand. Before bacterial immersion challenge tests, shrimp were acclimatized for two days in plastic tanks containing 15 liters (L) of artificial seawater with aeration. Shrimp were fed twice daily with a commercial pelleted feed.

Bacterial challenge tests

The immersion challenge tests were performed as described in Tran et al., 2013 and Joshi et al., 2014. Each type of bacteria and each bacterial concentration were performed in three replicates (ten shrimps a replicate). A selection for the optimal bacterial concentration was performed with a bacterial culture. Briefly, 10 shrimps were immersed for 15 minutes in 150-ml bacterial culture containing ~$10^6$, $10^7$and $10^8$ CFU/ml, and the immersed shrimp together with the bacterial culture were poured into 15-L of artificial seawater to obtain the final bacterial concentration of ~$10^4$, $10^5$ and $10^6$ CFU/ml, respectively. Instead of bacterial culture, the control shrimp were immersed in 1/100 dilution of sterile MHB in the same manner. Cumulative mortality (dead and moribund shrimp) was recorded. The optimal bacterial concentration in the 15-L artificial seawater of $10^5$ CFU/ml was used for the next immersion challenge tests with VH0, VH1 and 639.

Dot plaque assay

100-µl of an overnight bacterial culture was spread on MHA and was allowed to completely dry, prior to an application of a potential phage solution. To obtain a potential phage solution, 10-ml bacterial culture was incubated at 30°C with 250 rpm shaking for 16 hours and then put in 5000 rpm centrifugation for 10 minutes, subsequently followed by a filtration through a 0.45-µm filter membrane (Khemayan et al., 2012). A few 20-µl drops of filtered supernatant were applied onto the prepared bacterial lawns. The phage solution was allowed to absorb completely into agar with an overnight incubation at 30°C.

Growth curve

Overnight culture was inoculated into 100-ml of fresh medium to obtain the optical density ($OD_{600}$) of ~0.05, and then the inoculum was incubated at 30°C with 250 rpm shaking for 20

hours. $OD_{600}$ of the inoculum culture was monitored every hour during the culture. In addition, viable bacterial cell count on MHA was performed by spreading 100-µl of each dilution from 10-fold serial dilutions of cultures taken at each hour. Bacterial colonies on MHA were counted after incubation at 30ºC for 16 hours.

**Results and discussion**

Virulence levels of *Vibrio harveyi* strains 1114 and 639

Although VH1 and 639 reportedly caused a high mortality in shrimp, their virulence levels have never been systematically compared under the same experimental conditions. In this study, we used bacterial immersion challenge method to examine the susceptibility of *P. monodon* to VH0, VH1 and 639 and recorded dead and moribund shrimp for 24 hours post-immersion (hpi).

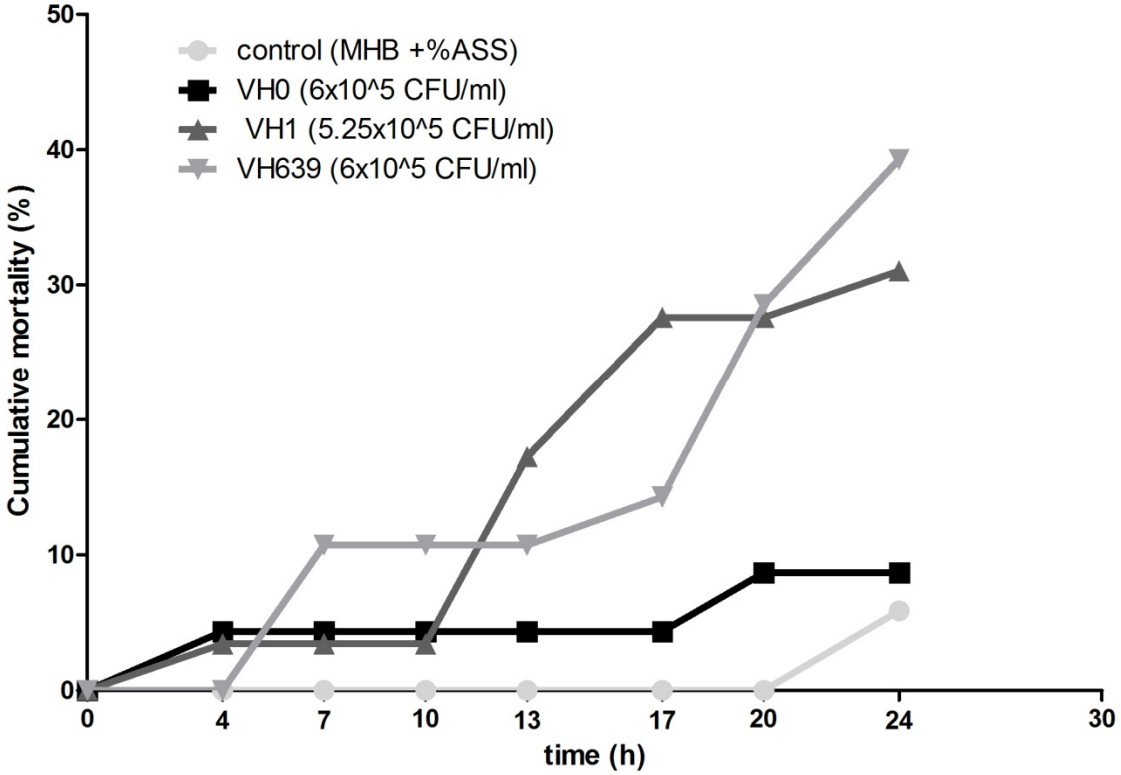


**Figure 1:** Cumulative shrimp mortality after immersion challenge with *V. harveyi* stains 1114 and 639 and 1/100 diluted sterile MHB.

During the first 10 hours, the cumulative mortalities of *P. monodon* challenged by both 639 and 1114 were similar (Figure 1). After 10 hpi, shrimp mortality rates in both 639- and VH1-challenged groups were increased quite rapidly, whereas a few shrimps in the VH0 group were dead. At 24 hpi, 639- and VH1-challenged groups showed a higher cumulative shrimp mortality (~40% and 30%, respectively) than the VH0-challenged group (10%). The result of shrimp mortalities suggested that 639 and VH1 have a comparable degree of virulence and are much more virulent than VH0. Figure 1 shows the cumulative mortalities that were calculated by combining all dead and moribund shrimp from the three replicates together. A similar result was also observed for the cumulative mortalities that were calculated by averaging the cumulative mortalities among the three replicates (data not shown).

Dot plaque assay

Figure 2 shows dot plaque assays performed with all possible combinations of bacterial lawns and supernatants of *V. harveyi* strains 1114 and 639. Clear lysis plaques were observed only in the combination of VH0 bacterial lawn applied by VH1 supernatant (a potential bacteriophage VHS1 solution; Figure 2b). No lysis plaques were observed for VH0 and VH1 lawns dropped by VH0 supernatant (Figure 2a and 2d) and for VH1 lawn dropped by VH1 supernatant (Fig. 2e). This result confirmed the previous finding that VH0 is lysed by VHS1 and that VH1 produces bacteriophage VHS1 and is resistant to subsequent VHS1 infection (Khemayan et al., 2006).

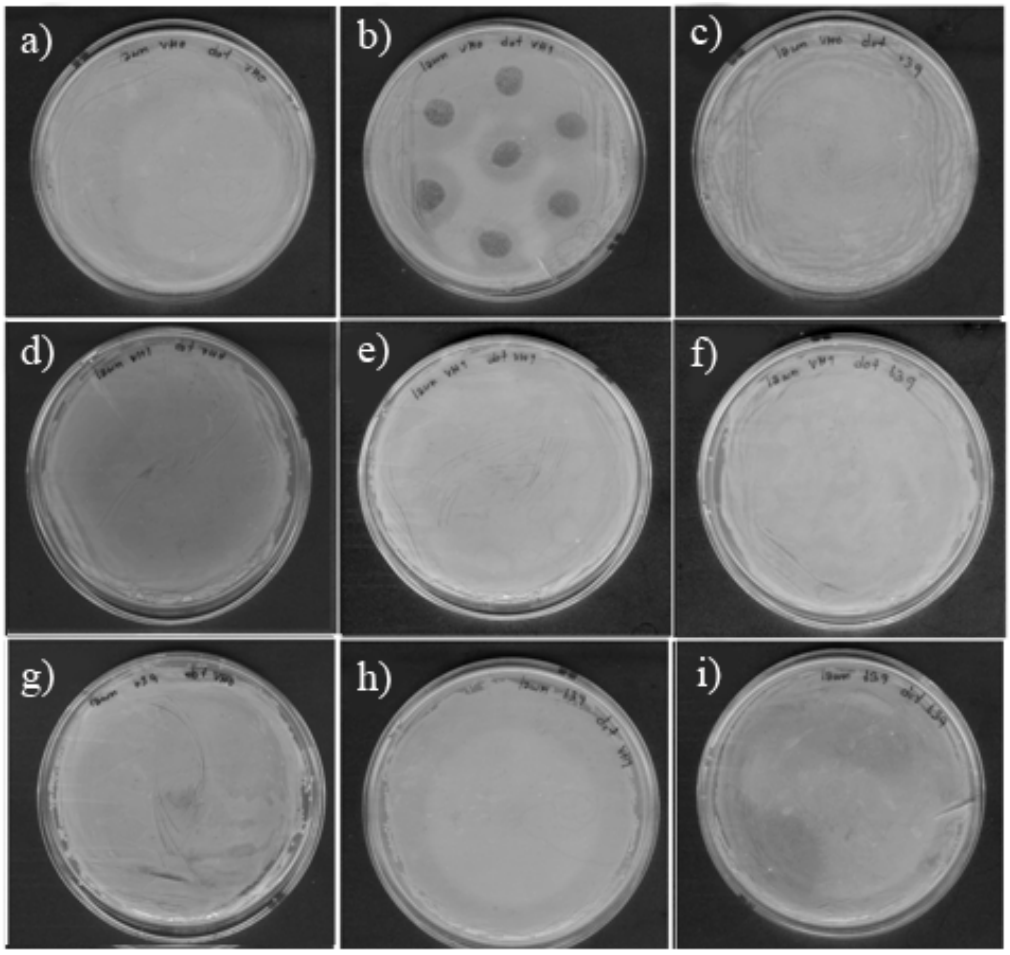


**Figure 2:** Results of dot plaque assay by dropping filtered supernatants from overnight bacterial cultures on bacterial lawns. (a) VH0 supernatant on VH0 lawn. (b) VH1 supernatant on VH0 lawn. (c) 639 supernatant on VH0 lawn. (d) VH0 supernatant on VH1 lawn. (e) VH1 supernatant on VH1 lawn. (f) 639 supernatant on VH1 lawn. (g) VH0 supernatant on 639 lawn. (h) VH1 supernatant on 639 lawn. (h) 639 supernatant on 639 lawn.

In contrast to VH0 and VH1, 639 lawns did not exhibit a lysis plaque when spotted with the filtered supernatant solutions from 639, VH0 and VH1 (Figure 2g, 2h and 2i). Similarly, VH0 and VH1 lawns spotted by 639 supernatant did not show a lysis plaque (Figure 2c and 2f). VHS1 DNA was observed by a PCR detection for both colony and supernatant of VH1, but no detectable VHS1 DNA was observed in either VH0 or 639 (data not shown). Hence, VH0 was free of VHS1 and lysed by VHS1 obtained in the VH1 supernatant. Although 639 was

also free of VHS1, it could not be infected and lysed by VHS1. According to the results, three scenarios of phage involvement in 639 are possible: 1) VHS1 obtained from VH1 supernatant was not specific to 639, 2) no other phages were associated with 639, or 3) 3) the 639 isolate used in this study was lysogenized (perhaps, by different phages), so no clear lysis plaque was observed. A future detailed genomic analysis of 639 will shed some light on whether a prophage is in the genome of 639.

Bacterial growth of *Vibrio harveyi* strains 1114 and 639

The growth of *V. harveyi* strains 1114 and 639 were measured by both optical density at 600 nm ($OD_{600}$) and viable cell count (Figure 3). For $OD_{600}$, both types of 1114 had a similar growth profile, whereas 639 grew slightly slower than 1114 did in an early log phase and began to grow slightly faster than 1114 after 5 hours post-inoculation (hpn) (Figure 3a). A similar growth profile during the first 20 hours was observed for the viable cell count (Figure 3b). After 15 hpn, 639 showed cell aggregation that affected on its $OD_{600}$ values (highly elevated values with a fluctuation for 15-20 hpn time points of each replicate; data not shown), but the cell aggregation was not observed for 1114 (Figure 3a). Nonetheless, the viable cell count confirmed the $OD_{600}$ result that 639 had higher viable cells than 1114 at 20 hpn (Figures 3a and 3b). Both measures suggested that the growth of 639 was slightly faster than that of 1114 (both VH0 and VH1). The observed bacterial aggregation on the flask surface for 639 suggested that the biofilm formation might occur in 639. Thus, the high virulence of 639 can be associated with the biofilm formation because biofilm formation enhances bacterial resistant to disinfectants and antibiotics (Austin and Zhang, 2006).

**Conclusion**

*V. harveyi* strains 1114 and 639 were examined their virulence levels to *P. monodon* under the same experimental conditions. Strain 639 and the lysogenized from of strain 1114 showed a comparable degree of high virulence. The pathogenicity of 1114 has been confirmed to be mediated by the bacteriophage *Vibrio harveyi* siphophage 1 (VHS1), but that of 639 remains still unclear. The results of dot plaque assay and PCR suggested that there was no interaction between 639 and the phage of 1114, but a possibility that 639 involves with different phages cannot be ruled out. In addition, the bacterial cell aggregation and cell colonization on the surface, properties of biofilm formation, were observed in 639 culture. Therefore, a further investigation is needed to determine whether virulence of 639 is associated with different bacteriophage(s) or uses a non-bacteriophage-mediated mechanism, e.g. biofilm formation.

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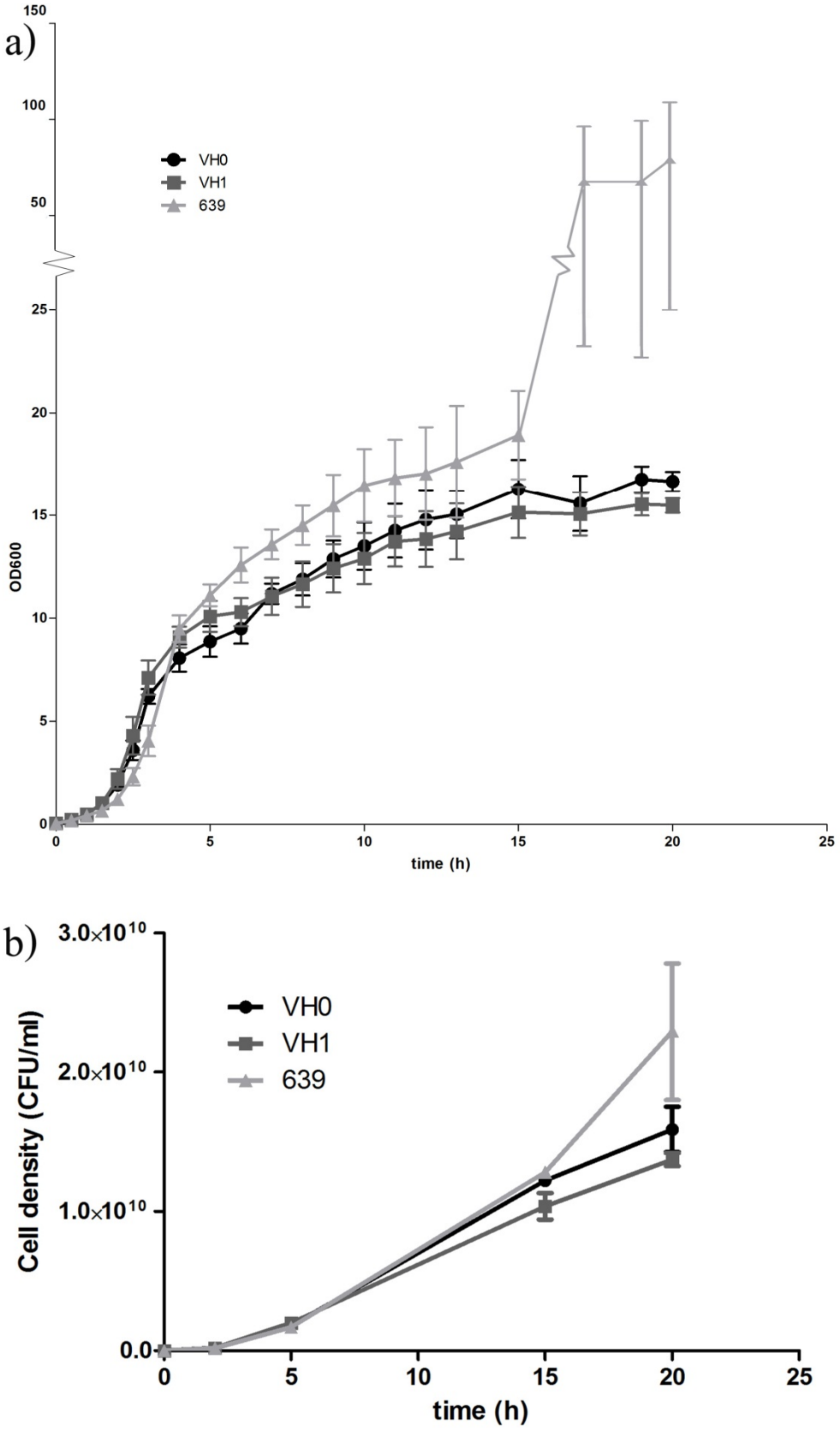


**Figure 3:** Growth curves of *V. harveyi* measured by (a) optical density at 600 nm ($OD_{600}$) and (b) viable bacterial cell count. Bars represent the standard error of the mean.

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