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Original Article
Özgün Araştırma

The effect of iron on the expression of hemolysin/cytolysin and growth of clinical and environmental strains of *Vibrio vulnificus*

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Abstract: Elevated levels of iron are strongly correlated with the growth and virulence of highly lethal human-pathogen *Vibrio vulnificus*. The present study examined the expression level of hemolysin/cytolysin-encoding gene (*vvhA*) and the growth rate of four clinical and four environmental strains of *V. vulnificus* under different iron concentrations (7, 10, 15, 30, and 50 µM). The expression levels of *vvhA* were determined according to the $2^{-\Delta\Delta CT}$ method. *vvhA* transcription was down-regulated as iron concentration increased. A significant difference was observed at the level of 30 and 50 µM ferric chloride concentrations compared to the lower concentrations in all the strains tested ($p < 0.001$). Conversely, elevated iron concentration was significantly correlated with the higher growth rates of all the isolates ($p < 0.05$). Increasing iron levels elevated the growth rate of the strains. Clinical strains appeared to be more correlated with high iron levels than environmental isolates. This study suggested that relatively increased growth rates of clinical strains of *V. vulnificus* under elevated iron concentrations might explain the higher virulence of this pathogen in patients with iron overload from an underlying disease.

Keywords: Growth, iron, pathogen, virulence, *vvhA*

Demirin *Vibrio vulnificus*'un klinik ve çevresel suşların büyümesi ve hemolizin/sitolizin ekspresyonu üzerine etkisi

Özet: Yüksek demir seviyeleri, oldukça ölümcül bir insan patojeni olan *Vibrio vulnificus*'un büyümesi ve virülansı ile güçlü bir şekilde ilişkilidir. Bu çalışma, farklı demir konsantrasyonları altında (7, 10, 15, 30 ve 50 µM) dört klinik ve dört çevresel *V. vulnificus* suşlarının, büyüme oranlarını ve hemolizin/sitolizin kodlayan genin (*vvhA*) ekspresyon seviyelerini incelemiştir. Ekspresyon seviyeleri $2^{-\Delta\Delta CT}$ yöntemine göre belirlenmiştir. Demir konsantrasyonu arttıkça *vvhA* transkripsiyon aşağı regülasyonu gözlenmiştir. Test edilen tüm suşlarda, 30 ve 50 µM ferrik klorür konsantrasyonları seviyesinde düşük konsantrasyonlara kıyasla anlamlı bir fark gözlenmiştir ($p < 0,001$). Artan demir konsantrasyonu, tüm izolatların daha hızlı büyüme oranları ile anlamlı derecede ilişkili bulunmuştur ($p < 0,05$). Demir seviyesinin artması, suşların büyüme oranını artırmıştır. Daha virülans etki gösteren klinik suşların demir ile korelasyonu çevresel izolatlardan daha yüksek olarak gözlenlenmiştir. Bu çalışma, yüksek demir konsantrasyonları altında *V. vulnificus*'un klinik suşlarının nispeten artmış büyüme oranlarının, altta yatan bir hastalıktan dolayı aşırı demir yükü olan hastalarda bu patojenin daha yüksek virülansını açıklayabileceğini düşündürmektedir.

Anahtar kelimeler: Büyüme, demir, patojen, virülans, *vvhA*

Introduction

Vibrio vulnificus is a species that causes very severe foodborne disease and accounts for the highest seafood-related fatalities in the United States (Baker-Austin and Oliver 2018). This highly fatal human pathogen, ubiquitous in coastal seawaters, has been isolated in large numbers from molluscan shellfish (Baker-Austin and Oliver 2018). Infections typically occur from the ingestions of contaminated raw shellfish consumptions, particularly oysters, and by contamination of pre-existing wounds during recreational activities (Horseman and Surani 2011). The most deadly disease is septicemia with a death rate of > 50% particularly in susceptible individuals having a high level of serum iron and immune deficiencies (Leng et al. 2019). This pathogen also leads to severe necrotizing wound infections in immunocompromised patients with a 25% mortality rate (Oliver 2005). Although rare, death occurs within an average of two days following the onset of disease (Jones and Oliver 2009).

Hemolysin/cytolysin (*vvhA*) is one of the putative virulence factors associated with the pathogenesis of *V. vulnificus*. *vvhA*-encoding exotoxin facilitates iron uptake by lyses of hemoglobin and is responsible for cytotoxic activity (Jones and Oliver 2009). The pathological role of extracellular hemolysin/cytolysin is under dispute. One study reported

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that the toxin is not responsible for the lethality of *V. vulnificus* since mutation of *vvhA* shows no difference in the 50% lethal dose in iron-loaded mice (Wright and Morris 1991). However, other researchers revealed the pathogenic significance of the toxin (Lee et al. 2005; Senoh et al. 2005; Jeong and Satchell 2012). Purified vvhA enhances vascular permeability, skin damage, and mortality rate in animal models (Gray and Kreger 1987), and submicrogram level of the pore-forming hemolysin/cytolysin was found to be lethal to mice (Park et al. 1996).

Iron is an essential growth factor for most microorganisms. Increased serum iron levels are highly correlated with the pathogenesis of *V. vulnificus* infections. Two hypotheses were put forth about how excess iron provides advantages to this flesh-eating pathogen: 1) Elevated iron levels significantly increase the growth rate of *V. vulnificus*, thus enhance host susceptibility to infection, and 2) decreases neutrophil activity (Jones and Oliver 2009). Injection of iron into mice significantly reduced LD_{50} and dramatically increased the mortality rate (Wright and Morris 1991). Moreover, a direct correlation was observed between host iron availability and the infectious dose of *V. vulnificus* (Jones and Oliver 2009). Interestingly, high iron content was found to be selective for more virulent clinical isolates of *V. vulnificus* (Çam and Brinkmeyer 2019), and a high correlation was observed between biofilm formation and increasing iron concentrations under in vitro conditions (Çam and Brinkmeyer 2020). Previous studies revealed that the presence of 16S rRNA *type B* and *vcgC* genes indicates 'clinical' origin while 16S rRNA *type A* and *vcgE* genes are significantly associated with environmental strains (Çam et al. 2019). In a previous study, the iron concentration of APW was found ~7 µM (Çam and Brinkmeyer 2019), therefore minimum iron concentration was 7 µM in this study. As proposed by several studies, iron is of utmost importance for the growth and virulence of this pathogen. The objective of this study was to examine the expression of the virulence gene, *vvhA*, in clinical and environmental strains of *V. vulnificus* under different iron concentrations.

Materials and Methods

Isolates and experimental design: A clinical (from a patient) strain was obtained from the US FDA. This clinical strain was obtained from the patient infected by *V. vulnificus* and considered as highly virulent. Three clinical and four environmental isolates were harvested from oyster and seawater, respectively (Table 1) and verified with a strain-specific gene-*vvhA* (Campbell and Wright 2003). To differentiate the clinical strains from environmental ones, 16S rRNA *type B/A* and *vcgC/E* genes were employed. Isolates were kept in Alkaline Peptone Water (APW) with 2% NaCl at 37 °C.

Table 1. *V. vulnificus* strains used in this study.

Strain ID	Type/Source	16S type/vcg
Clin-P	Clinical/Patient	B/C
Clin-O-1	Clinical/Oyster	B/C
Clin-O-2	Clinical/Oyster	B/C
Clin-S	Clinical/Seawater	B/C
Env-O-1	Environmental/Oyster	AB/E
Env-O-2	Environmental/Oyster	A/E
Env-S-1	Environmental/Seawater	A/E
Env-S-2	Environmental/Seawater	A/E

All isolates were confirmed with species-specific *vvhA* gene.

APW medium was supplemented with different concentrations of ferric chloride ($FeCl_3$) before inoculation. Final iron concentrations of incubation medium were 7, 10, 15, 30, and 50 µM. APW broth (2% NaCl, pH 7.4) was then inoculated with *V. vulnificus* strains (~1 × 10^6 cells ml^{-1}) separately in falcon tubes and incubated at 37 °C by shaking at 120 rpm. Aliquots were sampled at 8 h of growth to determine the expression of the virulence gene and the growth rates of *V. vulnificus* strains with increasing iron concentrations. All experiments were performed in duplicate with three replicates.

***RNA extraction and cDNA synthesis**:* CTAB (cetyltrimethylammonium bromide) protocol was followed for nucleic acid extraction (Çam et al. 2019). DNA was degraded with DNase I (Sigma). RNA concentrations were determined spectrophotometrically. High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) was employed to synthesize cDNA based on the protocol described by the manufacturer.

Determination of relative gene expression: The expression of *vvhA* was determined using probe assay with *vvhA* primers (Campbell and Wright 2003) (Table 2). Quantitative RT-PCRs were performed in a 25 µl volume of reaction mixture consisting of 1 × PCR Buffer, 1 µM of each primer, 250 nM probe, 200 nM dNTPs, 2.5 mM $MgCl_2$, 1 × BSA, PCR water, 1 U Taq DNA polymerase (ThermoFisher Scientific), and different concentrations of cDNA. Thermo-cycling conditions were set up at 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 60 s.

Table 2. Primers and probes used to determine expression levels of the genes in *V. vulnificus*.

Primer or Probe	Primer Sequence (5′-3′)	References
VvhA-F	TGTTTATGGTGAGAACGGTGACA	(Campbell and Wright 2003)
VvhA-R	TTCTTTATCTAGGCCCCAAACTTG	
VvhA-probe	(Texas Red)-CCGTTAACCGAACCACCCGCAA-(BHQ1)	
GyrB-F	GGTACCACGGTACGTTTCTG	(Bisharat et al. 2005)
GyrB-R	CTTTACGGCGTGTCATTTCAC	

The relative gene expressions were determined based on the threshold cycle according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The cycle threshold (Ct) values of the target genes were normalized to the GyrB house-keeping gene (DNA gyrase, subunit B) (Bisharat et al. 2005). In the $2^{-\Delta\Delta CT}$ method, the differences between the Ct values (ΔCt) of the *vvhA* gene and the GyrB house-keeping gene were calculated. The difference in ΔCt values between iron-treated and non-treated samples ΔΔCt was then calculated. The fold differences of the genes between the two samples were $2^{-\Delta\Delta CT}$. All real-time PCRs were performed in SmartCycler (ThermoFisher Scientific).

Growth rate: The growth rates of the isolates (generations/h) at various concentrations of ferric chloride were determined based on optical density (OD). OD was measured at 600 nm with a spectrophotometer (Biotek). OD readings were converted to cell counts by making standard curves for each strain, separately. Growth rates were calculated with the formula shown in the study (Çam and Brinkmeyer 2020). Cell count experiments were duplicated with three replicates.

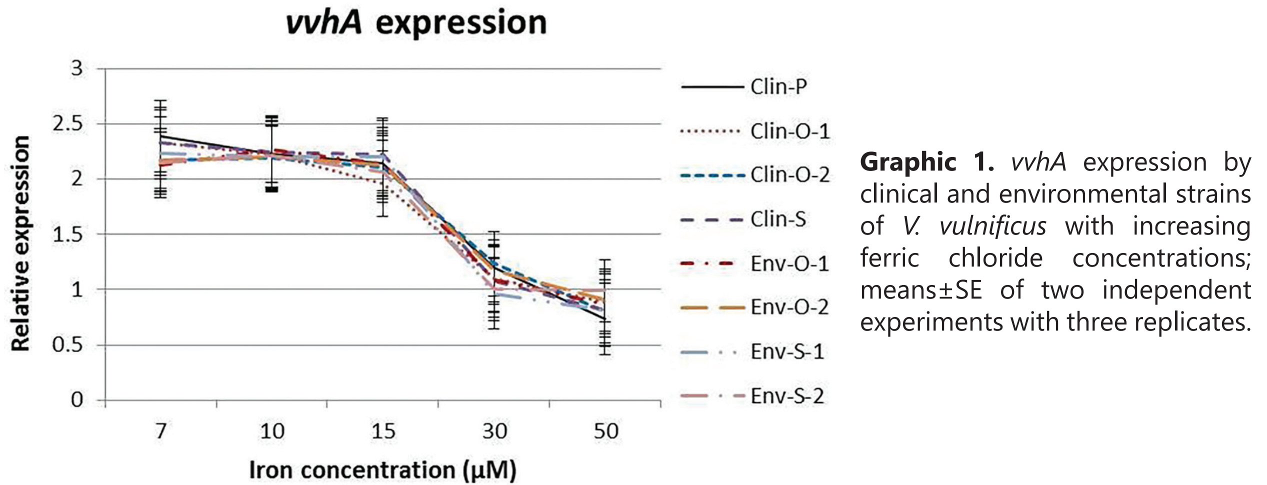
Statistical analysis: Bartlett's test was used for the equality of variances. One-way ANOVA and linear regression analysis were performed in SPSS statistics 24.0.

Results and Discussion

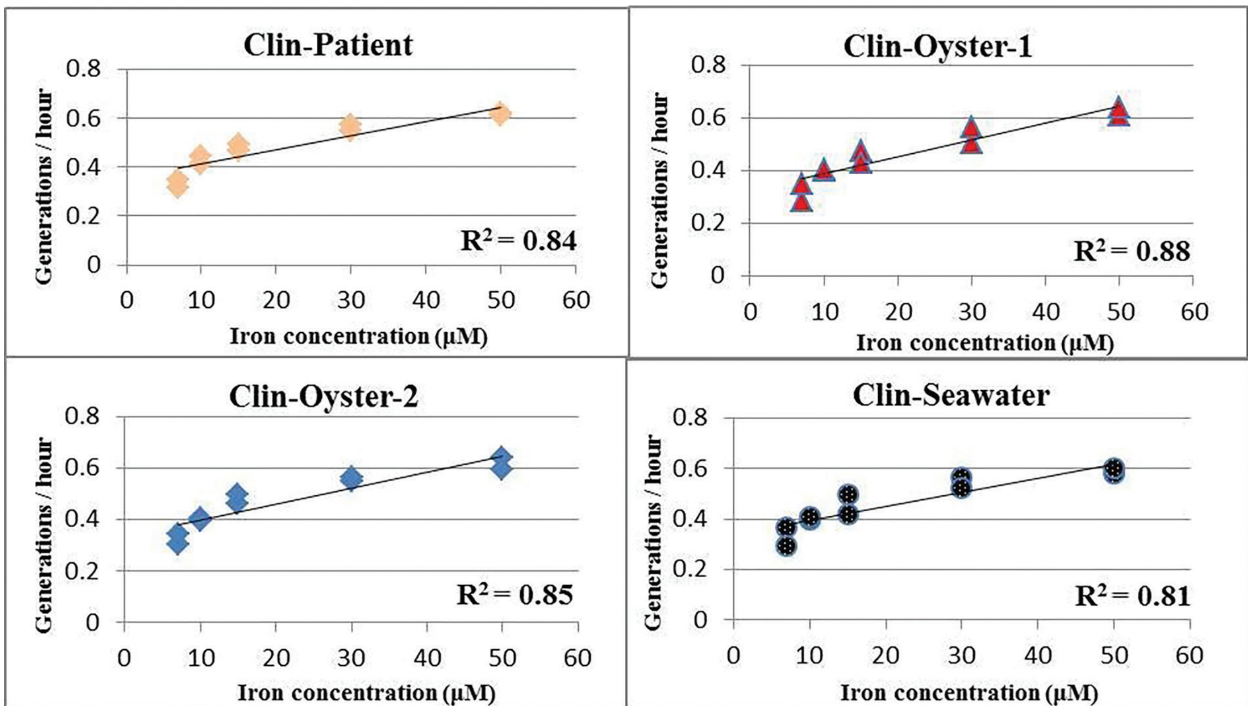
Iron is an essential factor for the pathogenesis of *V. vulnificus* in infected individuals. Excessive iron significantly decreases LD_{50} and dramatically increases the mortality rate of this pathogen in mice (Jones and Oliver 2009), therefore the current study aimed to investigate the increasing iron concentrations on *vvhA* expression and the growth rate of clinical and environmental strains of *V. vulnificus* under in vitro conditions.

Transcription of *vvhA* was down-regulated as iron concentration increased in all the strains tested (Graphic 1). The strains exhibited almost similar expression patterns in all iron levels. The groups exhibited significant differences (one-way ANOVA, $p < 0.001$). The level of *vvhA* transcription in all the isolates was significantly decreased at 30 and 50 µM compared to the lower iron concentrations (Bonferroni *post hoc* test, $p < 0.001$). These results show that *vvhA* transcription is repressed by increasing iron levels. The present finding is in good agreement with the study (Kim et al. 2009), suggesting that *vvhA* expression can be repressed by iron via Fur and LuxS quorum-sensing system. Quorum-sensing is a bacterial communication system that regulates gene expression levels in response to cell population density in a particular environment by releasing specific signaling molecules. In *V. vulnificus*, mutation analyses showed that LuxS quorum-sensing system plays important roles in virulence regulation along with other virulence regulators. Inactivation of *luxS* through mutations revealed that the transcriptional activity of *vvhA* is negatively controlled by *luxS* transcription (Kim et al. 2003). Besides, iron increases *LuxS* expression in *V. vulnificus* (Kawase et al. 2004). Therefore, in the current study, the down-regulation of *vvhA* in the presence of excessive iron might be attributed to increased activity of the LuxS quorum-sensing system.

The growth rates of clinical and environmental isolates were found to be significantly correlated with elevated iron concentrations (Linear regression, $p < 0.05$) (Graphic 2 and 3). Higher levels of ferric chloride enhanced the growth rates of the strains. Similar results were also obtained by the other studies (Hor et al. 2000; Starks et al. 2006; Kim et al. 2009), revealing that excess iron stimulates *V. vulnificus* growth. Iron serves as an important redox catalyst for various cellular processes such as respiration and DNA replication in microorganisms (Cassat and Skaar 2013). This pathogen is a ferrophilic microorganism and requires high concentrations of iron for growth (Kim et al. 2007); as a result, causing increased susceptibility of iron-overloaded patients to infections (Hor et al. 2000; Cassat and Skaar 2013). The patients with enhanced iron concentrations from an underlying disease were also found to be more susceptible to infections in other pathogens (Cassat and Skaar 2013).

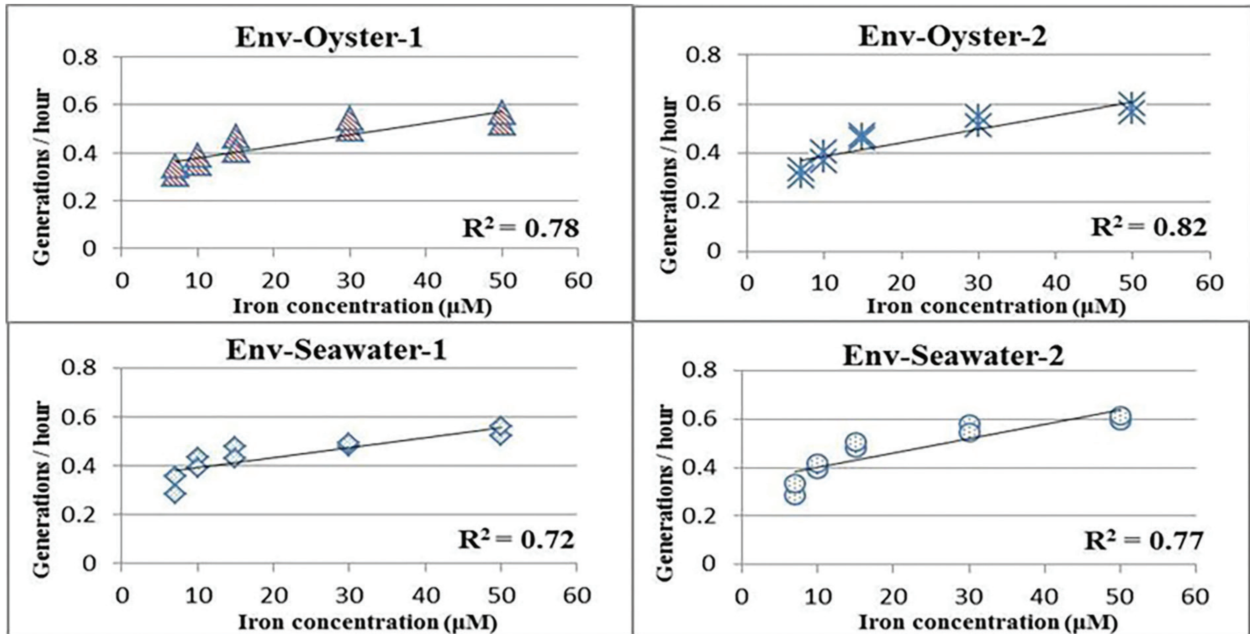


Graphic 1. *vvhA* expression by clinical and environmental strains of *V. vulnificus* with increasing ferric chloride concentrations; means±SE of two independent experiments with three replicates.



Graphic 2. Growth rates (generations/hour) of clinical strains of *V. vulnificus* with increasing ferric chloride concentrations (average of two independent experiments with three replicates) (Linear regression, $p < 0.05$).

Clinical strains displayed higher correlation rates than environmental strains under increasing iron concentrations (Graphic 2 and 3). R^2 values ranged from 0.81 to 0.88 in clinical strains and from 0.72 to 0.82 in environmental isolates. The highest correlations were observed in the clinical strains obtained from oysters, followed by another clinical strain from the patient. The lowest R^2 values (0.72 and 0.77) were found in environmental strains isolated from seawater. As observed in the current study, Starks et al. (2006) studied differential growth rates of clinical and environmental *V. vulnificus* isolates in the presence of excess iron in mice, indicating that an excessive amount of iron significantly increases the growth rates of clinical strains tested (Starks et al. 2006). The higher virulence of clinical strains might be explained by relatively higher growth and replication rates of the isolates at higher iron concentrations because the main reason for *V. vulnificus* infection is mostly attributed to increased serum iron concentrations in susceptible individuals (Hor et al. 2000; Kim et al. 2007; Kim et al. 2009).



Graphic 3. Growth rates (generations/hour) of environmental strains of *V. vulnificus* with increasing ferric chloride concentrations (average of two independent experiments with three replicates) (Linear regression, $p < 0.05$).

In conclusion, the present study revealed that excessive amounts of iron dramatically decreased transcription of *vvhA* and significantly increased the growth rates of *V. vulnificus* strains. The higher mortality rate of this microorganism in patients having an underlying disease such as hemochromatosis might be likely the increasing growth rates of this pathogen under high iron serum levels. Iron appeared to be more correlated with the growth rate of clinical strains rather than environmental isolates.

Use of laboratory animals Ethics Committee and other decisions of Ethics Committee and Permissions: No animal used.

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