Virulence Characterization of Viable but Nonculturable *Vibrio parahaemolyticus*

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Abstract (updated)

Under unfavorable conditions, some bacteria have demonstrated the ability to enter into a viable but nonculturable (VBNC) state, possibly as a survival mechanism. *Vibrio parahaemolyticus*, a foodborne pathogen commonly associated with seafood, could exist in VBNC state under nutrient starvation or low temperature conditions. Standard enumeration method of viable *V. parahaemolyticus* from food samples is dependent on the ability of the isolates to grow on laboratory media. However, VBNC cells of V. *parahaemolyticus* do not form colonies on standard media and therefore their presence is overlooked. VBNC cells may resuscitate and thus regain virulence under proper conditions, such as in the human GI track. Therefore, the goal of this study is to 1) optimize methods to enumerate VBNC cells of *V. parahaemolyticus*, and 2) to characterize and compare virulence between VBNC and vegetative cells. *V. parahaemolyticus* strain, RIMD2210633, a clinical O3:K6 isolate, was subjected to both nutrient (modified Morita mineral salt solution) and temperature (5°C) stresses to reach the VBNC state. Cells were considered nonculturable when no CFU was formed after plating 0.1 ml on Trypticase Soy Agar (TSAS) followed by incubation at 25°C. Live/Dead® BacLight™ kit was used to determine cell viability. The microscopic method was optimized to detect viable *V. parahaemolyticus* by adjusting the ratio of STYO 9 and propidium iodide from 1:1 to 1:3. The expression of virulence marker genes, *tdh2* and *escU,* were measured by using quantitative PCR. The levels of expression for each gene were normalized according to the geometric mean of results obtained for the control gene *pvsA*. Our study shows that VBNC cells expressed comparable levels of these virulence genes.

Introduction

- *Vibrio parahaemolyticus* is a major foodborne pathogen found in seafood that causes acute primary infections of the human gastrointestinal tract. Despite food safety regulations , *V. parahaemolyticus* is estimated to be responsible for 4500 illnesses annually in the United States.
- Under unfavorable conditions of nutrient and temperature stresses, *V. parahaemolyticus* has demonstrated the ability to enter the VBNC state, possibly as a survival mechanism. Bacteria in the VBNC state are alive but exhibit very low levels of metabolic activity. As such, they cannot grow in nutrient media as they are incapable of undergoing sustained cellular division.
- It is likely that *V. parahaemolyticus* cells existing in the VBNC state are undetected under standard laboratory detection methods, posing a risk to food safety.
- Previous studies showed inconsistent data on the virulence of *V. parahaemolyticus*. In this study, the expression of virulence markers, *tdh2* and *escU*, will be measured.
- The presence of *tdh2* is historically associated with the pathogenic potential of *V. parahaemolyticus*. It is almost exclusively found in clinical isolates and is the most recognized virulence factor.
- Another virulence marker is *escU*, which is present in the recently isolated pathogenic strains bearing O3:K6 serotype. It is involved in the type III secretion system.

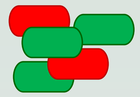
Methods

***Vibrio parahaemolyticus* Treatment**

Vegetative Cells: 25°C in TSB supplemented with 2% NaCl

VBNC Cells: 5°C in modified Morita mineral salt solution

Enumeration of Cells

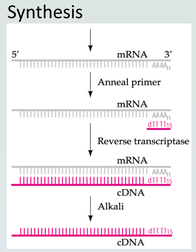


LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012

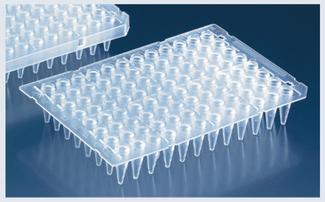
Standard Plate Count on TSA supplemented with 2% NaCl

RNA Extraction
TRIzol Method

Total RNA → cDNA
SuperScript II First Strand Synthesis



Quantitative PCR
DyNAmo ColorFlash SYBR Green qPCR kit



- **Genes**:
  - Housekeeping *pvsA*
  - Virulence factors *tdh2* and *escU*
- Performed in triplicate
- **Negative controls:**
  - No template control
  - No RT (RNA only)

Standard Curve:
- DNA extracted from o/n cultures
- 1:5 serial dilutions
- Concentrations: 25, 5, 1, 0.2, 0.04, 0.008, 0.0016, 0.00032 ng/µl

Conclusions

- Housekeeping gene *pvsA* and virulence genes *tdh2* (coding for the thermostable direct hemolysin) and *escU* (involved in type III secretion system) were expressed both in both culturable and viable but nonculturable *V. parahaemolyticus* cells.
- Relative expression levels of *tdh2* and *escU* is 1.28 and 1.00, respectively. This suggests VBNC cells expressed *tdh2* at a level that is 1.28-fold higher than culturable cells, where the expression level for *escU* was the same. These results are preliminary and will be repeated to confirm the increased expression of *tdh2* in VBNC cells.
- Our preliminary data suggest VBNC cells of *V. parahaemolyticus* retain the pathogenicity and are able to express virulence factors despite not growing in typical culture media. Ingestion of VBNC cells may therefore result in foodborne illnesses.

References

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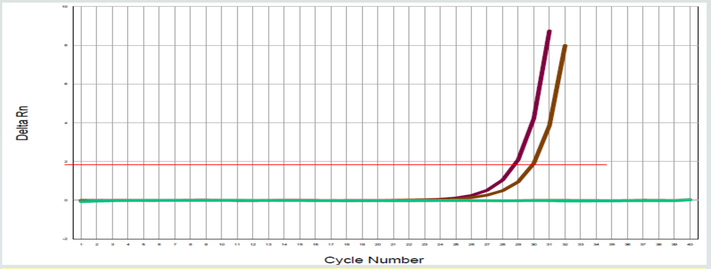
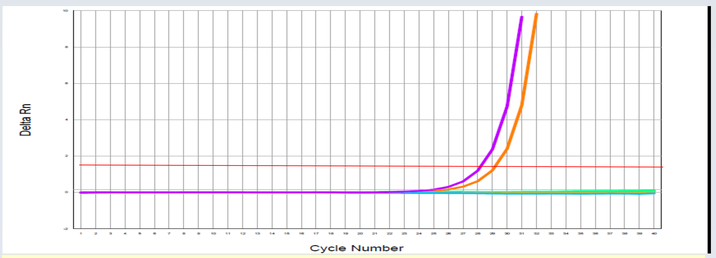
Results

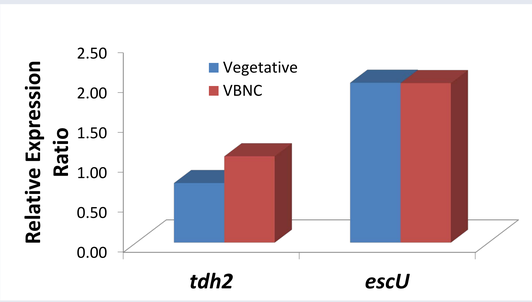
	CFU/mL	DMC/mL (Green cells)	DMC/mL (Red cells)	Total DMC/mL
Initial cell # to prepare standard curve	1×10^8	n/a	n/a	n/a
initial amount of vegetative (culturable) cells	1.5×10^7	3.7×10^8	5.2×10^7	4.2×10^8
initial amount of VBNC cells	<1	5.0×10^8	3.6×10^7	5.4×10^8



Expression profile of *tdh2* gene in culturable (left) and VBNC (right) state. Negative RNA only control is shown in green.



Expression profile of *escU* gene in culturable (purple) and VBNC (orange) state. Negative RNA only controls are shown in green and blue.



Mean relative expression ratios for *tdh2* and *escU* after temperature and nutrient stress. The levels of expression were normalized according to the geometric mean of results obtained for the control gene *pvsA*. The data are the normalized means of the results for one run, each with triplicate samples.

Gene	Slope	R-sq	Efficiency	Relative expression ratio
pvsA (reference gene)	-2.804	91.6	2.273	1
tdh2	-2.666	98.1	2.372	1.281
escU	-3.425	99.8	1.959	1.040

Values for slope were deduced from the standard cuves. Efficiency was calculated as $10^{(-1/\text{slope})}$. Relative expression ratio represents the fold increase (or decrease) in VBNC cells relative to culturabe cells.