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The thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*: Sequence variation and implications for detection and function

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ABSTRACT

Vibrio parahaemolyticus is a leading cause of bacterial food-related illness associated with the consumption of undercooked seafood. Only a small subset of strains is pathogenic. Most clinical strains encode for the thermostable direct hemolysin (TDH) and/or the TDH-related hemolysin (TRH). In this work, we amplify and sequence the *trh* gene from over 80 *trh* + strains of this bacterium and identify thirteen genetically distinct alleles, most of which have not been deposited in GenBank previously. Sequence data was used to design new primers for more reliable detection of *trh* by endpoint PCR. We also designed a new quantitative PCR assay to target a more conserved gene that is genetically-linked to *trh*. This gene, *ure*R, encodes the transcriptional regulator for the urease gene cluster immediately upstream of *trh*. We propose that this *ure*R assay can be a useful screening tool as a surrogate for direct detection of *trh* that circumvents challenges associated with *trh* sequence variation.
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1. Introduction

Vibrio parahaemolyticus is an autochthonous member of the microbial community in estuarine and marine waters globally and the leading cause of bacterial illness associated with seafood consumption (Newton et al., 2012; Raghunath, 2015). The illness is usually acquired through the ingestion of raw or undercooked seafood such as oysters that bioaccumulate the bacteria through filter feeding. Most *V. parahaemolyticus* infections manifest as mild to moderate gastroenteritis that is usually self-limiting. Skin infections also occur via open wound exposure to water containing *V. parahaemolyticus*. The illness, referred to as vibriosis, is significantly under-reported since the disease is usually not acute enough to require medical attention. According to the Centers for Disease Control and Prevention, for every reported *V. parahaemolyticus* illness in the United States, 140 cases go unreported, leading to an estimate of 35,000 cases per year (Scallan et al., 2011).

Only a small subset of *V. parahaemolyticus* strains is thought to be virulent, and early work suggested that *tdh*, a gene encoding for the thermostable direct hemolysin, was a reliable virulence marker. In fact, until the late 1980's, nearly all clinical isolates of this bacterium were found to elaborate TDH as evidenced by the lysis of erythrocytes on Wagatsuma blood agar, otherwise known as the Kanagawa phenomenon (Miyamoto et al., 1969). However in 1987, clinical *V. parahaemolyticus* isolates from the Republic of Maldives were found to be Kanagawa negative and also appeared to lack the *tdh* gene by DNA hybridization (Honda et al., 1987). It was later determined that these strains produced a homologous hemolysin, the TDH-related hemolysin (TRH) with a size and immunological activity similar to but distinct from TDH (Honda et al., 1988). The gene encoding this protein (*trh*) was subsequently cloned and sequenced and determined to be homologous (ca. 70% identity) with *tdh* (Nishibuchi et al., 1989). Yet another version of the *trh* gene was described in 1992 (*trh*2) that was 84% homologous to the original sequence (then renamed *trh*1) (Kishishita et al., 1992). The products of both *trh*1 and *trh*2 were reported to be antigenically related, and along with *tdh* are considered to be potential virulence markers (Ceccarelli et al., 2013).

Development of PCR-based assays for the reliable detection of *trh* has been a challenge due to sequence variation of this gene. A number of endpoint PCR assays have been published (Bej et al., 1999; West et al., 2013; Hossain et al., 2013; Tada et al., 1992), but in general, a systematic evaluation of these assays on *trh*1 and *trh*2 sequence variants has not been undertaken. Similarly, at least two probe-based real time PCR assays have been published (Nordstrom et al., 2007; Ward and Bej, 2006), but here again neither assay was systematically tested on a variety of known *trh* strain variants.

Our laboratory has previously sequenced the genomes of 19 *V. parahaemolyticus* strains. The GenBank accession numbers from this comparative genomics project are given in Table S1. Inspection of those draft genomes revealed that three strains initially screened as *trh*$^-$ using the established Biological Analytical Manual (BAM) endpoint PCR

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assay (U.S. Food and Drug Administration, 2004) were in fact *trh*$^+$, along with 10 other of the 19 strains chosen for sequencing, (see Table S1). In this work, we compare the complete *trh* gene sequence from these 13 draft genomes plus 68 additional *trh*$^+$ strains of both clinical and environmental origin. The results reveal the existence of a large number of sequence variants or sequevars (SVs) of both *trh*1 and *trh*2 that can lead to false negatives. To improve the reliability of *trh* detection by endpoint PCR, we propose a new primer pair. In addition, a previous study indicated urea hydrolysis to be a useful phenotype for identifying potentially virulent strains of *V. parahaemolyticus* in the Pacific Northwest (PNW) (Kaysner et al., 1994). Further, other reports have described a reliable genetic link between *trh* and the urease gene cluster in *trh*$^+$ stains of this bacterium (Iida et al., 1997; Suthienkul et al., 1995). This previous work along with sequence information from strains included in the comparative genomics project mentioned above, led to the design of a quantitative 5′ nuclease assay for qPCR detection of a gene in the urease gene cluster (*ure*R) as a surrogate for *trh* that avoids false negatives due to variation in the nucleotide sequence of the latter.

2. Materials and methods

2.1. Bacterial strains, culturing methodology, and DNA extraction

The strains of *V. parahaemolyticus* chosen for analysis in this study are described in Table S1. In addition to the 13 *trh*$^+$ strains from the previous comparative genomics project an additional 68 *trh*$^+$ strains were chosen for amplification and sequencing of the *trh* gene. These additional strains originated from both clinical (n = 39) and environmental (n = 30) sources and were collected between 1970 and 2011. The vast majority of these strains originated from the PNW of the United States. All strains were grown at 30 °C on trypticase soy agar amended with 1.5% NaCl (TSS). Isolated colonies were transferred to 3 mL of TSS broth and grown overnight at 37 °C on a Lab Line Cell-Gro Tissue Culture Rotator (Thermo Scientific, Waltham, MA) at 100 rpm. A 0.5 mL aliquot of overnight culture was centrifuged for 5 min at 5900 × *g* and resuspended in 1X PBS. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) protocol for cultured cells. The quality of the DNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), standardized to a final concentration of 20 ng/μl and stored at −20 °C.

2.2. PCR amplification and sequencing of the trh gene

Oligonucleotides used in this study (Table 1) were synthesized by Fisher Scientific (Pittsburgh, PA) unless otherwise stated. The draft genome of *V. parahaemolyticus* strain 10329 (Gonzalez-Escalona et al., 2011) and the 13 additional *trh*$^+$ *V. parahaemolyticus* isolates (Table S1) were used as reference sequences for the alignment of the *trh* gene as well as flanking noncoding sequence. This alignment (Fig. S1) was used to design of a primer pair (*trh*seqF and *trh*seqR) to amplify the complete *trh* gene. Cycling for this and all other endpoint assays in this work was carried out on an iCycler (Bio-Rad, Hercules, CA). Amplification of the *trh* gene used the following thermal profile: one cycle at 95 °C for 2.25 min, 35 cycles of 95 °C, 51 °C, and 72 °C each for 45 s, and a final extension at 72 °C for 9.25 min. Each 25 μl reaction consisted of both primers at 600 nM, 200 μM each dNTP, 2.5 μl of 10X buffer containing 20 mM $MgSO_4$, and 0.25 U of high fidelity Vent DNA polymerase (New England Biolabs, Ipswich, MA). PCR products were purified using the UltraClean PCR Clean-Up Kit (MoBio Laboratories, Solano Beach, CA) and submitted for Sanger sequencing of both strands with the primers *trh*seqF and *trh*seqR (Molecular Cloning Laboratories, South San Francisco, CA). The forward and reverse traces for each strain were aligned and edited using the Phrap algorithm in MacVector version 12.5.1 (MacVector Inc., Cary, NC).

2.3. Sequence and phylogenetic analyses

The *trh* sequences of all strains were compared using MacVector and those strains sharing identical sequences were assigned to the same sequence variant (SV) group. The phylogenetic relationship between SVs was inferred by the neighbor-joining method (Saitou and Nei, 1987) using MEGA version 6.06 (Tamura et al., 2013). The neighbor-joining tree was constructed with statistical support of 2000 bootstrap replications and includes all unique SVs from this work as well as all other unique *trh* sequences deposited in GenBank that cover the entire gene.

2.4. Detection of trh with existing and new primers

Based on alignments of all 13 SVs in this work, a new primer pair was designed for endpoint PCR detection of *trh* (trh_53F and trhdeg_531R, Table 1). Each 25 μl reaction contained 400 nM of each primer, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 5 μl of 5X GoTaq Flexi Buffer (Promega, Madison, WI) and 0.125 μl of GoTaq Flexi DNA Polymerase (also from Promega). Thermal cycling consisted of one cycle 95 °C for 2.5 min, 25 cycles of 95 °C, 53 °C, and 72 °C for 30 s and a final extension at 72 °C for 9.5 min. This assay was compared to the assay widely accepted for detection of *trh* (Bej et al., 1999) as recommended in the BAM Manual (U.S. Food and Drug Administration, 2004).

2.5. Real-time quantitative PCR (qPCR) analysis of ureR

We designed a quantitative 5′ nuclease assay for detection of *ure*R, which encodes for the transcriptional activator of the urease gene cluster located immediately upstream from *trh* (Park et al., 2000) and widely reported to be genetically-linked to *trh* (Iida et al., 1997; Suthienkul et al., 1995). The *ureR* gene sequence was determined in silico using the draft genomes of 10329 (Haendiges et al., 2015) and the 13 *trh*$^+$/*ureR*$^+$ reference genomes (Table 2). From the aligned sequences (Fig. S2), primers (*ure*R_139F and *ure*R_221R) and a probe (*ure*R_165/190P) targeting the *ureR* gene (Table 1) were designed using Primer Express Software v2.0

Table 1
Primers and probe used in this work.

Primer/probe	Sequence, 5′-3′ (label)	Target	Amplicon size (bp)	Reference
*trh*seqF	tgctttcctttatctcgagc	Upstream *trh*	~730–775	This work
*trh*seqR	catatgaaaacaaatgctttttag	*Downstream trh*		
trh-L	ttggcttcgatattttcagtatct	*trh*	486	BAM[a]
trh-R	cataacaaacatatgcccatttccg	*trh*		
*trh*_53F	cagtatctaaatcattcgcgattg	*trh*	478	This work
*trh*deg_531R	ggaaatrcacataacaaacatatg	*trh*		
*ure*R_1F	atggaatacaaaaacattcaatcatc	*ure*R	350	This work
*ure*R_350R	attatctgaaagttacgttcaacgc	*ure*R		
*ure*R_140F[b]	gcgtagtcatcgttccgaaatac	*ure*R	82	This work
*ure*R_221R[b]	aagtgagcctccattgattgtagag	*ure*R		
*ure*R_165/190P[b]	FAM/tcgcgtatc/ZEN/ctgcactctaacaccca/3IABkFQ/	*ure*R		

[a] http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm
[b] Synthesized by Integrated DNA Technologies, Coralville, IA.