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Short communication

Comparison of *toxR* and *tlh* based PCR assays for *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is a Gram-negative bacterium found in marine and estuarine environments and is globally the leading cause of bacterial seafood-related illness. A real-time PCR assay for *V. parahaemolyticus* was developed for the marker *toxR*, with a large-scale and direct comparison of its applicability as a species-specific marker compared to the *tlh* gene carried out. Assays for *tlh* and *toxR* were used for 255 presumptive *V. parahaemolyticus* strains from our strain library, utilising both real-time (*toxR*) and conventional PCR assays (*tlh*). Of the 255 strains test, 254 results were in concordance; 255 strains were identified as being *toxR* positive (100%) and 254 strains were *tlh* positive (99.6%). The single discordant strain (isolate V12/023) was of interest, because it represented a presumptive *V. parahaemolyticus* strain, isolated from a clinical case. Whole genome sequence analysis and multi locus sequence typing of this single discordant strain was carried out, which unambiguously identified that the isolate was indeed *V. parahaemolyticus.* Genome analysis identified mismatches in the primer binding sites for the established *tlh* assay is likely responsible for the assay failing on this particular strain. The identification of false-negative results in strains that are implicated in human infections using the *tlh* assay and clearly highlights the relevance of the comparison with a *toxR* assay which showed 100% identification for the *V. parahaemolyticus* strains tested.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative bacterium found naturally in marine and estuarine waters. This pathogen is a significant cause of foodborne disease, and is considered to be the leading agent responsible for bacterial illness associated with seafood consumption (Joseph, Colwell, & Kaper, 1982). The bacterium is found commonly in bivalve shellfish species such as oysters, clams and mussels. The clinical characteristics of *V. parahaemolyticus* infections include abdominal cramps, diarrhoea, nausea, headaches, fever, and chills (Honda & Iida, 1993). *V. parahaemolyticus* symptoms typically resolve in less than 72 h, and infections caused by *V. parahaemolyticus* are disproportionately self-limiting. However, they can be life-threatening in patients who suffer from underlying conditions such as liver dysfunction or suppressed immunity (Ottaviani et al., 2012). An estimated 40,000 people contract *V. parahaemolyticus* infections each year in the USA alone (Scallan et al., 2011), underlining the clinical burden associated with this pathogen. Indeed, epidemiological data from the USA has suggested that vibriosis is increasing compared to other foodborne pathogens (CDC, 2010), and infections caused by *V. parahaemolyticus* disproportionately contribute to the reported illness burden associated with Vibrios (Newton, Kendall, Vugia, Henao, & Mahon, 2012). Several large outbreaks of *V. parahaemolyticus* have also emerged in temperate regions in the last two decades, such as Alaska, Chile and Western Europe (Baker-Austin et al., 2013; Gonzalez-Escalona et al., 2005; Martinez-Urtaza et al., 2013; McLaughlin et al., 2005), highlighting the need for improved global surveillance systems. There is increased interest in the role of climate warming and extreme weather events such as heatwaves in modulating the risks associated with *V. parahaemolyticus* and other non-cholera vibrios associated with shellfish (Baker-Austin et al., 2016, 2013; Martinez-Urtaza et al., 2013).

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One of the established methods for identifying *V. parahaemolyticus* relies on the thermolabile haemolysin gene (*tlh*) (Bej et al., 1999). This target is used routinely in our laboratory as a species-specific testing method utilising a conventional PCR testing approach. However, previous studies have identified that *tlh* is present in some *V. alginolyticus* strains (Xie, Hu, Chen, Zhang, & Ren, 2005), potentially producing false-positive results. This led us to explore the potential of developing and comparing another molecular target to be used independently or in conjunction with the *tlh* assay. Several studies have demonstrated that *toxR* represents a reliable species-specific molecular target for the identification of *V. parahaemolyticus* (Croci et al., 2007; Kim et al., 1999). Although conventional PCR assays (Kim et al., 1999) and real-time PCR approaches (Lo et al., 2008) have been developed and published that target *toxR*, few studies provide direct side-by-side comparisons between *toxR* and *tlh*-targeted assays. A study by Croci et al. (2007) which compared conventional PCR assays for *toxR* and *tlh* indicated that the characteristics of accuracy expressed by the *toxR* identification method made it a suitable candidate as a reference method for the molecular identification of *V. parahaemolyticus* strains. More recently however, Li, Chiou, Chan, and Chen (2016) analysed 120 *V. parahaemolyticus* strains and found 100% congruence between tlh and toxR PCR tests. For laboratories that test bacterial pathogens present in seafood, it is essential that the molecular tests that are routinely used are as reliable, robust and accurate as possible. For this reason, during the process of replacing conventional PCR testing platforms in our laboratory with real-time methods, we decided to develop a *toxR* real-time PCR assay, then test, side-by-side this approach against both conventional and real-time PCR assays for *tlh* and *toxR* respectively. The comparison was carried out utilising a large number of tested strains from our strain bank, encompassing several hundred isolates including *V. parahaemolyticus,* closely related and distantly-related *Vibrio*s many of which were isolated from food samples and have diverse geographical origins. These data provide important performance characteristics of these two methods for species-specific discrimination of unknown isolates.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Presumptive *V. parahaemolyticus* strains were grown at 30 °C for 24 h on marine agar. Strains were streaked out to single colonies on plates prior to PCR testing (see below). A total of 335 bacterial strains (Table 1), including *V. parahaemolyticus* ($n = 255$), other *Vibrio* species ($n = 74$), and distantly related reference strains ($n = 6$) were used to assess the specificity of the oligonucleotide assays tested here.

Table 1
Percentage accuracy of assay.

	toxR	*tlh*
V. parahaemolyticus	255/255 (100% accurate)	254/255 (99.6% accurate)
Other *Vibrio* spp.		
V. fluvialis ($n = 1$)	ND	ND
V. vulnificus ($n = 28$)	ND	ND
V. cholerae ($n = 21$)	ND	ND
V. alginolyticus ($n = 24$)	ND	ND
Other bacteria		
S. paucimobilis (NCTC 11030)	ND	ND
P. aeruginosa (NCTC 10332)	ND	ND
P. mirabilis (NCTC 10975)	ND	ND
E. coli (NCTC 12241)	ND	ND
K. aerogenes (NCTC 9528)	ND	ND
E. faecalis (NCTC 775)	ND	ND

ND = not detected.

2.2. PCR and real-time PCR testing

The complete nucleotide sequences (open reading frame regions only) were analysed for all full length *toxR* nucleotide sequences, and were aligned using clustalW (Thompson, Higgins, & Gibson, 1994). A segment of the *toxR* genes was analysed using Primer Express software from DNASTAR (Madison, WI, USA). The probe and primers were subsequently assessed for species as well as strain specificity using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the GenBank database (Benson et al., 2013). The primer and probe sequences developed were forward primer (5′-3′ GAACCAGAAGCGCCAGTAGT), reverse primer (3′-5′ AAACAGCAGTACGCAAATCG) and probe (^{FAM}- TCACAGCA-GAAGCCACAGGTGC-^{TAMRA}). *Vibrio* isolates in the Cefas strain bank have been obtained from a variety of sources and encompass a combination of foodborne, clinical and environmental strains. These isolates were tested as part of routine housekeeping of the strain library which would incorporate *toxR* results into a database alongside other relevant data. A total of 255 individual putative *V. parahaemolyticus* isolates were grown on marine agar overnight at 30 °C. A single bacterial colony was emulsified in 300 μl of nuclease-free water and boiled at 99 °C for 5 min in 1.5 ml microcentrifuge tubes. The lysed samples were flash-spun and 5 μl was subsequently used as template in both conventional and real-time PCR assays. Strains of other *Vibrio*s, including *V. vulnificus* (28 isolates), *V. cholerae* (21 isolates) *V. fluvialis* (1 isolate) and *V. alginolyticus* (24 isolates) as well as more distantly related bacteria (6 species) were also included to assess exclusivity of the outlined *tlh* and *toxR* assays. For real-time PCR experiments, the assay comprised a total reaction of 25 μl, consisting of 1× TaqMan Universal PCR Master Mix (Applied Biosystems, UK), 900 nM *toxR* forward and reverse primers and 200 nM probe. Five microliters of template (boiled cell lysate) was subsequently added, and each reaction was performed in triplicate. *V. parahaemolyticus* strain NCTC 10885 was used as a positive control. Amplification was performed using a Stratagene© MxPro - Mx3000P system (Agilent, UK) with the following cycling parameters: 1 cycle at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 90 s. For each assay, samples generating a positive reaction result (sigmoid-shaped amplification curve rising above the threshold) in any replicate were considered positive. A positive control for *toxR* was made by lysing a single colony *V. parahaemolyticus* strain NCTC 10885. To evaluate the efficacy of the *toxR* assay using RT-PCR, the same strains were analysed for *tlh* using conventional PCR. The assay was optimised for *tlh* in a total volume of 50 μl consisting of 1× GoTaq Flexi Buffer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 μM *tlh* forward and reverse primers, 1.25 units GoTaq Flexi G2 DNA Polymerase and 5 μl template DNA. A thermal cycle protocol detailing the following commands was used: 1 cycle at 94 °C for 3 min; 30 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min; 1 cycle at 72 °C for 5 min; incubate at 4 °C indefinitely. PCR products were visualised on 2% agarose gels containing ethidium bromide and subjected to a 120 V, 400 mA electrophoresis run for 25–35 min. A positive control for *tlh* was made by lysing a single colony of *V. parahaemolyticus* strain NCTC 10885 which consistently gave strong bands in conventional PCR gel analyses. A genome copy number standard curve was created using tenfold serial dilutions of the NCTC 10885 boiled strain. The DNA concentration was quantified using the using the Quantifluor fluorimeter and ONE dsDNA reagents (Promega, UK) and genome copy numbers were calculated using the V. parahaemolyticus RIMD 2210633 reference genome length (5.17 Mbp) from the GenBank database.