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ScienceDirect
Int. J. Hyg. Environ. Health 211 (2008) 518–523
International Journal of Hygiene and Environmental Health
www.elsevier.de/ijheh

Efficiency of real-time polymerase chain reaction assay to detect *Vibrio vulnificus* in seawater

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Received 11 April 2007; received in revised form 17 September 2007; accepted 19 October 2007

Abstract

The growth of *Vibrio vulnificus* in an enriched culture of seawater during the summer in Japan was monitored by a plating technique used as the culture method and a real-time polymerase chain reaction (PCR) assay as the molecular method. *V. vulnificus* was detected by the real-time PCR assay in the samples of August and September but not by the culture method. *Vibrio parahaemolyticus*, however, was detected among all of the samples with both the culture method and real-time PCR assay. In the analysis of the bacterial populations in enrichment culture, it was demonstrated that the growth of *V. vulnificus* on agar media was inhibited by the rapid growth of *V. parahaemolyticus* after 4 h of incubation and the 100 times larger initial populations of bacteria other than *V. vulnificus* and *V. parahaemolyticus*. These findings demonstrate that *V. vulnificus* detection by culture methods is a failure, and molecular methods are effective and detect *V. vulnificus* accurately.
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Keywords: *Vibrio vulnificus*; Real-time PCR; Detection

Introduction

Vibrio vulnificus is widely distributed in coastal and estuarine waters throughout the world and infections are reported in many countries (DePaola et al., 1994; Oliver et al., 1983; Hoi et al., 1998). Seafood was found to be related to approximately 40 fatal cases of *V. vulnificus* per year in the United States (Hlady and Klontz, 1996). In a recent surveillance of emergency medicine physicians in Japan, 425 cases of *V. vulnificus* sepsis were estimated per year (Osaka et al., 2004). Oishi et al. (2006) studied the epidemiological and clinical characteristics of *V. vulnificus* infections reported in Japan from 1975 to 2005. They identified 185 cases using medical article search engines. Approximately 40% of the cases were reported in four prefectures around the Ariake Sea in Kyushu, which is in the southern part of Japan. Therefore, we have been trying to detect *V. vulnificus* from the seawater by culture methods and real-time polymerase chain reaction (PCR) assay. However, we noticed that *V. vulnificus* was not detected in seawater using a technique of enrichment followed by

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doi:10.1016/j.ijheh.2007.10.001

culture methods, but was detected by real-time PCR assay. Subsequently we have monitored the growth during enrichment by culture methods, but by a real-time PCR assay.

In this study, we sorted the bacteria in seawater samples into three classifications, *V. vulnificus*, *Vibrio parahaemolyticus* and other bacteria, and enumerated the bacteria numbers by using a culture method and real-time PCR assay (Lyon, 2000).

To detect or identify *Vibrio* species, several genes that reflect the phylogenetic relationship, such as the ToxR gene (*toxR*), are commonly used. The *toxR*, which codes for a trans-membrane DNA-binding regulatory protein, is present on the ancestral chromosome (Provenzano et al., 2000). However, a partial sequence of *toxR* is different among *Vibrio* species. The different sequence of *toxR* for each *Vibrio* species has been used as an effective marker for the identification of *V. parahaemolyticus* (Kim et al., 1999) and *V. vulnificus* (Takahashi et al., 2005a, b). In this study, real-time PCR assays for *V. vulnificus* (Takahashi et al., 2005a, b) and *V. parahaemolyticus* (Takahashi et al., 2005a, b) were used.

Because 9–15% of the *V. vulnificus* strains can ferment sucrose and form yellow colonies (Tamplin et al., 1982; Cerdà-Cuéllar et al., 2001) and the rest do not ferment sucrose but rather form green colonies on thiosulfate citrate bile salt agar (TCBS) medium, it is impossible to distinguish *V. vulnificus*. Recently, chromogenic agar media such as CHROMagar Vibrio (CV; CHROMagar, Paris, France) (Hara-Kudo et al., 2001) medium has been utilized for isolating *V. parahaemolyticus*. This medium is also able to distinguish *V. vulnificus* from other bacteria by forming a different colony color. In an elementary study using CV, TCBS and modified cellobiose-polymyxin B-colistin agar media for isolation of *V. vulnificus* from seafood, CV medium was better than the other media. Thus we used it to isolate *V. vulnificus* in this study.

Materials and methods

Bacterial strains

V. vulnificus (VV16; from short-neck clam) and *V. parahaemolyticus* (VP15; from scallop) were used in this study. The strains were incubated in APW at 35 °C for 8 h to use for quantification in TaqMan PCR.

Seawater samples

A total of eight seawater samples were obtained from Kumamoto and Shizuoka prefectures from July to September 2006 (Table 1). They were packed in polyethylene bottles, kept in a styrene foam box, and transferred to our laboratory at a room temperature. Culture was started within 2 days.

Culture

A portion (10 ml) of seawater sample was added to 90 ml of APW pre-warmed at 35 °C, and incubated at 35 °C for 0, 2, 4, 6, 8, 10, 14, 18 and 24 h. To quantify the number of bacteria of each time, the culture fluid was 10-fold diluted with phosphate buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 2% NaCl. Each dilution (0.1 ml) was plated onto CV agar. The plate was incubated at 35 °C for 22 h. Blue and purple colonies were suspected as *V. vulnificus* and *V. parahaemolyticus*, respectively. To identify each of the bacteria species, the PCR assay targeting for *toxR*, was carried out described below. Blue colonies and purple colonies on the CV agar plate were inoculated into triple sugar iron agar medium (OXOID Ltd., Basingstoke,Hampshire, UK), nutrient broth (Becton Dickinson, Sparks, MD), and nutrient broth supplemented with 3% and 8% NaCl.

Table 1. Seawater samples used in this study

Sample no.	Sampling date	Water temperature (°C)	Salt concentration (‰)	Detection of the *V. vulnificus* growth	
				Culture method	Real-time PCR assay
1	28 Jul.	30.0	13	+	+
2	4 Aug.	25.2	28	–	+
3	26 Aug.	26.4	12	–	+
4	30 Aug.	29.5	22	–	+
5	5 Sep.	28.0	25	–	+
6	12 Sep.	26.5	26	–	+
7	19 Sep.	25.5	25	–	+
8	26 Sep.	24.5	28	–	+

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