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Molecular and Cellular Probes 21 (2007) 312–315
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Short communication

Evaluation of an alkaline phosphatase-labeled oligonucleotide probe for detection and enumeration of *vibrio* spp. from shrimp hatchery environment

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Received 29 August 2006; accepted 8 March 2007
Available online 14 March 2007

Abstract

An alkaline phosphatase (AP)-labeled genus-specific oligonucleotide probe was developed to detect and enumerate vibrios in shrimp larvae and their surrounding environment. The probe was evaluated using 35 laboratory isolates of *Vibrio* species and 29 isolates of non-vibrio species. The probe was specific for the *Vibrio* species and no cross reaction was found with the non-vibrios included in the study. The total *Vibrio* counts obtained by plating on thiosulfate-citrate-bile salts-sucrose agar (TCBS) and using oligonucleotide probe were compared. Total *Vibrio* counts obtained by probe were comparatively higher than the counts obtained by plating on TCBS agar. The difference between the counts obtained by the probe and by plating on TCBS agar ranged from 2 to 21 times. The study reveals that the use of a non-selective medium such as T_1N_3 agar followed by detection using a genus-specific probe would help to precisely enumerate the total *Vibrio* load in the aquaculture environments.
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Keywords: Oligonucleotide probe; *Vibrio* spp.; Rapid detection

1. Introduction

The members of the genus *Vibrio* are widespread in the estuarine-marine environments and constitute the natural microbial flora of aquatic animals. Some *Vibrio* spp. are also considered important pathogens of aquatic invertebrates such as crustaceans and mollusks. Luminous *Vibrio* species are known to cause serious production loss in shrimp hatcheries and farms particularly in postlarvae and juvenile populations [1–4]. The *Vibrios* that are known to affect shrimp aquaculture are *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. fluvialis* and *V. anguillarum* [5–8]. Occurrence of shrimp mortalities in a shrimp farm are often accompanied by the presence of high numbers of *Vibrios* in the shrimp as well as in its surrounding environment. Hence the knowledge of total *Vibrio* population in aquaculture systems could help to predict the possibility of disease outbreaks.

Traditionally, estimation of total *Vibrio* count in the shrimp and the rearing water has been done by plating an aliquot of sample on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Some species of *Vibrio* grow poorly on TCBS agar and the medium has been found to be inhibitory at different levels to different *Vibrio* species [9,10]. Several researchers have reported that recovery of *Vibrios* on TCBS was low when compared to non-selective media like trypticase soya agar [11]. Therefore, alternative methods are required to estimate the levels of *Vibrio* spp. in shrimp aquaculture environments. We considered the possibility of using genus-specific oligonucleotide probe for enumeration of *Vibrios* by colony hybridization after plating on non-selective medium. Several investigators have recently described the use of sequence of *gyr*B gene, which encodes the subunit B protein of DNA gyrase for discrimination between closely related bacteria [12,13]. We compared the sequences of *gyr*B genes of *Vibrio* spp. available in Gen

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doi:10.1016/j.mcp.2007.03.003

Bank and identified a region that is conserved in all *Vibrio* spp. An oligonucleotide based on this sequence was synthesized, labeled with alkaline phosphatase (AP) and evaluated in this study.

2. Materials and methods

V. parahaemolyticus (AQ 4037), *V. vulnificus* (ATCC 27562), *V. cholerae* (ATCC 39315) and *V. harveyi* (laboratory isolate) were used as positive controls; *Aeromonas hydrophila* (laboratory isolate), *Pseudomonas* spp. (laboratory isolate), *Bacillus* spp. (laboratory isolate), *Moraxella* spp. (laboratory isolate), *Acinetobacter* spp. (laboratory isolate) and *Salmonella enterica enterica* serovar Typhi (ATCC 12225) were used as negative controls. Other cultures used in this study were isolated from environmental samples such as shrimp farm water, sediment, estuarine water, cultured shrimp and shrimp larvae and identified using a battery of biochemical tests [14]. All the cultures were maintained as glycerol stock at −80 °C. The cultures were retrieved from −80 °C stock and maintained on agar slants during this study. All the Vibrio cultures were maintained on T_1N_1 agar slants (1% tryptone, 1% NaCl and 2% agar) at room temperature. Non-vibrio species were maintained on tryptone soya agar (TSA) slants at room temperature. A list of the isolates used in this study is given in Table 1.

Table 1
Bacterial strains used for determining specificity of the probe

Name of organism	Number of strains	Source
Vibrio parahaemolyticus	02	SW
	03	SS
Vibrio vulnificus	03	SW
	02	CS
Vibrio cholerae 01	01	Seawater
Vibrio cholerae non-01	03	SW
Vibrio harveyi	04	Shrimp larvae
	03	SW
Vibrio alginolyticus	02	SW
	02	SS
Vibrio fischeri	03	SW
Vibrio mimicus	01	SW
Vibrio pelagicus	01	SS
Vibrio orientalis	01	SW
Vibrio cincinnatiensis	01	SS
Vibrio hollisae	01	SW
Vibrio fluvialis	01	SS
Vibrio anguillarum	01	SW
Salmonella Typhi (ATCC 12225)	01	Unknown
Pseudomonas spp.	04	EW
Bacillus spp.	03	EW
Escherichia coli	03	CS
Aeromonas hydrophila	03	Fish
Moraxella spp.	05	CS
Acinetobacter spp.	05	CS
Moritella marina	02	SS
Photobacterium damselae	03	SW

SW: shrimp farm water; SS: shrimp farm sediment; CS: cultured shrimp; EW: estuarine water.

A 32-mer oligonucleotide sequence (TGTCAGGAA-AAAGATCCTGCACTGTCTGAACT) that is unique to the gyrase B sub unit (*gyr*B) of *Vibrio* spp. was synthesized and labeled with the AP enzyme by DNA technology A/S (Aarhus, Denmark). The probe was evaluated using 35 isolates of different *Vibrio* species and 29 isolates of non-vibrio species. All the isolates were spot inoculated on T_1N_1 agar plates and incubated at 37 °C overnight. Colony lifts were performed onto Whatman No. 541 filter papers and colony hybridization was carried out as described in the FDA Bacteriological Analytical Manual [14], except that hybridization and washing temperatures were varied with the optimal temperature being 56 °C.

Twenty samples which included 12 shrimp larval samples, 4 water samples and 4 sediment samples were collected from the aquaculture ponds in Kundapura and Udupi district on the south west coast of India during the period from March 2006 to April 2006. The samples were processed within 2 h of collection. Shrimp larvae (10–15) were homogenized and 1:1, 1:10 and 1:100 dilutions of the homogenate and sediment samples were prepared in physiological saline. Water samples were diluted 1:10 and 1:100 in physiological saline. In the case of shrimp larvae and sediment samples, a 0.2 g of the 1:1 dilution and 100 µl of the 1:10 and 1:100 dilutions were surface spread in parallel on TCBS agar plates and T_1N_3 agar plates (1% tryptone, 3% NaCl, 2% agar, pH 7.4) in duplicate. In the case of water samples, 100 µl each of undiluted sample as well as 1:10 and 1:100 dilutions were surface spread in duplicate. All the plates were incubated at 37 °C overnight. Total *Vibrio* counts were obtained by counting green and yellow colonies on TCBS agar. Colony lifts were performed on T_1N_3 agar plates with 100–1000 colonies onto Whatman No. 541 filter paper. Colony hybridization was carried out as described in the FDA Bacteriological Analytical Manual [14], except that in the present study, hybridization and washing were performed at an optimal temperature of 56 °C. The total *Vibrio* counts obtained by direct enumeration on TCBS agar and by using AP-labeled oligonucleotide probe were compared. Further, to assess the specificity of the probe at least 20 colonies that reacted with the probe were recovered from each sample and subjected to a battery of biochemical tests such as oxidase test, production of acid from glucose fermentation in Kligler iron agar (KIA), sensitivity to vibrio static agent (150 µg) and the capability of growth at 3% NaCl for the identification of isolates upto genus level [15,16]. All the biochemical tests were performed strictly as per the protocol described by Ottaviani et al. [16]. Over 400 colonies reacting with the probe were identified by biochemical reactions.

3. Results and discussion

The probe was evaluated using 35 isolates of different *Vibrio* species and 29 isolates of non-vibrio species. The probe reacted only with the *Vibrio* spp and none of the non-vibrio species such as *A. hydrophila*, *Pseudomonas*

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