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APPLICATION OF A RAPID METHOD FOR DETECTING *VIBRIO PARAHAEMOLYTICUS* IN SEAFOOD

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ABSTRACT

A double-layer agar plate (DLAP) method was used to detect *Vibrio parahaemolyticus* in culture broth and seafood samples, which were added with certain amount of *V. parahaemolyticus* and then subjected to freezing treatment. The results of DLAP, which were compared with the most probable number (MPN) method and direct-plating methods such as Bio-chrome Vibrio medium (BCVM) and thiosulfate–citrate–bile salts–sucrose agar (TCBS), showed that they were not significantly different ($P > 0.05$) from each other under the normal experimental conditions. However, if the bacterium was inactive or injured, the results of DLAP were as effective as MPN and more accurate than those of BCVM and TCBS. Furthermore, DLAP showed the total agreement compared with the MPN method by examining the presence of *V. parahaemolyticus* in naturally contaminated seafood samples. Because of its sensitivity and exactness, DLAP offers a broad application for fast screening and detecting of *V. parahaemolyticus* in seafood.

PRACTICAL APPLICATIONS

Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *Vibrio parahaemolyticus* may lead to the development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever. Now, this bacterium is recognized as an important seafood-borne pathogen throughout the world. Usually, 5 to 7 days are required for *V. parahaemolyticus* detection by the most commonly used procedure – the most probable number method, which is very time-consuming and labor-intensive. Some time-saving alternatives such as polymerase chain reaction or DNA methods are not economically appealing. The considerations demand for a rapid method for *V. parahaemolyticus* detection that is at least as sensitive and accurate as the methods used currently. A double-layer agar plate method described here can satisfy these criteria and can be used for rapid detection of *V. parahaemolyticus* in artificially and naturally contaminated samples in laboratory testing.

INTRODUCTION

Vibrio parahaemolyticus, a halophilic inhabitant of estuarine and coastal environments in temperate zones, has been isolated from many kinds of seafood and salted food, particularly in filter-feeding molluscan shellfish. The microorganism was first identified as a foodborne pathogen in Japan in the 1950s (Fujino *et al.* 1953; U.S. Food and Drug Administration (FDA) 2005) and is mainly responsible for acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low-grade fever (Sakazaki 2002; Cho *et al.* 2008). Now in China, this pathogen is a common cause of foodborne disease associated with the consumption of raw or insufficiently cooked seafood and included as a key indicator in the national monitoring plan for seafood, especially for shellfish (Wang *et al.* 2010).

The most commonly used procedure for detecting *V. parahaemolyticus* in food is the most probable number

(MPN) method described in Bacteriological Analytical Manual Online (U.S. Food and Drug Administration (FDA) 2004). The main disadvantage of it is that thiosulfate–citrate–bile salts–sucrose agar (TCBS) cannot differentiate *V. parahaemolyticus* from some strains of *Vibrio vulnificus* or *Vibrio mimicus* (Su and Liu 2007), and the presumptive positive colonies formed on TCBS need to be confirmed by biochemical identification tests. Results of MPN method may be available for 5–7 days (Di Pinto *et al.* 2008), which is very time-consuming and labor-intensive. To overcome the above disadvantages, some procedures for the specific detection of total or virulent *V. parahaemolyticus* in food have been reported, such as polymerase chain reaction (PCR) (Wang and Levin 2004; Ward and Bej 2006; Rosec *et al.* 2009) and DNA–DNA hybridization methods (Gooch *et al.* 2001; Banerjee *et al.* 2002) and loop-mediated isothermal amplification assays (Zhao *et al.* 2010). The above methods greatly reduced the time and efforts required for sample analysis, but cannot be widely applied in the field of monitoring *V. parahaemolyticus* in seafood because most of them require skilled persons and expensive apparatus and materials to conduct the tests.

Recently, Bio-chrome Vibrio medium (BCVM) was developed for selective and specific detection of *V. parahaemolyticus* (Abad *et al.* 2009). Like TCBS, BCVM is also a chromogenic medium, which can differentiate *V. parahaemolyticus* from other *Vibrio* strains with the formation of unique purple colonies on it (Su *et al.* 2005). A study comparing BCVM with TCBS in detecting *V. parahaemolyticus* in 296 oysters and environmental samples showed that the selectivity and specificities of BCVM and TCBS for *V. parahaemolyticus* were 94 and 77%, respectively (Duan and Su 2005). BCVM is more specific and accurate than TCBS. Subsequently, some researchers (Duan *et al.* 2006) developed a double-layer agar plate (DLAP) based on the thin agar layer (TAL) method (Kang and Fung 2000) for direct enumeration of *V. parahaemolyticus* cells, which was prepared with BCVM bottom layer overlaid with a nonselective medium (tryptone soy agar [TSA] supplemented with 3% NaCl). The study showed that chromogenic medium may not allow heat-injured *V. parahaemolyticus* to grow well on it, but DLAP was as effective as the MPN method.

In this study, we further verified the efficiency of DLAP method as previously described. Through adding certain amount of *V. parahaemolyticus* in cultures and three kinds of seafood samples, and applying freezing treatments to make *V. parahaemolyticus* inactive or injured, the results of DLAP in artificially contaminated samples were compared with the MPN and direct-plating methods on BCVM and TCBS. After verifying the efficiency of DLAP, we applied DLAP for quick screening and testing of *V. parahaemolyticus* in naturally contaminated seafood samples.

MATERIALS AND METHODS

Bacterial Strains and Media

V. parahaemolyticus (laboratory collection strains from Food College of Shanghai Ocean University, numbered *V.p*1515 and *V.p*1516). *Escherichia coli* (BYK00105-01-01), *Listeria monocytogenes* (BYK00089-01-01) and *Salmonella* (BYK000423-01-01) were received from Aquatic Pathogen Collection Centre of Ministry of Agriculture, P. R. China.

TSA, tryptone soy broth (TSB) and TCBS were bought from Shanghai City Disease Prevention and Control Center, P. R. China. BCVM was bought from CHROMagar Biotechnology Company (Shanghai), P. R. China.

Preparations of Experimental Materials

About 10 mL of sterile BCVM was poured into a sterile Petri dish and allowed to solidify at room temperature in a biological safety cabinet. An equal volume of TSA containing 3% NaCl, which had been autoclaved and tempered to about 50C, was then poured into the BCVM layer and allowed to solidify in the biological safety cabinet. It was the double-layer agar plate.

Two strains of *V. parahaemolyticus* were cultured together in TSB containing 3% NaCl. Non-*Vibrio* bacteria that included *E. coli*, *Listeria monocytogenes* and *Salmonella* were cultured in TSB. All cultures were individually enriched at 37C for 18 to 24 h. Further, 10 mL of enrichments of *V. parahaemolyticus*, the bacterial amount of which was detected by direct-plating method on TSA–3% NaCl, was mixed with 10 mL of enrichments of non-*Vibrio* bacteria to produce the original mixed bacterial cultures.

All seafood samples were bought from fish markets in Shanghai, P. R. China. The samples were transported in portable coolers at ambient temperature and were analyzed immediately upon arrival to the laboratory. Twenty-five grams of the edible parts of the samples was collected into sterile cans with 225 mL of alkaline peptone water containing 3% NaCl to obtain a 1:10 dilution and subjected to further homogenization for 60 s. Oyster and prawn and codfish samples, which were confirmed negative for *V. parahaemolyticus* through the conventional method, were selected for adding *V. parahaemolyticus* at an amount of 10^4 colony-forming units (cfu/g) as the original experimental samples.

Evaluation of DLAP for Detecting *V. Parahaemolyticus* in Culture Broth

The capability of DLAP for detecting *V. parahaemolyticus* in bacterial cultures was determined both in ideal experimental conditions and in injured conditions after freezing treatments. The results were compared with MPN and

direct-plating methods on TCBS and BCVM plates. The mixed cultures contained *V. parahaemolyticus* more than 10^6 cfu/mL and were diluted to a suitable concentration to be applied in a 3×3 tube MPN method and surfaced-plated on DLAP, TCBS and BCVM. After that, the cultures were frozen-treated in a refrigerator at −18C for over 24 h. Populations of *V. parahaemolyticus* in the culture broth were determined with MPN and direct-plating methods on DLAP, TCBS and BCVM plates as previously described.

The results of the MPN method were obtained by checking MPN statistical table after a series of identification tests. Growth of *V. parahaemolyticus* on DLAP and BCVM was examined by the formation of purple colonies, while that on TCBS was blue-green colonies after incubation at 37C for 24 h. The results obtained by the MPN and direct-plating methods on DLAP, TCBS and BCVM plates were compared.

Evaluation of DLAP for Detecting *V. Parahaemolyticus* in Artificially Contaminated Seafood Samples

To evaluate the capability of DLAP for detecting *V. parahaemolyticus* in samples, three kinds of seafood samples were prepared, which were artificially added with *V. parahaemolyticus* at an amount of 10^4 cfu/g. Similar to the tests previously described, the above samples were also subjected to the freezing treatments at −18C for over 24 h. The populations of *V. parahaemolyticus* both in untreated and in frozen-treated samples were determined with the MPN and direct-plating methods on DLAP, TCBS and BCVM plates, and the results obtained by different methods in different samples were further analyzed.

Application of DLAP for Detecting *V. Parahaemolyticus* in Naturally Contaminated Seafood Samples

After evaluating the efficiency of DLAP in spiked cultures and samples, we applied DLAP for detecting *V. parahaemolyticus* in naturally contaminated seafood samples compared with the MPN method. Populations of *V. parahaemolyticus* in samples were determined with the MPN and direct-plating method on DLAP. The results and test cycle of the two methods were analyzed and compared.

Statistical Analysis

The results of microbiological tests were transformed into log values for statistical analysis. Comparisons of *V. parahaemolyticus* populations obtained by different methods were conducted using Student's *t*-test at a significance level of $P = 0.05$.

RESULTS AND DISCUSSION

Evaluation of DLAP for Detecting *V. Parahaemolyticus* in Culture Broth

Four methods, MPN and direct plating on DLAP and TCBS and BCVM medium, were conducted to determine the populations of *V. parahaemolyticus* in mixed bacterial cultures under the normal and the frozen-treated conditions. Growth of *V. parahaemolyticus* on different media appeared in unique colony colors, such as purple colonies in the DLAP or BCVM medium and blue-green colonies in the TCBS medium. Only *V. parahaemolyticus* was able to produce purple colonies in DLAP, while *E. coli* produced light green colonies and *L. monocytogenes* and *Salmonella* produced blue and brown colonies, respectively (Duan *et al.* 2006). Growth of non-*Vibrio* bacteria on DLAP did not affect the purple colonies' specificity and characteristics produced by *V. parahaemolyticus*.

Figure 1 shows the determination of *V. parahaemolyticus* in original cultures (Fig. 1A) and in frozen-treated cultures

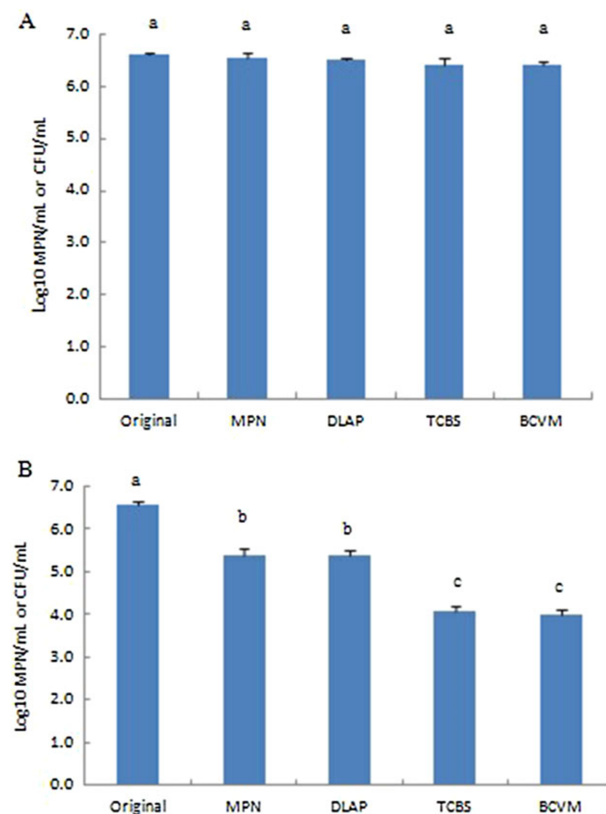

FIG. 1. DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN ORIGINAL CULTURES (A) AND IN FROZEN-TREATED CULTURES (B) BY FOUR METHODS
Data were reported as mean ± SD ($n = 3$). Bars with different letters were significantly different ($P < 0.05$).

TABLE 1. DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN NORMAL AND FROZEN-TREATED BIOLOGICAL SAMPLES

Methods (log_{10} MPN/g or cfu/g)	Oyster		Prawn		Codfish	
	Normal	Frozen-treated	Normal	Frozen-treated	Normal	Frozen-treated
Original	4.70 ± 0.09^a	–	4.70 ± 0.09^a	–	4.70 ± 0.09^a	–
MPN	4.70 ± 0.06^a	3.63 ± 0.05^b	4.73 ± 0.01^a	3.57 ± 0.13^b	4.65 ± 0.02^a	3.04 ± 0.00^b
DLAP	4.69 ± 0.15^a	3.56 ± 0.07^b	4.70 ± 0.03^a	3.53 ± 0.21^b	4.64 ± 0.04^a	3.02 ± 0.03^b
TCBS	4.83 ± 0.11^a	2.75 ± 0.18^c	4.79 ± 0.08^a	2.69 ± 0.10^c	4.78 ± 0.05^a	2.10 ± 0.02^c
BCVM	4.69 ± 0.02^a	2.63 ± 0.24^c	4.69 ± 0.02^a	2.71 ± 0.01^c	4.68 ± 0.01^a	2.14 ± 0.04^c

Data were reported as mean ± SD ($n = 3$). Means with the same letter in the same column were not significantly different ($P > 0.05$).
BCVM, Bio-chrome Vibrio medium; DLAP, double-layer agar plate; MPN, most probable number; TCBS, thiosulfate–citrate–bile salts–sucrose agar.

(Fig. 1B). The added amount of *V. parahaemolyticus* in original cultures was 6.59 log_{10} cfu/mL. The results of *V. parahaemolyticus* obtained by the MPN and direct-plating methods in the cultures were 6.54 log_{10} cfu/mL (MPN), 6.50 log_{10} cfu/mL (DLAP), 6.41 log_{10} CFU/mL (TCBS) and 6.41 log_{10} cfu/mL (BCVM), which were not significantly different ($P > 0.05$) from each other. Thus, under the normal conditions or when the bacterium is in optimum cellular conditions, it is practicable to detect *V. parahaemolyticus* in culture broth by DLAP method.

On the contrary, it is known that *V. parahaemolyticus* may be partly dead or injured upon exposure to high or low temperature (Johnston and Brown 2002; Wong *et al.* 2004). Figure 1B shows that overnight freezing treatment could create large numbers of injured cells and reduce the total *V. parahaemolyticus* populations by more than 1.18 log_{10} cfu/mL. There was no significant difference between the results obtained by MPN (5.39 log_{10} cfu/mL) and DLAP (5.41 log_{10} cfu/mL) methods, which were better than those of direct plating on TCBS (4.09 log_{10} cfu/mL) or BCVM (4.01 log_{10} cfu/mL) medium. The populations of *V. parahaemolyticus* obtained by DLAP were 1.32 and 1.40 log higher than those obtained by TCBS or BCVM medium. This is because the injured or inactive bacteria cannot produce metabolites normally, which reacts with the selective agents of the medium to display the colonies' color. However, DLAP was as effective as MPN method for recovering the cold-injured *V. parahaemolyticus* cells because the top TSA layer of DLAP could provide a favorable environment to injured cells to resuscitate before the selective agents of BCVM diffused to the TSA surface. DLAP had the same effects with MPN and is capable of detecting injured or inactive *V. parahaemolyticus* in culture broth.

Evaluation of DLAP for Detecting *V. Parahaemolyticus* in Artificially Contaminated Seafood Samples

A further experiment was performed to evaluate the capability of DLAP method for detecting *V. parahaemolyticus* in different artificially contaminated seafood samples under the normal and frozen-treated conditions. The three samples were added with *V. parahaemolyticus* at an amount of over 10^4 cfu/g.

Similar to the above results for detecting *V. parahaemolyticus* in cultures, the freezing treatments also reduced the total *V. parahaemolyticus* populations by more than 1.07 log_{10} cfu/g in different seafood samples (Table 1). Similarities in the three samples indicated that results obtained by MPN and DLAP methods had no significant difference ($P > 0.05$) from each other and higher than those obtained by TCBS and BCVM plates. The DLAP method was able to overcome the disadvantages that injured or inactive *V. parahaemolyticus* could not grow directly on a strong inhibitory medium. The results of DLAP, which agreed with those of MPN, showed better stability and repeatability in different samples.

Application of DLAP for Detecting *V. Parahaemolyticus* in Naturally Contaminated Seafood Samples

We applied DLAP for detecting *V. parahaemolyticus* in naturally contaminated seafood samples compared with the MPN method. Table 2 shows the basic agreement of the results of two methods for detecting *V. parahaemolyticus* in different seafood samples. Because the MPN method needed enrichment of *V. parahaemolyticus* before testing and biochemical tests by VITEK automicrobial evaluation instrument for confirmation, test cycle of MPN was longer than that of DLAP by about 24 h. DLAP, which was as sensitive and accurate as the MPN method, could be used as a simple and fast procedure for detecting *V. parahaemolyticus* in seafood samples in different conditions.

CONCLUSIONS

This study demonstrates that DLAP method is specific for fast screening of *V. parahaemolyticus* in culture broth and seafood samples; the results of which are as effective and accurate as the MPN method. DLAP method has the advantages for recovering the injured or inactive

TABLE 2. DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN DIFFERENT SEAFOOD SAMPLES WITH DLAP AND MPN METHODS

Sample name	DLAP		MPN		Significance
	Result ($\log_{10}$ cfu/g)	Test cycle (h)	Result ($\log_{10}$ cfu/g)	Test cycle (h)	(P for t-test)
Mussel	3.56 ± 0.07	60	3.63 ± 0.05	84	$P > 0.05$
Ruditapes philippinarum	2.10 ± 0.02	60	2.18 ± 0.04	84	$P > 0.05$
Scapharca subcrenata	Undetected	60	Undetected	84	–
Litopenaeus vannamei	Undetected	60	Undetected	84	–
Portunus trituberculatus	Undetected	60	Undetected	84	–
Sea bass	Undetected	60	Undetected	84	–
Salmon	Undetected	60	Undetected	84	–
Octopus	Undetected	60	Undetected	84	–

Data were reported as mean ± SD ($n = 3$).
DLAP, double-layer agar plate; MPN, most probable number.

V. parahaemolyticus cells and can also report the real number of the bacterium in different samples, which is easier to operate with no additional skills and is more time-saving than the MPN method. DLAP method showed better stability and repeatability and can be used as an alternative procedure for the MPN method for wide applications in laboratory testing of *V. parahaemolyticus* in seafood.

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