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Comparison of Different Methods for Isolation of Bacterial DNA from Retail Oyster Tissues

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Abstract

Oysters are filter feeders that bioaccumulate bacteria in water while feeding. To evaluate the bacterial genomic DNA extracted from retail oyster tissues, including the gills and digestive glands, four isolation methods were used. Genomic DNA extraction was performed using the Allmag™ Blood Genomic DNA (Allrun, Shanghai, China), the MiniBEST Bacterial Genomic DNA Extraction kits (Takara, Dalian, China), and the phenol-chloroform and boiling lysis methods. The concentration of the genomic DNA was measured using a spectrophotometer. The purity of the genomic DNA was evaluated by PCR amplification of 16S rDNA followed by determining the cloning efficiency of the amplicon into the pMD19-T vector. Furthermore, the bacterial DNA quality was also evaluated by PCR assays using a pair of species-specific primers for *Vibrio parahaemolyticus*. Our results showed that the two commercial kits produced the highest purity of DNA, but with the lowest yields. The phenol-chloroform method produced the highest yield although it was time-consuming. The boiling lysis method was simple and cost effective; however, it was only suitable to isolate genomic DNA from bacteria present in retail samples following an enrichment step. The two commercial kits were good candidates for genomic DNA extraction from retail oyster tissues without enrichment.

Keywords: Extraction methods; Bacterial DNA isolation and evaluation; Oyster; Molecular assay

Introduction

It is well known that oysters filter large volumes of water during feeding and are able to bio-accumulate bacteria including food-borne pathogens from the surrounding water [1,2], therefore, oysters may be an important vehicle for dissemination of food-borne pathogens [3-6]. In the coastal cities of China, more than 60% of seafood tested positive for bacterial pathogens [7,8]. The fact that pathogens could be maintained in oysters for at least one month [9] imposes a huge risk for human health [3,4]. *Vibrio* spp. represented the predominant species among the bacteria in oysters [2,10,11].

Detection and identification of pathogens in oysters have relied on culture-based methods, which are time-consuming and labor intensive [12,13]. To improve efficiency, more rapid assays were developed for detection of these pathogens in oysters using molecular approaches [7,14-16] and immunoassays [17,18]. The molecular assays such as PCR have been used widely because of their high sensitivity, speed, and convenience [7,19,20]. Efficient amounts of sample DNA of high quality is of critical importance for molecular assays. So far, different methods have been used to extract the DNA samples, including commercial kits [19,21,22], the phenol-chloroform method [15,23] and the boiling lysis method [10,24,25].

Most molecular assays have focused on the detection of bacterial pathogens in oysters after enrichment, rather than direct detection [7,26,27]. It was reported that *V. parahaemolyticus* could be detected without enrichment, but no further data were reported [20,24]. Generally, seafood samples are subjected to enrichment overnight to allow growth of pathogenic bacteria [19,28-30] prior to detection assays. The oyster is very different from common agricultural produce due to its bio-accumulating nature. The densities of *V. parahaemolyticus* in oysters were > $10\text{-}10^4$ CFU/g in oysters [24]. Over 107 CFU of aerobic bacteria per gram were found in retail oysters after 1 week storage at 4°C [31]. Furthermore, it has been reported that the bacteria were mainly accumulated in digestive glands and gills [2,6,10,11]. Therefore, more bacteria should be accumulated in those tissues compared to other types of oyster tissues. There is very little information about the detection of food-borne pathogens in oysters without enrichment. To address this issue, four methods were compared for bacterial DNA isolation from retail oyster tissues for detection of bacteria. The quality of the DNA was evaluated to determine a good candidate method for DNA extraction.

Materials and Methods

Oyster samples

Field oysters (n=50) were collected randomly from the Jiangyang market in Shanghai in May 2011. Five field oysters (n=5) were collected randomly and opened with sterilized knives as previously described [2,10]. Oyster tissues (~150 grams) were homogenized using a sterile grinder at 12000 rpm for 60 sec with 600 ml (1:4) of sterile physiological saline. In a separate procedure, five oysters were dissected and specific tissues, including digestive glands and gills, were collected (3.0 g) and grounded with sterile tissue grinders as described previously [2,5].

Samples were centrifuged at 300 g for 3 min at 4°C. The supernatants (0.1 mL) were plated onto ChromAgar™ *Vibrio* plates after making 10-fold serial dilutions. The plates were incubated at 37°C for 16 h. In addition, 25 mL of supernatant from oysters were added into 225

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mL tryptic soy broth (TSB) (Sparks, MD, USA) medium for sample enrichment, and incubated at 37°C for 10 h with shaking at 160 rpm. Samples were taken every two hours and plated onto ChromAgar™ *Vibrio* plates. After incubation at 37°C overnight, the violet colonies were enumerated. All of the supernatants (0.5 mL) were stored in 20% sterile glycerol at -80°C until further use.

Genomic DNA extraction

Pre-treated supernatants (0.2 mL) were centrifuged at 10,000 g for 5 min at 4°C, including supernatants from whole oysters and isolated tissues, and sample enrichment. The pellets were washed twice with sterile physiological saline and resuspended in 0.1 mL sterile physiological saline. Genomic DNA was extracted by four separate methods as follows: 1) Commercial kits: the extraction protocols of the Allmag™ Blood Genomic DNA kit (Allrun, Shanghai, China) and the MiniBEST Bacterial Genomic DNA Extraction kit (Takara, Dalian, China) were performed in accordance to the manufacturer's instructions; 2) Phenol-chloroform method: extraction was performed as previously described [32]; 3) Boiling lysis method – extraction was performed as previously described [10]. The total DNA was eluted or diluted with 0.1 mL sterile water. The concentration of extracted DNA was measured using the Nanodrop 2000C (Thermo Scientific, DE, USA) using sterile physiological saline as the blanking solution. The quality of DNA was evaluated by OD260/OD280 ratios (>1.8). In addition, the DNA samples were also visualized on 0.8% agarose gel. All bacterial DNA was stored at -80°C until further use.

Amplification of 16S rDNA genes and cloning of PCR products

To evaluate the quality of the DNA extracted by different methods, 16S rDNA genes were amplified by Polymerase Chain Reaction (PCR) in a PTC-200 thermocycler (MJ research, CA, USA). The bacterial DNA from different tissues and enrichment samples was amplified using the 27F and 1492R primers [33]. The PCR mixture consisted of 1.0 µl of template, 2.0 U TaqE (Fermentas, Vilnius, Lithuania), 5.0 µl PCR buffer (with Mg^{2+}), 1.0 µL of 10.0 mmol/L dNTPs, 1.0 µL of 10.0 µmol/L for each primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-GGTTACCTTG TTACGACTT-3') [33] and deionized water for a total reaction volume of 50.0 µl. The cycling conditions were as follows: initial denaturation at 94°C for 5 min; 35 cycles consisting of template denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 2 min; and then a final extension at 72°C for 15 min. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. All PCR reactions were performed three times.

The PCR products were recovered using the Gel DNA Recovery kit (Takara, Dalian, China) and cloned into the pMD19-T vector (Takara, Dalian, China) according to the manufacturer's instructions. The ligation products were transformed into JM109 competent cells, which were then plated onto Luria-Bertani (LB, OXIOD, Ltd, England) agar plates with 50 µg/mL ampicillin (Sigma, MO, USA), X-gal and isopropyl β-D-1-thiogalactopyranoside. The white colonies were enumerated after incubation at 37°C for 16 h.

Detection of *V. parahaemolyticus* by PCR

To confirm that the genomic DNA from oyster tissues could be used to detect foodborne pathogens, a species-specific gene (irgB) of *V. parahaemolyticus* was used as the target for the PCR as previously described [15].

Statistical analysis

Each experiment was repeated three times (N=3) with triplicates in each experiment (n=3). One way analysis of variance (ANOVA) or Tukey test was used for statistical analysis by Origin 8.0 (OriginLab, MA, USA).

Results and Discussion

This study evaluated extraction of bacterial genomic DNA from oyster tissues by different methods. Our results indicated that the boiling lysis method produced the highest concentration of DNA from oyster samples compared with the other three methods (Table 1). The concentration of DNA was highest from digestive glands regardless of the method used (Table 1). The DNA yields were lower with commercial kits compared to the other two methods. There was no significant difference ($P>0.05$) in the recovery of DNA between the two kits whereas significant differences were found between the other methods (Table 1). There was a significant difference between the quantities of DNA recovered from the gills and other tissues. In addition, there was no significant difference ($p>0.05$) between the quantities of DNA recovered from isolated digestive glands and the whole oyster.

After extraction by the various methods, the DNA was amplified by PCR using 16S rDNA universal primers that were designed specifically for bacterial detection [33]. The PCR products were cloned into the pMD19-T vector, and the numbers of white colonies on the selective plates were enumerated. Although DNA from oyster tissues extracted by the boiling lysis method has the highest yield, it could not be amplified by the primers specific for bacterial 16S rDNA (data not shown). This indicates that the DNA from samples without enrichment did not contain enough bacterial DNA to be amplified by PCR. Alternatively, the DNA samples may have contained some PCR inhibitors that may have blocked PCR amplification. The DNA samples produced by the MiniBEST Bacterial Genomic DNA Extraction kit resulted in the highest number of transformed clones (Table 2). There were significant differences in the number of transformed colonies ($P<0.05$) among all methods and all tissues (Table 2), but no significant difference ($P>0.05$) was found among all methods and all tissues after enrichment. Furthermore, the results indicated that both the quantity and quality of DNA extracted from digestive glands was the highest amongst the tissues tested.

Our previous work showed that *Vibrio* spp. was found to be predominant in oysters [11]. All genomic DNA extracted by different methods from different tissues was further evaluated by PCR using primers for the species-specific gene (irgB) of *V. parahaemolyticus* [15]. Genomic DNA purified by the two commercial kits produced positive results for PCR without enrichment (data not shown). The presence of *V. parahaemolyticus* in oysters was also confirmed by plating onto ChromAgar™ *Vibrio* plates. After enrichment, *V. parahaemolyticus* was detected in all samples by PCR, regardless of the DNA extraction

Method	Gills (µg/sample)	Digestive glands (µg/sample)	Oyster (µg/sample)
Allmag™ Blood Genomic DNA kit	55.4 ± 4.3^{aA}	65.6 ± 2.2^{bA}	60.0 ± 2.9^{abA}
MiniBEST Bacterial Genomic DNA Extraction kit	57.3 ± 2.2^{A}	63.5 ± 2.5^{A}	57.5 ± 3.4^{A}
Phenol-chloroform method	132.9 ± 6.2^{aB}	154.3 ± 8.2^{bB}	143.9 ± 7.0^{abB}
Boiling lysis method	187.7 ± 10.7^{aC}	267.8 ± 10.8^{bC}	271.4 ± 8.8^{bC}

$^{a-c}$: statistical analysis on rows; $^{A-C}$: statistical analysis on column

Table 1: Yields of DNA from oyster tissues by four different isolation methods.

Method	Gills (clone number)	Digestive glands (clone number)	Oyster (clone number)	Enrichment (clone number)
Allmag™ Blood Genomic DNA kit	196 ± 7^{aA}	238 ± 7^{bA}	146 ± 13^{cA}	1633 ± 40^{dA}
MiniBEST Bacterial Genomic DNA Extraction kit	234 ± 13^{aB}	265 ± 11^{bB}	135 ± 6^{cA}	1636 ± 38^{dA}
Phenol-chloroform method	63 ± 7^{aC}	82 ± 6^{bC}	84 ± 6^{bB}	1623 ± 45^{cA}
Boiling lysis method	0^{aD}	0^{aD}	0^{aC}	1660 ± 36^{bA}

$^{a-d}$: statistical analysis on rows; $^{A-D}$: statistical analysis on columns.

Table 2: Numbers of colonies from PCR cloning.

Methods	Time (h)	Cost ($)
Allmag™ Blood Genomic DNA kit	0.5	1.0
MiniBEST Bacterial Genomic DNA Extraction kit	2.0	1.9
Phenol-chloroform method	4.0	<0.5
Boiling lysis method	0.15	<0.17

Table 3: Time and cost comparisons among four DNA isolation methods.

methods used. Our results indicated that the pathogen could be detected in retail oysters with enrichment. Thus, the four methods could be used to recover the bacterial DNA for detection by the PCR after enrichment.

The boiling lysis method has been used extensively in food pathogen detection and clinical diagnosis due to its high yield of DNA, convenience, and low cost [7,10,34]. Our results suggest that the method was not suitable for molecular detection if the retail oyster samples were not subjected to enrichment (Table 2). It has been reported that only 6.1% of the samples were positive for *V. parahaemolyticus* without enrichment [24]. In the absence of any treatment, PCR inhibitors may be present in the oyster, which interfere with PCR amplification [19,35]. Many investigators have developed protocols to reduce the presence of PCR inhibitors for testing for the safety of seafood [22,36-38]. However, the boiling method is simple and efficient, and still remains a good candidate for extraction of bacterial DNA with sample enrichment. Another major problem with detecting pathogenic bacteria in shellfish tissue without enrichment is the high level of shellfish DNase present in the tissue which must be removed before cell lysis. These problems have been previously addressed [39,40].

The phenol-chloroform method is simple and has been widely used for several decades. The yield is generally higher than the absorption-based kits (Table 1); however the results are often not reproducible, and the procedure involves the use of several toxic reagents. For all of the four methods, the cost and working time were evaluated (Table 3). The boiling lysis method was relatively fast and cost effective. In contrast, the MiniBEST Bacterial Genomic DNA Extraction kit method was relatively time-consuming. Although the commercial kits were more expensive relative to the other methods, they offer superior DNA quality. Our results show that the MiniBEST Bacterial Genomic DNA Extraction kit took almost 4 hours to perform, while the Allmag™ Blood Genomic DNA kit took only 30 minutes to complete, and required fewer reagents. The Allmag™ Blood Genomic DNA kit, which utilizes magnetic silica in the extraction procedure produced high DNA quality that was suitable for subsequent molecular assays, which is consistent with the previous reports [37,38], and this could be used for extraction of DNA from oyster samples.

Our data show that among DNA yields and cloning efficiencies of PCR products generated from various tissues, the digestive glands gave the highest yields by methods used (Tables 1 and 2), suggesting this tissue is a better candidate for the detection of pathogenic bacteria in oysters. Oysters easily bio-accumulate pathogens in the course of feeding in seawater [5,6,41,42]. The digestive glands could be used as target tissues for food safety monitoring of shellfish [5,6]. These results were consistent with our previous studies [2,5,11,35]. In summary, four DNA isolation methods were compared, and the digestive glands of oysters were also identified as a suitable tissue for pathogen detection. Our work enhances the ability to detect pathogens in seafood and may benefit the seafood industry and consumers.

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