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Quantitative detection of *Plesiomonas shigelloides* in clam and oyster tissue by PCR

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Abstract

A quantitative assay for *Plesiomonas shigelloides* in clams and oysters based on the conventional polymerase chain reaction was developed. The assay involved the treatment of homogenized tissue samples with 4.0% formaldehyde that presumably denatured DNases and proteases present in the tissue which would otherwise inactivate the PCR reaction. The level of detection of *P. shigelloides* in clam tissue without enrichment was 200 CFU/g. The addition of 0.1% bovine serum albumin (BSA) to PCR reactions or the DNA purification system reduced the level of detection to 60 CFU/g. Formaldehyde had no effect on the level of detection with clam tissue. The level of detection of *P. shigelloides* in oyster tissue without enrichment was 6×10^5 CFU/g. The addition of 4.0% formaldehyde to oyster tissue homogenates reduced the level of detection to 6×10^2 CFU/g in contrast to the addition of 0.1% BSA to PCR reactions or the DNA purification system which reduced the level of detection to only 2×10^5 CFU/g. The combination of formaldehyde plus BSA, formaldehyde plus DNA purification, or formaldehyde plus BSA plus DNA purification all gave a detection level of 2×10^2 CFU/g of oyster tissue. With clam tissue, the linear range for detection of *P. shigelloides* was 60 to 2×10^4 CFU/g. With oyster tissue, the linear range for detection of *P. shigelloides* was 2×10^2 to 6×10^4 CFU/g.

Keywords: *Plesiomonas shigelloides*; Quantitative PCR; PCR inhibitors; Clams; Oysters

1. Introduction

Plesiomonas shigelloides has been recognized as a potential human and animal pathogen for more than 50 years. The organism is a Gram-negative, motile, non-spore-forming, facultatively anaerobic and oxidase positive bacterium (Miller and Koburger, 1985). *P. shigelloides* has been isolated from a variety of environmental sources and is distributed worldwide (Cooper and Brown, 1968; Zakhariev, 1971). However, the aquatic environment is the primary reservoir of this bacterium (de Mondino et al., 1995; Aldova et al., 1999). Seafoods, such as salted fish (Hori and Hayashi, 1966), crabs (Claesson et al., 1984), and oysters (Rutala et al., 1982), etc., have been implicated in outbreaks of *P. shigelloides* enteritis.

Traditional techniques for the detection and identification of this pathogen include culture-based methods and biochemical tests, which are time consuming. The polymerase chain reaction (PCR) is a highly sensitive, specific and rapid method for detecting bacteria in pure culture and natural waters (Joshi et al., 1991; Bej et al., 1990). However, when applied to food samples the PCR can be inhibited or its sensitivity reduced severely (Gerard et al., 1996; Hopfer et al., 1995; Verkooyen et al., 1996; Al-Soud et al., 1998; Al-Soud et al., 2000). PCR inhibitors may act through one or more of the following mechanisms: (1) interference with the cell lysis step (2) degradation or capture of the nucleic acids or (3) inactivation of the thermostable DNA polymerase (Wilson, 1997; Al-Soud et al., 1998).

It is often difficult to identify individual PCR-inhibitory substances in complex biological samples, although in some instances, specific PCR inhibitors have been recognized. The components in biological samples that have been identified to be PCR inhibitors are immunoglobulin G, haemoglobin, lactoferrin, and proteases (Al-Soud and Rädström, 1998, 2001; Al-Soud et al., 1998, 2000).

Various techniques have been employed to reduce the effect of PCR inhibitors and/or to separate the target microorganism from the PCR inhibitors. For example, aqueous two phase-systems, boiling, density gradient centrifugation, dilution, DNA extraction methods, enrichment media, filtration, and immunological

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techniques have been used to facilitate the PCR (Kroll, 1993; Lantz et al., 1994). Besides the use of various pre-PCR treatments, the addition of amplification facilitators has been used as well as alternate DNA polymerases more resistant to PCR inhibitors than conventional *Taq* polymerase (Al-Soud and Rädström, 1998, 2000).

There is presently no report concerning the quantitative PCR detection of *P. shigelloides* either in pure culture or in tissue. The purpose of this study was to establish a rapid and efficient procedure for quantitative detection of *P. shigelloides* in clams and oysters by PCR. The method used in this paper was based on a combination of differential centrifugation, addition of formaldehyde and bovine serum albumin (BSA), and purification of DNA using a commercial DNA clean-up system. The effect of nonselective enrichment on the sensitivity of detection of *P. shigelloides* in clam and oyster tissue was also investigated.

2. Material and methods

2.1. Strain of P. shigelloides and culture conditions

The strain of *P. shigelloides* used was ATCC 51572. All media were from Difco (Detroit, MI, USA). The bacterium was stored at 4 °C on slants of Tryptic Soy Agar supplemented with 0.25% glucose, 0.5% NaCl, and 0.5% yeast extract (TSA^+). To obtain exponential phase cultures, one loop of the bacterium was transferred to a tube containing 9 ml of Tryptic Soy Broth similarly supplemented (TSB^+), followed by overnight incubation at 37 °C with rotary agitation (250 rpm). The entire 9 ml culture was then transferred to 100 ml of TSB^+ in a 250 ml flask, followed by incubation for 4 h at 37 °C with rotary agitation at 250 rpm. Five milliliters of this culture was then inoculated into 100 ml of TSB^+ and incubated for 3 h at 37 °C with rotary agitation at 250 rpm. This resulted in mid-log phase cultures having cell densities of $\sim 1.9\times10^9$ CFU/ml, corresponding to an absorbance value at 600 nm in 1 cm path length cuvets of ~3.8. CFU were determined by spread plating 0.1 ml of appropriate decimal dilutions in 1.5% NaCl onto duplicate plates of TSA^+. The cell suspension was then used to seed blended clam and oyster tissue samples for quantitative PCR detection of *P. shigelloides.*

2.2. Preparation of shellfish tissue samples

Three batches of littleneck clams (*Protothaca staminea*) and Canadian oysters (*Crassostrea gigas*) were purchased at different times from local retail sources. Shellfish tissue (100 g) was blended with 900 ml TSB^+ for each experiment. The blended samples were used immediately or kept frozen at −20 °C for later use. Each batch of shellfish was proven to be free of *P. shigelloides* via enrichment followed by PCR as described below.

2.3. Extraction of seeded bacteria from tissue without enrichment

Mid-log exponential phase cell suspensions (0.1 ml) containing 1.2×10^3, 4×10^3, 1.2×10^4, 4×10^4, 1.2×10^5, 4×10^5, 1.2×10^6, 4×10^6,1.2×10^7, or 4×10^7 CFU of *P. shigelloides* were seeded into 200 ml centrifuge bottles containing 100 ml of clam or oyster tissue homogenates. These bottles were then centrifuged at 1000 *g* for 5 min, and 50 ml of each supernatant were removed to a fresh centrifuge bottle and centrifuged at 10,000 *g* for 10 min to pellet bacteria. The pellet at the bottom of the bottle was resuspended in 50 ml of 0.85% NaCl and centrifuged at 10,000 *g* for another 10 min. After discarding the supernatant, the pellet was resuspended in 1 ml 0.85% NaCl and transferred to a 1.5 ml centrifuge tube. The cell suspension was then centrifuged at 10,000 *g* for 10 min. After discarding the supernatant, the pellet was resuspended in deionized water, with vortexing to obtain a volume of 0.5 ml. The cell suspensions were used for lysis as described below or kept frozen at −20 °C. A sample without seeded *P. shigelloides* served as the negative control.

2.4. Extraction of seeded bacteria from tissue following enrichment

Cell suspensions (0.1 ml) containing 4, 12, 40, 120, 400, 1200, 4000, or 12,000 CFU of *P. shigelloides* were seeded into 250 ml flasks containing 100 ml of blended clam or oyster tissue. These flasks were then incubated at 37 °C for 7 h with rotary agitation at 250 rpm for enrichment of *P. shigelloides*. After enrichment, the contents of each flask were removed to 200 ml centrifuge bottles. Each enrichment culture (100 ml) was then centrifuged at 1000 *g* for 5 min. Fifty milliliters of the supernatant in each bottle was removed to a fresh centrifuge bottle and centrifuged at 10,000 *g* for 10 min. The pellet at the bottom of the bottle was resuspended in 50 ml of 0.85% NaCl and centrifuged at 10,000 *g* for 10 min. After removal of the supernatant, the pellet was resuspended in 1 ml of 0.85% NaCl and transferred to a 1.5 ml micro-centrifuge tube. The cell preparation was then centrifuged at 10,000 *g* for 10 min to pellet the cells. After discarding the supernatant, the pellet was resuspended as above. The cell suspensions were lysed as described below. A sample without seeded *P. shigelloides* served as a negative control.

2.5. Formaldehyde treatment

When samples were not enriched, formaldehyde (cat. no. F-79, Fisher Scientific Company, Fair lawn, NJ, USA) was added to 100 ml of blended shellfish tissue samples after seeding of blended samples with *P. shigelloides* and before initiating differential centrifugation as described above. After 7 h of nonselective enrichment, formaldehyde was added to 100 ml of the enrichment cultures before initiating differential centrifugation as described above. The final concentration of formaldehyde added to the samples was 4.0%. After mixing, the samples with formaldehyde were kept at room temperature for 5 min, and then subjected to differential centrifugation as described above.

2.6. DNA template preparation

TZ lysing solution (Abolmaaty et al., 2000) was used for cell lysis. TZ consisted of 2.0% Triton X-100 plus 2.5 mg/ml of