Development of a real-time PCR method for detection and quantification of the parasitic protozoan *Perkinsus olseni*

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**ABSTRACT**

The objective of this study was to develop a real-time PCR method for the rapid detection and quantification of the protozoan pathogen *Perkinsus olseni* using a TaqMan probe. For the standard, genomic DNA was extracted from $10^5$ in vitro-cultured *P. olseni* trophozoites, and then 10-fold serial dilutions to the level of a single cell were prepared. To test the reliability of the technique, triplicates of genomic DNA were extracted from $5 \times 10^4$ cells and 10-fold serial dilutions to the level of 5 cells were prepared. The standards and samples were analyzed in duplicate using an Exicycler™ 96 real-time quantitative thermal block. For quantification, the threshold cycle ($C_T$) values of samples were compared with those obtained from standard dilutions. There was a strong linear relationship between the $C_T$ value and the log concentration of cells in the standard ($r^2 = 0.996$). Detection of DNA at a concentration as low as the equivalent of a single cell showed that the assay was sensitive enough to detect a single cell of *P. olseni*. The estimated number of *P. olseni* cells was similar to the original cell concentrations, indicating the reliability of *P. olseni* quantification by real-time PCR. Accordingly, the designed primers and probe may be used for the rapid detection and quantification of *P. olseni* from clam tissue, environmental water, and sediment samples.

**Key words:** *Perkinsus olseni*, real-time PCR, TaqMan probe, quantification

**Introduction**

*Perkinsus olseni* is a pathogenic parasitic protozoan occurring in the Manila clam (*Ruditapes philippinarum*) and Venus clam (*Protothaca jedoensis*) in Korean waters (Park *et al*., 2005; Park *et al*., 2006a). Park and Choi (2001) reported a higher prevalence and infection intensity in commercial clam beds located on the south and the west coasts of Korea in the early fall season. It has been reported that mass mortality of Manila clams in late summer was associated with extremely high levels of *P. olseni* infection (Park *et al*., 2006b).

The classic method of detection and quantification of *Perkinsus* spp. parasites involves Ray’s Fluid Thioglycollate Medium (RFTM) incubation, subsequent staining with Lugol’s iodine, and laborious microscopic counting (Ray, 1954; Mackin, 1962). Choi *et al.* (1989) described the body burden technique; its modification, described by Ragone Calvo *et al.* (2003), enables detection and quantification of *Perkinsus* spp. More recently, standard PCR methods have enabled more sensitive and specific detection of parasites through species-specific primers (Fong *et al*., 1993; Marsh *et al*., 1995; Hamaguchi *et al*., 1998; Robledo *et al*., 1998; Robledo *et al*., 2002; Coss *et al*., 2001; Casas *et al*., 2004). In addition, the development of PCR enzyme-linked immunosorbent assays (Elandalloussi *et al*., 2004) and immunodetection through flow cytometry (Ragone Calvo *et al*., 2003) have enabled more sensitive and specific detection and enumeration of parasites. However, Bushek *et al.* (2002) highlighted drawbacks associated with immunochemistry, related to the cross-reactivity of polyclonal antibodies with other...
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free-living phototrophic and parasitic dinoflagellates; thus, overestimation may occur when detection is performed using flow cytometric methods.

In general, the aforementioned methods are time consuming and associated with difficulty in the specificity of the target species. To overcome these problems, a real-time PCR technique has been developed as an alternative technique. The principle involved in this real-time PCR method is the detection of an increasing fluorescence signal as the PCR reaction occurs in real time, such that the initial target levels of RNA or DNA can be quantified (see review by Espy et al., 2006). Advancements in this technique have been useful in clinical microbiology, in terms of detecting and quantifying viruses, fungi, parasites, bacteria, and mycobacteria with high sensitivity and specificity, and in relatively less time (Abe et al., 1999; Costa et al., 2001; Blessmann et al., 2002; Drago et al., 2002). Indeed, successful detection and quantification of *Perkinsus marinus* in various substances has been achieved using the real-time PCR technique (Audemard et al., 2004; Audemard et al., 2006; Gauthier et al., 2006).

The objective of this study was to develop a rapid detection and quantification method for *P. olseni* and to test the reliability of the quantification of *P. olseni* with this real-time PCR method using a TaqMan probe.

**Material and Methods**

1. **Cell culture**

   Laboratory maintained, in vitro-cultured trophozoites were obtained for the experiment. Briefly, trophozoites were raised in medium containing Dulbecco’s modified Eagle medium (Sigma) and Ham’s F-12 (Gibco) 1:2, buffered with 5 mM HEPES (Gibco) and 3.5 mM sodium bicarbonate (Sigma), supplemented with 5% fetal bovine serum (Sigma), and fortified with 30 μL/mL penicillin-streptomycin (10,000 units/mL; Gibco), 20 μL/mL gentamicin (1 mg/mL; Sigma), 10 μL/mL nystatin (0.1 g/10 mL; Sigma), and 10 μL/mL chloramphenicol (0.1 g/10 mL; Sigma) to prevent microbial contamination. The culture conditions were 35 psu, pH 7.2-7.4, and 26°C.

2. **Cell enumeration and DNA extraction**

   In vitro-cultured cells were harvested and permeated once via a 23-gauge needle with a 10 mL syringe to segregate cell clumps. The cells were washed with 0.01 M phosphate-buffered saline (PBS; Sigma) 3 times and filtered through a 10 μm sieve to obtain unicelated cells. From the filtered cell suspension, a 10 μL aliquot was obtained, which was diluted to 100 μL with 0.01 M PBS and neutral red staining. The stained parasite cells were counted under a light microscope at 400× magnification with a hemocytometer and adjusted to 100,000 cells. For standard curve construction, genomic DNA from 100,000 *P. olseni* cells was extracted, and the DNA concentration was determined using Tecan NanoQuant Infinite 2000. Then, 10-fold serial dilution down to a 1 cell concentration was conducted. Similarly, triplicates of genomic DNA extracted from 50,000 cells were 10-fold serially diluted down to a 5 cell concentration. DNA from both standards and samples was extracted using a QIAGEN DNeasy Blood & Tissue kit according to the manufacturer’s protocol. Elution was performed 3 times with 100 μL of elution buffer (AE), with 5 min incubation at each time-point.

3. **Oligonucleotide and real-time PCR conditions**

   Novel PK-ITS forward (5′ CAGAATTCGTGAA CCAATCTGAGA-3′) and reverse (5′ TGTGCCTCTTCTT CCGGATA-3′) primers and a TaqMan probe (5′ FAM-TCAACGCATACTGCACAAAGGGGA-3′-BHQ1) were designed and synthesized in the sequence of the 5.8S and internal transcribed spacer 2 ribosomal RNA regions (Accession no: AF473840) using Bioneer modified oligodesign software (Primer3. cgi V.0.6; Daejeon, South Korea). BIO-RP-purified primers and an HPLC-purified TaqMan probe were synthesized by the Bioneer Company (Deajon, South Korea). Gradient PCR was performed from 45°C to 65°C to obtain the optimal annealing temperature. The highest florescence emission and lowest threshold cycle (Ct) value was recorded at 56°C, and this temperature was used for subsequent reactions. For the PCR reaction, AccuPower™ DualStar qPCR
PreMix (Bioneer, Deajon, South Korea) was used with 10 pmol of probe, 10 pmol of each primer, the DNA template, and PCR-grade water for a final volume of 20 L. The standards, samples, and negative control were analyzed in duplicate using an Exicycler™ 96 real-time quantitative thermal block (Bioneer, Deajon, South Korea). The negative control contained no template DNA. The thermal cycle protocol consisted of pre-incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 10s and 56°C for 30s. For quantification, the CT values of samples were compared with those obtained from standard dilutions. The experiment was performed 3 times to measure the intra-reproducibility of the test. The CT values of each standard curve in 3 different experiments were used to determine the coefficient of variation (CV).

Results and Discussion

Two factors contribute to emission of the fluorescence signal during the PCR reaction, namely, SYBR Green chemistry and PCR sequence-specific probe chemistry. Of the real-time methods, the TaqMan probe is a commonly used PCR sequence-specific probe, which anneals to the DNA sequence between primers of the target gene of interest, resulting in cleavage during the extension process due to exogenous activity of the Taq polymerase enzyme while emitting a fluorescence signal (Holland et al., 1991). The advantage of the TaqMan probe is that the probe is designed in the unique gene sequence, which allows sensitive, specific, and accurate determination without non-specific binding, whereas the use of SYBR Green dye is prone to producing false-positive results due to non-specific binding (see review by Espy et al., 2006). The TaqMan probe-based real-time PCR method has been used for the detection and quantification of parasites from shellfish (Gauthier et al., 2006; Faveri et al., 2009; Nagle et al., 2009; Xie et al., 2009) and environmental water samples (Bertrand et al., 2004).

The primer and probe used in the present study targeted the 97 bp region of 5.8S and the ITS-2 region of *P. olseni*, making the probe specific to *Perkinsus* spp. Manila clams in Asian countries are reported to be susceptible to *P. olseni* infection in Korea, Japan, and China and *P. honshuensis* in Japan (Dungan and Reece, 2006; Park et al., 2006a). Regarding the possibility of *P. honshuensis* infection in Korea, Yang et al. (2011) investigated Manila clams from 25 locations along the coast of Korea and reported that *P. honshuensis* was not found in any collected samples, suggesting that perkinsosis in Korean waters is caused solely by *P. olseni*. The specificity of the primer and probe used in the present study was tested using MPX (Manila clam parasite unknown), which is morphologically similar to trophozoites of *P. olseni* in the Manila clam; the test was negative (Fig. 1). In addition, the lack of fluorescence in the negative template control confirmed the absence of contaminations in the PCR reaction mixture. Thus, these findings suggest that the probe developed in the present study is suitable for detecting and quantifying *P. olseni* in Manila clams.
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Table 1. Sensitivity, linearity, and reproducibility of the standard curve

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>DNA concentration</th>
<th>1st (n = 3)</th>
<th>2nd (n = 3)</th>
<th>3rd (n = 3)</th>
<th>Grand mean</th>
<th>Coefficient of variation (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>3 ng</td>
<td>18.95 ± 0.11</td>
<td>18.14 ± 0.18</td>
<td>18.70 ± 0.42</td>
<td>18.60 ± 0.23</td>
<td>1.2</td>
</tr>
<tr>
<td>10,000</td>
<td>300 pg</td>
<td>21.66 ± 0.31</td>
<td>21.11 ± 0.13</td>
<td>21.52 ± 0.01</td>
<td>21.42 ± 0.15</td>
<td>0.7</td>
</tr>
<tr>
<td>1,000</td>
<td>30 pg</td>
<td>24.87 ± 0.17</td>
<td>24.11 ± 0.13</td>
<td>24.60 ± 0.01</td>
<td>24.53 ± 0.10</td>
<td>0.4</td>
</tr>
<tr>
<td>100</td>
<td>3 pg</td>
<td>27.62 ± 0.23</td>
<td>27.50 ± 0.10</td>
<td>27.85 ± 0.35</td>
<td>27.66 ± 0.23</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>300 fg</td>
<td>30.96 ± 0.08</td>
<td>31.01 ± 0.02</td>
<td>30.53 ± 0.68</td>
<td>30.83 ± 0.26</td>
<td>0.8</td>
</tr>
<tr>
<td>1</td>
<td>30 fg</td>
<td>33.01 ± 0.16</td>
<td>33.14 ± 0.00</td>
<td>32.65 ± 0.01</td>
<td>32.93 ± 0.06</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Slope = −1.25 −1.34 −1.24 −1.27
Intercept = 33.38 33.55 33.12 33.35
R² = 0.997 0.996 0.996 0.997

for the detection of *P. olseni* in Korean waters.

Data in Table 1 illustrate the sensitivity, linearity, and reproducibility of standards on 3 different occasions. Sensitivity of the present method was observed at 30 fg of *P. olseni* DNA, which is equivalent to a single cell of the pathogen (Table 1). CV values ranged from 0.1 to 1.2, signifying the reproducibility and stability of the DNA template, enabling repeated testing from 1 set of DNA extraction. Among the experiments using standards, the 2nd experiment was selected for producing the standard curve due to the highest slop value. The standard curve showed a strong linear relationship between CT values and log concentrations of cells, with a square regression coefficient (R²) of 0.996 (Table 1, Fig. 2). The calculated number of cells of individual samples and the mean value produced by plotting against the standard curve were not significantly different from the original cell counts (Table 2). For instance, the average number of cells obtained by real-time PCR was 46,803 ± 5,676, 4,086 ± 824, 438 ± 101, 30 ± 5, and 4.6 ± 5 for original cell counts of 50,000, 5,000, 500, 50, and 5, respectively (Table 2).

For reliable quantification of target molecules from environmental waters using real-time PCR, the removal of PCR inhibitors and efficient recovery of DNA are 2 major concerns. According to Wilson (1997), environmental water samples can potentially contain inhibitors of DNA extraction and PCR amplification. Therefore, Audermard *et al.* (2004) used 2 types of DNA extraction kits manufactured by QIAGEN, namely, the tissue kit and the stool kit. The stool kit is specially designed for extracting DNA while adsorbing inhibitory substances. Audermard *et al.* (2004) reported that the stool kit showed more efficient DNA recovery than the tissue kit, due to the removal of inhibitors in environmental waters. However, they reported that the tissue kit showed more efficient and minimized variations in DNA

![Fig. 2](image-url). A standard curve of the threshold cycle generated for 10-fold serially diluted DNA concentrations extracted from 10⁵ cells.
recovery when triplicate DNA elution was performed. Accordingly, we also conducted triplicate DNA elution and successfully quantified in vitro-cultured *P. olseni* cells in culture media. Although we were able to quantify *P. olseni* successfully using real-time PCR in the present study, the efficiency and reliability of DNA recovery of *P. olseni* in host tissues and the environmental water column or sediment in Korean waters should be examined prior to the widespread application of this technique, because various PCR inhibitors may exist in the Korean marine environment (Wilson, 1997; Frostegard et al., 1999; Watson et al., 2000).

In conclusion, we have developed novel primers and a probe and optimized a technique for the rapid and sensitive detection and quantification of *P. olseni*. This technique may be used to further our understanding of the transmission dynamics of the organism.

**Acknowledgement**

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**References**


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**Table 2. Estimated cell concentrations against the standard curve using real-time PCR**

<table>
<thead>
<tr>
<th>Known # of cells</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>41,554</td>
<td>52,827</td>
<td>46,030</td>
<td>46,803 ± 5,676</td>
</tr>
<tr>
<td>5,000</td>
<td>4,197</td>
<td>4,850</td>
<td>3,212</td>
<td>4,086 ± 824</td>
</tr>
<tr>
<td>500</td>
<td>330</td>
<td>530</td>
<td>455</td>
<td>438 ± 101</td>
</tr>
<tr>
<td>50</td>
<td>31</td>
<td>25</td>
<td>36</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4.6 ± 0.5</td>
</tr>
</tbody>
</table>

P-value: P = 0.31, P = 0.40, P = 0.21
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of *Perkinsus olseni* and *Perkinsus honshuensis* in Manila clam *Ruditapes philippinarum* in Korean waters using molecular markers. The 9th Asian Fisheries and Aquaculture Forum, Book of Abstracts. Shanghai Ocean University, China, p. 79.