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

Dinesh Gajamange, Jong-Man Yoon and Kyung-Il Park

[1]*Department of Aquatic Life Medicine, Kunsan National University, Gunsan 573-701, Republic of Korea*

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 5×10^4 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^{TM} 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

Received: December 14, 2011; Accepted: December 28, 2011
Corresponding author: Kyung-Il Park
Tel: +82 (63) 469-1881 e-mail: kipark@kunsan.ac.kr
1225-3480/24417

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℃.

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 unicleated 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′ CAGAATTCCGTGAA CCAGTAGA-3′) and reverse (5′ TGTCGCTCTTCTT CCCGATA-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℃ to 65℃ to obtain the optimal annealing temperature. The highest florescence emission and lowest threshold cycle (C_T) value was recorded at 56℃, and this temperature was used for subsequent reactions. For the PCR reaction, AccuPower^{TM} 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℃ for 10 min, followed by 40 cycles of 95℃ for 10s and 56℃ for 30s. For quantification, the C_T 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 C_T 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 (Hollend *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

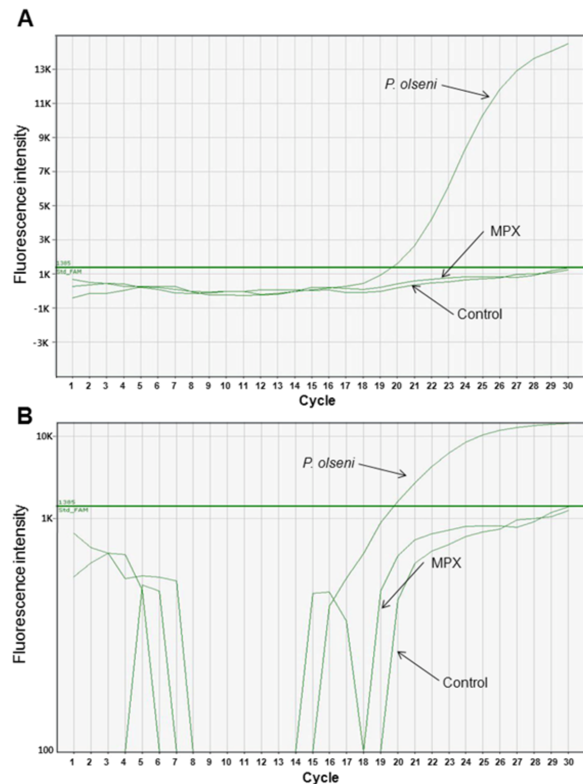

Fig. 1. Amplification plots of real-time PCR for *Perkinsus olseni*, MPX, and control using a *Perkinsus* spp.-specific probe. Linear curve (**A**) and log scale curve (**B**)

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

Table 1. Sensitivity, linearity, and reproducibility of the standard curve

Cell concentration	DNA concentration	C_T value (mean ± SD)				Coefficient of variation (CV)
		1st (n = 3)	2nd (n = 3)	3rd (n = 3)	Grand mean	
100,000	3 ng	18.95 ± 0.11	18.14 ± 0.18	18.70 ± 0.42	18.60 ± 0.23	1.2
10,000	300 pg	21.66 ± 0.31	21.11 ± 0.13	21.52 ± 0.01	21.42 ± 0.15	0.7
1,000	30 pg	24.87 ± 0.17	24.11 ± 0.13	24.60 ± 0.01	24.53 ± 0.10	0.4
100	3 pg	27.62 ± 0.23	27.50 ± 0.10	27.85 ± 0.35	27.66 ± 0.23	0.8
10	300 fg	30.96 ± 0.08	31.01 ± 0.02	30.53 ± 0.68	30.83 ± 0.26	0.8
1	30 fg	33.01 ± 0.16	33.14 ± 0.00	32.65 ± 0.01	32.93 ± 0.06	0.1
Slope		−1.25	−1.34	−1.24	−1.27	
Intercept		33.38	33.55	33.12	33.35	
R^2		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

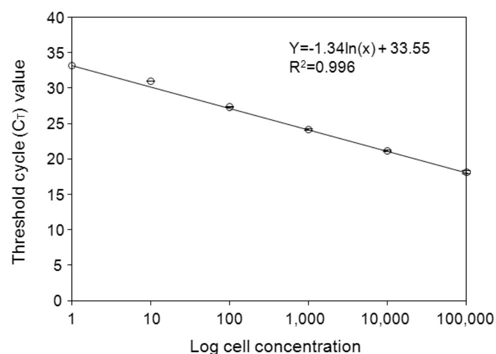

Fig. 2. A standard curve of the threshold cycle generated for 10-fold serially diluted DNA concentrations extracted from 10^5 cells.

between C_T values and log concentrations of cells, with a square regression coefficient (R^2) 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

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

Known # of cells	Sample A	Sample B	Sample C	Mean ± SD
50,000	41,554	52,827	46,030	46,803 ± 5,676
5,000	4,197	4,850	3,212	4,086 ± 824
500	330	530	455	438 ± 101
50	31	25	36	30 ± 5
5	5	4	5	4.6 ± 0.5
P-value	P = 0.31	P = 0.40	P = 0.21	-

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

This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0013304).

References

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba M. and Kohara, M. (1999) Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *Journal of Clinical Microbiology*, **37**: 2899-2903.

Audemard, C., Ragone Calvo, L. M., Paynter, K. T., Reece, K. S. and Burreson, E.M. (2006) Real-time PCR investigation of parasite ecology: In situ determination of oyster parasite *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. *Parasitology*, **132**: 827-842.

Audemard, C., Reece, K.S. and Burreson, E.M., (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. *Applied Environmental Microbiology*, **70**: 6611-6618.

Bertrand, I., Gantzer, C., Chesnot, T. and Schwartzbrod, J. (2004) Improved specificity for *Giardia lamblia* cyst quantification in wastewater by development of a real-time PCR method. *Journal of Microbiological Methods*, **57**: 41-53

Blessmann, J., Buss, H., Nu, P.A., Dinh, B.T., Ngo, Q.T., Van, A.L., Alla, M.D., Jackson, T.F., Ravdin, J.I. and Tannich, E. (2002) Real-time PCR for detection and differentiation of Entamoeba histolytica and Entamoeba dispar in fecal samples. *Journal of Clinical Microbiology*, **40**: 4413-4417.

Bushek, D., Dungan, C.F. and Lewitus, A. J. (2002) Serological affinities of the oyster pathogen *Perkinsus marinus* (Apicomplexa) with some dinoflagellates (Dinophyceae). *Journal of Eukaryotic Microbiology*, **49**: 11-16.

Casas, S.M., Grau, A., Reece, K.S., Apakupakul, K., Azevedo, C. and Villalba, A. (2004) *Perkinsus mediterraneus* n. sp., a protistan parasite of the European flat oyster *Ostrea edulis* from the Balearic Islands, Mediterranean Sea. *Disease of Aquatic Organisms*, **58**: 231-244.

Choi, K.S., Wilson, E.A., Lewis, D.H., Powell, E.N. and Ray, S.M. (1989). The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. *Journal of Shellfish Research*, **8**: 125-131.

Coss, C.A., Robledo, J.A., Ruiz, G.M. and Vasta. G.R. (2001) Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of species-specific PCR-based diagnostic assay. *Journal of Eukaryotic Microbiology*, **48**: 52-61.

Costa, C., Vidaud, D., Olivi, M.E., Bart-Delabesse, E., Vidaud, M. and Bretagne, S. (2001) Development of two real-time quantitative TaqMan PCR assays to detect circulating *Aspergillus fumigatus* DNA in serum. *Journal of Microbiological Methods*, **44**: 263-269.

Dungan, C.F. and Reece, K.S. (2006) In vitro propagation of two *Perkinsus* spp. Parasites from Japanese Manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. *Journal of Eukaryotic Microbiology*, **53**: 316-326.

Drago, L., Lombardi, A., De Vecchi, E. and Gismondo, M.R. (2002) Real- Time PCR assay for rapid detection of *Bacillus anthracis* spores in clinical samples. *Journal of Clinical Microbiology*, **40**: 4399

Elandalloussi, L.M., Leite, R., Afonso, R., Nunes, P., Robledo, J.A, Vasta, G.R. and Cancela, L. M. (2004) Development of a PCR-ELISA assay for diagnosis of *Perkinsus marinus* and *Perkinsus atlanticus* infection in bivalve molluscs. *Molecular and Cellular Probes*, **18**: 89-96.

Espy, M.J., Uhl J.R., Sloan L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengewack, N.L., Rosenblatt, J.E., Cockerill, III. and Smith, T.F. (2006) Real-Time PCR in clinical microbiology: applications for routine laboratory testing. *Clinical Microbiology Review*, **19**: 165-256.

Faveri, De.J., Somolowitz, R.M. and Roberts, S.B. (2009) Development and validation of a real-time quantitative PCR assay for the detection and quantification of *Perkinsus marinus* in the eastern oyster *Crassostrea virginica*. *Journal of Shellfish Research*, **28**: 459-464.

Fong, D., Rodriguez, R., Koo, K., Sun, J., Sogin, M., Bushek, D., Littlewood, D.T. and Ford, S. (1993) Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkisnus marinus*. *Molecular Marine Biology and Biotechnology*, **2**: 346-350.

Frostegard, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. and Simonet, P. (1999) Quantification of bias to the extraction of DNA directly from soils. *Applied and Environmental Microbiology*, **65**: 5409-5420.

Gauthier, J.D., Miller, C.R. and Wilbur, A.E. (2006) TaqMan MGB Real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus* spp. on oysters. *Journal of Shellfish Research*, **20**: 619-624.

Hamaguchi, M.N., Suzuki, N., Usuki, H. and Ishioka, H. (1998) *Perkinsus* protozoan infection in short-necked clam *Tapes* (*=Ruditapes*) *philippinarum* in Japan. *Fish Pathology*, **33**: 473-480.

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Science USA*, **88**: 7276-7280.

Mackin, J.G. (1962) Oyster disease caused by Dermocystidiun marinum and other microorganisms in Louisiana. *Publication of the Institute of Marine Science. University of Texas*, **7**: 132-229.

Marsh, A., Gauthier, J.D. and Vasta, G.R. (1995) A semiquantitative PCR assay for assessing *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica*. *Jounal of Parasitology*, **81**: 577-583.

Nagle, L., Place, A.R., Schott, E.J., Jagus, R., Messick, G. and Pitula, J.S. (2009) Real-time PCR-based assay for quantitative detection of *Hematodinium* sp. in the blue crab *Callinectes sapidus*. *Disease of Aquatic Organisms*, **84**: 79-87.

Park, K.I. and Choi, K.S. (2001) Spatial distribution and infection intensity of the protozoan parasite *Perkinsus* sp. in the Manila clam *Ruditapes philippinarum* in Korea. *Aquaculture*, **203**: 9-22.

Park, K.I., Park, J.K., Lee, J. and Choi, K.S. (2005) Use of DNA sequences to determine the identity of *Perkinsus* sp. found in Manila clam, *Ruditapes philippinarum* in Korean water. *Disease of Aquatic Organisms*, **66**: 255-263.

Park, K.I., Ngo, T.T.T., Choi, S.D., Cho, M. and Choi, K.S. (2006a) Occurrence of *Perkinsus olseni* in the Venus clam *Protothaca jedoensis* in Korean waters. *Journal of Invertebrate Pathology*, **93**: 81-87.

Park, K.I., Figueras, A. and Choi, K.S. (2006b) Application of enzyme-linked immunosorbent assay (ELISA) for the study of reproduction in the Manila clam *Ruditapes philippinarum*: (Mollusca: Bivalvia): II. Impacts of *Perkinsus olseni* on clam reproduction. *Aquaculture*, **251**: 182-191.

Ragone Calvo, L.M.., Dungan, C.F., Roberson, B.S. and Burreson, E.M. (2003) Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. *Disease of Aquatic Organisms*, **56**: 75-86.

Ray, S.M. (1954) Experimental studies on the transmission and pathogenicity of *Dermocytidium marinum*, a fungus parasite of oyster. *Journal of Parasitology*, **40**: 235

Robledo, J.A.F., Gauthier, J.D, Coss, C.A., Wright, A.C. and Vasta, G.R. (1998) Species- specificity and sensitivity of a PCR- based Assay for *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*: A comparison with the fluid thioglycollate assay. *Journal of Parasitology*, **85**: 1237-1244.

Robledo, J.A.F., P. Nunes, P.A, Cancela, M.L. and Vasta G.R. (2002) Development of an in vitro clonal culture and characterization of the rRNA gene cluster of *Perkinsus atlanticus*, a protozoan parasite of the clam *Tapes decussatus*. *Journal of Eukaryotic Microbiology*, **49**: 414-422.

Watson, R. J., and B. Blackwell. 2000. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. *Canadian Journal of Microbiology*, **46**: 633-642.

Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, **63**: 3741-3751.

Xie, Z.X., Xie, L.J., Liu, J.B., Xie, Z.Q., Pang, Y.W. and Deng, X.W. (2009) Development and application of a real-time PCR assay for detection of *Perkinsus olseni*. *Journal of Pathogen Biology*, **4**: 517-519.

Yang, H.S., Kang, H.S, and Choi, K.S. (2011) Detection

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.