# Development of Clamping Probe for Rare DNA Detection using Universal Primers

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## Abstract

PCR amplification with universal primer is a useful tool for speciation of symbionts in marine eukaryote coupled with robust separation method such as denaturing high performance chromatography (DHPLC). To overcome the biased amplification, clamping PCR is recommended to suppress the amplification of host gene. In this study, we evaluated the efficiency of rare gene detection for two kinds of clamping probes which were successfully utilized for eukaryotic symbiont analysis: C3 linked nucleotide (C3) and peptide nucleic acid (PNA). PNA was 3-4 orders of magnitude higher than that of C3 tested in clamping efficiency and rare gene detection. This represented that PNA could be a more competent clamping probe for the enhancement of PCR amplification for rare symbiont genes.

**Key words:** Peptide nucleic acid (PNA), Clamping PCR, Symbiont, Clamping efficiency

## Introduction

Use of universal primer targeted conserved regions in small subunit ribosomal RNA (SSU rRNA) gene has been utilized as a useful tool for identifying species and amplifying unknown functional gene (Pineau et al., 2004) as well. In 1989, Bottger (1989) first demonstrated that a portion of the 16S rRNA gene from Legjonella pneumophila, *Escherichia* coli or Mycobacterium tuberculosis can be amplified by using one set of universal PCR primers and then sequenced to identify these bacteria. Several targets are available for each taxonomy now using 16S rDNA for bacteria (Baker et al., 2003; Tian et al., 2005), 18S rDNA for eukaryote (Blankenship and Yayanos, 2005), 23S rDNA for marine algae and cyanobacteria (Sherwood and Presting, 2007), and 28S rDNA and ITS for fungi (Mohlenhoff et al., 2001; Iwen et al., 2002). In spite of convenience of the universal primer with ease of application to most species, separation of the amplicons from unknown sample mostly relied on a special treatment such as restriction enzyme (Lin et al., 2004) and denaturing high performance liquid chromatograph (DHPLC) (Troedsson et al., 2008a; Troedsson et al., 2008b). Universal primer, however, have a drawback such as underestimation of rare parasites in parasitic diagnosis because of a biased amplification of dominant gene (Bass and Cavalier-Smith, 2004). To overcome this obstacle, PCR amplification of dominant gene is intentionally suppressed by clamping probes such as the peptide nucleic acids (PNAs) (Troedsson et al., 2008b), locked nucleic acids (LNAs) (Karkare and Bhatnagar, 2006) and C3 oligonucleotide (Vestheim and Jarman, 2008) and GC clamp (Jury et al., 2007).

PNA is an oligonucleotide analogue with polyamide backbone. Because of strong binding with DNA, this mimic DNA has been exploited to produce powerful bimolecular tools, antisense and antigene agents, molecular probes and biosen-

sors (Nielsen and Egholm, 1999). Since PNA cannot extended by the polymerase, PNA has been also successfully utilized for clamping PCR amplification in mutation detection (Orum, 2000), and parasite detection (Troedsson et al., 2008b).

Recently, Vestheim and Jarman (2008) has proposed that a 3'-modified oligonucleotide, C3 spacer, can also be a useful clamping probe for PCR amplification of dominant gene. Inserted propyl group cannot be recognized by polymerase and thus isn't accessible for further reactions. C3 is a cost-effective nucleotide. Comparing to PNA, C3 is much cheaper and relatively easy to synthesize. If C3 has a competitive clamping efficiency for dominant gene to PNA, it is favorable to use as a clamping probe for DHPLC assay.

Therefore, two different clamping probes were tested with same sequence, C3 spacer and PNA, in terms of clamping efficiency of dominant gene and enhancement of PCR amplification of rare genes using two distinct model assays, blue crab and grass shrimp.

## Materials and Methods

### Model systems

A blue crab model (CS model), *Callinectes sapidus* coupled with dinoflagate parasite, *Hematodinium* sp. and grass shrimp model (PP model), *Palaemonetes pugio*, coupled with trematode, *Microphallus turgidus*, and bopyrid isopoda, *Probopyrus pandalicola*, were applied for the test, respectively. From each animal, nearly-full-length 18S rRNA was PCR amplified by universal primer set: UnivF-15 (5′-CTG CCA GTA GTC ATA TGC-3′) (Frishcher et al., 2002) and UnivR-1765 (5′-ACC TTG TTA CGA CTT TAC-3′) (Firscher et al., 2000; Troedsson et al., 2008b). For standard templates of the model, purified plasmid DNAs were prepared by transforming the PCR amplicon into plasmid vector pCR™ 4-TOPO (Invitrogen, Carlsbad, CA, USA) and then purified using High Pure plasmid isolation kit (Roche, Mannheim, Germany). This plasmid DNAs were used as templates for all PCR reactions.

### Clamping probe design

The clamping probe was designed to prime onto antisense strand of the target gene and to prevent elongation of forward PCR primer. To avoid the any false priming to its parasitic gene fragment and primer dimer with PCR primers, candidate of probe sequence were analyzed by Primer premiere 5.0 (Premier Biosoft International, Palo Alto, CA, USA). C3 spacer was synthesized from Integrated DNA Technologies (Coralville, IA, USA): BC-1361F-C3, 5′- GGT GTC CAG TTC GCA G/C3SP/-3′ for blue crab, *C. sapidus*; PP-1417F-C3, 5′- CCG TTC GAC ACA GTC T/C3SP/-3′ for grass shrimp, *P. pugio*. After qualitative confirmation with C3 probe, PNA was synthesized from Panagene (Daejeon, Ko-

rea): BC18S-1361F-PNA, H-GGT GTC CAG TTC GCA G-NH$_2$ for blue crab, PP-1417F-PNA, Lys-CCG TTC GAC ACA GTC T-NH$_2$ for grass shrimp, respectively.

### Clamping efficiency

To investigate the variation of clamping efficiency depending on clamping probe concentration, clamping-PCR was performed, with varying concentration (0 - 2 μM) of each probes, by a set of universal primer: Univ18s-1131F (5′-AAA CTY AAAGRA ATT GAC CC-3′) (Troedsson et al., 2008a) and Univ18s-1629R (5′-GAC GGG CGG TGT GTR C-3′) (Gruebl et al., 2002). The twenty μl of PCR reaction mix was constituted by 1X Discoverase™ PCR buffer (Invitrogen), 0.3U of Discoverase™ DNA Polymerase (Invitrogen), 0.5 μM of each Primer, varying concentration of clamping probe, 200 μM of dNTP, 2 mM of MgSO$_4$ and 2 μl of templates. Thermal cycling condition was set as: 30 s at 94℃; 25 cycles of 30s at 94℃, 30s at clamping probe's annealing temperature, 30s at 54℃, 30s at 68℃; and finally 5min at 68℃. The annealing temperature for clamping probe was set to 66℃ for PNA and 48℃ for C3 in blue crab, 65℃ and 46.4℃ for grass shrimp, respectively. All clamping-PCR were carried out in these conditions. After clamping-PCR, PCR products were applied to DHPLC to quantify the amplicon concentration.
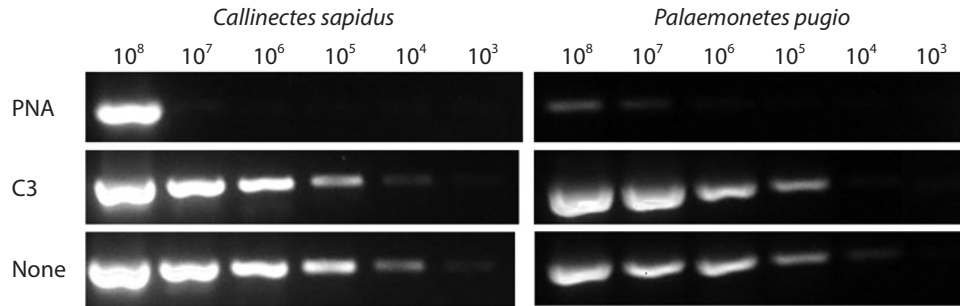
Clamping efficiency of each type of probe was investigated using serially diluted plasmid DNAs as templates ($10^8$ -$10^3$ copies). For this, each clamping-PCR were performed with previously described mixture adding 1 μM of clamping probe. The amplicons were eluted on 1.5% agarose gel.

### Detection of rare gene by DHPLC

To investigate the competency of rare gene detection, clamping-PCR was carried out to amplify the rare gene with clamping the amplification of dominant gene with clamping probe. A 3 by 7 factorial matrix mixture was used as a template. For detection of rare gene, PCR amplicon was further separated by DNASep HT Cartridge (Transgenomic, Omaha, NE) with linear gradient elution of WAVE® Optimized Buffers. Separation condition was tested by varying oven temperature and buffer B% (Troedsson et al., 2008a; Cho et al., Unpublished) and for 10 min. Chromatographic analysis was carried out by HSX-3500 fluorescence detector (Ex = 495 nm and Em = 537 nm) after SYBR Gold staining (Molecular Probe Inc.) with Navigator software version 1.6.2 (build 12) (Transgenomic, Omaha, NE).

### Statistical analysis

All data given in mean value and standard deviation from, at least, duplicate data. The significance of clamping efficiency depending on probe concentration was analyzed by analysis of variance (ANOVA) and tukey's test for post-hoc test for mean

**Fig. 1.** Clamping efficiency of PNA and C3. Bloking efficiency of each probe was tested by serial diluted plasmid DNA of each target 18S rRNA gene. PNA showed 3-4 order magnitude reduction of amplification in comparison with C3.
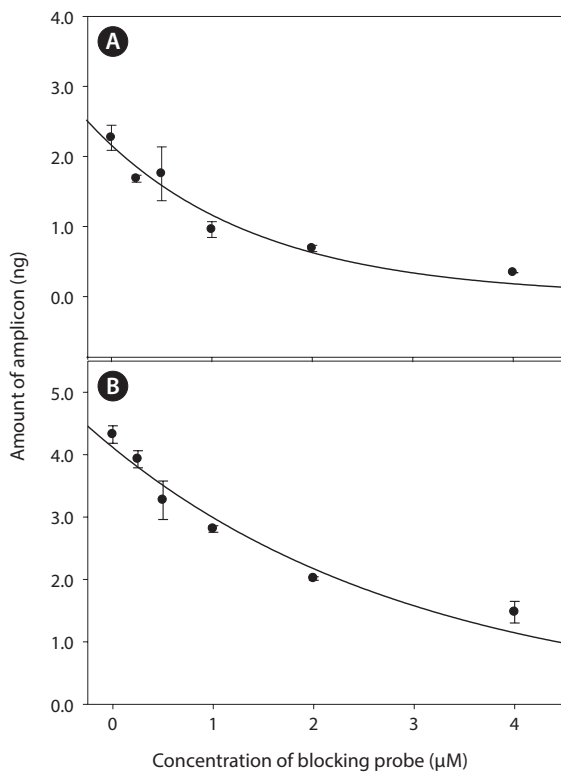


**Fig. 2.** Changes in amount of target-amplicons in response to different concentration of clamping probe. (A) Blue crab (CS) model and (B) Grass shrimp (PP) model with PNA. Bar=standard deviation. Amount of target-amplicon was significantly decreased in response to increase of clamping probe concentration after 25 cycles of clamping-PCR amplification ($r^2$=0.9352, $F$=73.1739 and P=0.0010 for CS model; $r^2$=0.9414, $F$=81.3607 and P=0.0008 for PP model). Amount of amplicon was quantified using peak height by 5 μL injection to DHPLC.

difference. Nonlinear regression analysis was also carried out for amount of target-amplicon from varying concentration of clamping probe. All statistical analyses were performed by statistical software, Sigmastat 3.11 (Systat Software, Inc.).

## Results

To quantitatively estimate the clamping efficiency for each type of clamping probe, serially diluted plasmid DNAs were applied to clamping PCR with/without 1 μM of clamping probe. In CS model, PNA completely blocked amplification of target amplicon up to $10^6$ copies of template, which were 5 orders of magnitude than PCR amplification without clamping probe. In other hand, C3 rarely showed clamping efficiency. In PP model, up to $10^5$ copies, PNA completely blocked and even slightly in $10^8$ and $10^7$ copies, band intensity of target amplicon was significantly smaller than those of none clamping probe PCR condition and C3 amplification (Fig. 1). The clamping efficiency of PNA was always superior to C3 spacer by more than 3 orders magnitude.

Optimal concentration for clamping probe was tested by varying concentration of clamping probes from 0 μM to 2 μM. PCR amplicons were further injected to DHPLC to quantify the amount of target amplicons in response to probe concentration. With increase of clamping probe, amplification of target-amplicon was obviously suppressed in both two model system ($P$<0.001) (Fig. 2) with exponential decrease ($r^2$=0.9352, $F$=73.1739 and $P$=0.0010). At 2 μM, amplification of target amplicon was significantly reduced by 85% for CS model and 66% for PP model, respectively.

Optimization of dHPLC assay was carried out with varying buffer B% and oven temperature conditions. For CS model, separation was optimized at 59.5℃ of oven temperature and 60-70% of linear gradient buffer B at 3.5 mL/min of flow rate (Troedsson et al., 2008a) while, for PP model, 57.5℃ and 62-68% at 0.45 mL/min (Cho, 2012). Retention time of each host and parasite peaks were highly reproducible (Fig. 3). WE applied these assay for further separation of PCR amplicons from pDNA mixture to measure the enhancement of rare gene amplification.

Detection of rare gene was highly improved by clamping-PCR, especially by PNA- clamping PCR. In CS model, the parasite, *Hematodinium* sp. was detected up to 1:$10^6$ ratios, which were 5 orders magnitude higher than non-clamping
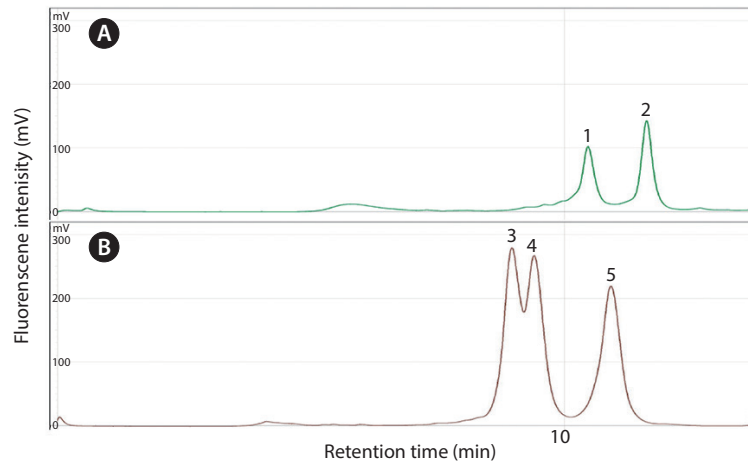
**Fig. 3.** Optimization of DHPLC assay for two distinct model assays for rare gene detection. (A) blue crab, (B) grass shrimp. 1: *Hematodinium* sp., 2: *Callinectes sapidus*, 3: *Microphallus turgidus*, 4: *Palaemonetes pugio*, and 5: *Probopyrus pandalicola*.

PCR and 2 orders magnitude than C3-clamping PCR. In PP model, PNA-clamping PCR was effective up to 1:$10^4$ ratios for detection of rare gene, which was 4 orders magnitude better than non-clamping PCR and 3 orders magnitude than C3-clamping PCR. In all model, PNA-clamping PCR was superior to C3-clamping PCR for detection of rare gene (Table 1).

## Discussion

In aquatic ecosystem, animals are always exposed to relatively large biomass of parasitesand co-existent with diverse symbionts in various ways (Kuris et al., 2008). Except epidemic case, detection of unknown symbionts is highly time- and cost-consuming work because of their rare intensities. Recently, denaturing high-performance liquid chromatography

**Table 1.** Detection of rare gene (%) with clamping probes in multi template plasmid DNA mixtures by PNA-PCR, C3-PCR and PCR coupled with DHPLC assay

| Ratio[1] | n[2] | CS[3] | | | PP[4] | | |
|---|---|---|---|---|---|---|---|
| | | PNA | C3 | None | PNA | C3 | None |
| 1:$10^1$ | 3 | 100 | 100 | 100 | 100 | 100 | 33 |
| 1:$10^2$ | 3 | 100 | 100 | 0 | 100 | 0 | 0 |
| 1:$10^3$ | 3 | 100 | 100 | 0 | 66 | 0 | 0 |
| 1:$10^4$ | 3 | 100 | 33 | 0 | 66 | 0 | 0 |
| 1:$10^5$ | 3 | 100 | 0 | 0 | 0 | 0 | 0 |
| 1:$10^6$ | 3 | 66 | 0 | 0 | 0 | 0 | 0 |
| 1:$10^7$ | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

[1]Ratio between parasite and host in gene copy numbers. [2]Number of independent assays in 8 by 3 factorial experiments. [3]*Callinectes sapidus*. [4]*Palaemonetes pugio*.

(DHPLC) has been utilized as a new diagnostic technique for detecting parasitism in marine eukaryotic organism (Troedsson et al., 2008a; Troedsson et al., 2008b). The potential of this new technology is now expanding its application for marine biology and ecology. PCR-based DHPLC assay has been turned out a versatile and efficient model for detecting low ranged parasites and , with universal primers targeted highly conserved region, successfully utilized to investigate the symbiont composition in 18s rRNA for eukaryotes (Schmidt and Relman, 1994) and 16s rRNA for bacteria (Amann and Ludwig, 2000). To achieve the amplification of rare parasitic gene, however, it is necessary to suppress the amplification of the dominant host gene to avoid a biased PCR amplification of the dominant host gene.

C3 (Dames et al., 2007; Vestheim and Jarman, 2008) and PNA (Orum, 2000; Shakeel et al., 2006; Troedsson et al., 2008b) have been used as effective clamping probes and further enhancer of PCR amplification of rare gene. The clamping efficiency of PNA was obviously superior to C3 spacer by 2-3 orders of magnitude (Fig. 1). PNA has a strong binding independent of salt concentration and also greater specificity in binding to complementary DNA (Demidov and Frank-Kamenetskii, 2004). This high specificity and strong binding may facilitate strong hybridization on intra-strand of target DNA and prevent polymerase to displace the PNA molecules from the template (Shakeel et al., 2006).

In this study, the extension temperature (68℃) is much higher than annealing temperature of any C3 probes. During extension, the polymerase may displace C3 probe from the template, and then extend towards completion of the reaction (Shakeel et al., 2006). When the binding position of clamping probe is closer to PCR primer, the DNA/DNA binding activity may be relatively stronger than further one before thermal cycling temperature stabilized.

PNA binding should precede PCR primer binding. Tm of the PNA and DNA is, therefore, the most important variables that affect binding order, which should be between 65℃ and 75℃ (Orum, 2000). Mostly, PNA can meet this condition with relatively short sequence because of its stronger PNA/DNA binding. As a general rule, A Tm of a PNA/DNA duplex is 1℃ higher per base pair than Tm of the corresponding DNA/DNA duplex (in 100 mmol dm$^{-3}$ NaCl) (Shakeel et al., 2006). To make C3 spacers of PNA's Tm, the sequence should be prolonged much more than PNA length. Considering interaction with PCR primers and various symbiont and host genes, long clamping probe may possibly generate non-specific annealing and a dimer with PCR primers, which eventually decrease the detection efficiency of clamping-PCR. On the order hand, PNA can reach the Tm by only 12-18 bases with much stronger binding activity. Therefore, PNA can be a more efficient clamping probe for enhancement of PCR amplification of rare gene than C3 spacer.

The ratio of clamping probe to template is constantly varied with each cycles and kinetic of PNA annealing are constantly changing in the reaction (Demers et al., 1995). Therefore, the optimal concentration of clamping probe should be determined carefully and fully tested with various conditions. In the study, amount of target PCR amplicon was exponentially decreased with increase of clamping probe concentration (Fig. 2) in accordance with previous study (Demers et al., 1995). One μM of PNA concentration has been successfully utilized for parasitic gene detection in wild blue crab samples ((Troedsson et al., 2008a; Troedsson et al., 2008b) and showed over 50% inhibition efficiency in target gene amplification in the study. Therefore, this concentration was applied to further experiment for clamping efficiency.

A significant difference was observed at clamping efficiency between two PNA probes. In CS model, BC18S-1361-PNA completely blocked amplification of pDNA of *C. sapidus* up to 10$^7$ copies, while PP18S-1417-PNA was only up to 10$^5$ copies in PP model. The possible cause for these differences might be attributed to difference of sequence, DNA structure, clamping position and difference of binding activity etc. Although not being explained quantitatively, an apparent improvement of clamping efficiency was observed when the clamping probe bound at closer position to extension reverse primer (data not shown) regardless of Tm. This may attribute to thermodynamic characteristics of polymerase activity.

Estuarine and coastal environments can be good habitats and nursery ground for many aquatic biotas and also be a sink tank for land-driven compounds. Monitoring of these contaminants dynamics is crucial not only for concern of human health but also conservation of ecosystem homeostasis. Input of contaminants may make hosts more susceptible to parasitism and increase mortality rates of infected hosts in the ecosystem (Möller, 1987; Lafferty and Kuris, 1999), although being disagreements also available on this matter (Lafferty and Kuris, 1999). Tremendous effort has, so far, been made

to understand the interaction between environment contamination and parasitic epidemics in aquatic ecosystem (Lafferty and Kuris, 1999; Kim et al., 2008). Detection or discovery of parasitism is probably a crucial prerequisite condition to understand the impact of environmental stress on aquatic ecosystem. During the past decades, noticeably developed diagnostic techniques have enabled detection and quantification of a variety of parasites, parasitoids and pathogens including molecular diagnosis (Lightner and Redman, 1998; Cunningham, 2002; Mothershed and Whitney, 2006; Thakur et al., 2008). DHPLC is also one of those versatile and competent tools for monitoring parasitic/symbiont composition. In order to facilitate the detection of rare symbiont gene, clamping-PCR is a promised tool to overcome the biased amplification of PCR amplification. Therefore, PNA can be the most useful and prominent tool for clamping-PCR over its high price and complexity of synthesis.

## Acknowledgments

## References

Amann R and Ludwig W. 2000. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol Rev 24, 555-565.

Baker GC, Smith JJ and Cowan DA. 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Meth 55, 541-555.

Bass D and Cavalier-Smith T. 2004. Phylum-specific environmental DNA analysis reveals remarkably high global biodiversity of Cercozoa (Protozoa). Int J Syst Evol Microbiol 54, 2393-2404.

Blankenship LE and Yayanos AA. 2005. Universal primers and PCR of gut contents to study marine invertebrate diets. Mol Ecol 14, 891-899.

Bottger EC. 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol Lett 65, 171-176.

Cho SM. 2012. Development of Denaturing High-Performance Liquid Chromatography (DHPLC) Assay to detect parasite infection in Grass shrimp, *Palaemonetes pugio*. Fish Aquat Sci 15, 107-115.

Cunningham CO. 2002. Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206, 19-55.

Dames S, Margraf RL, Pattison DC, Wittwer CT and Voelkerding KV. 2007. Characterization of aberrant melting peaks in unlabeled probe assays. J Mol Diagn 9, 290-296.

Demers DB, Curry ET, Egholm M and Sozer AC. 1995. Enhanced PCR Amplification of VNTR Locus D1s80 Using Peptide Nucleic-Acid

(PNA). Nucleic Acids Res 23, 3050-3055.

Demidov VV and Frank-Kamenetskii MD. 2004. Two sides of the coin: affinity and specificity of nucleic acid interactions. Trends Biochem Sci 29, 62-71.

Frischer, ME, Danforth JM and Tyner LC. 2000. Development of an Argopecten-specific 18S rRNA targeted genetic probe. Mar Biotechnol 2, 11-20.

Frishcer, M, Hansen A, Wylie J, Wimbush J, Murray J and Nierzwicki-Bauer S. 2002. Specific amplification of the 18S rRNA gene as a method to detect zebra museel (Dreissena polymorpha) larvae in plankton samples. Hydrobiol 487, 33-44.

Gruebl T, Frischer ME, Sheppard M, Neumann M, Maurer AN and Lee RF. 2002. Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite Hematodinium sp. Dis Aquat Organ 49, 61-70.

Iwen PC, Hinrichs SH and Rupp ME. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol 40, 87-109.

Jury F, Bueid A, Ollier W, Fox A and Upton M. 2007. Analysis of mixed microbial species in clinically relevant biofilms using denaturing HPLC. In. ICC, Munich, Germany, pp. S394.

Karkare S and Bhatnagar D. 2006. Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. Appl Microbiol Biotechnol 71, 575-586.

Kim Y, Powell EN, Wade TL and Presley BJ. 2008. Relationship of parasites and pathologies to contaminant body burden in sentinel bivalves: NOAA Status and Trends 'Mussel Watch' Program. Mar Environ Res 65, 101-127.

Kuris AM, Hechinger RF, Shaw JC, Whitney KL, Aguirre-Macedo L, Boch CA, Dobson AP, Dunham EJ, Fredensborg BL, Huspeni TC, Lorda J, Mababa L, Mancini FT, Mora AB, Pickering M, Talhouk NL, Torchin ME and Lafferty KD. 2008. Ecosystem energetic implications of parasite and free-living biomass in three estuaries. Nature 454, 515-518.

Lafferty KD and Kuris AM. 1999. How environmental stress affects the impacts of parasites. Limnol Oceanogr 44, 925-931.

Lightner DV and Redman RM. 1998. Shrimp diseases and current diagnostic methods. Aquaculture 164, 201.

Lin HW, Yin JH, Lo JP, Wang YH, Lee SY and Lu JJ. 2004. Use of universal polymerase chain reaction assay and endonuclease digestion for rapid detection of Neisseria meningitides. J Microbiol Immunol Infect 37, 371-374.

Möller H. 1987. Pollution and parasitism in the aquatic environment. Int J Parasitol 17, 353-361.

Mohlenhoff P, Muller L, Gorbushina AA and Petersen K. 2001. Molecular approach to the characterisation of fungal communities: methods for DNA extraction, PCR amplification and DGGE analysis of painted art objects. FEMS Microbiol Lett 195, 169-173.

Mothershed EA and Whitney AM. 2006. Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. Clin Chim Acta 363, 206-220.

Nielsen PE and Egholm M. 1999. An Introduction to Peptide Nucleic Acid. Current Issues Molec Biol 1, 89-104.

Orum H. 2000. PCR clamping. Curr Issues Mol Biol 2, 27-30.

Pineau P, Henry M, Suspe`Ne R, Marchio A, Dettai A, Debruyne R, Petit T, Le´Cu A, Moisson P, Dejean A, Wain-Hobson S and Vartanian J-P. 2004. A Universal Primer Set for PCR Amplification of Nuclear Histone H4 Genes from All Animal Species. Mol Biol Evol 22, 582-588.

Schmidt TM and Relman DA. 1994. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. Methods Enzymol 235, 205-222.

Shakeel S, Karim S and Ali A. 2006. Peptide nucleic acid (PNA) - a review. J Chem Technol Biot 81, 892-899.

Sherwood AR and Presting GG. 2007. Universal primers amplify a 23s rDNA plastid marker in eukaryotic algae and cyanobacteria. J Phycol 43, 605-608.

Thakur NL, Jain R, Natalio F, Hamer B, Thakur AN and Muller WE. 2008. Marine molecular biology: an emerging field of biological sciences. Biotechnol Adv 26, 233-245.

Tian YJ, Yang H, Wu XJ and Li DT. 2005. Molecular analysis of microbial community in a groundwater sample polluted by landfill leachate and seawater. J Zhejiang Univ Sci B 6, 165-170.

Troedsson C, Lee RF, Stokes V, Walters TL, Simonelli P and Frischer ME. 2008a. Development of a denaturing high-performance liquid chromatography method for detection of protist parasites of metazoans. Appl Environ Microbiol 74, 4336-4345.

Troedsson C, Lee RF, Walters T, Stokes V, Brinkley K, Naegele V and Frischer ME. 2008b. Detection and discovery of crustacean parasites in blue crabs (Callinectes sapidus) by using 18S rRNA gene-targeted denaturing high-performance liquid chromatography. Appl Environ Microbiol 74, 4346-4353.

Vestheim H and Jarman SN. 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. Front Zool 5, 12.