アサリに寄生するPerkinsus olseniとP. honshuensisのPCR-RFLPによる識別法の開発

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| 著者       | 高橋, 美希  
             | 良永, 知義  
             | 下川, 潤  
             | 小川, 和夫 |
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Development of a PCR-RFLP Method for Differentiation of Perkinsus olseni and P. honshuensis in the Manila Clam Ruditapes philippinarum

Miki Takahashi, Tomoyoshi Yoshinaga*, Tsukasa Waki, Jun Shimokawa and Kazuo Ogawa

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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ABSTRACT—We developed a method using PCR-RFLP to differentiate Perkinsus olseni and P. honshuensis, the latter of which was recently discovered as a new species in Mie, Japan, in the Manila clam Ruditapes philippinarum. In normal gill samples spiked with cultured trophozoites of the parasites, the minimum infection levels that could be detected by this method were 100 cells per 10 mg sample for P. olseni and one cell for P. honshuensis. Using this method, we found that clams from the western Seto Inland Sea were infected with both species of the parasites. This is the first report of P. honshuensis from areas outside the type locality of this species.

Key words: Perkinsus olseni, Perkinsus honshuensis, Ruditapes philippinarum, PCR-RFLP

Perkinsus olseni is a protozoan parasite that infects clams, abalones and several other bivalves, and gastropods. Infection of the Manila clam Ruditapes philippinarum with P. olseni has been reported in the Far East, including Japan, Korea, China, and southwestern Europe. The majority of Manila clam populations in Japan are infected with P. olseni. Furthermore, P. olseni is thought to have caused the decline in Manila clam production in Japan and Korea in the 1980s. Recently, a new species of parasite, P. honshuensis, was described in Manila clams in Mie Prefecture. These two parasite species are morphologically similar. Thus, species identification is confounded as it requires establishment of clonal strains and sequencing of several genomic regions of clones, including internal transcribed spacer regions (ITS). To investigate the distribution of these parasites, a fast and convenient method for identification is needed. Furthermore, we had already launched a study on Perkinsus infection in Manila clams in the western Seto Inland Sea when P. honshuensis was first described. Thus, it was an urgent need to reveal whether the clam populations we investigated were infected with P. olseni only or co-infected with the both species.

Abollo et al. (2006) developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to differentiate Perkinsus chesapeaki, P. marinus, P. olseni and P. mediterraneus. In this study, we developed a PCR-RFLP method to differentiate P. olseni and P. honshuensis. We then tested infection of the parasites in Manila clams from several localities in Japan.

Materials and Methods

We obtained clonal strains of P. olseni and P. honshuensis from the American Type Culture Collection (ATCC # PRA 181 and PRA 177, respectively). The trophozoite cells of them were subcultured using a routine method in Perkinsus broth medium (ATCC medium 1886) according to the instruction by American Type Culture Collection.

We obtained Manila clams that were infected with P. olseni and/or P. honshuensis from three localities for PCR-RFLP analysis developed in this study. Two of the localities, a clam farm in Hiroshima Prefecture and a tidal flat in Oita Prefecture, are located in the western Seto Inland Sea. The third locality is a tidal flat located in Yatsushiro Bay, Kumamoto Prefecture. Based on our preliminary examinations, the clams from the western Seto Inland Sea were heavily infected with Perkinsus (prevalence 100%, geometric mean intensity of infection > 10^6 cells/g gill weight), while those from Yatsushiro Bay were very lightly infected (prevalence 44%, geometric mean intensity of infection ~10^4 cells/g gill weight). The numbers of clams applied for the analysis were 3, 4 and 3 for the clam farm in Hiroshima, the tidal flat in Oita and the tidal flat in Kumamoto, respectively. We also obtained uninfected clams from Akkeshi, Hokkaido. We confirmed the absence of infection in clams from this locality during a preliminary examination using Ray’s fluid thioglycolate medium method and Perkinsus genus-specific PCR.

We extracted DNA from cultured trophozoites and sample clams using a QIAmp DNA Mini Kit (Qiagen, USA), following the manufacturer’s protocol. First, we centrifuged (300 x g, 5 min) 10-day-old cultures of trophozoites of the two Perkinsus strains and extracted DNA from each pellet (~10^5 cells/pellet). As trophozoites of the two Perkinsus species frequently formed massive clusters in the medium, the cell density was measured as follows: 10 µL of the pellet was resuspended in 90 µL of PBS and passed through a 25G hypodermic
needle to break up the cell clusters. The suspension was then diluted 100 times with 100% ethanol. Following this, we mounted 0.5 or 1 µL of the suspension onto a slide and stained the trophozoite nuclei with DNA-binding dye (Hoechst 33342) diluted in SlowFade Gold antifade reagent (Invitrogen, USA). The cells were then counted under a fluorescence microscope. Second, we extracted DNA from normal (= uninfected) gill samples (10 mg each) spiked with 1–1 x 10⁶ cultured trophozoites. Last, we extracted DNA from 10 mg of the gills of cultured or wild Manila clams (shell length 7–18 mm) obtained from the two localities in the western Seto Inland Sea and one locality in Yatsushiro Bay.

For PCR amplification, we used following Perkinsus specific primers: PerkITS-85 and PerkITS-750. We compared the nucleotide sequences of the ITS region of congeneric species registered in GenBank (P. olseni, DQ516703-DQ516715; P. honshuensis, DQ516667-DQ516702). Based on this comparison, we concluded that P. olseni and P. honshuensis could be differentiated by RFLP analysis using the restriction enzyme Dde I.

We performed PCR amplification in a total volume of 20 µL containing 16 µL of the DNA extract, dNTP Mixture (0.2 mM per nucleotide, TaKaRa, Japan), 0.75 µM of each primer, and 0.025 units/µL TaKaRa Ex Taq™ (Hot start version; TaKaRa). The extract of cultured cells was diluted, and the amounts of DNA were adjusted to 10⁻⁶–10 ng for each PCR. The thermal conditions were 94°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. This was followed by a single step at 72°C for 10 min.

For RFLP analyses, 5 µL of the PCR products were digested with 5–7 units of a restriction enzyme (Hinf I, Rsa I, or Dde I: Toyobo, Japan) in buffers provided from the manufacturer in a final volume of 10 µL for 4 h at 37°C. Following digestion the fragments were loaded onto a 1.5% agarose gel, stained with SYBR safe DNA gel stain (Invitrogen) and visualized with Safe Imager (Invitrogen). The fragments obtained from cultured trophozoites were purified using a DNA purification kit (QiAquick PCR purification kit, Qiagen) before being loaded on the gel, while those from the gill samples spiked with trophozoites and from the collected clams were loaded without purification.

**Results**

RFLP band patterns after Rsa I and Hinf I digestions that were deduced from nucleotide sequences were very similar between P. olseni and P. honshuensis. Expected fragment lengths after Rsa I digestion were 406, 193 and 74 bp for P. olseni and 413, 194 and 59 bp for P. honshuensis. Those after Hinf I were 363 and 159–151 pb (a combined band) for P. olseni and 371 and 150–145 (a combined band) for P. honshuensis.
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A

M P 1 2 3 4 5 6 7 8 9 10 N

B

M P 1 2 3 4 5 6 7 8 9 10 N

Fig. 2. PCR-RFLP electrophoretic profiles of PCR products of extracts from normal (= uninfected) gill samples spiked with *P. olseni* (A) and *P. honshuensis* (B) trophozoites. M, 100 bp marker; P, trophozoites only; N, negative control. The number of the trophozoites subjected to extraction in lane 1 was $10^9$ (serially diluted 1:10).

Fig. 3. PCR-RFLP analysis of Manila clams from three localities. M, 100 bp marker; O, positive control (*P. olseni*); H, positive control (*P. honshuensis*); 1–3, Hiroshima; 4–6, Kumamoto; 7–10, Oita.

tidal flat in Kumamoto, and *P. honshuensis* was not found in any clams from this site (Fig. 3).

Discussion

*P. olseni* can be distinguished from congeneric species by conducting PCR-RFLP with *Hinf I* and *Rsa I*. However, *P. olseni* and *P. honshuensis* were not distinguished using these enzymes. We developed a PCR-RFLP method using the *Dde I* restriction enzyme that was used to distinguish these two species. We found that the intensity of *P. olseni* and *P. honshuensis* infection in Manila clams was approximated using the electrophoretic profile generated by PCR-RFLP. However, it was not achieved to accurately quantify the number of *Perkinsus* cells using this method, because the trophozoites formed massive clusters in medium. Quantitative surveillance of infection intensity are needed to estimate the impact of these parasites on clam populations. Given this, future research should focus on the development of methods for quantification. Very recently, species-specific mixed primers for *P. marinus* and *P. honshuensis* were developed by Moss according to the OIE manual. Species-specific PCR primer could be developed for *P. honshuensis* as well. By using species specific primers, semi-quantitative assays will become possible.

We used the new method to confirm the presence of parasites in Manila clams from several different localities around Japan. We found that Manila clams in the western Seto Inland Sea were infected with both *P. olseni* and *P. honshuensis*, though predominantly with *P. olseni*. Previously, *P. honshuensis* was reported only from Mie Prefecture. Detection of *P. honshuensis* had not been attempted in areas other than the original locality of the parasite until this study. Thus, this is the first report of *P. honshuensis* outside Mie. *P. olseni* may be widely distributed in other areas in Japan and in the Far East, as previously suggested. However, more detailed investigations are needed to accurately determine the distribution of the two species. The Manila clams from Yatsushiro Bay appeared to be infected with *P. olseni* only. However, considering that the low infection levels of *Perkinsus* in the clams from the site and the limited number of samples, the absence of *P. honshuensis* in Yatsushiro Bay is not definitive.

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References

M. Takahashi, T. Yoshinaga, T. Waki, J. Shimokawa and K. Ogawa

分子進化に伴い変化するIHNVの病原性
高月万美子・金 亨雄・塚井久会

2006年、分離されたIHNVの日本株の3ゲンバン質遺伝子を解析した結果、IHNV日本株に長鎖および短鎖系が存在し、これらは日本のニジマス養殖環境で今なお進化していることが示された。2006年分離株のニジマスに対する病原性を比較した結果、短鎖系での累積死亡率は76%以上であったが、長鎖系株では20〜40%。また、比較に用いた1976年の分離株では10%以下と、各株の病原性に差異が認められた。従って、ニジマス養殖環境でのIHNVの分子進化に伴い、IHNVのニジマスに対する病原性も変化していると考えられた。

魚病研究, 44 (4), 159-165 (2009)

生体染色と感染実験によるEnteromyxum leeiの海水
中における生残能力の推定
横山 博・高田裕美・木下哲彦・中川和夫

粘液脂子虫性や病原性菌の1種Enteromyxum leeiの栄養体を蛻光色素で生体染色することにより、in vitroでのE. leeiの生死判定法を開発した。この染色法と未感染トラフまたはカスファへの感染実験を併用して、E. leeiの海水井における生残能力を調べた。In vitroでは20°Cで12〜24時間以内に失活したが、感染実験では24時間後で感染力を保持している場合もあった。E. leeiの海水井での生残時間は、条件にもよりが約24時間以内であると推定された。

魚病研究, 44 (4), 172-177 (2009)

エイ卵の水カビ病における塩化カリウムの抗真菌効果
三浦正之・田井喜司雄・大野平祐

KCIの水カビ病原因菌への影響ならびにエイ卵の水カビ病発生防除効果について検討した。0.24%までのKCIは、水カビ病原因菌の伸長および遊泳の発育を抑制しない一方で、遊泳子の運動を0.03%で1分以内に顕著に抑制した。KCI溶液中で、E. pulicariaを感染後から発育期まで飼育した結果、0.06%及び0.12%では水カビ病の発生が抑制された。また、この濃度で発育率、卵化率及び奇形率に悪影響はみられなかった。KCIはエイ卵の水カビ病発生防除に有効であると考えられた。

魚病研究, 44 (4), 166-171 (2009)

水温法によるヒラメ稚魚へのTenacibaculum maritimum
実験感染法の検討
西岡豊弘・渡辺太一・佐野元彦

ヒラメ稚魚に対する感染実験によるT. maritimumの実験感染のためについて検討した。攻撃菌濃度が10⁸ CFU/mLの時、いずれの試験水温（15°C, 20°C, 25°C）でも60%以上の死亡率となった。10⁸, 10⁹CFU/mL攻撃ではどの水温でも死亡率はそれぞれ20〜40%。0%と低かった。死亡魚には卵巣の発赤や体表のびらんが認められ、雄魚より卵巣が再分離された。魚体通過させ、シードロット法で保存した細菌を使用したことが、強い感染力と再現性のある結果をもたらしたと考える。

魚病研究, 44 (4), 178-181 (2009)

ワガ話におけるサケ・マス類の病原ウイルス、特に伝染性サケ腎症ウイルスの検出状況
塚井久会・岩脇周平・吉水 守

伝染性サケ腎症ウイルス（ISAV）の検出状況を明らかにすることを目的に、2005〜2007年にかけてサケ科魚類8種5,967尾の肉汁から体腔液を採取し、ASKおよびASE細胞を用いてウイルスの分離培養を試みた。その結果、ISAVはいずれの検体からも分離されなかった。3魚種と16尾から伝染性腎臓障害症ウイルス（IHNV）が、また3魚種と1尾から伝染性腎臓障害症ウイルス（IPNV）が分離された。従って、ISAVは日本産魚種であると考えられた。

魚病研究, 44 (4), 182-184 (2009)

アサリに寄生するPerkinsus olseniとP. honshuensisのPCR-RFLPによる識別法の開発
高橋美希・良知義也・寺下川 璃・小川和夫

アサリに寄生するサビキサツ属原虫2種のPCR-RFLPによる識別法を開発した。未感染のアサリの飼育で培養した栄養体を混合した試料では、P. olseniiとP. honshuensisの検出限界は、アサリ飼10 mgあたりそれぞれ100細胞と1細胞であった。この方法によって、室戸内海西部のアサリにこの2種の混合感染が確認された。

魚病研究, 44 (4), 185-188 (2009)