Available online at www.sciencedirect.com
ScienceDirect
Molecular and Cellular Probes 20 (2006) 323–329
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Differential diagnosis of *Perkinsus* species by polymerase chain reaction-restriction fragment length polymorphism assay

Elvira Abollo[a,*], Sandra M. Casas[a], Giuseppe Ceschia[b], Antonio Villalba[a]

[a]*Centro de Investigacions Mariñas, Conselleria de Pesca e Asuntos Maritimos, Xunta de Galicia, Apartado 13, 36620 Vilanova de Arousa, Spain*
[b]*Istituto Zooprofilattico Sperimentale delle Venezie, Laboratorio Patologia Molluschi. Via della Roggia 102, 33030 Basaldella di Campoformido (Udine), Italy*

Received 17 February 2006; accepted 3 April 2006
Available online 17 April 2006

Abstract

Perkinsosis is an infection of marine molluscs caused by the protistan parasites of the genus *Perkinsus*, which has been classified by the OIE as a disease that warrants notification. In the present study, we have applied a molecular genetic approach to develop an optional method for the specific identification of *Perkinsus* species.

A species-specific polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay of the rRNA ITS region was developed to identify and distinguish among *Perkinsus* species. A taxonomic key was established that allows successful identification of *Perkinsus* species using a single restriction enzyme (*Rsa* I) to discriminate *P. chesapeaki* and *P. marinus* or by a combination of two endonucleases (*Rsa* I plus *Hinf* I) to discriminate *P. olseni* and *P. mediterraneus*. In order to validate the RFLP assay, the PCR products were cloned and sequenced, and its phylogenetic affinity was determined. Phylogenetic analysis confirmed the specific identification carried out by RFLPs. Herein is the first report of *P. olseni* in Manila clams from the NW Adriatic Sea (Italy), which we identified by employing this method. The PCR-RFLP assay herein described may be useful to provide accurate, rapid and inexpensive identification of *Perkinsus* species, and may aid in ongoing epizooetiological studies and diseases control programmes.

Keywords: *Perkinsus*; PCR-RFLP; Species identification; Genetic key; Molluscs

1. Introduction

Species of the genus *Perkinsus* are parasites of a wide variety of mollusks around the world, and have been associated with severe mortalities and significant economic losses [1]. In fact, *P. olseni* and *P. marinus* are pathogen species included in the list of notifiable diseases to the OIE (Office International des Epizooties), having their distribution implications in the movement of shellfish internationally. Traditionally, *Perkinsus* infections have been diagnosed by histology or incubating mollusk tissues in a Ray's fluid thioglycollate medium (RFTM), in which the parasite cells enlarge, thus making their detection easier [2]. The RFTM assay has been proven more sensitive than histology for diagnosis of *P. olseni* and *P. marinus* infections [3–7], and is a technique commonly used for routine surveillance and determination of prevalence in host populations despite some limitations in specificity [8,9] and sensitivity [4,7].

Polymerase chain reaction (PCR)-based approaches utilizing appropriate genetic markers have provided useful tools to overcome the limitations for the diagnosis of *Perkinsus* species. Sequences analysis of the internal transcribed spacers (including ITS1, ITS2 and 5.8S) [10–16], the small subunit (18S) [17,18] and non-transcribed spacer (NTS) [19–23] of the rRNA have been used for species discrimination and phylogenetic studies. Moreover, PCR primers based on ITS region sequences have been developed to be genus-specific and species-specific [12,24–26]. Recent molecular studies have recognized five species of the genus *Perkinsus* as valid: *P. marinus, P. olseni, P. mediterraneus, P. qugwadi* and *P. chesapeaki* [1,23,27]. The allocation of *P. qugwadi* to the genus

*Corresponding author. Tel.: +34 986 500 155; fax: +34 986 506 788.
E-mail address: eabollo@cimacoron.org (E. Abollo).

0890-8508/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2006.04.001

Perkinsus is controversial and the comparison of DNA sequences shows significant dissimilarities between this and the other known species of the genus [1,23]. Currently, *P. olseni*/*P. atlanticus* and *P. chesapeaki*/*P. andrewsi* are considered conspecific, with the names *P. olseni* and *P. chesapeaki* given priority in accordance with the International Code of Zoological Nomenclature rules [23,27].

The present study reports a rapid and inexpensive method for the specific identification of *P. olseni*, *P. mediterraneus*, *P. marinus* and *P. chesapeaki* by PCR-RFLP assay. This technique represents a rapid and sensitive diagnostic system, which has been widely used in the specific identification and detection of pathogens belonging to different taxa [28–37]. The accuracy of this method allow us to report for the first time the specific identification of *Perkinsus* like organisms infecting the Manila clam *Ruditapes philippinarum* from the NW Adriatic Sea (Italy).

2. Materials and methods

2.1. Samples

Two *P. olseni* isolates (PaG-1f, PaG-5f), three *P. mediterraneus* isolates (PmG2, PmG3, PmHt2), two *P. chesapeaki* isolates (CRMA.J-44/E3, CRTP-17/A10) and one *P. marinus* isolate (P1) were used to develop in vitro no-clonal cultures. *P. olseni* cultures were established from the gills of *T. decussatus* from Galicia (NW Spain) [13]. *P. mediterraneus* cultures were established from the gills (PmG2, PmG3) and heart (PmHt2) of *Ostrea edulis* from the Balearic Island, following the method of La Peyre et al. [38]. *P. chesapeaki* cultures were obtained from Chris Dungan (Maryland Department of Natural Resources, Cooperative Oxford Laboratory). CRMA.J-44/E3 (ATCC 50864) was established from the labial palp of *Mya arenaria* from the Choptank River in Maryland (E USA), and CRTP-17/A10 was established from the labial palp of *Tagelus plebeius* from the same area [14]. *P. marinus* culture was obtained from Jerome F. La Peyre (Department of Veterinary Science, Louisiana State University Agricultural Center). P1 culture was established from the heart of *Crassostrea virginica* from the Rappahannock River in Virginia (E USA) [38]. All cultures were maintained in JL-ODRP-2A, 25 ppt, except for *P. mediterraneus* cultured in JL-ODRP-2A, 35 ppt [39]. Cultures in stationary phase were centrifuged at $1000 \times g$, 10 min. The supernatants were discarded and the pellets containing the cells were washed twice in Tris EDTA (TE) buffer and frozen to −20 °C.

A batch of Manila clam *Ruditapes philippinarum* seed with a mean length of 14.5 mm was introduced in The Marano lagoon (N Adriatic Sea, Italy) for on growing. The batch was sampled every ten days and the prevalence of perkinsosis was analysed through histology and incubation in fluid thioglycollate medium. In June 2005, pieces of gill tissue from 60 clams (30.9 mm in mean length; 8.1 g in mean weight) were excised and sub-divided. One piece of gills was incubated in RFTM for a week [2], stained with Lugol's iodine and examined under a dissecting microscopy. A second gill piece was fixed in 95% ethanol to be used as a source for later DNA extraction.

2.2. Genomic DNA extraction and PCR amplification

DNA extractions of cell cultures and tissues were performed employing the DNAzol reagent® (Invitrogen Life Technologies™) according to the manufactures' instructions. rRNA ITS region was amplified using the PerkITS-85 and PerkITS-750 primers [12]. These primers were designed to target the ITS region of all *Perkinsus* species except *P. qugwadi*. PCR reactions were performed in total volume of 25 μl containing 1 μl (= 250–280 ng) of genomic DNA, PCR buffer at 1 × concentration, $MgCl_2$ at 1.25 mM, nucleotides at 0.4 mM (Amersham Biosciences), primers at 0.1 μM and Taq DNA polymerase at 0.025 units/μl (Amersham Biosciences). The cycling protocol was 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, followed by 72 °C for 10 min. A negative control (no DNA) was included in all PCR reactions. PCR products were separated on a 1.5% agarose (in 1X Tris–acetic EDTA buffer) gel, stained with ethidium bromide and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories).

2.3. RFLP profiles

Banding patterns expected by digestions of *Perkinsus* spp. ITS region sequences with various restriction enzymes were determined using Restriction Mapper V. 3.0. (http://www.restrictionmapper.org/). The sequences of *Perkinsus* species deposited in the GenBank database used to construct simulation restriction maps are shown in Table 1. All restriction reactions were carried out in a final volume of 20 μl containing 10 μl of DNA, 2 μl of enzyme buffer, 1 μl of restriction enzyme (*Hinf* I and *Rsa* I) (Amersham Biosciences) and 7 μl of sterilized distilled water. The digestions were performed during 2 h at 37 °C, and 20 min at 65 °C to inactivate the enzymes. To visualize the restriction patterns, aliquots of 10 μl digested samples mixed with 1 μl loading buffer were subject to electrophoresis through 2% MS-4 agarose gel (Pronadisa, Hispanlab, Spain) stained with ethidium bromide and run at 60 V for 1 h. A 50 bp ladder (Amersham Biosciences) was included as molecular weight marker.

2.4. DNA cloning and sequencing

Fresh PCR products from the ITS region amplifications from *Perkinsus* sp. cultures were ligated into cloning vector pCR2.1 (Invitrogen Life Technologies™) at 14 °C overnight and transformed into *E. coli* One Shot Top 10F′ Chemically Competent (Invitrogen Life Technologies™). Transformed cells were screened by PCR using the vector's