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NOTE

# Shellfish tissues evaluated for *Perkinsus* spp. using the Ray's fluid thioglycollate medium culture assay can be used for downstream molecular assays

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ABSTRACT: Ray's fluid thioglycollate medium (RFTM) culture assay is the standard, recommended method for surveillance of *Perkinsus* spp. infections in marine molluscs. In this assay, shellfish tissues are incubated in RFTM, stained with Lugol's iodine solution to render *Perkinsus* spp. cells blue-black, and evaluated microscopically to rate infection intensities. A limitation of this assay, however, is the lack of pathogen species specificity. Generally, identification of *Perkinsus* spp. requires DNA sequence analysis of parallel or additional samples since the exposure to iodine is believed to hamper DNA amplification from samples processed by the RFTM assay. However, we show that *P. marinus* DNA can be successfully amplified by PCR from *Crassostrea virginica* tissues cultured in RFTM and stained with Lugol's iodine. The beneficial consequence is that, where necessary, DNA sequence data may be obtained from RFTM-cultured tissues, allowing the identification of the *Perkinsus* sp. responsible for an observed infection. This would obviate further sampling, representing gain of time and reduction in cost, where a *Perkinsus* sp. is unexpectedly observed in new host(s) or location(s) but where parallel samples are not available for molecular diagnostics. Laboratories without molecular diagnostic tools for *Perkinsus* spp. may fix presumptive *Perkinsus* sp.-positive culture material in 95% ethanol for transport to, and subsequent analysis by, a laboratory that does have this capacity.

KEY WORDS: *Perkinsus* · Dermo disease · RFTM · Lugol's iodine · DNA · PCR



## INTRODUCTION

Protistan parasites in the genus *Perkinsus* infect diverse marine molluscs worldwide including oysters, clams, scallops, and abalone. Currently, 7 *Perkinsus* species are recognized: *P. marinus*, infecting the oyster *Crassostrea virginica* along the Atlantic and Gulf of Mexico Coasts of the USA (Mackin et al. 1950); *P. olseni*, described from the abalone *Haliotis rubra* in Australia but currently known to infect hosts from Asia, New Zealand, South America, and Europe (Lester & Davis 1981, Elston et al. 2003, Villalba et al. 2004); *P. ugwadi*, in the scallop *Patinopecten yessoensis* in British Columbia, Canada (Blackbourn et al. 1998); *P. chesapeaki*, infecting an array of clams (and *C. virginica*) in the mid-Atlantic region of the USA (Burreson et al. 2005); *P. mediterraneus*, from the oyster *Ostrea edulis* in the Balearic Islands (Spain) of the Mediterranean Sea (Casas et al. 2004); *P. honshuensis*, from Japanese Manila clams (Dungan & Reece 2006); and *P. beihaiensis*, from Chinese *Crassostrea* spp. oysters (Moss et al. 2008). Among these species, *P. marinus* and *P. olseni* are economically significant pathogens listed as 'notifiable' by the World Organisation for Animal Health (OIE).

The standard diagnostic method recommended for surveillance of *Perkinsus* spp. infections is Ray's fluid

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thioglycollate medium (RFTM) culture assay. This assay was developed in the early 1950s (Ray 1952, 1966), after *P. marinus* was described in *Crassostrea virginica* in the Gulf of Mexico (Mackin et al. 1950). In this assay, pieces of animal tissue, usually mantle and/or rectum, are incubated in RFTM for several days during which *Perkinsus* spp. cells enlarge from 2–10 µm to 50–70 µm, forming hypnospores. After incubation, the enlarged cell walls are stained blue-black by the addition of Lugol's iodine, and stained *Perkinsus* spp. cells can be easily observed under a microscope. This assay presents advantages of low cost and simplicity relative to histology, and the infection intensity data collected are semi-quantitative. Furthermore, this diagnostic assay is more sensitive than histological methods (McLaughlin & Faisal 1999). One of the major limitations of the RFTM assay, however, is that it does not allow discrimination among *Perkinsus* spp. In fact, even *Perkinsus* spp. observed histologically cannot be identified to species based on morphological characteristics alone. Rather, identification of *Perkinsus* spp. requires the analysis of DNA sequences, in particular, ribosomal DNA non-transcribed spacer (NTS) and/or internal transcribed spacer (ITS) sequences (Goggin 1994, Marsh et al. 1995, Robledo et al. 1998, Casas et al. 2004, Burreson et al. 2005).

Samples for molecular analysis and DNA-based species identifications by laboratories that have such capabilities are generally preserved from recently shucked animals, while separate tissue samples are used for RFTM assay determination of infection prevalence and intensity; additional tissue samples may be fixed and processed for histology. It is often the case with microparasites like *Perkinsus* spp., however, that the few parasite cells present in a very light infection may be captured in one tissue sample, but not in others. Such animals may be, among other possible outcomes, RFTM-positive for a *Perkinsus* sp. but histology- and PCR-negative. In such cases, the identity of a *Perkinsus* sp. observed by RFTM cannot definitively be resolved. It is also the case that many laboratories are not equipped for molecular diagnostics, and therefore use only RFTM assays, the OIE-recommended gold-standard tool, and histology for surveillance of *Perkinsus* spp. infections. While this may reasonably suffice for routine detection of a *Perkinsus* sp. in a known host from a locality in which a particular *Perkinsus* sp. is known to be enzootic, this is not adequate for establishing new host records, or for drawing conclusions about a parasite's possible geographic range expansion. In examples such as these, it is unfortunate that definitive parasite species identifications cannot be established from RFTM-assayed tissue samples.

To our knowledge, there have been no attempts to PCR-amplify *Perkinsus* sp. DNA from RFTM-cultured, Lugol's iodine-stained material. This may be due to the common belief that the exposure to iodine may compromise downstream PCR amplification (Godhe et al. 2002). This has never been tested, however, in the case of RFTM-iodine processed tissues. If such amplifications were possible, identification of a *Perkinsus* sp. parasite to species would be possible even if parallel samples were not preserved specifically for molecular work, and without the time and expense of resampling a host population. In the reported study, our objective was to determine whether *P. marinus* in tissues processed for RFTM assays and stained with Lugol's iodine would be PCR-amplifiable following microscopic observation.

## MATERIALS AND METHODS

**Samples analyzed.** The oyster samples were *Crassostrea virginica* (n = 67) collected during the fall of 2007 from 2 sites located in the *Perkinsus marinus*-enzootic Virginia portion of the Chesapeake Bay (USA), Tangier Sound (n = 45) and Pocomoke Sound (n = 22).

**RFTM assays.** Upon arrival at the laboratory, oysters were processed for RFTM assays as follows. After oysters had been cleaned and measured, they were shucked, with pieces (5 to 10 $mm^2$) of gill, mantle and rectum excised and immersed in RFTM. Antimycotics and antibiotics comprising 200 units of mycostatin (Nystatin), 500 units penicillin G, and 500 mg dihydrostreptomycin per ml of media were added before placing the tubes into an incubator at 22 to 25°C. After 5 to 7 d, tissues were removed from the RFTM and placed on a glass slide. Then, 1 to 2 drops of 20% (v/v) Lugol's iodine solution were added, and tissues were macerated using a scalpel blade before being coverslipped. Scalpel blades were immersed in 95% EtOH and then flamed between each sample to prevent cross-contamination. The preparation was examined under a microscope, and *Perkinsus* sp. infection intensities were ranked based on the scale of Mackin (Ray 1954): no detection; rare (1 to 2 parasites found in entire preparation); very light (3 to 10 parasites found in entire tissue preparation); light (11 to 100 cells in entire preparation); light–moderate (some areas free of parasites but other areas showing localized concentrations of 24 to 50 cells, or cells uniformly distributed such that 2 to 3 cells occur in each 100× field); moderate (parasites so numerous that >3 cells are present in all fields at 100×, but masses of >50 cells are still more or less localized, tissue not showing blue/black color macroscopically); moderate–heavy (parasite cells present in large num-

bers in all tissues, but less than half of the tissue showing a blue/black color macroscopically); or heavy (parasite cells in enormous numbers, with the major part of tissue appearing blue/black macroscopically).

**DNA extraction.** Once the preparations had been examined under a microscope and *Perkinsus* sp. infections had been rated, the coverslip from each sample was removed using a scalpel, and tissues were scraped into a tube containing 10 volumes of 95% ethanol per 1 volume of tissue. As described earlier, scalpel blades were flamed between samples to minimize the risks of contamination from sample to sample. DNA was extracted from ethanol-preserved tissue using a DNeasy Tissue Kit (Qiagen). The volumes of lysis buffer and Proteinase K added were adjusted to the volume of oyster tissues preserved so all the tissue collected from each RFTM slide could be lysed. In general, the volume of lysis buffer provided by the kit was 720 µl and the volume of Proteinase K was 80 µl. After lysis, a subsample of 400 µl of the lysate was processed for DNA extraction following the manufacturer's protocol. DNA was quantified with a GeneQuant Pro spectrophotometer (Amersham Biosciences).

**PCR assays.** PCR was performed on each of the extracted DNA samples using *Perkinsus* genus ITS-1 rDNA-specific primers PerkITS (Audemard et al. 2004), *P. marinus* ITS-1 rDNA-specific primers PmarITS (Audemard et al. 2004), and *P. chesapeaki* ITS-1 rDNA-specific primers PchesITS (Burreson et al. 2005). The sizes of the amplified PCR fragments were 703, 509, and 670 base pairs, respectively. Reaction and amplification conditions were as previously described (Audemard et al. 2004, Burreson et al. 2005). The volume of DNA per reaction ranged from 0.5 to 2.0 µl depending on the DNA concentration of the sample, such that the amount of DNA template per PCR reaction was ~200 ng. Each PCR experiment included a known *Perkinsus* sp.-positive sample as a positive control, and a no-template sample (DNA replaced with water) as a negative control. Amplification products were electrophoresed in 2% agarose gels (in 1× Tris-Acetate EDTA buffer), stained with ethidium bromide, and visualized under UV light.

## RESULTS

### RFTM assay results

Among the oysters analyzed, infection intensities based on RFTM assay results ranged from rare to heavy, with most of the infected oysters (37/67) characterized by light–moderate infections. *Perkinsus* sp. was not detected by RFTM assays in 9 individuals (Table 1).

Table 1. Results from RFTM assays (number of oysters according to categories of infection intensity) and subsequent PCR assays on the same individuals with the number (percentage) of PCR positive per infection category. *Perkinsus chesapeaki* was not detected in any sample

| RFTM infection intensity | No. of oysters | No. of PCR positive (%) *Perkinsus* spp. | *P. marinus* |
|---|---|---|---|
| Negative | 9 | 9 (100) | 8 (88) |
| Rare | 5 | 5 (100) | 3 (60) |
| Very light | 6 | 6 (100) | 6 (100) |
| Light | 12 | 12 (100) | 11 (92) |
| Light–moderate | 25 | 25 (100) | 25 (100) |
| Moderate | 7 | 7 (100) | 7 (100) |
| Moderate–heavy | 2 | 2 (100) | 2 (100) |
| Heavy | 1 | 1 (100) | 1 (100) |

### DNA concentrations

DNA extracted from the all RFTM-Lugol's iodine processed samples could be quantified, with DNA concentrations ranging from 28 to 520 ng $\mu l^{-1}$.

### PCR results

Using the *Perkinsus* genus-specific primers (PerkITS), *Perkinsus* DNA was amplified by PCR in each of the tissue samples testing positive by RFTM assays (n = 58, Table 1). Interestingly, PCR amplifications were also observed in all tissue samples that were negative by RFTM assays (9/9).

When the same DNAs were tested using the *Perkinsus marinus*-specific PCR assay, amplifications were observed for a majority of samples (55/58) testing positive by RFTM assays (Table 1). As observed with PerkITS primers, *P. marinus* was also detected by PCR in samples found negative by RFTM assays (8/9 samples). Finally, with the *P. chesapeaki*-specific PCR assay, *P. chesapeaki* was not detected in any of these samples.

## DISCUSSION

We have demonstrated that DNA can be extracted and PCR-amplified successfully from a majority of RFTM-Lugol's iodine-processed samples. To our knowledge, this study was the first to investigate whether molecular tools could be applied to shellfish tissues processed by RFTM and stained with Lugol's iodine. Although a previous study showed that DNA could be extracted and amplified by PCR from tissues incubated in RFTM, it did not investigate the potential use of molecular tools on shellfish tissues exposed to iodine solution (Novoa et al. 2002). The potential bind-

ing of iodine to DNA, thereby making DNA inaccessible to amplification, has been postulated as a reason for the absence of PCR amplification observed for some Lugol's iodine-fixed samples (Marin et al. 2001, Godhe et al. 2002). However, other studies have shown that PCR amplification could be obtained after exposure of dinoflagellates or nematode eggs to Lugol's iodine (Tengs et al. 2001, Galluzzi et al. 2004, Harmon et al. 2007). Discrepancies between these studies may be due to the type of sample analyzed or the duration of the fixation in Lugol's iodine. In our case, the exposure to Lugol's iodine was less than 3 h, which may have prevented excessive DNA damage.

Using the same tissue sample, it was possible to first detect a *Perkinsus* spp. infection using RFTM-Lugol's iodine, and then to identify the *Perkinsus* species responsible for this infection by PCR. The detection by PCR of *P. marinus* but not of the sympatric *P. chesapeaki* suggests that the *Crassostrea virginica* analyzed in this study were only infected by *P. marinus*, a *P. marinus* host specificity that was previously postulated by La Peyre et al. (2006). Using the PerkITS assay, amplifications were observed for all of the samples found positive by RFTM assays as well as for the samples testing negative by RFTM assays. Using the PmarITS assay, amplifications were observed for all the samples testing positive by RFTM assays except for a few rare or light infections. Using this PCR assay, *P. marinus* DNA was also detected in a majority of samples testing negative by RFTM assays. This could be due to a higher sensitivity of these PCR assays, and of the PerkITS assay in particular, compared to RFTM assays, as demonstrated for other PCR assays for *P. marinus* (Marsh et al. 1995, Robledo et al. 1998, Yarnall et al. 2000, Gauthier et al. 2006). As previously suggested (Audemard et al. 2004), the PerkITS assay may be slightly more sensitive than the PmarITS assay, since the overall number of positive PCR results was higher with PerkITS than with PmarITS. Nevertheless, most of the infections detected by RFTM assays were associated with PCR amplification using PmarITS primers.

Being able to apply PCR to DNAs from RFTM-Lugol's iodine-processed samples could be particularly useful in the context of a *Perkinsus* sp. survey in a new host species or outside the known geographical range of a particular *Perkinsus* species. In such studies (e.g. Park & Choi 2001, Leethochavalit et al. 2004), RFTM assays were used to detect *Perkinsus* spp. infections due to low cost, simplicity of use, and sensitivity. The identification of the species responsible for the detected infections, however, necessitated further sampling or further studies where tissue samples were specifically collected for molecular analysis (Park et al. 2005). Here, we demonstrated that when such surveys are conducted, tissues found positive by RFTM-Lugol's iodine assays can be preserved in 95% ethanol for further molecular analysis, providing a gain of time and a reduction of the cost since only the positive samples for RFTM assay need to be processed using the costly molecular tools. The DNA extracted from these tissues can then be analyzed by PCR and sequenced if necessary. Preservation in 95% ethanol in particular will allow laboratories without molecular biological capabilities to store post-RFTM assays *Perkinsus* sp.-positive tissues stably and indefinitely, until they can be transported to a suitable laboratory for further characterization.

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