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AN IMPROVED PROCEDURE TO COUNT *PERKINSUS MARINUS* IN EASTERN OYSTER HEMOLYMPH

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ABSTRACT *Perkinsus marinus* infection intensity in *Crassostrea virginica* can be quantified without killing of oysters by determining parasite density in hemolymph samples incubated in fluid thioglycollate medium (FTM). The goal of this study was to improve existing protocols for counting of *P. marinus* in oyster hemolymph. Specifically, the objectives were to examine the effects on parasite number and diameter of: 1) adding supplements to FTM such as lipid and oyster extract; 2) incubating with various FTM preparations with and without agar or beef extract; 3) incubating with various hemocyte densities (10^5, 10^6, and 10^7 hemocytes/mL of FTM) in a constant FTM volume; 4) incubating with different volumes of FTM (0.2 mL, 1.0 mL, 5.0 mL, and 25.0 mL); and 5) sodium hydroxide digestion of cellular debris. From these results, an improved hemolymph protocol was developed. The diameters and numbers of enlarged parasites or hypnospores in hemolymph of 20 oysters measured by the improved protocol and the standard FTM hemolymph assay of Gauthier and Fisher were compared. Finally, the standard and improved protocols were compared with the FTM body burden assay. The diameter of hypnospores from samples processed with the improved protocol (26 ± 13 μm) was significantly greater than the diameters from samples processed with the standard protocol (10 ± 4 μm). The number of hypnospores in samples processed with the improved protocol ($8.6 \times 10^3 \pm 3.3 \times 10^3$) was significantly greater than the numbers in samples processed with the standard protocol ($1.9 \times 10^3 \pm 3.4 \times 10^3$). Results of the body burden assay were significantly correlated with results of the standard hemolymph assay and with results of the improved hemolymph assay. The coefficient of determination ($r^2 = 0.7602$) and slope (0.91189) of the regression of the FTM body burden assay against the improved FTM hemolymph assay was improved from the coefficient of determination (0.5543) and slope (0.61257) of the regression of the FTM body burden assay against of the standard FTM hemolymph assay.

KEY WORDS: dermo, *Perkinsus marinus*, FTM hemolymph diagnostic assay, *Crassostrea virginica*

INTRODUCTION

Use of a protocol to quantify the presence of the pathogenic protozoan *Perkinsus marinus* by determining the number of enlarged parasites, or hypnospores, in hemolymph samples of eastern oysters, *Crassostrea virginica*, allows estimation of infection intensity without killing oysters (Gauthier & Fisher 1990). Therefore, infection intensity in the same animal can be monitored over time. In the existing protocol, hemolymph from the adductor muscle sinus is removed, centrifuged, and the cell pellets containing oyster hemocytes and parasites are incubated in fluid thioglycollate medium (FTM) for 1 week. After incubation, cellular debris is digested with sodium hydroxide (NaOH). The hypnospores are stained with Lugol's solution and their numbers per mL determined. This FTM hemolymph assay has been useful to follow the progression of the disease in individual oysters under different environmental conditions (Fisher et al. 1992, Ragone Calvo & Burreson 1994).

Gauthier and Fisher (1990) proposed additional advantages of the FTM hemolymph assay, including the detection of early infections and the measurement of systemic infections rather than localized infections. In their initial study, the FTM hemolymph assay detected many infections misdiagnosed as negative by Ray's FTM tissue assay. In Ray's FTM tissue assay, a piece of oyster tissue (e.g., mantle tissue in Gauthier and Fisher's 1990 study) is incubated in FTM for about a week, the tissue is then smeared on a slide, and the parasites stained with Lugol's solution and the intensity of infection are estimated using a semi-quantitative scale (Ray et al. 1953, Ray 1954a, Ray 1954b). Bushek et al. (1994), however, found no evidence that the FTM hemolymph assay was more sensitive than the FTM tissue assay in detecting low *P. marinus* infections when both mantle and rectal tissues were used in the tissue assay. Moreover, correlations between FTM tissue and FTM body burden assays were always higher than correlations between FTM hemolymph and FTM body burden assays. The body burden assay measures the number of parasites in the whole oyster and is considered the most sensitive and accurate diagnostic assay for *P. marinus* (Bushek et al. 1994, Fisher & Oliver 1996, Oliver et al. 1998).

A quantitative competitive polymerase chain reaction (QCPCR) assay for *P. marinus* was recently shown to be more sensitive than the FTM hemolymph assay, detecting infections in 24 oysters compared with 22 infections with the FTM hemolymph assay (Yarnall et al. 2000). Diagnosis of *P. marinus* in oyster hemolymph by QCPCR detected as many infections as with the FTM body burden assay, suggesting that hemolymph is suitable for determining *P. marinus* infection in oysters. Whereas QCPCR is an effective assay and specific for *P. marinus*, it is also expensive and requires technical expertise and equipment in molecular biology to perform as indicated by Yarnall et al. (2000). The FTM hemolymph assay in contrast is easy and inexpensive to perform. There is thus a need to improve the sensitivity and accuracy of the FTM hemolymph assay.

Potential problems with FTM assays have been previously noted and include insufficient parasite enlargement, parasite clumping, and parasite adherence to the walls of centrifuge tubes, each of which can lower parasite counts (Bushek et al. 1994, Fisher & Oliver 1996). Poor parasite enlargement may be caused

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by exhaustion of necessary nutrients in FTM or tissue (Ray 1954a, Bushek et al. 1994, Fisher & Oliver 1996). In addition, the protocols used in FTM hemolymph assay varied greatly among researchers in sample preparation (e.g., centrifugation speed), sample processing, and in parasite counting protocols (Gauthier & Fisher 1990, Ragone Calvo & Burreson 1994, Bushek et al. 1994, Oliver et al. 1998). It is likely that these recognized problems and differences in protocols account for the poor performance (i.e., sensitivity and accuracy) of the FTM hemolymph assay.

The goal of this study was to improve the protocol for counting of *P. marinus* in oyster hemolymph through systematic examination of components of the standard protocol described by Gauthier and Fisher (1990). The objectives were to examine the effects of: (1) adding supplements to FTM, including lipid and oyster extract; (2) adding various FTM preparations (with and without agar or beef extract); (3) incubating with various hemocyte densities (10^5, 10^6, and 10^7 hemocytes/mL of FTM) in a constant FTM volume; (4) incubation of hemocytes in different volumes of FTM (0.2 mL, 1.0 mL, 5.0 mL, and 25.0 mL); and (5) NaOH digestion of cellular debris on hypnospore diameter and number.

MATERIALS AND METHODS

Oysters

Eastern oysters were collected from Hackberry Bay (29°23′54″N, 90°28″W) in the spring of 1999, placed in 16 mm mesh shellfish cages (Aquatic Eco-Systems, Inc., Apopka, FL), and hung in the water from docks at the Louisiana Sea Grant Oyster Hatchery (29°12′30″N, 90°02′30″W) in Grand Isle, Louisiana, an area enzootic for *P. marinus*. In the summer and fall of 1999, oysters were transported to the Department of Veterinary Science at Louisiana State University, Baton Rouge, and were placed in an indoor recirculating system equipped with 1 μm and 10 μm cotton filters in polypropylene filter cartridges and an ultraviolet light. Water was maintained at 15 ppt with hw-Marinemix *Professional* sea salts (Hawaiian Marine Imports Inc., Houston, TX) and 25°C.

General Procedures

All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. A notch was ground into the edge of oyster shells near the adductor muscle and 3 mL of hemolymph were withdrawn from the adductor muscle sinus. Hemolymph samples were transferred to 3 mL snap-cap tubes and immediately placed on ice to prevent hemocyte clumping. The number of hemocytes per mL of hemolymph was determined using a Neubauer Bright-Line hemocytometer (Reichert, Buffalo, NY). Hemolymph samples from each oyster, containing 10^6 hemocytes, were added to 1.5 mL microcentrifuge tubes, centrifuged at 800 × g for 10 min, and the supernatant was discarded. All samples were prepared in triplicate. Pellets were resuspended in 1 mL of alternative fluid thioglycollate medium (AFTM) (Sigma number A 0465) prepared according to the manufacturer's instructions and supplemented with 16 g/L of hw-Marine *Professional* sea salts and 50 μg/mL chloramphenicol. This solution, referred to as AFTM throughout this report, did not contain agar, unlike FTM (Sigma number T 9032), which contains 0.75 g/L of agar. Samples were layered with 10 μL of nystatin (5,000 Units/mL) to prevent fungal growth. Test tubes were stored in the dark at room temperature for seven days, allowing parasites within hemocytes to enlarge to hypnospores. After incubation, samples were centrifuged at 1500 × g for ten min and the AFTM supernatant was discarded. Pellets were resuspended in 1 mL of 2 N NaOH and incubated in a 60°C water bath for 1 to 2 h to digest hemocyte debris. Samples were centrifuged to remove NaOH and hypnospores were rinsed three times with 0.1 M phosphate-buffered saline (PBS) containing 0.5 mg/mL bovine serum albumin (BSA). In a previous study, it was found that BSA decreased parasite clumping and improved parasite recovery by reducing the number of parasite that adhered to the test tube walls (Coates et al. 1999). Samples were stored at 4°C in 1 mL of PBS supplemented with BSA and 2 mg/mL of sodium azide. At the time of counting, samples were centrifuged, and 900 μL of supernatant were removed. Each 100-μL sample was transferred to a separate well of a 96-well tissue culture plate. Fifty microliters of each sample was transferred to wells containing 50 μL of PBS with BSA to form a 1:1 dilution. Samples were serially diluted in this manner until a 1:64 dilution was reached. Lugol's solution (50 μL; 0.012 g/mL of potassium iodide and 0.008 g/mL of iodine in water) was added to each well to stain the samples. Tissue culture plates containing samples were centrifuged at 200 × g for 5 min to form a monolayer of hypnospores on the plate bottoms to facilitate counting. Numbers of hypnospores were recorded at 200-X magnification using an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) from wells containing 100 to 400 hypnospores. Cells were counted at 400× when they were too small to identify at 200×. The diameter (μm) of 50 hypnospores from each sample was measured with an ocular micrometer, and the number of hypnospores per 10^6 hemocytes was calculated.

Experiments

Effects of Supplemented AFTM on Hypnospore Diameter and Number

Hemolymph was collected from five oysters as described above. AFTM, supplemented with 5% lipid concentrate (Gibco, Gaithersburg, MD), 5% oyster extract, or 5% oyster saline, was added to 10^6 hemocytes from each oyster. Oyster extract was prepared by homogenizing whole oyster tissue in oyster saline at a concentration of 0.2 g wet tissue per mL, centrifuging at 10,000 × g for 15 min, removing the oyster extract (supernatant), and storing it at −20°C. Oyster saline (0.95 g/L $CaCl_2 \cdot 2H_2O$, 1.46 g/L $MgSO_4$, 2.18 g/L $MgCl_2 \cdot 6H_2O$, 0.67 g/L KCl, 11.61 g/L NaCl, and 0.35 g/L $NaHCO_3$) was included as a control. Samples were incubated, processed, counted, and measured as described above. Lipid was found to increase hypnospore diameter and was added to FTM in all further experiments.

Effect of FTM Types on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above and five formulations of FTM, supplemented with 16 g/L of hw-Marine *Professional* sea salts, 50 μg/mL chloramphenicol, and 5% lipid concentrate, were added to 10^6 hemocytes samples. The formulations compared were Bacto fluid thioglycollate medium (Becton Dickinson and Company, Franklin Lakes, NJ, Difco number 0256154), Bacto thioglycollate medium with K agar (Difco number 0607178), Bacto fluid thioglycollate medium with beef extract (Difco number 0697179), alternative fluid thioglycollate medium (Sigma number A 0465), and thioglycollate medium (Sigma num-

ber T 9032) (Table 1). Samples were incubated, processed, measured, and counted as described above.

Effect of Hemocyte Density on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above and 10^5, 10^6, and 10^7 hemocytes from each oyster were incubated in 1 mL of AFTM supplemented with 5% lipid concentrate. For this experiment, the number of hypnospores per hemocyte was calculated, and hypnospore diameter was measured as described above.

Effect of AFTM Volume on Hypnospore Diameter and Number

Hemolymph was collected from 15 oysters as described above. Hemocytes (10^6) were incubated in 0.2, 1.0, 5.0, or 25.0 mL of AFTM supplemented with 5% lipid concentrate. Samples were processed, measured, and counted as described above.

Effect of NaOH Digestion of Samples on Hypnospore Diameter and Number

Hemolymph samples from 15 oysters were processed with and without NaOH digestion after incubation in AFTM supplemented with 5% lipid concentrate. Samples processed without NaOH digestion were centrifuged at 1500 × g for 10 min to remove the AFTM supernatant. Cell pellets were rinsed three times with sterile artificial seawater (hw-Marinemix *Professional*) at 15 ppt and containing 0.5 mg/mL BSA. Samples processed with NaOH digestion were treated as described in the General Procedures section.

Effect of Protocol on Hypnospore Diameter and Number

From the results of the above experiments, an improved protocol for counting of *P. marinus* in oyster hemolymph was developed and compared with the 'standard' protocol of Gauthier and Fisher (1990). Major differences between the standard and improved protocols are summarized in Table 2. Briefly, hemolymph samples were collected from 20 oysters infected with *P. marinus*. Hemolymph samples were divided into two equal aliquots, one aliquot to be processed with the standard protocol and the other to be processed with the improved protocol. Hemocytes (10^6) from samples processed with the standard protocol were incubated for seven days in 1 mL of FTM (Sigma number T 9032) supplemented with 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol, and layered with 10 μL of nystatin (5000 Units/ml). Hemocyte debris was digested with 1 mL of 2 N NaOH, and hypnospores were rinsed three times with 1 mL of distilled water. Samples were centrifuged, 900 μL of supernatant were removed, and each 100-μL sample was transferred to a separate well of a 96-well plate, where it was serially diluted in distilled water and stained with Lugol's solution. Standard protocol centrifugation speeds were increased to the centrifugation speeds of the improved protocol to exclude their effects on parasite recovery. Hemocytes (10^6) from samples processed with the improved protocol were incubated for seven days in 1 ml of AFTM (Sigma number A 0465) supplemented with 5% lipid, 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol, and layered with 10 μL of nystatin (5000 Units/mL). Hemocyte debris was digested with 1 mL of 2 N NaOH and hypnospores were rinsed once with 1 mL of distilled water supplemented with 0.5 mg/mL BSA and twice with 1 mL PBS supplemented with 0.5 mg/mL BSA. Samples were centrifuged, 900 μL of supernatant were removed, and each 100-μL sample was transferred to a separate well of a 96-well plate, where it was serially diluted in PBS supplemented with BSA and stained with Lugol's solution. After processing samples with both protocols, the number of hypnospores was counted 200× magnification in wells containing 100 to 400 hyp-

TABLE 2.

Differences between the standard protocol and the improved protocol to count *Perkinsus marinus* in oyster hemolymph.

Component	Standard	Improved
Type of FTM	FTM (with agar)	Alternative FTM (without agar)
FTM Supplement	None	5% lipid concentrate
Rinsing solution	Distilled water	Phosphate-buffered saline (0.1 M) with 0.5 mg/mL bovine serum albumin

TABLE 1.

Components of different brands of fluid thioglycollate media.

	Difco[a]			Sigma[a]	
Component	Bacto FTM	Bacto FTM With Potassium Agar	Bacto FTM With Beef Extract	Alternative FTM	FTM
Beef extract	—	—	5 g	—	—
Yeast extract	5 g	5 g	5 g	5.00 g	5.0 g
Casein digest	15 g	15 g	15 g	15.00 g	15.0 g
Dextrose	5.5 g	5 g	5.500 g	5.50 g	5.5 g
Sodium chloride	2.5 g	—	2.5 g	2.50 g	2.5 g
Potassium chloride	—	2.5 g	—	—	—
L-Cystine	0.5 g	0.5 g	0.5 g	0.50 g	0.5 g
Sodium thioglycollate	0.5 g	0.3 mL[b]	0.5 g	0.50 g	0.50 g
Agar	0.75 g	0.45 g	0.75 g	—	0.75 g
Resazurin	0.001 g	0.001 g	0.001 g	—	0.001 g

[a] Decimal places reported as suggested by manufacturer.
[b] Thioglycollic acid.

nospores after dilutions. The diameter of 50 hypnospores was measured from each hemocyte sample.

Comparison of Standard and Improved FTM Hemolymph Assay Against FTM Body Burden Assay

The standard and improved FTM protocols for counting *P. marinus* in oyster hemolymph (as described above) were compared with the FTM body burden assay for determining the number of parasites in whole oyster and a regression line was calculated for each comparison. The regression lines were compared with determine if infection intensities obtained from the improved hemolymph protocol were more closely correlated with infection intensities obtained from the body burden assay than those obtained from the standard hemolymph protocol.

The 20 oysters from which hemolymph was removed for comparison of the standard and improved hemolymph protocols, were processed using modification of the body burden assay of Fisher and Oliver (1996) (Coates et al. 1999). Briefly, each oyster was removed from the shell, blotted dry, and homogenized with a hand-held Biohomogenizer, a stainless-steel rotor/stator emulsifying instrument, (Biospec Products, Inc., Batlesville, OK, catalog number 1281) in 20 mL of sterile artificial seawater prepared at 15 ppt with hw-Marine *Professional* sea salts. One milliliter of homogenate was transferred to 9 mL of AFTM (Sigma number A 0465) supplemented with 16 g/L of hw-Marinemix *Professional* sea salts, 50 μg/mL of chloramphenicol 5% lipid, and layered with 100 μL of nystatin (5000 Units/mL), and incubated for seven days. Samples were centrifuged, the supernatant removed, and 10 mL of 2 N NaOH added for 4 to 5 h at 60°C to digest the oyster tissue. Hypnospores were rinsed once with 10 mL of distilled water supplemented with 0.5 mg/mL BSA and twice with 10 mL PBS supplemented with 0.5 mg/mL BSA. Samples were centrifuged, 5 mL of supernatant were removed, 50 μL of each sample were transferred to a separate well of a 96-well plate, where it was serially diluted in PBS supplemented with 0.5 mg/mL BSA and stained with Lugol's solution. The number of hypnospores was counted (200× magnification) in wells containing 100 to 400 hypnospores after dilutions. The number of hypnospores per gram of oyster tissue was calculated for each oyster and compared with the number of hypnospores per 10^6 hemocytes determined by the standard and improved hemolymph protocols using linear regression analysis.

Statistical Analysis

Statistical analysis was performed using SAS Version 8.0 software (SAS Institute, Inc. Cary, NC). Only hemolymph samples containing at least 50 hypnospores (per triplicate) were used data analysis. Data were log transformed and analyzed with a randomized block design (blocked by oyster). Tukey's post-ANOVA test was used to examine differences among treatments. Differences were considered significant at $P < 0.05$. All data were reported as mean ± standard deviation. To compare the standard and improved FTM hemolymph assays against the FTM body burden assay, two linear regressions were calculated using log-transformed data.

RESULTS

Effects of Supplemented AFTM on Hypnospore Diameter and Number

The diameter of hypnospores from samples incubated in AFTM supplemented with lipid concentrate (27.3 ± 11.5 μm) was significantly greater ($P < 0.0001$) than the diameter of hypnospores from samples incubated in AFTM supplemented with oyster extract (18.8 ± 9.4 μm) or with saline (16.5 ± 10.2 μm) (Fig. 1). No significant difference in hypnospore diameter was found between samples incubated in AFTM supplemented with oyster extract or with saline. No significant difference was found in the hypnospore numbers between samples incubated in AFTM supplemented with lipid concentrate ($1.9 \times 10^5 \pm 7.1 \times 10^4$), oyster extract ($1.8 \times 10^5 \pm 4.7 \times 10^4$) or the saline control ($2.2 \times 10^5 \pm 6.6 \times 10^4$).

Effect of FTM Types on Hypnospore Diameter and Number

No significant differences were found for hypnospore diameter or hypnospore number among samples incubated in different formulations of FTM (Table 3). The absence of agar in the Sigma alternative fluid thioglycollate medium facilitated sample processing because the viscous layer that generally collected over hypnospore pellets after centrifugation when samples were incubated in FTM with agar was eliminated.

Effect of Hemocyte Density on Hypnospore Diameter and Number

The diameter of hypnospores from samples that received 10^5 hemocytes per ml of AFTM (19.9 ± 8.8 μm) was significantly greater ($P < 0.001$) than the diameter of hypnospores from samples that received 10^6 hemocytes per ml (15.3 ± 8.7 μm) and 10^7 hemocytes per ml (15.2 ± 12.1 μm) (Fig. 2). No significant difference was found in hypnospore diameters between samples that received 10^6 hemocytes per ml and 10^7 hemocytes per ml. No significant difference was found in the number of hypnospores per hemocyte among samples that received 10^5 hemocytes per ml (0.06 ± 0.15 hypnospores/hemocyte), 10^6 hemocytes per ml (0.67 ± 2.26 hypnospores/hemocyte) and 10^7 hemocytes per ml (0.03 ± 0.07 hypnospores/hemocyte).

Effect of AFTM Volume on Hypnospore Diameter and Number

The diameter of hypnospores from samples that were incubated in 5.0 mL (24 ± 12 μm) and 25.0 mL (28 ± 13 μm) of AFTM was significantly greater ($P < 0.0001$) than the diameter of hypnospores from samples incubated in 0.2 mL (13 ± 6 μm) and 1.0 mL (16 ± 8 μm) of AFTM (Fig. 3A). However, the number of hypnospores in samples incubated in 25.0 mL of AFTM ($4.5 \times 10^4 \pm 6.9 \times 10^4$) was significantly lower ($P < 0.0002$) than the number of hypnospores in samples incubated in 0.2 mL ($9.3 \times 10^4 \pm 1.1 \times 10^5$), 1.0

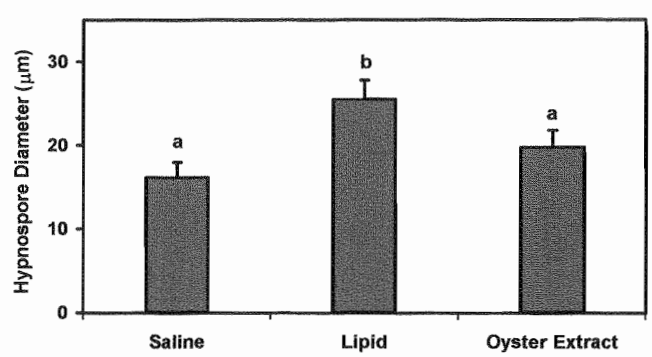

Figure 1. Hypnospore diameter (mean ± SD, $n = 5$) after incubation in AFTM supplemented with lipid concentrate, oyster extract, and saline (control). Hemocytes (10^6) were incubated for 7 days in 1 mL of supplemented AFTM. Treatments sharing a letter were not significantly different.

TABLE 3.

Average values (mean ± SD, $n = 9$) of hypnospore diameter and number of hypnospores per 10^6 hemocytes after incubation in different types of FTM.

Type of FTM	Hypnospore Diameter (μm)	Number of Hypnospores
Difco Bacto FTM	21 ± 11	$8.3 \times 10^4 \pm 1.1 \times 10^5$
Difco Bacto FTM with K agar	20 ± 12	$1.8 \times 10^5 \pm 3.3 \times 10^5$
Difco Bacto FTM with beef extract	19 ± 11	$1.2 \times 10^5 \pm 1.7 \times 10^5$
Sigma alternative FTM	18 ± 11	$2.0 \times 10^5 \pm 4.0 \times 10^5$
Sigma FTM	22 ± 13	$1.8 \times 10^5 \pm 3.1 \times 10^5$

mL ($1.8 \times 10^5 \pm 2.7 \times 10^5$), and 5.0 mL ($1.2 \times 10^5 \pm 1.5 \times 10^5$) of AFTM (Fig. 3B). No significant difference was found in the number of hypnospores among samples incubated in 0.2, 1.0, and 5.0 mL of AFTM.

Effect of NaOH Digestion of Samples on Hypnospore Diameter and Number

No significant difference was found in hypnospore diameter between samples processed with NaOH digestion (24 ± 17 μm) or without it (19 ± 9 μm). No significant difference was found in the number of hypnospores between samples processed with NaOH digestion ($1.8 \times 10^4 \pm 2.6 \times 10^4$) or without it ($2.6 \times 10^4 \pm 3.8 \times 10^4$). Sodium hydroxide facilitated counting by reducing cellular debris.

Effect of Protocol on Hypnospore Diameter and Number

Hypnospore diameter from samples processed with the improved protocol (26 ± 13 μm) was significantly greater ($P < 0.0001$; $n = 20$) than the diameter of hypnospores from samples processed with the standard protocol (10 ± 4 μm). Hypnospore numbers in samples processed with the improved protocol ($8.6 \times 10^3 \pm 3.3 \times 10^3$) were significantly greater ($P < 0.0001$) than the number of hypnospores in samples processed with the standard protocol ($1.9 \times 10^3 \pm 3.4 \times 10^3$). Hypnospores from hemocytes processed with the improved protocol readily settled to the plate bottom and could be easily counted. Hypnospores processed with the standard protocol were difficult to count because they remained suspended in the wells and the media column had to be scanned vertically as a consequence (Fig. 4).

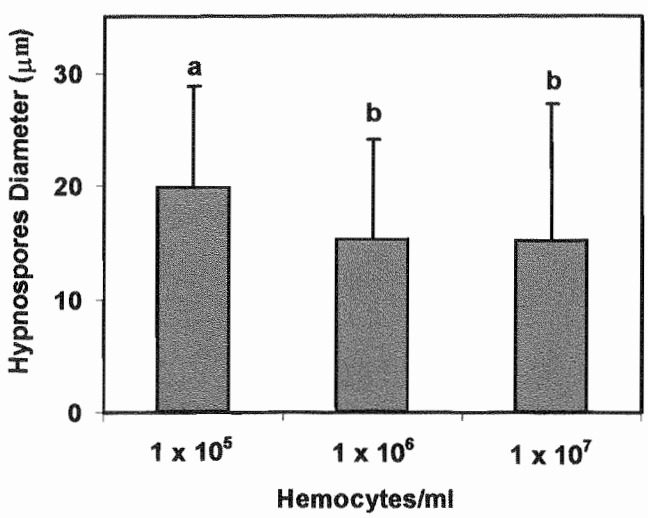

Figure 2. Effect of hemocyte density (10^5, 10^6, or 10^7 hemocytes per ml of AFTM) on hypnospore diameter (mean ± SD, $n = 13$). Hemocytes were incubated in 1 mL of AFTM supplemented with 5% lipid for seven days. Means sharing a letter were not significantly different.

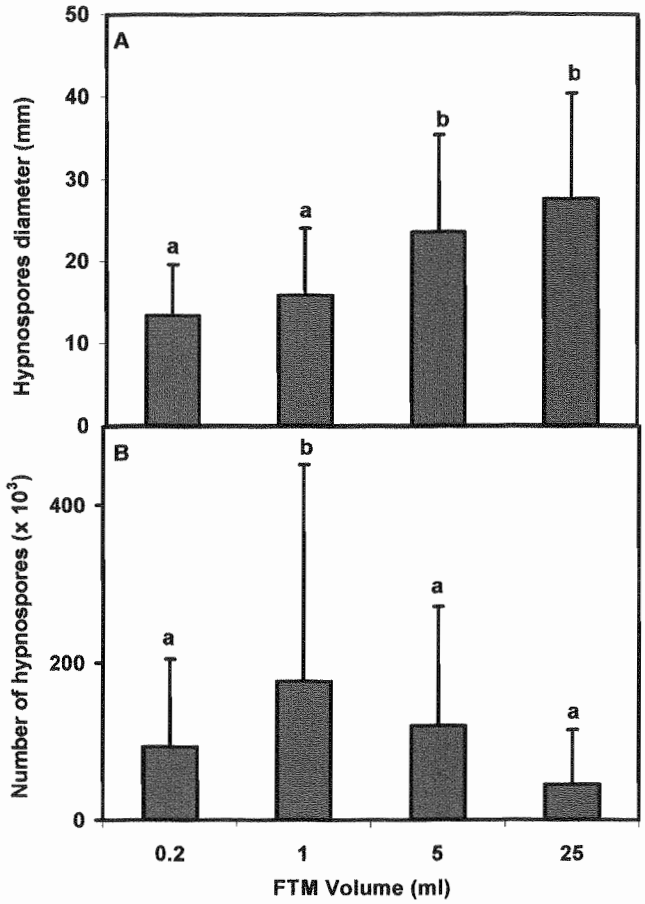

Figure 3. Hypnospore diameter (A) and number (B) (mean ± SD, $n = 10$) after incubation in 0.2, 1.0, 5.0, and 25.0 mL of AFTM. Hemocytes (10^6) were incubated for seven days in 1 mL of AFTM supplemented with 5% lipid. Means sharing a letter in each panel were not significantly different.

Comparison of Standard and Improved FTM Hemolymph Assays with FTM Body Burden Assay

Using linear regression, comparison of the infection intensity determined by the body burden assay (log of hypnospores per gram of oyster tissue) and the infection intensity determined by the standard hemolymph assay (log of hypnospores per 10^6 hemocytes) showed that the correlation was highly significant ($P < 0.0001$) with a coefficient of determination (r^2) of 0.5543 and a slope for the regression line of 0.6126 (Fig. 5A). Using linear regression, comparison of the infection intensity determined by the body burden assay and the infection intensity determined by the improved hemolymph assay showed a highly significant correlation ($P < 0.0001$) with an improved coefficient of determination ($r^2 = 0.7602$) and slope (0.9119) (Fig. 5B). This increased coefficient of determination indicated a reduction in variance with the improved FTM hemolymph assay.

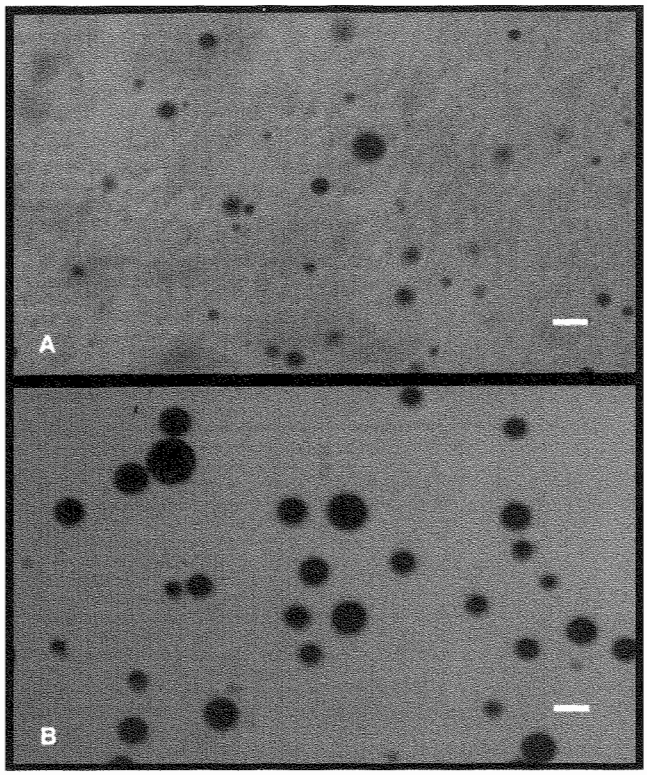

Figure 4. Light micrographs of *Perkinsus marinus* hypnospores after incubation and processing with the standard and improved protocols at 200× magnification. Bar represents 30 µm. A, Hypnospores were small (7–13 µm) and not clearly visible after incubation and processing with the standard protocol. B, Hypnospores were larger (15–44 µm) and more visible after incubation and processing with the improved protocol.

DISCUSSION

Use of the improved protocol to count *P. marinus* in hemolymph resulted in a 167% increase in hypnospore diameter and a 358% increase in hypnospore number over the standard protocol. The improved protocol also facilitated sample processing and counting. These improvements were caused by modifications of the standard hemolymph protocol, such as the addition of lipid concentrate to AFTM, the use of FTM without agar, and the addition of BSA to rinsing solutions. These modifications resulted in larger hypnospores, decreased sample viscosity, increased hypnospore recovery, and reduced cellular debris. Because processing, identification, and counting of hypnospores were facilitated, the improved protocol was simpler and more accurate than the standard protocol for counting of hypnospores in hemolymph. As a result, the coefficient of determination (r^2) of the linear regression between *P. marinus* $\log_{10}$ numbers in oyster body and *P. marinus* $\log_{10}$ numbers in hemolymph increased from 0.554 to 0.760 when the improved hemolymph protocol instead of the standard hemolymph protocol was used to count *P. marinus* in hemolymph.

Results from previous studies have suggested that nutrients from FTM and oyster tissues are used by enlarging parasites and that the ratio of FTM to oyster tissue may be critical for optimal hypnospore enlargement (Ray 1954a, Bushek et al. 1994, Fisher & Oliver 1996). The failure of parasites in highly infected oyster tissue to fully enlarge in FTM was proposed to be a result of exhaustion of necessary substances in medium or tissue (Ray 1954a). Hypnospore enlargement was also reported to be greatest in the FTM tissue assay, intermediate in the FTM hemolymph assay and least in the FTM body burden assays and therefore related to the volume of oysters sampled per ml of FTM (Bushek et al. 1994). Moreover, parasite enlargement was inversely related to infection intensity. Using *P. marinus* cultured *in vitro,* parasite enlargement in FTM can be significantly increased by addition of various nutrients including lipids (Wagner et al. 2001). Despite these observations, the effects of supplementing FTM with nutrients such as lipid or oyster extract and the effects of the ratio of FTM to oyster nutrients (e.g., from hemocytes) on parasite enlargement and numbers had not been studied and therefore needed to be investigated to improve the FTM hemolymph assay.

Addition of lipids to AFTM significantly increased hypnospore diameter by 66% over the saline control, and facilitated sample processing and counting because larger hypnospores were more easily pelleted during centrifugation. Lipids were added because the accumulation of numerous lipid droplets in hypnospores is characteristic of this life stage and may be critical to enlargement (Perkins & Menzel 1966, Perkins 1996). It was recently confirmed that lipids are taken up and stored in hypnospores (Soudant et al. 2000). This study found that fluorescent lipid analogs were primarily stored in cytoplasmic lipid droplet after 24 h of incubation but that after 24 h, fluorescence appeared in the membrane and cytosol of hypnospores.

The mean diameter of hypnospores in AFTM supplemented with 5% oyster extract was only 14% greater than the diameter of hypnospores in AFTM supplemented with oyster saline. It was surprising that the addition of oyster extract did not induce significant cell enlargement over saline as oyster extract would be expected to contain high concentrations of nutrients. Although the components of the oyster extract were not examined, it is likely that the water-soluble extract contained only small amounts of oyster lipids, which may account for the minimal enlargement of hypnospores.

Determining the effects of the ratio of infected hemocyte number to AFTM volume on hypnospore formation helped formulate the optimal combination of nutrients, from oyster and AFTM, to promote maximal parasite enlargement. In this study the number of infected hemocytes incubated in a constant volume of AFTM was adjusted, as was the volume of AFTM that had a constant number of infected hemocytes. Hypnospores from the lowest hemocyte density (10^5 cells per ml of AFTM) had the greatest enlargement indicating hypnospores in this treatment had more nutrients available from AFTM. Unfortunately, this low density may not provide an adequate sample size for determining parasite infection intensity. Using a higher number of hemocytes while maintaining hemocyte density (e.g., 10^6 per 10 mL) requires a larger volume of FTM, which in turn may affect the assay. To address this potential effect of volume on the assay, 10^6 hemocytes were incubated in various volumes of AFTM. Hypnospores from 10^6 hemocytes incubated in the largest AFTM volumes (5 and 25 mL) had the greatest enlargement, but there was also a significant decrease in the number of hypnospores from samples incubated in 25 mL of AFTM, indicating that smaller cells may be lost during processing of these volumes. Moreover, large volumes of AFTM requires the purchase of larger amounts of media and antibiotics, which increases cost. The most practical and optimal ratio was set at 10^6 hemocytes to 1 mL of AFTM. At this ratio of infected hemocytes to AFTM volume, parasites had adequate nutrients available for

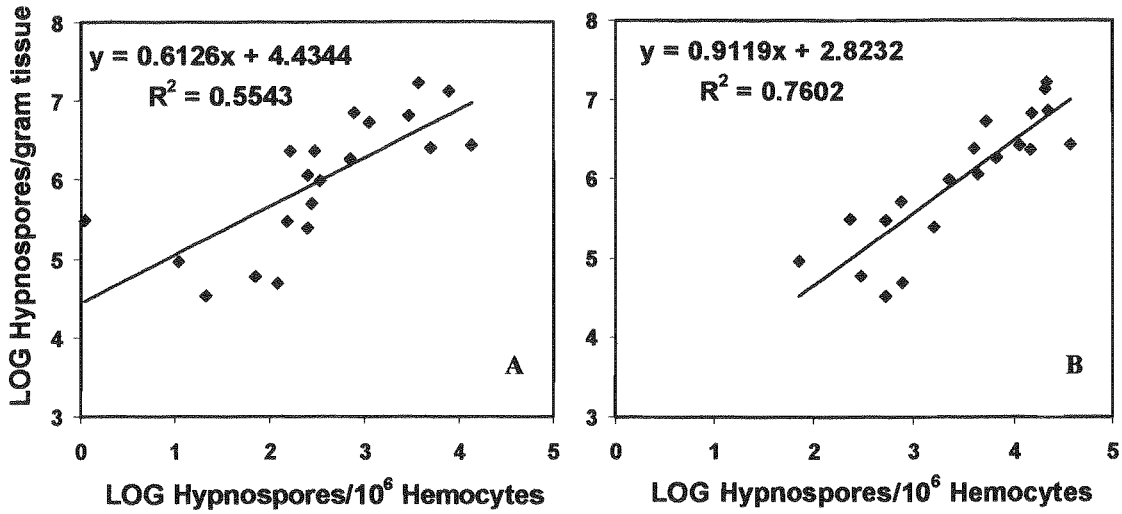

Figure 5. Linear regression lines, formulas, and coefficient of determinations for the comparison of the log of the number of hypnospores per 10^6 hemocytes determined by the standard FTM hemolymph protocol and the log of the number of hypnospores per gram of oyster tissue determined by the body burden assay (A), and for the comparison of the log of the number of hypnospores per 10^6 hemocytes determined by the improved FTM hemolymph protocol and the log of the number of hypnospores per gram of oyster tissue determined by the body burden assay (B).

enlargement in a small AFTM volume while minimizing protocol cost and hypnospore loss.

Perkinsus marinus in hemolymph has exclusively been reported in numbers of parasite per ml of hemolymph (Gauthier & Fisher 1990, Bushek et al. 1994, Oliver et al. 1998, Yarnall et al. 2000). The volume of hemolymph collected per oyster for use in the assay in these past studies varied by as much as a factor of 10, affecting the assay performance. Most parasites, however, are found within the phagosomes of hemocytes (Ray 1954a, Perkins 1996) and the density of hemocytes in hemolymph of bivalves can vary considerably with factors such as temperature, disease and feeding (Feng et al. 1977, Ford & Tripp 1996). *Perkinsus* may, therefore, be better expressed in number of parasites per number of hemocytes (e.g., per 10^6 hemocytes).

The coefficient of determination (r^2) of the linear regression of *P. marinus* $\log_{10}$ numbers in oyster body on *P. marinus* $\log_{10}$ numbers expressed per 10^6 hemocytes was 0.760 (n = 20). When the numbers of *P. marinus* for the same hemolymph samples were expressed per ml of hemolymph instead of 10^6 hemocytes, the coefficient of determination was 0.776 (n = 20) and higher than all, except for one previously reported r^2. An r^2 of 0.53 (n = 12) for Texas oysters sacrificed 24 h after collection and an r^2 of 0.89 (n = 12) for animals held for ten days at high temperature and salinity before sampling were reported by Gauthier and Fisher (1990). An r^2 of 0.675 (n = 25) was reported by Yarnall et al. (2000). An r^2 of 0.241 (n = 100) for oysters from Apalachicola Bay, Florida and an r^2 of 0.771 (n = 100) and 0.738 (n = 100) for oysters from Virginia and New York were reported by Oliver et al. (1998).

The use of FTM hemolymph assay is not recommended for predicting the number of parasites in whole oyster with light *P. marinus* infections (<1,000 parasite/g tissue) (Bushek et al. 1994, Oliver et al. 1998). In our study, the intersects (i.e., y when x = 0) of the linear regression for the number of parasites in whole oyster was 665 ($\log_{10}$ 665 = 2.823) or 1834 ($\log_{10}$ 1834 = 3.2635) depending on whether hemolymph parasite number was expressed per hemocyte or per ml. Hence, parasite numbers per g tissue in whole oyster would need to be greater than these numbers for the hemolymph FTM assay to be able to detect any parasite. This is in agreement with a Bushek et al. (1994) study in which a high percentage of oysters with parasite numbers below 1,000 *P. marinus* per g (wet) tissue were diagnosed as negative with the FTM hemolymph assay. The limitation in sensitivity of the FTM hemolymph assay is less of a concern for Gulf coast oysters than for oysters from the Northeast and central Atlantic coast because *P. marinus* prevalence in Gulf oysters in most sites is close to 100% as determined by the FTM tissue assay. This assay typically detects infection intensities when they are greater than 1,000 parasite/g wet tissue (Bushek et al. 1994, Soniat 1996, Fisher et al. 1996).

No statistical differences in hypnospore diameters or numbers were found among the FTM formulations tested, indicating that none of the FTM formulations provided more nutrients for parasite uptake than others. After incubation of parasites in media composed of individual FTM components, Ray (1954a) found that yeast extract combined with dextrose or casitone were the nutrients responsible for parasite enlargement. All FTM formulations tested in the present study contained approximately equal concentrations of yeast extract (5.0 g/L), dextrose (5.5 g/L), and casitone (15.0 g/L), which explains why no increase in hypnospore diameter or number was found among the FTM formulations tested. Incubation of hemocytes in FTM without agar, or AFTM, did simplify the processing and counting of samples. A viscous layer often forms over the cell pellets during rinsing steps before and after NaOH digestion when FTM with agar is used to enlarge *P. marinus* cells (La Peyre, personal observation, Oliver et al. 1998). This layer is made of indigestible agar because the use of AFTM eliminated this layer, thereby simplifying sample processing and reducing the risk of losing hypnospores in the discarded layer. Sample counting was also greatly simplified because 1) debris including indigestible agar that can interfere with hypnospore counting was eliminated, and 2) hypnospores readily settled on the bottom of wells of tissue culture plates forming monolayers and could be easily counted. In contrast to earlier studies, the need to scan for parasites vertically

through the column was eliminated (Choi et al.1989, Gauthier & Fisher 1990, Bushek et al. 1994). Moreover, parasite suspensions could be serially diluted in 96-well plates and counted at the appropriate dilution faster than they could be prepared at the appropriate dilution and counted on filter paper by the technique of Oliver and Fisher (1996) or counted with hemacytometers as used by Choi et al. (1989). Although agar is traditionally used in the microbiological medium FTM to preserve anaerobic conditions and keep bacteria suspended throughout the media for maximizing the use of nutrients (Hitchens 1921), it was clear from our study that the conditions produced by this viscosity are not necessary to enlarge *P. marinus* parasites. AFTM was therefore selected for parasite enlargement in the improved FTM hemolymph protocol.

In an evaluation of methods to diagnose *P. marinus*, NaOH digestion was used to process hemolymph and oyster tissue. It was found that NaOH digestion facilitated the counting of hypnospores from oyster tissues and from hemolymph, but it was noted that NaOH may not be needed for the hemolymph technique because it adds time and labor to the assay (Bushek et al. 1994, Fisher & Oliver 1996). In our study, NaOH digestion of hemolymph samples simplified and reduced the time spent counting. By eliminating debris, cells formed a monolayer on cell culture plate bottoms eliminating the need to scan vertically for hypnospores caught in debris. In past studies, it was noted that hypnospores became sticky forming clumps and adhering to the sides of test tubes after NaOH digestion and upon rinsing (Choi et al. 1989, Bushek et al. 1994, Fisher & Oliver 1996). In our study, the loss of hypnospores as a result of clumping and adherence to the side of test tubes was alleviated by adding BSA to rinsing solutions as recommended by Coates et al. (1999).

In conclusion, simple modifications to the standard protocol for counting of *P. marinus* in oyster hemolymph improved the technique. The addition of lipid to AFTM, the use of FTM without agar, and the use of BSA in rinsing solution facilitated sample processing and counting. The availability of an improved protocol to count *P. marinus* in hemolymph will be useful in monitoring the dynamic change of parasites in hemolymph in infected individual oysters under various conditions. The role of hemocytes in the infection process or in the elimination of the parasites (e.g., hemocyte killing assay) can also be investigated more accurately with this assay.

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