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THE DEVELOPMENT OF A POSITIVE NON-INFECTIOUS CONTROL FOR THE DETECTION OF *PERKINSUS* USING THE RAY TEST

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ABSTRACT To establish a noncontagious control for the Ray thioglycollate test for the detection of *Perkinsus* in mollusks we evaluated nonviable stages of *P. olseni* for enlargement of hypnospores and blue/black iodine stain. Trophozoites made nonviable with formalin, irradiation or colchicine failed to swell in thioglycollate. They remained small and did not differentially stain in iodine. Trophozoites that had already developed into hypnospores in thioglycollate were rendered inactive by freezing, ethanol or formalin immersion. They retained their iodinophilic properties and thus could provide a partial control for the Ray Test.

KEY WORDS: *Perkinsus*, abalone, fluid thioglycollate medium

INTRODUCTION

Members of the genus *Perkinsus* are protozoan parasites found exclusively in mollusks. *Perkinsus* parasites have long been recognized as a cause of mortality in commercially important mollusks (Ray & Chandler 1955). *Perkinsus marinus* has been demonstrated to have profound effects on the oyster *Crassostrea virginica*, leading to reduced growth, reduced fecundity, and increased mortality (Menzel & Hopkins 1955, Mackin 1962). In Australia, *P. olseni* infections result in deep abscesses and soft yellow pustules in commercially important abalone, *Haliotis rubra* and *H. laevigata*. This is of great concern to the endemic abalone industry as infected individuals are unacceptable for processing (Lester & Davis 1981). Transmission appears to occur through zoospores that develop via hypnospores (Goggin et al. 1989). Hypnospores can be found within the abscesses (Goggin & Lester 1995) and may also be produced by culturing trophozoites in fluid thioglycollate medium (FTM) (Ray 1952). Other species of *Perkinsus* produce hypnospores though the role of zoospores in transmission between mollusks is not clear (Perkins 1996).

Diagnosis of *Perkinsus* infections is commonly accomplished by the FTM assay (Ray 1952). Trophozoites in infected tissue develop into usually much larger hypnospores that stain blue-black with Lugol's iodine (Ray 1952). The reaction with iodine relies on the development of hypnospores; trophozoites remain brown (Ray 1952, Stein & Mackin 1957). Prior to the development of this technique, diagnosis relied on fresh tissue smears or histologic sections. These methods are labor intensive, use expensive materials, rely on a high degree of expertise, and lack sensitivity to detect low levels of infection (Ray 1954).

Infected hosts frequently show no overt sign of disease, and as such, infected tissue can easily reach processing plants undetected (Goggin et al. 1990). Here they may be examined for the presence of *Perkinsus* with the FTM assay, however, due to inexperience in recognizing the parasite or through errors in the formulation of the culture media, investigators may fail to detect infections.

The development of positive reference material to assist *Perkinsus* diagnosis in fisheries laboratories would be an invaluable tool to help minimize misidentifications and false negatives. The material needs to be noncontagious to prevent cross-infection into local mollusk populations during shipping or in disposal after use, while maintaining the key attributes of the FTM assay: enlargement of hypnospores in FTM and uptake of the iodine stain (Ray 1952).

Fisher and Oliver (1996) stated that dead trophozoites of *P. marinus* fail to enlarge in FTM. We sought to make *P. olseni* material nonviable to see if swelling would still occur. We also sought to determine whether hypnospores, following enlargement in FTM, could be rendered inactive while retaining their iodinophilic properties.

MATERIALS AND METHODS

Trophozoite Inactivation

Pieces of infected mantle tissue from three blood cockles, *Anadara trapezia*, infected with *P. olseni* (Murrell et al. 2002) were exposed to either dilute formalin, irradiation, and colchicine to attenuate the viability of hypnospores while retaining the characteristics of the Ray Test. Several tests were run on tissues from one animal to enable the results from different dose levels to be compared. Tissue from animal 1 was immersed in one of four dilute formalin solutions [1:4 × 10^3, 1:4 × 10^4; 1:4 × 10^5; 1:4 × 10^6 formalin:seawater (approximately 34‰)]. For a period of 30 minutes tissue from animal 2 was treated with gamma irradiation. The self-contained gamma radiation source of ^{60}Co had a dose rate of 670 Gy h^{-1}. Duplicate wet tissue samples were placed in glass petri dishes and irradiated to a maximum absorbed dose rate of 600 Gy in increments of 200 Gy. Variations in absorbed dose were minimized by placing thin tissue samples within a uniform portion of the radiated field. Two tissue samples from animal 3 were placed in FTM to which was added colchicine, at one of two concentrations: 10^{-4} M and 10^{-6} M for six hours, after which they were rinsed in seawater.

After treatment all tissues were placed in FTM, supplemented with 200 mg chloromycetin and 200 units of mycostatin to reduce fungal and bacterial contamination (Ray 1966), and incubated at 25°C for five days. A second pair of samples from animal 3 was left in the colchicine-supplemented FTM for the full period of incubation (5 days).

Following incubation, a portion of infected tissue was examined and hypnospore abundance counted, using a compound microscope at ×40 magnification in five fields of view. To facilitate easy enumeration, part of the tissue sample was stained with iodine prior to counting. Hypnospores were teased out of the unstained tissue and transferred to a glass petri dish containing seawater. The seawater in the dish was replaced twice daily. Hypnospores that adhered to the dish were allowed to develop; a process that typi-

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TABLE 1.

Effects of formalin, irradiation and colchicine treatment on trophozoite enlargement, resulting hypnospore viability, and iodinophilia.

Treatment	Duration of Treatment	Swelling Evident After FTM Culture (Y/N)	No. Enlarged Hypnospores Present (5 Fields 40×)	% Hypnospores Viable	Presence of Iodinophilia (Y/N)
Control	—	Y	480	80%	Y
1:4 × 10^3 formalin:seawater	1 h	N	0	~	~
1:4 × 10^4 formalin:seawater	1 h	N	0	~	~
1:4 × 10^5 formalin:seawater	1 h	N	0	~	~
1:4 × 10^6 formalin:seawater	1 h	Y	510	90%	Y
Control (no treatment)	—	Y	520	90%	Y
Gamma 200 Gy	—	Y	400	100%	Y
Gamma 400 Gy	—	Y	30	85.7%	Y
Gamma 600 Gy	—	Y	4	100%	Y
Control	—	Y	90	100%	Y
10^{-4} M Colchicine	6 h	Y	80	83.3%	Y
10^{-6} M Colchicine	6 h	Y	70	100%	Y
10^{-4} M Colchicine	entire incubation	Y	170	93.3%	Y
10^{-6} M Colchicine	entire incubation	Y	200	100%	Y

cally took 1–5 days. Parasites were inspected under a dissecting microscope and were deemed viable only if cell division occurred within five days. To assess whether treated cells retained their iodinophilic properties irrespective of viability, individual hypnospores were isolated from the treated tissue and transferred to a separate petri dish, where they were stained with 3–4 drops of Lugol's iodine. Iodinophilia was based qualitatively on the uptake of stain by the hypnospore and was assessed approximately three minutes after application (Quick 1972). Control tissues from all three mollusks were placed directly into FTM and incubated for the equivalent period of time to confirm that they were infected with *Perkinsus*.

Hypnospore Inactivation

To obtain hypnospores, pieces of mantle, foot, digestive gland, and gill from *A. trapezia*, from Wynnum, Queensland and from *H. rubra* and *H. laevigata* collected from South Australia, were incubated in 20 ml FTM at room temperature (approximately 24°C) for 4–6 days. The medium was supplemented with 200 mg chloromycetin and 200 units of mycostatin (Ray 1966). The incubated tissues containing resulting hypnospores were then subjected to various treatments: freezing at –20°C; immersion in 10% formalin; and immersion in 70% ethanol. The effectiveness of each treatment on hypnospore viability and iodinophilia was assessed after 24, 48, and 72 h. The viability of hypnospores prior to treatment was confirmed by viability testing of a random subset of enlarged cells.

RESULTS

Trophozoite Inactivation

In all treatments, trophozoites that developed into hypnospores in FTM were capable of further development and were evidently

TABLE 2.

Effects of various treatments on hypnospore viability and iodinophilia.

Host	Treatment	Duration of Treatment	% Hypnospores Viable	Presence of Iodinophilia (Y/N)
A. trapezia	Control	—	80%	Y
H. rubra	Control	—	80%	Y
H. laevigata	Control	—	100%	Y
A. trapezia	10% formalin	24 h	0%	Y
A. trapezia	10% formalin	48 h	0%	Y
A. trapezia	10% formalin	72 h	0%	Y
H. rubra	10% formalin	72 h	~	~
H. laevigata	10% formalin	72 h	0%	Y
A. trapezia	70% ethanol	24 h	0%	Y
A. trapezia	70% ethanol	48 h	0%	Y
A. trapezia	70% ethanol	72 h	0%	Y
H. rubra	70% ethanol	72 h	0%	Y
H. laevigata	70% ethanol	72 h	0%	Y
A. trapezia	Freezing (–20°C)	24 h	0%	Y
A. trapezia	Freezing (–20°C)	48 h	0%	Y
A. trapezia	Freezing (–20°C)	72 h	0%	Y
H. rubra	Freezing (–20°C)	72 h	0%	Y
H. laevigata	Freezing (–20°C)	72 h	0%	Y

viable (Table 1). Treatment had little effect on the parasitic stages that had enlarged. No hypnospores were evident in tissues exposed to formalin concentrations of $1{:}4 \times 10^3$, $1{:}4 \times 10^4$; $1{:}4 \times 10^5$. Tissues exposed to $1{:}4 \times 10^6$ formalin:seawater provided similar hypnospore numbers to those of the control (Table 1). Trophozoites exposed to gamma irradiation showed a progressive decline in hypnospore numbers with increasing radiation exposure (200 Gy, 400 Gy, and 600 Gy) (Table 1). Colchicine treatment had little effect on hypnospore enlargement and viability, irrespective of treatment concentration. The majority of hypnospores retained from incubation appeared viable, with motile zoospores evident after five days examination (Table 1).

Hypnospore inactivation

All treatments killed hypnospores within 24 h of treatment. Nevertheless, all the dead hypnospores exhibited clear iodinophilia, staining a deep blue on application of Lugol's iodine (Table 2).

DISCUSSION

The negative correlation between treatment intensity (or duration) and hypnospore abundance, particularly for the irradiated tissue, followed by the successful development of recovered hypnospores indicate that nonviable trophozoite stages fail to enlarge in FTM. This is in agreement with Fisher and Oliver (1996), who stated that enlargement of *P. marinus* trophozoites in FTM only occurs if the parasite is viable. Formalin treatment at concentrations $\geq 1{:}4 \times 10^5$ formalin:seawater appeared to prevent hypnospore enlargement, with no hypnospores recovered from these concentrations. The extremely weak formalin ($1{:}4 \times 10^6$ formalin:seawater) appeared to have little effect on trophozoite viability, with treated tissues exhibiting a similar enlarged hypnospore abundance to that of the control. Similarly, colchicine concentrations of 10^{-4} M or 10^{-6} M had little effect on parasite viability. Colchicine has been demonstrated to be a potent microtubule inhibitor at such concentrations (Wiest et al. 1993), thus preventing cell division. The fact that parasites treated with colchicine remained viable and underwent division once out of the treatment is consistent with microscopic observations that no cell division occurs during trophozoite differentiation to hypnospores and cell enlargement. Swelling of *Perkinsus* cells in FTM had been suggested to occur as a result of the direct uptake of the media (Ray 1952), although the mechanisms of this process are not yet described.

As nonviable trophozoites failed to enlarge in FTM, and thus failed to become iodinophilic, we tested whether hypnospore stages could be killed and still retain their iodinophilic properties. All treatments tested killed hypnospores. The sensitivity of hypnospores to low temperature is in accordance with Chu and Greene (1989), who observed that hypnospores of *P. marinus* exhibited 100% mortality at 0°C for 24 h. Goggin et al. (1990) observed motile zoospores from cultured tissues previously chilled at 4°C, 0°C and frozen at −20°C, showing that trophozoites are more tolerant to low temperatures than hypnospores.

In all cases, nonviable hypnospores were iodinophilic. Thus, such tissue could be transported risk-free to processing plants to serve as a positive reference for sample comparison. Although this is not a control for FTM development, it still supplies users with a positive control that is safe, noncontagious, and simple to use.

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