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A COMPARISON OF PARAFFIN HISTOLOGY AND HEMOLYMPH ANALYSIS FOR THE DIAGNOSIS OF *HAPLOSPORIDIUM NELSONI* (MSX) IN *CRASSOSTREA VIRGINICA* (GMELIN)

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ABSTRACT Diagnosis of the oyster pathogen *Haplosporidium nelsoni* (MSX) by paraffin histology is compared with a technique in which hemolymph drawn from the oyster adductor muscle sinus is examined for parasite plasmodia. Oysters from seed beds of the James River, Virginia imported to an MSX endemic area in May, 1986 were sampled monthly through December, 1986 and in February, 1987. A sample of 25 oysters was bled each month and then processed for sectioning. Of the 200 oysters sampled, 89 (44.5%) were diagnosed as infected using histology and 61 (30.5%) were diagnosed as infected using hemolymph examination. All the heavy and moderate infections diagnosed by paraffin histology were also diagnosed by hemolymph, but only 64.3% of the light infections and only 43.5% of the rare infections were diagnosed by hemolymph analysis. However, 92.3% of the undetected rare infections and 60.0% of the undetected light infections were localized in gills and plasmodia had not entered the circulatory system. The hemolymph technique, which takes about 4 h, detected 89.7% of the systemic infections diagnosed by paraffin histology.

KEY WORDS: *Haplosporidium nelsoni,* MSX, oyster, diagnosis, techniques

INTRODUCTION

Traditionally, protozoan parasites of oysters have been diagnosed by paraffin histological techniques (Farley 1967; Andrews and Frierman 1974; Ford and Haskin 1982). This technique is accurate, but time consuming when rapid diagnosis is needed. Although promising new diagnostic procedures utilizing enzyme immunoassay, monoclonal antibody techniques and nucleic acid probes are under development in most areas of the world where oyster diseases significantly reduce the harvest, none are currently available. A simple, rapid technique for diagnosis of bivalve diseases has been developed by C. Austin Farley. The technique relies upon the presence of parasites in host hemolymph that occurs in systemic infections.

The purpose of this study was to compare diagnosis of *Haplosporidium nelsoni* (Haskin, Stauber and Mackin) through an annual infection period using traditional paraffin histology and hemolymph analysis. Because of a prolonged drought and record high salinity in the Chesapeake Bay, Virginia during 1986, oysters had the highest levels of *H. nelsoni* ever recorded. These high prevalences allowed good comparisons of the two techniques.

MATERIALS AND METHODS

Oysters were dredged in May, 1986 at Horsehead Rock in the James River, Virginia. Oysters from this rock are known to be highly susceptible to MSX and have been used as controls for over 25 years for disease monitoring programs conducted in Chesapeake Bay by the Virginia Institute of Marine Science and in Delaware Bay by Rutgers University. A 0.6 m by 1.2 m (2 by 4 feet) tray containing 378 oysters was suspended from a pier at VIMS in the lower York River, an MSX endemic area. Two additional trays of 400 oysters each were placed at the usual monitoring location about 1 km upriver. The MSX infection period typically begins in May each year (Andrews and Frierman 1974), therefore oysters for disease monitoring are transplanted to trays each year at that time. A sample of 25 oysters analyzed for *H. nelsoni* at the time of transplantation was negative for the parasite. Samples of 25 oysters were taken from the pier tray in late May and approximately every 30 days through November, 1986. No oysters remained in the tray after the November sample was removed so an additional sample in February, 1987 was taken from one of the other monitoring trays. All oysters were analyzed for the presence of *H. nelsoni* by both hemolymph analysis and paraffin histology.

Hemolymph Analysis

Immediately after sampling oysters from the tray, each oyster was numbered and the shell notched opposite the adductor muscle with a hand-held grinding tool. Using a 22 ga needle, 0.1 to 0.2 ml of hemolymph was drawn from the adductor muscle sinus into a 3 cc syringe containing 2.0 ml of cold 15 ppt artificial seawater containing 0.05 gm/l phenol red. If too much hemolymph is withdrawn from the oyster a thick cell layer results after settling and the slide may be difficult to diagnosis. Contents of the syringe were gently expressed into Farley chambers and hemocytes allowed to settle for one hour. These chambers were developed by C. Austin Farley, NMFS, Oxford, Maryland and consisted of plastic tissue-embedding rings sanded flat to

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prevent leakage and held to numbered microscope slides with an elastic "pony-tail" band. Rubber bands are not satisfactory because they are easily cut by the edges of the glass slide. After one hour, chambers were removed and the slide with attached cell monolayer was fixed for 5 min in Dietrich's AFA. Slides were stained with Harris' hematoxylin and eosin, coverslipped, and examined for the presence of plasmodia.

Slides were initially scanned at 100× in their entirety or until plasmodia were observed. Later, plasmodia were counted in each of 5 100× fields randomly slected using a microslide field finder. In heavy infections five 450× fields were counted and converted to 100× counts by multiplying by a factor of 20. Average counts per five 100× fields are given in Table 1; a + symbol indicates that plasmodia were observed on the slide, but none was present in the randomly selected fields. Plasmodia counts could not be standardized to number of hemocytes because of high variability in number of hemocytes, even in uninfected oysters, and because heavy infections increased hemocytosis. Generally, there were relatively more hemocytes in heavy infections.

Paraffin Histology

After hemolymph was withdrawn, oysters were opened and an approximately 5 mm thick section of tissue through the visceral mass that included mantle, gills, stomach, intestine and digestive diverticula was excised and fixed in Davidson's AFA for 24 h. Tissue was embedded in paraffin, sectioned at 6 μm and stained with Harris' hematoxylin and eosin. Oyster tissue was not trimmed before or after embedding and sections from only one oyster were placed on each slide. Sections were diagnosed, without reference to hemolymph preparations, by technician Juanita Walker who has been responsible for MSX diagnosis at VIMS for over 20 years.

Rating of infection intensity was as follows: rare (R)-less than 10 plasmodia in entire section, not limited to gill epithelium; rare localized (RL)-rare to light infection restricted to gill epithelium; light (L)-less than 2 plasmodia per 450× field but greater than 10 in entire section; light localized (LL)-many plasmodia but infection restricted to gill epithelium; moderate (M)-2 to 5 plasmodia per 450× field; heavy (H)-greater than 5 plasmodia per 450× field; sporulation (S)-any infection when spores were present.

TABLE 1.

Comparison of prevalence and intensity of *H. nelsoni* in *C. virginica* determined by paraffin histology (P) and hemolymph analysis (A) in samples of 25 oysters. Hemolymph analysis values are average number of plasmodia per five 100× microscope fields.

Oyster number	27 May 86		27 Jun 86		29 July 86		28 Aug 86		25 Sep 86		27 Oct 86		1 Dec 86		27 Feb 87	
	P	A	P	A	P	A	P	A	P	A	P	A	P	A	P	A
1	U	0	U	0	U	0	U	0	R	+	U	0	L	0	U	0
2	U	0	U	0	LL	0	L	10	U	0	U	0	U	0	H	55
3	U	0	U	0	U	0	U	0	RL	0	R	+	L	16	L	1
4	U	0	U	0	U	0	U	0	L	0	RL	0	U	0	U	0
5	U	0	U	0	U	0	RL	0	H	64	L	+	U	0	M	130
6	U	0	U	0	U	0	RL	0	U	0	U	0	RL	0	L	+
7	U	0	U	0	U	0	U	0	U	0	M	34	LL	0	L	3
8	U	0	U	0	H	384	U	0	U	0	U	0	L	3	U	0
9	U	0	U	0	H	528	M	8	U	0	R	1	RL	0	L	4
10	U	0	U	0	U	0	R	+	L	0	U	0	U	0	U	0
11	U	0	U	0	U	0	U	0	L	3	RL	0	L	18	M	+
12	U	0	U	0	U	0	L	0	M	7	U	0	U	0	RL	0
13	U	0	U	0	U	0	U	0	U	0	U	0	U	0	U	0
14	U	0	U	0	LL	0	U	0	H	161	U	0	L	2	L	+
15	U	0	U	0	U	0	U	0	L	1	R	3	R	+	L	+
16	U	0	U	0	LL	0	M	28	L	1	L	4	U	0	M	6
17	U	0	LL	0	L	3	M	20	L	0	H	1816	H	176	L	+
18	U	0	U	0	U	0	U	0	U	0	U	0	U	0	L	1
19	U	0	U	0	M	15	H	736	H	41	R	3	U	0	M	1
20	U	0	U	0	L	+	L	5	L	1	R	2	RL	0	L	3
21	U	0	U	0	U	0	L	2	R	0	R	+	LL	0	U	0
22	U	0	U	0	M	+	RL	0	S	+	M	52	LL	0	L	+
23	U	0	U	0	U	0	L	1	H	496	U	0	L	+	U	0
24	U	0	U	0	LL	0	U	0	U	0	R	+	U	0	U	0
25	U	0	U	0	RL	0	L	0	U	0	RL	0	LL	0	M	+
Total No. infected	0	0	1	0	11	6	14	9	16	11	15	12	15	7	17	16
Prevalence (%)	0	0	4.0	0	44.0	24.0	56.0	36.0	64.0	44.0	60.0	48.0	60.0	28.0	68.0	64.0

H = heavy; L = light; LL = light, localized; M = moderate; R = rare; RL = rare, localized; S = spores; U = uninfected; + = plasmodia present on slide, but none in random fields; 0 = no plasmodia on slide.

RESULTS

Of the 200 oysters analyzed for *H. nelsoni,* 89 (44.5%) were infected based on paraffin histology (Table 1). Prevalence of infection gradually increased through September and then remained at approximately 60% through fall and winter. Hemolymph analysis detected 61 infections in the 200 oysters (30.5%), or 68.5% of the total detected by paraffin histology. Hemolymph analysis detected all the heavy and moderate infections as determined by paraffin histology, but only 64.3% (27/42) of the light infections and 43.5% (10/23) of the rare infections. However, of the 15 light infections not detected by hemolymph analysis, 9 cases (60.0%) were localized infections and of the 13 undetected rare infections, 12 (92.3%) cases were localized infections (Table 1). Thus, hemolymph analysis detected 37 of the 44 (84.1%) rare and light systemic infections. As expected, no localized infections were detected by hemolymph analysis because plasmodia had not entered the circulatory system. If the localized infections are removed from consideration, hemolymph analysis detected 61 of the 68 (89.7%) systemic infections.

Plasmodia counts from hemolymph analysis sorted relatively well into rare-light, moderate and heavy infections, although there was some overlap (Table 2). Counts from hemolymph analysis for rare and light infections as determined by histology ranged from 1 plasmodium on the slide to about 5 plasmodia per 100× field, with much overlap between rare and light counts. Counts for moderate infections ranged from a few plasmodia on the slide to 130 plasmodia per 100× field and counts for heavy infection ranged from 41 to over 1800 plasmodia per 100× field. The oyster with spores in the digestive diverticula had a very low number of plasmodia in the hemolymph.

Size of plasmodia in hemolymph preparations depended upon the intensity of infection. In rare and light infections, plasmodia were usually small, between 5 and 20 μm in diameter, with 2 to 15 nuclei per plasmodium. In moderate and heavy infections plasmodia ranged from 5 to 70 μm in diameter and large plasmodia often had over 100 nuclei (Figure 1a). Most nuclei were about 2 μm in diameter with an obvious eccentric endosome and dark-staining bar of "Kernstab", but large metaphase nuclei, up to 11 μm long, were also present in many plasmodia. Plasmotomy appeared to be occurring in all large plasmodia and in many small plasmodia. This process appeared to commence with gradual concentration of cytoplasm in from two to seven areas at the periphery of the plasmodium (Figure 1b) with subsequent fragmentation of the original plasmodium into smaller plasmodia (Figure 1c). Plasmodia less than 15 μm in diameter were often observed in phagocytes.

TABLE 2.

Summary of hemolymph plasmodia counts for the four intensity categories determined by paraffin histology.

Paraffin histology intensity category				
Heavy	Moderate	Light		Rare
1816	130	18	1	3
736	52	16	1	3
528	34	10	+	2
496	28	5	+	1
384	20	4	+	+
176	15	4	+	+
161	8	3	+	+
55	7	3	+	+
41	6	3	0	+
	1	3	0	+
	+	2	0	0
	+	2	0	
	+	1	0	
		1	0	
		1		

DISCUSSION

Results of this study suggest that hemolymph diagnosis of *H. nelsoni* may be an acceptable and preferable alternative to paraffin histology diagnosis, depending upon the objective of the diagnoses. If a rapid survey of a large number of oysters is required for oyster mortality predictions, then hemolymph analysis is probably the technique of choice. All heavy and moderate infections and 84.1% of rare and light systemic infections were diagnosed by this technique. Similar results were obtained by Ford and Kanaley (1988) using hemolymph diagnosis. The hemolymph technique did not detect localized gill epithelial infections, but investigators disagree on the fate of these infections. If, as some believe, gill epithelial infections always develop into systemic infections, then the hemolymph technique may seriously underestimate the actual prevalence, and potential mortality, because localized gill infections may account for almost 50% of the infections in certain months (Table 1, July and December, for example). If, as others believe, gill epithelial infections do not develop into systemic infections, failure to detect localized infections may not be a serious disadvantage if the goal is to predict mortality. Unfortunately, until the life cycle of MSX is solved, there is no way to know if all systemic infections begin as localized infections in the gills. Thus, periodic calibration with paraffin histology should be incorporated into any monitoring program using hemolymph diagnosis.

The main advantage of the hemolymph technique is rapid diagnosis of *H. nelsoni*—approximately 4 h for a sample of 25 oysters as compared to more than 48 h for paraffin histology. The hemolymph technique may also detect other parasites, such as *Perkinsus marinus,* but no comparisons with paraffin histology or thioglycollate culture have been made for this parasite. The disadvantages of the hemolymph technique are primarily related to the fact that no permanent section of oyster tissue is obtained as it is in paraffin histology. Thus, there is no record of oyster tissue response to *H. nelsoni* infection and no record of

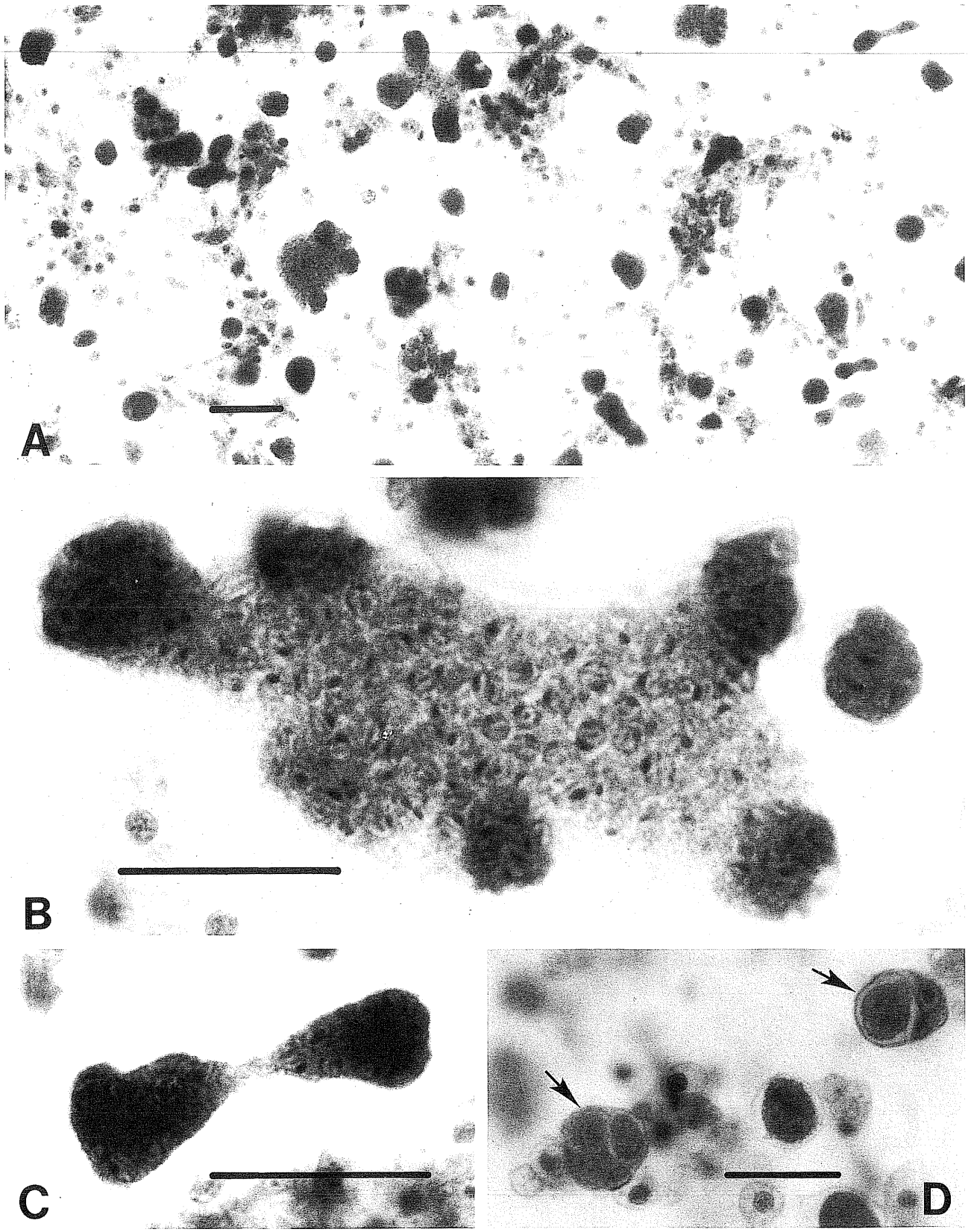

Figure 1. MSX plasmodia in hemolymph preparations. (A) Low magnification of heavy *H. nelsoni* infection illustrating various sizes and shapes of plasmodia. (B) A large plasmodium in the process of fragmenting. (C) Plasmodium that has almost completed separation into two smaller plasmodia. (D) Phagocytized plasmodia (arrows). The phagocyte on the left has engulfed at least two plasmodia. All bars = 20 μm.

other parasites present. These may or may not be important considerations, depending upon the objective.

The process of plasmotomy described may be an artifact of the technique in which plasmodia are allowed to settle onto a glass slide, but it clearly indicates that plasmodia are capable of fragmentation as suggested by Farley (1967).

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