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Author: KINGSCOTE, BARBARA F.

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LEPTOSPIRES IN FINGER NAIL CLAMS*

BARBARA F. KINGSCOTE**, Section of Zoonoses and Wildlife Diseases, Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

Abstract: Clams of the Genus Sphaerium were collected on an island where *Leptospira interrogans* serotype *pomona*, infection was enzootic in deer. Leptospires were isolated from both dry, estivating and aquatic, active clams. The isolates did not resemble parasitic leptospires antigenically, and they did not produce disease in gerbils. Clams excreted leptospires into water. Uninfected clams collected from a different source were exposed to serotype *pomona* in their aquarium. Serotype *pomona* was recovered from the water after 2 days. Leptospires isolated from fluid in the mantle cavity, digestive gland, and other tissues of the clams appeared to be antigenically different from *L. pomona* and from each other, and to resemble serotype *biflexa* Patoc I, serotype *semaranga*, and a New England strain of water leptospire. It is possible that the molluscan environment modified the expression of genes directing the structure of surface antigens of serotype *pomona*, and that this modification was stable.

INTRODUCTION

Pathogenic bacteria and viruses have been isolated from marine bivalve molluscs following natural and experimental infection. Liu[7] drew attention to the digestive diverticula of molluscs as sites of virus concentration. Prescott et al.[11] reviewed studies showing that the coliform counts in oysters exceeded those in the water around them in warm weather, implying either cumulative retention or proliferation of the bacteria in the oysters.

This paper reports the isolation of leptospires from small, fresh-water clams commonly called finger nail clams, of the Genus *Sphaerium*. The work was part of an epizootiological study on Navy Island[6] where leptospirosis was enzootic in the white-tailed deer population. The second phase of the study was designed to assess the potential of clams to maintain and excrete parasitic leptospires, under controlled conditions.

MATERIALS AND METHODS

Navy Island occupies half a square mile in the Niagara River in Ontario. It is almost covered with hardwood and shrubs growing on poorly drained clay soil overlying dolomite. When the water which covers most of the island in spring dries up, vast numbers of finger nail clams *(Sphaerium occidentale)* estivate in dry clay and damp leaves in shallow drainage ditches and depressions in the woods. These two habitats and a pond were the sources of clams in which natural infection by leptospires was studied.

S. occidentale, like other sphaeriids, bears live young. It requires two years to complete its life cycle, and therefore adults as well as young must survive all climatic extremes. It feeds on diatoms, bacteria, and possibly organic matter, which it removes from suspension by the muco-ciliary action of the gills, transports to the digestive diverticula, and

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** Present address: Department of Veterinary Microbiology and Immunology, Ontario Veterinary College.

digests in phagocytic cells of the digestive gland.[9] The surface tension of particles and the ambient temperature greatly influence phagocytosis, on which molluscan digestion, excretion, and defense largely depend.

a) Natural infection

Clams were collected in August, 1968 and June, 1970. Three habitats were sampled: a dry drainage ditch, damp woodland depressions, and a pond. In the laboratory, the clams were kept at 20 to 23C in the substratum from which they had been collected. Those in earth were held in plastic bags kept partly open to allow air to circulate; those in pond material were held in an open jar.

Clams in groups of five to ten were separated from the substratum and dried on paper to ensure that no leptospires from the external environment were carried over into the cultures. They were rehydrated in aged tap water and held for 6 hours to 3 days. This environmental water was cultured to detect leptospiral excretion. The clams were dried again and dissected under 6X magnification. In the group collected in August, 1968, mantle fluid and a homogenate of the soft tissues of the clams were cultured separately. In the group collected in July 1970, mantle fluid absorbed on filter paper, digestive gland, and the remaining soft tissues, were collected separately into Ellinghausen - McCullough (EM) medium,[2] held about an hour at room temperature, observed by direct dark-field microscopy and congo red negative staining, and cultured. Figure 1 shows the external appearance of the clams, and the internal anatomy referred to above.

Culture media included Cox agar,[1] Korthof medium, and EM medium. Both water samples and tissue and mantle fluid suspensions were passed through a membrane filter of porosity 0.22μ before inoculation into culture media. Cultures were incubated at 25C and 30C. Serological identification of leptospiral isolates was attempted as soon as the cultures reached suitable density to serve as antigen in the Microscopic Agglutination Test with live antigen (MAL test).[13] They were tested against rabbit antisera to serotypes *pomona* and *hardjo*, and leptospires isolated from Navy Island water, also against the sera of raccoon and deer collected on Navy Island. The criterion used to denote a positive reaction was 50% clearance of antigen by antiserum at a dilution of 1/60 or higher.

b) Experimental infection

Clams of the Genus *Sphaerium* were collected from a woodland pond in June. They were extremely abundant and active, and the population included animals of all ages from juvenile to maturity. The clams were maintained in their native water until they were used for experiment. Samples of this water and of three groups of five clams were tested for the presence of leptospires by the method described above. Then groups of 30 clams were prepared for exposure to serotype *pomona*.

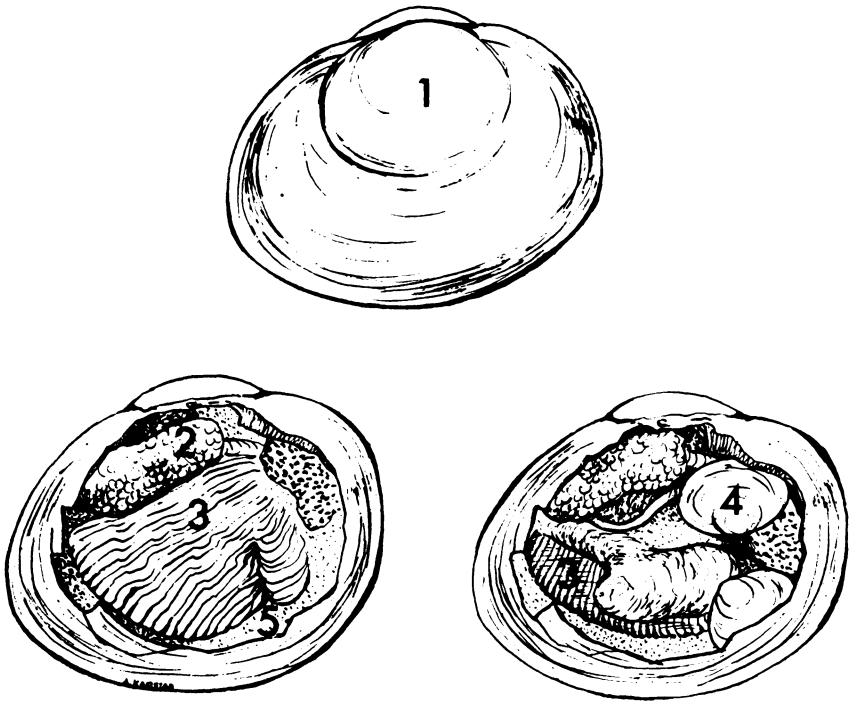
They were dried for 1½ hours at room temperature and then placed in 35 ml of aged tap water. After they resumed activity the water was sampled, filtered, and cultured to determine if the clams had excreted leptospires. Pre - exposure cultures were examined weekly for 6 weeks. Meanwhile experimental exposure of the clams was conducted. Two ml of a culture in logarithmic growth phase, of a porcine strain of serotype *pomona*, were added to the environment of the clams. The aquarium was left exposed to the air and no attempt was made to control microbial contamination. After 1 and 2 days the water was examined by direct microscopy and culture of filtrates to assess the bacterial population.

To determine whether the clams had become infected with serotype *pomona*, groups of ten specimens after 1 and 2 days of exposure were examined by the method described above. An attempt was made to learn whether serotype *pomona* would survive in estivating clams, by keeping infected groups dry for several days before dissection. However, unlike the naturally estivating clams, which could withstand repeated drying and rehydration, these animals failed to survive. Their bodies underwent desiccation in 3 or 4 days.

Leptospiral isolates derived from the above cultures were injected intraperitoneally into weanling gerbils at a dose of 1 ml, to test their infectivity and antigenicity. To confirm their identity they were tested against serotype *pomona* antiserum by the MAL test. Because some of these tests were negative, the serological testing was expanded and repeated using antisera to serotypes *pomona, hardjo, semaranga, biflexa sao paulo, biflexa patoc,* and four leptospiral isolates from New England waters.

FIGURE 1

FINGER NAIL CLAM (Sphaerium sp.). VIEWS OF SHELL, SUPERFICIAL DISSECTION, AND DEEP DISSECTION. (13 x natural size)



LEGEND

1 Umbo
2 Digestive gland
3 Gill
4 Unborn clam
5 Mantle cavity

RESULTS

a) Natural infection

From the collection made in August, 1968, three of nine groups of clams, containing a total of 22 individuals, yielded leptospiral isolates. Two of the nine groups excreted leptospires into the environmental water. Both drainage ditch and forest depression habitats were represented. The isolates were labile and difficult to grow up to antigen density. All grew at first in Korthof medium at 30C but not in serum-free medium. The isolate from clam tissue reacted weakly to serotype *hardjo* antiserum, more strongly to antiserum to a Navy Island water isolate, but not to serotype *pomona* antiserum.

A gerbil injected with a culture from environmental water developed a homologous titer of 4860. Neither isolates from water nor clam tissue produced cultural or histological evidence of infectivity in gerbils, although several blind serial passages were made.

The collection made in June, 1970 yielded 2 positive cultures from a single group out of approximately 15 groups of five to 10 clams, representing all the habitats sampled. In the positive group, both digestive gland and other tissues, but not mantle water nor environmental water, contained leptospires. The two cultures (designated D and OT) grew prolifically at first at 25C in EM medium, but not in Korthof medium, in contrast to the 1968 cultures. After two subcultures the OT strain was difficult to propagate, and after it resumed a moderate rate of growth it could be propagated in serum-containing medium as well as EM medium.

The first transfer of this isolate to Korthof medium produced pleomorphic growth in which both hooked and unhooked leptospires, thick spirochaete-like forms, and contaminants were seen by dark-field microscopy. Electron microscopy confirmed the presence of contamination, also of hooked and straight leptospires, and showed the thick forms to be leptospires closely entwined. Electron-dense inclusions appeared in the protoplasmic cylinders of leptospires, maybe analogous to the lamellar bodies shown by Ritchie and Ellinghausen[12] and Nauman et el.[9] The cells also appeared to have two axial filaments entwined in opposite directions around the helical protoplast. Figure 2 shows the inclusions and the axial filaments.

Gerbils inoculated with OT culture showed neither serological nor cultural evidence of infection. The isolates D and OT were tested by the MAL test against 15 sera including antisera to parasitic and water leptospires and field sera from raccoons and deer shot on Navy Island. The OT culture was weakly reactive to antisera to three strains of New England *Leptospira* soon after isolation, but this reactivity was lost during adaptation to laboratory medium.

b) Experimental infection

No evidence was found to indicate that the experimental clams were infected with leptospires from their native ecosystem, and none of the cultures of pond water from which they had been taken produced leptospires.

Serotype *pomona* survived in water in the model system for 2 or 3 days, in the presence of numerous microbial contaminants. Later these predominated and overwhelmed the leptospires. Leptospires were present in clams after exposure for 1 or 2 days to serotype *pomona,* in numbers large enough to be readily seen by direct dark-field microscopy of fluid and tissue suspensions. In the bodies of clams, leptospires clearly predominated over contaminants. A rough comparison of bacterial counts in water, diluted mantle fluid, and tissue suspensions, negatively stained with congo red[3] showed that when the ratio of leptospires to other bacteria in water was 1:25, the ratios in suspensions of mantle fluid, digestive gland, and other tissues were 1:24, 5:0, and 20:0 respectively. When these suspensions were incubated without filtration, the contaminants rapidly overgrew the leptospires.

Five populations of leptospires derived from one clam infection experiment were serologically tested. They were subcultured every 2 months for 10 months, then retested. The results are summarized

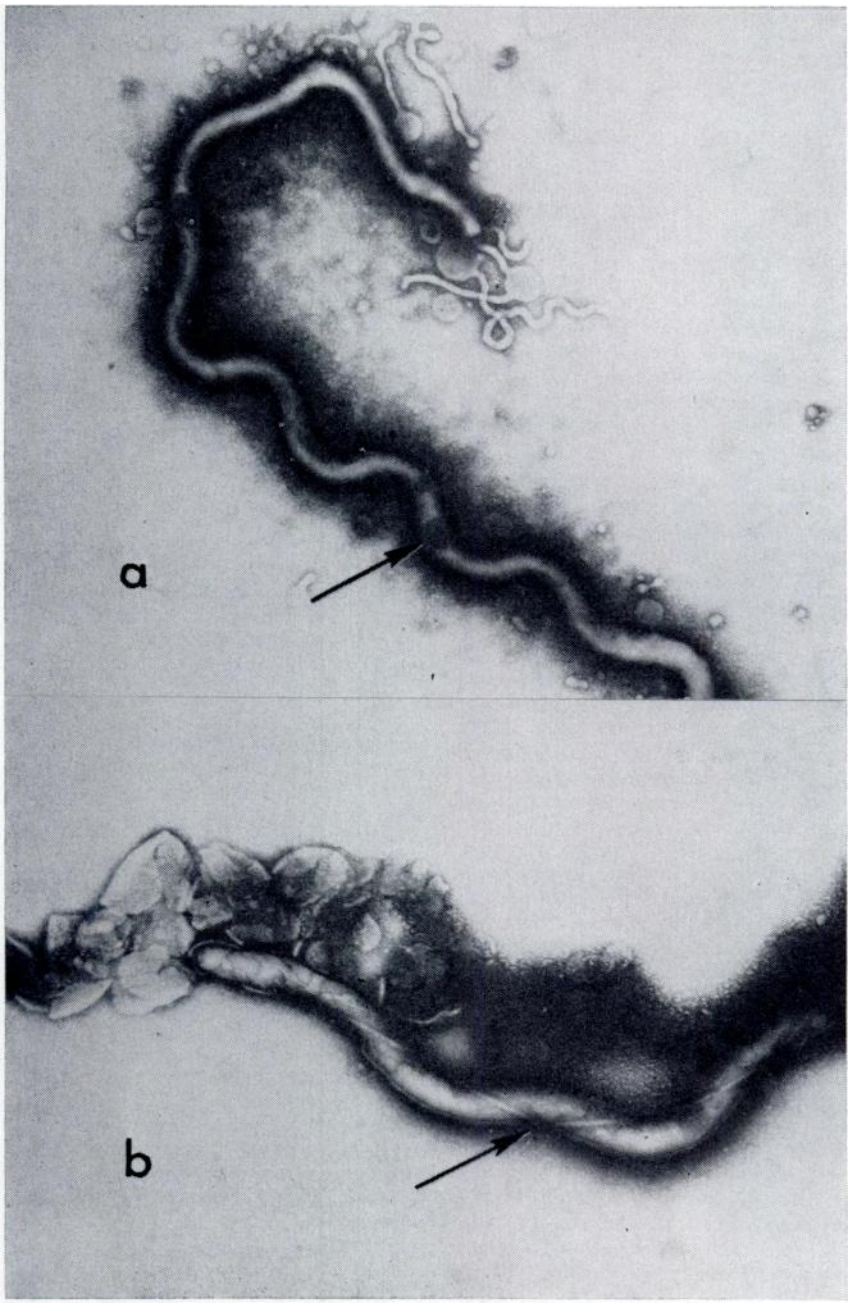


FIGURE 2. Leptospires isolated from pooled tissues of clam
a) cytoplasmic inclusion b) axial filaments

in Table 1. They indicate that the leptospires in the water after 1 and 2 days were antigenically serotype *pomona*. They also show that the clam cultures M, D, and OT, from mantle fluid, digestive gland, and other tissues respectively, did not resemble antigenically the infecting strain, and were not identical with each other. M and D cross-reacted with *semaranga*, D with *patoc*, and OT strongly with B2, a water isolate from New England.[4] B2 antiserum immobilized four cultures without agglutinating them, and agglutinated OT.

The results of gerbil inoculation with the five cultures supported the observation that serotype *pomona* remained in the water, but that the cultures derived from the clams differed antigenically from each other and from *pomona*. M elicited a very feeble antibody response, D evoked a strong response to M and D antigens, and OT evoked a strong homologous response. Renal lesions characteristic of leptospirosis were produced by the cultures derived from the water, but not by those derived from the clams. These results are compiled in Table 2.

TABLE 1. Serological reactions (MAL Test*) of leptospires recovered from clams or their aquarium water after exposure to serotype **pomona**.

	Antigen **					
Antiserum	W1	W2	M	D	OT	231
pomona	>2160##	>2160	—	—	—	
	26,000	26,000	—	—	—	26,000
hardjo	—	—	—	—	60	
	—	—	—	—	—	—
semaranga	—	—	180	180	—	
	—	—	60	—	—	—
biflexa sao paulo	—	—	—	—	60	
	—	—	—	—	—	—
biflexa patoc	—	—	2160	180	—	
	—	—	>540	60	—	—
B 2 New England water	1080#	2160#	2160#	1080#	6480	
	540#	180#	1620#	1620#	3240	180#
B 11 New England water	—	—	—	—	—	
	—	—	—	—	—	—
B 16 New England water	—	—	60	60	180	
	—	—	60	—	60	—
H 16 New England water	—	—	—	—	—	
	—	—	—	—	—	—

* Microscopic agglutination test with live antigen.[13]

** W1—culture of aquarium water 1 day after infection with serotype *pomona*
W2—culture of aquarium water 2 days after infection with serotype *pomona*
M—culture of mantle fluid of clams
D—culture of digestive gland of clams
OT—culture of pool of other tissues of clams
231—strain of serotype *pomona* used to infect clams

Titer is expressed as the reciprocal of the highest dilution of antiserum producing 50% clearance of antigen. Results of first and second series of tests appear above and below respectively.

Titer of antiserum in first and second series of tests, producing immobilization of leptospires without agglutination with one exception. M was immobilized by B2 up to 1620, where agglutination also occurred.

TABLE 2. Effects on gerbils of leptospiral cultures derived from exposure of clams to serotype **pomona**.

Culture injected, 1 ml intraperitoneally	Gerbil Response		
	Serological * titer	Cultural results	Histological lesions
W1, aquarium water 1 day after addition of leptospires	180 homologous 180 *pomona* 231 180 W2	positive	Nephritis suggesting leptospirosis
W2, aquarium water 2 days after addition of leptospires	180 homologous 180 *pomona* 231 180 W1	positive	Nephritis suggesting leptospirosis
M, mantle fluid of clams	negative	negative	negative
D, digestive gland of clams	2160 homologous 2160 M	negative	negative
OT, other tissues of clams	2160 homologous	negative	negative

* Microscopic agglutination test[13] with live antigen. Titer is expressed as the reciprocal of the highest dilution producing 50% clearance of the antigen named.

DISCUSSION

The ability of *S. occidentale* to retain leptospires through a period of drying and then to excrete them into water, offers an ideal means of survival for this very drought-sensitive organism. Considering that several weeks elapsed between collection and final dissection of the clams during which the leptospires remained viable, it seems possible that leptospires could overwinter in clams also.

Although part of the food of *S. occidentale* is bacteria, it appears that leptospires are not digested. This selectivity makes the internal *milieu* of the clam a less competitive habitat for leptospires than the external aquatic environment. Also it suggests that there is a basic difference in surface structure between leptospires and some other bacteria. The results of experimental exposure of clams to serotype *pomona* show that leptospires are selectively retained by them, from a mixed population of bacteria in the water. The experiment was too brief to indicate whether leptospires could reproduce in clams.

Two aspects of lamellibranch physiology, their excretion of nitrogenous wastes as ammonium, amine, or urea, and the possibility of their using the gastropod method of phagocytosing calcium carbonate to buffer the contents of the digestive diverticula, are relevant to the need of leptospires for ammonium[2] and calcium.[5]

The variety of strains of leptospires isolated from clams experimentally exposed to *pomona* may have been due to prior undetected infection, considering the low rate of isolation from naturally infected clams. However, one would expect leptospires from the prior population to have contaminated the parasitic strain in the water and overgrown it in subsequent culture. This did not occur. Another possibility is that the internal milieu of the clams induced a permanent variation analogous to the S-R variation in other bacteria. Differences between M, D and OT may reflect selective pressure by different microenvironments in the clam acting on a mixed population or a single multipotent genotype. The immobilization of leptospiral populations W1, W2, M and D and the agglutination of OT by B_2 antiserum, poses two questions. Did the immobilized cultures possess only axial filament antigens in common with B_2? Did OT gain, express, lose, or suppress antigenic determiners?

According to Nauman et al.,[9] leptospires contain two independent axial filaments, one originating at each end of the cell. The clam isolate reported here appears to have two filaments overlapping and coiled oppositely over the whole length of the cell to the subterminal points of insertion.

Noguchi[10] made a detailed study of *Cristispira*, a genus of spirochetes living in the crystalline styles of North American oysters, and he reviewed the literature on spiral organisms found in lamellibranchs. He did not mention *Leptospira*. *Cristispira* can be distinguished from *Leptospira* using dark field microscopy, by their greater size and lack of rigidity. In smears of clam tissue suspensions, negatively stained with congo red, forms similar to *Cristispira* were seen. No such forms appeared in the cultures, and the small porosity of the filter membranes may account for this.

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