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Short Communication

Atkinsiella infection in the rotifer *Brachionus plicatilis*

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***Atkinsiella* infection in the rotifer *Brachionus plicatilis* has not hitherto been reported. The first known case of this infection is described herein. The fungal growth in vitro was inhibited by 0.05 μg/ml of malachite green or 15.6 ppm of formalin. It was experimentally demonstrated that the fungus is pathogenic to the swimming crab, *Portunus trituberculatus* in the zoeal stage.**

Key Words——*Atkinsiella* sp.; fungal infection; rotifer.

Some fungi belonging to the order Lagenidiales were previously found to be parasites of "rotifers." They were identified as *Olpidium gregarium* Schroet. and *Myzocytium zoophthorum* Sparrow (Sparrow, 1936), and *Lagenidium oophilum* Sparrow (Sparrow, 1939), although the scientific names of the rotifers were not given. Later, *M. microsporum* (Karling) Sparrow and *L. distylae* Karling were reported as parasites of *Distyla* sp. (Karling, 1944). Karling (1944) also found *L. parthenosporum* Karling in various rotifers including *Distyla* sp., *Philodina* sp. and *Heterodera* sp.

Recently, Barron (1989) reported *Lagenidium oviparasiticum* Barron as a parasite in eggs of *Adineta* sp. Furthermore, Comps et al. (1993) found a *Lagenidium*-like fungus in *Brachionus plicatilis* Müller. In this case, 85-90% of the rotifers examined were infected with the fungus.

All of the above fungi which have been reported as parasites in rotifers, except for *Olpidium gregarium*, were characterized by vesicle formation when they produced zoospores. In Japan, no fungal infection caused by fungi of Lagenidiales has yet been reported in rotifers.

From February to May 1992, the rotifer, *Brachionus plicatilis*, which was being reared at Chiba Prefectural Tokyo Bay Sea Farming Center, Chiba Prefecture, Japan, did not increase in number. It was bred in a concrete tank as the first food supply for seed production of crustaceans and fishes. Because protozoa were observed microscopically on the surface of rotifers, a bath treatment with 25 ppm formalin was first conducted to solve the problem in the tank. However, no increase in the number of rotifers in the tank was found following the treatment. Further detailed microscopical observation revealed thick, non-septate hyphae measuring about 10 μm diam in the eggs and bodies of many rotifers examined. Discharge tubes were extended outside of the rotifers (Figs. 1, 2), and zoospores with lateral biflagella were released into the seawater through the tubes. Vesicles were not formed at the tip of the discharge tubes. From these observations, the infecting fungus was concluded to be a species belonging to the order Lagenidiales.

The infection occurred in a concrete tank in which the seawater was kept at a constant temperature of 28°C by heating during the course of seed production. Final mortality due to the infection was approximately 100%.

Fungi were isolated by inoculating three infected rotifers onto PYGS agar and incubating at 20°C. The agar consisted of 1.25 g of peptone, 1.25 g of yeast extract, 3.0 g of glucose, 37.6 g of artificial seawater (Aqua-Ocean®, Japan Pet Drugs) and 12 g of agar in 1,000 ml of distilled water. For inhibition of bacterial growth, the addition of 500 μg/ml each of streptomycin sulphate and ampicillin to the medium was required. Growing fungal colonies were subcultured onto PYGS agar to obtain pure cultures. One of the isolated strains, NJM 9231, was used for all experiments.

The fungal colony was yellowish, moist and raised slightly at the center. For morphological observation, the strain was incubated in PYGS broth at 25°C for three days to grow mycelia. The mycelia were then washed twice with sterilized artificial seawater, resuspended in sterilized artificial seawater and incubated at 25°C to induce zoospore formation. Thalli in PYGS broth were at first thick, non-septate, stout and irregular. They formed septa in the broth (Fig. 3), then divided into subthalli. When these were incubated in sterilized artificial seawater, each subthallus changed into a zoosporangium. Zoospore formation occurred within 24 h at 25°C after mycelia were transferred into seawater. Each zoosporangium formed one to several discharge tubes (Fig. 4), then zoospores were produced in the zoosporangium and discharge tubes, and released through the

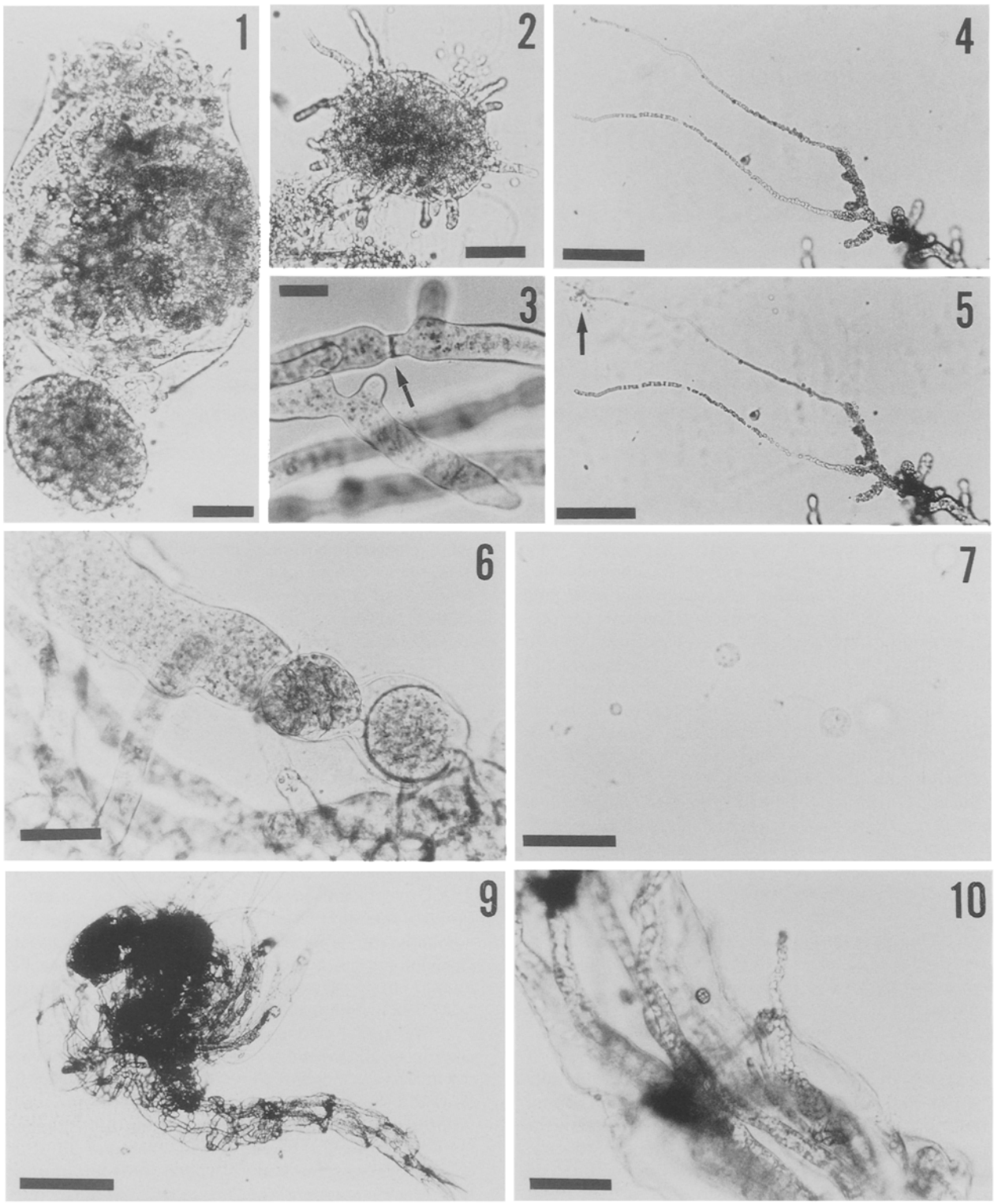

Fig. 1. Gross appearance of the rotifer naturally infected with a fungal infectio. Scale: 50 μm.
Fig. 2. Discharge tubes extending from the egg of a rotifer with fungal infection. Scale: 50 μm.
Fig. 3. Mature thallus with septum (arrow) dividing subthalli. Scale: 30 μm.
Fig. 4. Sporangium with two discharge tubes. Scale: 100 μm.
Fig. 5. Zoospores released through the orifice of upper discharge tube (arrow). Scale: 100 μm.
Fig. 6. Gemmae in PYGS broth after 3 days of incubation at 25°C. Scale: 50 μm.
Fig. 7. Encysted spores with a hair-like filament. Scale: 30 μm.
Fig. 9. A zoea of the swimming crab *Portunus trituberculatus*, artificially infected with *Atkinsiella* sp. NJM 9231. Scale: 0.3 mm
Fig. 10. Discharge tube with zoospores extending from a zoea artificially infected with *Atkinsiella* sp. NJM 9231. Scale: 50 μm.

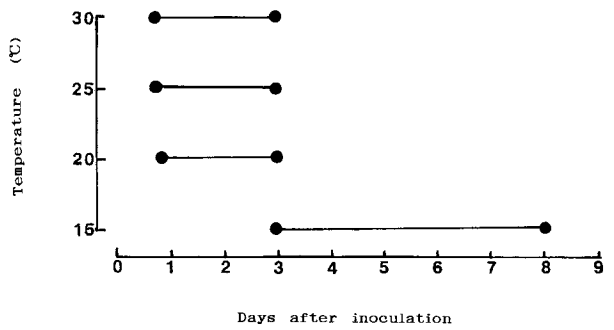

Fig. 8. Effect of temperature on the period of zoospore production of strain NJM 9231 after mycelial colonies were transferred into sterilized seawater.

orifices of the discharge tubes into the seawater (Fig. 5). Zoospores had lateral biflagella, and were isokont, pyriform and monoplanetic. Gemmae were also produced (Fig. 6) and changed to zoosporangia. Encysted zoospores were spherical and produced a hair-like filament. Later a small thallus was produced at the top of the filament (Fig. 7). No sexual reproduction was observed. From these morphological characteristics, the present strain could be classified as a species of the genus *Atkinsiella* according to Karling (1981). The strain NJM 9231 was maintained at 25°C on PYGS agar, and transferred to fresh PYGS agar monthly. The detailed and formal description of *Atkinsiella* sp. isolated from the rotifer *Brachionus plicatilis* will be presented in another paper.

As zoospore production was thought to be affected by incubation temperature, the effect of temperature on zoospore production was investigated as follows. Zoospore production in sterilized seawater was tested at four different temperatures (15, 20, 25 and 30°C). When ten mycelial colonies were transferred into sterilized seawater, zoospores were produced after 18 h at 30°C, 18-21 h at 25°C and 21-24 h at 20°C (Fig. 8).

Zoospore production was continuously observed for up to 3 days when thalli were incubated at 20-30°C, after which the thalli became empty. When the thalli were incubated at 15°C, zoospore production was observed 3 days after the start of incubation and continued for 5 days. However, some subthalli were not emptied after zoospore production had finished. Thus, it was demonstrated that zoospore production was affected by temperature, as zoospores were quickly produced at higher temperatures.

The effects of two chemicals, malachite green and formalin, on growth of the strain were determined. The concentrations of malachite green and formalin tested ranged from 0.0125 to 6.25 μg/ml and from 2.0 to 100 ppm, respectively. Broth for these tests consisted of 3.125 g of peptone, 3.125 g of yeast extract, 7.5 g of glucose, and 37.6 g of artificial seawater in 1,000 ml of distilled water. Tests were made in Multiwell Tissue Culture Plate (Falcon®, Becton Dickinson) with a mixture of 0.8 ml of broth, 0.2 ml of diluted chemical solution and 1 ml of zoospore suspension containing 3.0×10^3 spores/ml. The plates were incubated at 25°C. The growth of the fungus was checked 2 and 7 days after inoculation under a microscope and with the naked eye. In the positive control without chemicals, germination and fungal growth were observed after 2 days and 7 days of inoculation, respectively. The test revealed that both germination and fungal growth were inhibited by 0.05 μg/ml of malachite green, while formalin inhibited the germination and fungal growth at the concentrations of 7.8 ppm and 15.6 ppm, respectively.

Hamasaki and Hatai (1993) reported that *Atkinsiella* sp. isolated from zoeae of the mud crab *Scylla serrata* Forsskål was inhibited at the concentration of 25 ppm formalin in vitro, and bath treatment with 25 ppm formalin was also effective against fungal infection due to fungi of the order Lagenidiales in eggs and newly hatched larvae of swimming crabs *Portunus trituberculatus* Miers.

Rotifers are bred in a concrete tank as the first food supply for seed production of crustaceans and fishes at the Chiba Prefectural Tokyo Bay Sea Farming Center. It was feared that the *Atkinsiella* sp. might cause a fungal infection in larvae of those aquatic animals. The pathogenicity of the strain NJM 9231 toward other marine animals was tested by using zoeal stage larvae of the swimming crab. Zoeal larvae were first dipped into sterilized seawater including 500 μg/ml each of streptomycin sulphate and ampicillin to decrease bacterial cells on the surface. Fifty zoeal larvae were then put into a 100 ml beaker with 72 ml of sterilized seawater without any antibiotics and 8 ml of zoospore suspension adjusted to 1.0×10^3 spores/ml. Another 50 zoeal larvae were also placed in a beaker with 80 ml of seawater as a control. During the course of artificial infection, zoeal larvae were not fed and the water temperature was approximately 20°C. Weak aeration was provided in each beaker. After exposure to the zoospores, zoeal larvae in the beaker were examined daily under a microscope to determine whether infection had occurred. Encysted zoospores were first mainly observed around the eyes and swimmerets 1 day after inoculation. Mycelia were found in the bodies of the zoeal larvae artificially infected with the fungus after 4 days (Fig. 9), and zoospore formation was observed after 5 days (Fig. 10).

Mortality of zoeae caused by the fungal infection was about 40%. No mortalities occurred in the zoeae in the control beaker. As a result, the fungus isolated from the rotifer was demonstrated to be pathogenic to swimming crabs. Pathogenicity toward rotifers was also tested, but the test was not successful. This was attributed to the presence of many bacteria inside healthy rotifers. It was interesting that the fungal infection could develop under natural conditions.

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