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Research brief

Octosporea bayeri: fumidil B inhibits vertical transmission in *Daphnia magna*

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Abstract

Microsporidia are a highly successful and ecologically diverse group of parasites, and thus represent interesting model systems for research on host–parasite interactions. However, such research often requires the ability to cure hosts of infections, a difficult task, given the short lifespan of most invertebrates and the efficient vertical transmission of some parasites. To our knowledge, few treatments are available to cure microsporidiosis in invertebrate hosts, and protocols have not yet been developed to inhibit vertical transmission and thereby cure host lines. We present a protocol for inhibiting vertical transmission of the microsporidian parasite *Octosporea bayeri* in the freshwater crustacean *Daphnia magna*. We used 100 mg/L Fumidil B dissolved in the culture medium of the host. This technique allowed *Daphnia* to survive and reproduce and inhibited vertical transmission of the parasite. The method presented here may be of general interest for other aquatic host–parasite systems involving microsporidia.

Keywords: *Daphnia*; Microsporidia; Fumagillin; Fumidil B; Vertical transmission; Curing

1. Introduction

Microsporidia are a very successful group of parasites (Dunn and Smith, 2001). They infect a wide range of host species, from protists to nearly all invertebrate phyla and all vertebrate classes, and exhibit a large diversity in transmission strategies and life cycles. Consequently microsporidiosis can serve as a model system of host–parasite interactions for epidemiologists, ecologists, and evolutionary biologists (Agnew and Koella, 1999; Dunn et al., 1998; Ebert, 1998; Streett et al., 1993; Terry et al., 1997). But one man's meat is another man's poison: microsporidia are often a significant problem in cultures of animals, whether scientific or economic (e.g., aquaculture or apiculture) (Burges, 1973; Fries et al., 1984; Kent, 2000).

Microsporidia are obligate intracellular parasites with two morphological forms: a meront or proliferative within-cell stage and the spore or transmission stage. The life-cycles of microsporidia can be direct or indirect. Parasite transmission may occur horizontally (between infected and susceptible hosts) or vertically (from parent to offspring), and many species of microsporidia use both (Dunn and Smith, 2001). Vertical transmission is primarily maternal and often highly efficient (Andreadis and Vossbrinck, 2002; Dunn et al., 2001; Hunt et al., 1972; Streett et al., 1993).

Fumagillin is one of the few drugs known to be active against microsporidia (McCowen et al., 1951), The drug is derived from *Aspergillus fumigatus* (Bailey, 1953; Conteas et al., 2000; Didier, 1997; Webster, 1994; Wilson, 1974) and it has been used to treat *Nosema apis* in honey bees (Bailey, 1953), by feeding bees with fumagillin dissolved in sugar syrup. This approach has also been adapted for other insect species (Flint et al., 1972; Whittington and Winston, 2003; Wilson, 1974). Fumagillin administered in the diet, has also been used to treat microsporidiosis in fish (Hedrick et al., 1991; Kent and Dawe, 1994). Here we present a method for treating microsporidiosis in aquatic invertebrates. Using dissolved fumagillin in the culture medium, we cured clonal lines of *Daphnia magna* of infection by the parasite *Octosporea bayeri*.

Octosporea bayeri Jírovec (1936) is a microsporidian parasite of *D. magna* that infects the fat-cells and ovaries of its host and transmits both vertically and horizontally. Horizontal transmission occurs via spores released from decaying dead hosts. Vertical transmission is highly efficient in *O. bayeri*, with 100% of the clonal offspring, and 84% of the sexual young being infected (Vizoso et al., in press).

The host, *D. magna*, is a filter-feeding freshwater cladoceran typically inhabiting eutrophic ponds. It shows cyclical parthenogenesis, with clonal reproduction in good conditions and sexual reproduction when conditions are less favorable. Sexual reproduction results in the formation of resting eggs (ephippia). These eggs can survive extreme environmental conditions like drying or winter cold, and hatch when conditions become better. In a meta-population of *D. magna* in Tvärminne (southern Finland) *O. bayeri* is highly prevalent.

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Forty-five percentage of the *Daphnia* populations harbor the parasite, and the prevalence within some populations may reach 100% (Ebert et al., 2001, D. Ebert pers. observation).

2. Materials, methods, and results

2.1. Fumidil B

Fumagillin *by itself* is not water-soluble, but Fumidil B *is soluble*[100 g Fumidil B contain 2 g of fumagilin as salt with bicyclohexylammonium plus excipient (Sanofi; Ceva santé animale, la ballastière, 33501 Libourne Cedex, France)]. Fumagillin B (Medivet Pharmaceuticals, Canada) a *Nosema* treatment that basically contains the same agents but has a considerably higher solubility (in sugar syrup) is an alternative to Fumidil B (Webster, 1994).

2.2. Toxicity of Fumidil B

To find a concentration of Fumidil B that allowed the host to survive and reproduce, we performed a series of assays with varying concentrations of the antibiotic. *D. magna* (aged 48–72 h) from a single clone and vertically infected with the microsporidian parasite *O. bayeri* were placed singly in 80 ml artificial culture medium (modified after: Ebert et al., 1998; Klüttgen et al., 1994). Fumidil B was dissolved in the medium at the following concentrations: 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 4000 mg/L before *Daphnia* were added. Three individual *Daphnia* were used at each concentration, and the treatment medium was renewed three times a week for the lifespan of the treated *Daphnia*. All animals were kept at a constant temperature of 20 °C in a climate chamber (light:dark; 16:8 h). Cultures were checked daily for survival and reproduction, and fed with 2×10^6 cells of the green algae *Scenedesmus* sp. The experiment ended after 6 weeks, when all treated animals had died.

All *Daphnia* survived and reproduced in concentrations up to 500 mg/L Fumidil B in artificial culture medium. The animals died before reproduction at a concentration of 1000 mg/L, and died within three days in 4000 mg/L. A problem with the assay was that some of the drug precipitated and clogged the filter apparatus of the *Daphnia*. This effect was more pronounced in higher doses of Fumidil B. As a consequence of either this clogging effect and/or the toxicity of Fumidil B itself, newborn *Daphnia* in the 500 mg/L treatment had a very low survival in the treatment medium. They usually died within the first days if not removed and placed in medium without antibiotic.

2.3. Toxicity and curing effects of Fumidil B

In a second assay we repeated the first trial with Fumidil B concentrations of 0, 100, and 200 mg/L. This time, the drug was dissolved in a 1:1 mixture of artificial culture medium and deionized water. This was intended to reduce the problems with the low solubility of Fumidil B. All other conditions were the same as in the first experiment. Survival and reproduction were checked daily, and newborn *Daphnia* were removed and placed in culture medium without antibiotic and raised. When these young released their first young (c. day 12–15 post hatching) four of them, as well as their treated mother were sacrificed and checked for the presence of spores under a light microscope (Leica DMLB, magn.: 400×).

All *Daphnia* survived and reproduced in both 100 and 200 mg/L. The clogging effect appeared less pronounced in the second assay, especially in the 100 mg/L treatment. Still most newborn animals died if left in the treatment medium.

Light microscopy revealed that all treated *Daphnia* mothers still contained spores of *O. bayeri* (c. day 40). However, we could not find spores in any of the examined offspring of the two Fumidil B treatments. An examination of descendants of the cured individuals about six weeks later (*c.* 4 asexual *Daphnia* generations) still showed no visually detectable infections. All offspring of the control treatments were infected.

2.4. Curing Daphnia clones from O. bayeri

Two juvenile infected *Daphnia* (3-day old) from 18 different clones infected with *O. bayeri* were singly placed in artificial medium with 100 mg/L Fumidil B. The treatment medium was exchanged every Monday, Wednesday, and Friday, and the animals were fed with 2×10^6 algae cells per day for the first seven days, and then with 5×10^6 algae. Newborn offspring of the second (F1.2) and third clutch (F1.3) of the treated females were separated from their mothers and placed in pure artificial medium and raised to reproduction. Adult offspring of the F1.2 and F1.3 females were scanned for *O. bayeri* using real-time PCR. To do so, DNA was extracted from homogenized animals using the EZNA Tissue DNA kit (Peqlab, Erlangen, Germany). Real-time PCR was performed on the Rotor-Gene 2000 (Corbett Research, Mortlake, Australia) using SYBR Green I chemistry (Applied Biosystems, Rotkreuz, Switzerland). Species specific primers obi1fwd (agcatgtaggagcggaagag) and obi1rev (gatcaccggaaacgtactgg) were used to amplify a 112 bp fragment in the small subunit of the ribosomal RNA gene. Reaction volume was 20 µl with 2 µl of template DNA and 600 nM of each primer. Amplification was performed with an initial incubation step of 5 min at 95 °C and 50 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C (where fluorescence was acquired). A melt with constant fluorescence acquisition was done from 60 to 90 °C, in order to detect non-target amplifications. To ensure the accuracy of the PCR data, we added uninfected controls, water controls and a series of positive controls (dilutions of an *O. bayeri* infected individual) to every run of the real-time PCR machine. We were able to reliably detect parasite DNA in samples with a 10^4-fold dilution of normally infected positive controls.

In total, we examined one offspring from each of 36 F1.2 *Daphnia* and one offspring from each of 36 F1.3 *Daphnia* for infection using PCR. *Daphnia* from the F1 generation were not directly tested because, if healthy, they were used later for an experiment and therefore could not be killed to assess infection.

In 94.4% (34 out of 36) of the F1.2 offspring the quantitative PCR did not amplify DNA of *O. bayeri*. Light microscopy revealed that the F1.2 mothers of the two infected animals had been infected as well, indicating that curing had not worked in these 2 replicates. These two infected lines came from different clones, and the second clutch was born within 10 days after the beginning of the treatment. Neither of the F1.3 descendents (total of 36) showed evidence for infection. The parasite negative lines (one per clone) had subsequently been kept in culture for more than 10 asexual *Daphnia* generations and none developed signs of infection, indicating that vertical transmission had ceased.

3. Discussion

By keeping *D. magna* in a culture medium with 100 mg Fumidil B per liter, we inhibited vertical transmission of the microsporidian parasite *O. bayeri*. In the two cases where treatment was not successful, the clutch may have been produced too soon after exposure to Fumidil. The process of egg production takes between 5 and 8 days (Ebert, 1992), and in both infected lines the second brood was produced within 10 days from the start of the experiment. We therefore suggest that broods produced 10 days after initiation of the drug are reliably cured of infection.

Since vertical transmission in untreated lines occurs with an estimated efficiency of 100% (Vizoso et al., in press), Fumidil B treatment is so far the only available method for curing host lines. Cure is desirable for three reasons: first, cured *Daphnia* are much easier to maintain in laboratory cultures and do not run the risk of contamination of other cultures. Second, samples used for experiments can be truly

randomized, without discarding infected individuals. Finally, the possibility of curing clonal lines (or individuals) opens the door for interesting experiments regarding the ecology, epidemiology, and evolution of host–parasite interactions. For example, ecological and evolutionary consequences of infection can be assessed for the same host clones in sick and healthy replicates.

In addition to the lethality of high concentrations of Fumidil to *Daphnia* and the apparent clogging effect of the filter apparatus on freshly hatched *Daphnia*, we also observed that treated *Daphnia* did not grow well and reproduced later than controls. It could take up to three weeks to produce the first clutch of young in mothers treated with 100 mg/L Fumidil compared to 10–12 days in untreated individuals.

All *Daphnia* survived and reproduced at a concentration of 100 mg/L Fumidil B, allowing us to cure clonal lines. At this concentration we were able to use standard *Daphnia* medium instead of a 1:1 mixture of medium and deionized water. Given the observed effects of Fumidil on adult and young *Daphnia*, we suggest keeping cured lines for at least one further generation under controlled laboratory conditions prior to use in experiments. Although parasite-free offspring of mothers that were treated with Fumidil B were in good condition, maternal effects (e.g. Lynch and Ennis, 1983) may reduce their fitness relative to offspring from untreated mothers.

The mechanism by which fumagillin inhibits microsporidian replication is not very well understood. There is evidence that fumagillin inhibits RNA synthesis, which could explain its effect on the parasite, but as well indicates why fumagillin is toxic to treated hosts (Jaronski, 1972; Webster, 1994). This also explains why mature parasite spores may not be affected (Bailey, 1953). Consequently, spores left in the host body may continue the horizontal spread of infection after the treatment has stopped. Nevertheless, Fumagillin has been shown to have effects on the spores itself. Irregularly shaped spores of *Encephalitozoon cuniculi* were found by Shadduck (1980), and spores with a reduced infectivity in *Encephalitozoon intestinalis* had been reported by Didier (1997).

Our protocol did cure the original host *Daphnia*. All examined Fumidil B-treated animals did contain spores, even after more than 3 weeks of exposure to Fumidil B. Only their offspring was free of parasites, while all control offspring were infected. This indicates that Fumidil B might not be active against spores (Bailey, 1953), but only against the replicating parasite. Alternatively, Fumidil might cause the production of non-infective spores. If vertical transmission in our system occurs by spores actively infecting oocytes or yolk cells as is found in other invertebrate systems (Dunn et al., 2001), the production of non-infective spores would result in no vertical transmission despite the presence of seemingly normal spores.

The aim of the present study was to develop a method to cure *Daphnia* from infection by *O. bayeri*. Effects of the drug on spore quantity and quality in treated hosts, as well as the exact mechanism for preventing vertical transmission remains unclear.

Even though the method presented here is specific to a certain host–parasite system, it may also be valuable to other systems, e.g., microsporidiosis in gammariids or mosquitos (Agnew and Koella, 1999; Kellen et al., 1966; Kelly et al., 2003; MacNeil et al., 2003). To our knowledge, this is the first method to treat microsporidiosis in aquatic hosts to which drugs cannot be administered as food. Moreover, in systems where mature spores are excreted continuously (e.g., microsporidian gut parasites) or in longer-lived hosts, it may be possible to cure individuals rather than inhibiting vertical transmission.

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References

Agnew, P., Koella, J.C., 1999. Life history interactions with environmental conditions in a host–parasite relationship and the parasite's mode of transmission. Evolutionary Ecology 13, 67–89.

Andreadis, T.G., Vossbrinck, C.R., 2002. Life cycle, ultrastructure and molecular phylogeny of *Hyalinocysta chapmani* (Microsporidia: Thelohaniidae), a parasite of *Culiseta melanura* (Diptera: Culicidae) and *Orthocyclops modestus* (Copepoda: Cyclopidae). Journal of Eukaryotic Microbiology 49, 350–364.

Bailey, L., 1953. Effect of fumagillin upon *Nosema apis* (Zander). Nature 171, 212–213.

Burges, H.D., 1973. Enzootic diseases of insects. Annals of the New York Academy of Sciences 217, 31–49.

Conteas, C.N., Berlin, O.G.W., Ash, L.R., Pruthi, J.S., 2000. Therapy for human gastrointestinal microsporidiosis. American Journal of Tropical Medicine and Hygiene 63, 121–127.

Didier, E.S., 1997. Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication in vitro. Antimicrobial Agents and Chemotherapy 41, 1541–1546.

Dunn, A.M., Smith, J.E., 2001. Microsporidian life cycles and diversity: the relationship between virulence and transmission. Microbes and Infection 3, 381–388.

Dunn, A.M., Terry, R.S., Smith, J.E., 2001. Transovarial transmission in the microsporidia. Advances in Parasitology 48, 57–100.

Dunn, A.M., Terry, R.S., Taneyhill, D.E., 1998. Within-host transmission strategies of transovarial, feminizing parasites of *Gammarus duebeni*. Parasitology 117, 21–30.

Ebert, D., 1992. A food independent maturation threshold and size at maturity in *Daphnia magna*. Limnology and Oceanography 37, 878–881.

Ebert, D., 1998. Infectivity, multiple infections, and the genetic correlation between within-host growth and parasite virulence: a reply to Hochberg. Evolution 52, 1869–1871.

Ebert, D., Hottinger, J.W., Pajunen, V.I., 2001. Temporal and spatial dynamics of parasites in a *Daphnia* metapopulation: which factors explain parasite richness?. Ecology 82, 3417–3434.

Ebert, D., Zschokke-Rohringer, C.D., Carius, H.J., 1998. Within- and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. Proceedings of the Royal Society of London B *Octosporea bayeri* 265, 2127–2134.

Flint, H.M., Eaton, J., Klassen, W., 1972. Use of Fumidil-B to reduce microsporidian disease in colonies of boll-weevil (Coleoptera: Curculionidae). Annals of the Entomological Society of America 65, 942.

Fries, I., Ekbohm, G., Villumstad, E., 1984. *Nosema apis*, sampling techniques and honey yield. Journal of Apicultural Research 23, 102–105.

Hedrick, R.P., Groff, J.M., Baxa, D.V., 1991. Experimental infections with *Enterocytozoon salmonis* (Chilmonczyk, Cox, Hedrick (Microsporea))—an intranuclear microsporidium from chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 10, 103–108.

Hunt, R.D., Foster, H.L., King, N.W., 1972. Encephalitozoonosis—evidence for vertical transmission. Journal of Infectious Diseases 126, 212–214.

Jaronski, S.T., 1972. Cytochemical evidence for RNA synthesis inhibition by Fumagillin. Journal of Antibiotics 25, 327–331.

Jírovec, O., 1936. Über einige in *Daphnia magna* parasitierenden Mikrosporidien. Zoologischer Anzeiger #116, 136–142.

Kellen, W.R., Chapman, H.C., Clarke, T.B., Lindegren, J.E., 1966. Transovarian transmission of some *Thelohania* (Nosematidae: Microsporidia) in mosquitoes of California and Louisiana. Journal of Invertebrate Pathology 8, 355–359.

Kelly, A., Hatcher, M.J., Dunn, A.M., 2003. The impact of a vertically transmitted microsporidian, *Nosema granulosis* on the fitness of its *Gammarus duebeni* host under stressful environmental conditions. Parasitology 126, 119–124.

Kent, M.L., 2000. Marine netpen farming leads to infections with some unusual parasites. International Journal for Parasitology 30, 321–326.

Kent, M.L., Dawe, S.C., 1994. Efficacy of fumagillin Dch# against experimentally-induced *Loma salmonae* (Microsporea) infections in chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 20, 231–233.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Research 28, 743–746.

Lynch, M., Ennis, R., 1983. Resource availability, maternal effects, and longevity. Experimental Gerontology 18, 147–165.

MacNeil, C., Dick, J.T.A., Hatcher, M.J., Terry, R.S., Smith, J.E., Dunn, A.M., 2003. Parasite-mediated predation between native and invasive amphipods. Proceedings of the Royal Society of London, B 270, 1309–1314.

McCowen, M.C., Callender, M.E., Lawlis, J.F., 1951. Fumagillin (H-3), a new antibiotic with amebicidal properties. Science 113, 202–203.

Shadduck, J.A., 1980. Effect of Fumagillin on in vitro multiplication of *Encephalitozoon cuniculi*. Journal of Protozoology 27, 202–208.

Streett, D.A., Woods, S.A., Onsager, J.A., 1993. Vertical transmission of a *Nosema* sp. (Microsporida, Nosematidae) infecting a grasshopper, *Chorthippus curtipennis* (Orthoptera, Acrididae). Environmental Entomology 22, 1031–1034.

Terry, R.S., Dunn, A.M., Smith, J.E., 1997. Cellular distribution of a feminizing microsporidian parasite: a strategy for transovarial transmission. Parasitology 115, 157–163.

Vizoso, D.B., Lass, S., Ebert, D., in press. Characteristics of the microsporidium *Octosporea bayeri* and its routes of transmission. Parasitology.

Webster, T.C., 1994. Fumagillin affects *Nosema apis* and honey-bees (Hymenoptera: Apidae). Journal of Economic Entomology 87, 601–604.

Whittington, R., Winston, M.L., 2003. Effects of *Nosema bombi* and its treatment fumagillin on bumble bee (*Bombus occidentalis*) colonies. Journal of Invertebrate Pathology 84, 54–58.

Wilson, G.G., 1974. Use of Fumidil B to suppress microsporidan *Nosema fumiferanae* in stock cultures of spruce budworm, *Choristoneura fumiferana* (Lepidoptera-Tortricidae). Canadian Entomologist 106, 995–996.