Bulletin of the National Salmon
Resources Center, No. 5: 1-6
December 2002

さけ・ます資源管理センター
研究報告　第 5 号　1-6頁
2002年12月

Investigation of the Transmission Stage of the Microsporidian *Kabatana takedai* in Salmonids

Isao Fujiyama[*1], Shigehiko Urawa[*2#],
Hiroshi Yokoyama[*1], and Kazuo Ogawa[*1]

[*1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan*
[*2]*Research Division, National Salmon Resources Center, 2-2 Nakanoshima, Toyohira-ku, Sapporo 062-0922, Japan*

Abstract. – In order to examine the transmission route of the microsporidian *Kabatana takedai* to salmonid fish, experimental challenges to rainbow trout (*Oncorhynchus mykiss*) was tried by three methods (oral intubation, intraperitoneal injection, and immersion) using *K. takedai* mature spores isolated from infected fish. However, the experimental infection was not successful by any methods. To estimate the size of the transmission stage, masu salmon (*O. masou*) were exposed to infectious river water filtered by three sets of nylon meshes with openings of 300 μm, 100 μm and 40 μm. As a result, *K. takedai* infections occurred among all fish groups. These results suggest that the transmission of *K. takedai* to fish is not due to the direct spore transmission, but might due to the unknown infectious stage smaller than 40 μm.

Key words : Microsporidia, *Kabatana takedai*, transmission, plankton net

Introduction

The microsporidian parasite *Kabatana takedai* (Awakura 1974) is known as an important and endemic pathogen of wild and cultured salmonid fish. This disease has been reported only in the Chitose River (Takeda 1933), Lake Akan and Tokitonuma of Hokkaido, Japan (Awakura et al. 1966; Awakura 1978) and in the Taranay and Bryanka Rivers of Sakhalin, Russia (Vyalova and Voronin 1987; Vyalova 1999).

The parasite infecting the heart and trunk muscle was first described by Takeda (1933) from rainbow trout (*Oncorhynchus mykiss*) in the Chitose Hatchery. Since it was evident that the disease occurred when river water temperature exceeded 15℃ from July to September (Awakura 1974; Urawa 1989), the disease had been controlled by keeping water temperature under 13℃ adding spring water in the hatchery. However, Urawa (2001) found that *K. takedai* could form cysts at 13℃, and the result of other experiment in 2001 indicated that the cyst formation occurred even at 11℃ (Fujiyama, unpublished data).

Generally, it is a common concept that the infection of microsporidian is due to direct transmission of spores released into the water from dead host organisms (Lom and Dyková 1992), which supported by some experimental evidences. *Glugea plecoglossi* established the infection by intubation, injection and immersion with spores (Takahashi 1981), and *Loma salmonae* formed xenoma by the ingestion of infected tissue (Kent et al. 1995). Similarly, *K. takedai* was reported to be successful in experimental transmission by ingestion of the feed-mixed spores or immersion with spores, although the prevalence was only 10% and 22%, respectively (Awakura 1974).

Awakura (1974) also examined whether salmonid fish could be infected with *K. takedai* through intermediate hosts. Juvenile sockeye salmon (*O. nerka*) was exposed to the Chitose River water filtered by four kinds of plankton nets with openings of 2.0 × 2.0 mm, 0.65-0.80 × 0.70-0.80 mm, 0.20-0.33 × 0.23-0.30 mm and 0.12-0.18 × 0.04-0.12 mm for 75 days in the infective season. As a result,

[#]Corresponding author (urawa@salmon.affrc.go.jp)
Contribution A No. 27 from the National Salmon Resources Center.

only the finest net prohibited the infection with *K. takedai*. He suspected that fish were not infected with spores directly, but through the involvement of small aquatic organisms as a vector, which were trapped on 0.12-0.18 × 0.04-0.12 mm-net, but passed through 0.20-0.33 × 0.23-0.30 mm-net.

We performed infection experiments similar to those of Awakura (1974) with some improvement in order to determine the transmission stage of *K. takedai* to fish.

Materials and Methods

Experimental fish

Juvenile rainbow trout used in the experimental infection trial (trial 1) were obtained from Yamanashi Prefectural Fisheries Technology Center and maintained in dechlorinated tap water at 16℃ before the beginning of the trial. Underyearling masu salmon in the plankton net trial (trial 2) were provided from the Chitose Hatchery, where the fish had been reared in the pond supplied with spring water, which were not contaminated with *K. takedai*. The fish were held in flow-through tanks with well water at 11℃ before the beginning of the trial. These fish were fed commercial dry pellets.

Spore preparation

Mature spores used in the trial 1 were prepared by the following methods. Cysts of *K. takedai* were isolated from masu salmon reared in the Chitose Hatchery, squashed and passed through a steel mesh (Cell Dissociation Sieve-Tissue Grinder Kit, Sigma, U.S.A.) and a nylon mesh series (100 μm, 75 μm, 50 μm, 25 μm, 10 μm, and 5 μm) to remove tissue debris. The filtrate was layered onto equal volume of 50% Percoll and centrifuged at 750×g for 15 min. The pellet was resuspended in distilled water and stored at 4℃ with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, Gibco. BRL, U.S.A.) until used. Spore viability defined as the extrusion rate of polar tube stimulated by 30% hydrogen peroxide was about 60%.

Spore infection experiment (trial 1)

Juvenile rainbow trout (mean body length, 6.9 cm) were challenged with *K. takedai* spores by the following methods.

Intubation: Twenty three fish were anesthetized with 50 ppm tricainemethanesulphonate (MS222, Sigma, U.S.A.), and then intubated orally into the stomach with *K. takedai* spores (1×10^7 spores/fish) using a 1 ml tipped with a 5 cm sterilized silicon tube (1 mm inner diameter and 2 mm outer diameter).

Injection: Twenty three fish were anesthetized and injected with spore suspensions (1×10^7 spores/fish) intraperitoneally.

Immersion: Twenty three fish were exposed to 2 l of spore suspension (2.6×10^3 spores/fish) in a 3-l plastic container at 18℃ for 24 hours with a gentle aeration.

Unexposed control: Twenty three fish were maintained in the 3-l plastic container with 2 l of dechlorinated tap water at 18℃ for 24 hours with a gentle aeration.

The experimental fish were transferred to each of three 60-l glass tanks with a circulated filtration unit maintained at 18±2℃, and fed with dry pellets.

Table 1. Combination of three kinds of nets to filtrate river water in four test groups for infection experiments with *K. takedai*.

Group	Openig size of net (μm)			Total number of nets
	300	100	40	
A				0
B	○			1
C	○	○		2
D	○	○	○	3

Plankton net experiment (trial 2)

From July 29 to August 12 in 2002, 30 juvenile masu salmon were kept in each of the four 120-l flow-through tanks supplied with Chitose River water filtrated by combination of three sets of nylon mesh with openings of 300 μm, 100 μm and 40 μm at the mean flow rate of 6.4 (5.2-9.0 in range) l/min (Table 1). As a positive control group, 30 fish were kept in a tank supplied with unfiltered river water. The river water temperature during the exposure was 16.0-17.4℃. Plankton nets were washed once in two or three days in order to prevent the nets from clogging. Then the experimental fish were stocked in flow-through tanks supplied with parasite-free well water at 18℃, and fed with commercial dry pellets for 5 weeks.

Sampling and parasitological examination

Trial 1: Half of the surviving fish in each tank were removed and sacrificed with MS222 overdosed at 22 days post exposure (PE). The heart and trunk muscle were examined to detect *K. takedai* cysts using a dissection microscope. At 33 days PE, all remaining surviving fish were sacrificed and examined as described above.

Trial 2: After maintained under spring water for 5 weeks, the fish were weighed, measured and examined immediately for *K. takedai* cysts as described above. The number of parasite cysts was recorded for infected fish.

Statistical analysis

Statistical comparisons were performed between the four groups for trial 2. A comparison of the prevalence (percentage) and mean number of cysts in the heart and trunk muscle of infected fish were performed using chi-square analysis and Kruskal-Wallis ANOVA, respectively. Significant differences were reported at the α=0.05 level of probability.

Results

Spore infection experiment

The cysts of *K. takedai* were not observed in the heart and trunk muscle of rainbow trout challenged using any of the three methods (Table 2). While a few fish died during this experiment, the cause of death was not considered to be *K. takedai* infection by gross observation.

Plankton net experiment

The prevalence of *K. takedai* was 71.4-90.0% in 4 test groups (Table 3). The mean number of cysts in the heart and trunk muscle of the infected fish was 1.3-2.0 and 14.9-17.5 cysts per fish, respectively. There were no significant differences in the prevalence nor number of cysts among all test groups. A few fish were died in Group B, C and D at the beginning of this experiment, but the cause of these deaths was not considered to be *K. takedai* infection by gross observation.

Table 2. Prevalence of infection with *Kabatana takedai* in the heat and trunk muscle of rainbow trout by three experimental infection methods, 23 and 33days post exposure (PE).

Infection method	23 days PE		33 days PE	
	Heart	Trunk	Heart	Trunk
Intubation	0/8*	0/8	0/8	0/8
Injection	0/10	0/10	0/10	0/10
Immersion	0/7	0/7	0/8	0/8
Control	0/9	0/9	0/10	0/10

* Number of fish infected / number of fish examined.

Table 3. The prevalence and intensity of infection with *Kabatana takedai* in the heart and trunk muscle of masu salmon reared in the river water filtrated by different opening sizes of plankton nets. The opening size and combination of nets used for each test group were shown in Table 1.

Test group	Number of fish examined	Prevalence (%)*[1]	Intensity*[2]	
			Heart	Trunk
A	30	90.0	2.0 ± 1.3 (1-5)	16.0 ± 13.4 (1-57)
B	27	88.9	2.0 ± 1.0 (1-4)	14.9 ± 11.3 (3-45)
C	27	81.5	1.8 ± 0.7 (1-3)	17.5 ± 10.6 (3-52)
D	21	71.4	1.3 ± 0.8 (1-3)	15.5 ± 15.0 (3-46)

*[1] Number of fish infected with *K. takedai* / Number of fish examined.
*[2] Mean number ± SD (range) of cysts in the infected fish.

Discussion

Since *K. takedai* was first described by Takeda (1933), several aspects of studies on this parasite have been reported; the temperature-dependency of the parasite's development, the infection route, the host response, and the epizootiology in the Chitose River and Chitose Hatchery (Awakura and Kurahashi 1967; Awakura 1974; Urawa 1989, 2001). However, the prevention method of *K. takedai* infection in the Chitose Hatchery has not been established yet, because the life cycle and critical temperature requirement in the development of this parasite are not elucidated yet.

We could not establish the artificial infection by any exposure methods using *K. takedai* spores. Awakura (1974) reported that *K. takedai* could succeed in the transmission and forming of cysts by ingestion of the feed added spores or immersion with spore suspensions. However, Awakura (1974) also mentioned that there might be some problems with the fluctuating water temperature during the experiment and the challenge method. In this study, the water temperature was fixed at 18℃ and the quantitative method of infection was used. Replicated experiments under the similar condition to that of Awakura (1974) could not reproduce the infection with *K. takedai* (Awakura, unpublished data). The present study also suggests that spores are not the infectious stage to fish.

Most of the fish were infected with *K. takedai* within a short period (2 weeks) exposing to the river water. Awakura (1974) presumed that the rotifer *Euchlanis dilatata* which flowed from the upstream of the Chitose River might act as the intermediate organism in natural transmission through the field and experimental studies. The size of *E. dilatata* is about 200-270 μm (Mizuno 1991), therefore the rotifer had to be trapped on 100 μm or 40 μm-net. If *E. dilatata* was the intermediate organism, the fish of Group C or D would have been expected to be uninfected with *K. takedai*. Awakura (1974) reported that the fish exposed to the river water filtrated by 0.12-0.18×0.04-0.12 mm net were uninfected with *K. takedai* at all, but also mentioned that the flow rate of the river water through the finest net (about 3 l/min) was a quarter of the other groups because of the clogging of the net. It is suspected that this clogging was one of the reasons why the fish were uninfected. During the fish was exposed to river waters in this experiment, the organisms trapped on each net were fixed by 10% neutral buffered formalin and observed. As a result, *E. dilatata* were found in both the 100 μm and 40 μm nets. The four test groups resulted in high prevalence of infection with *K. takedai*, which were not significantly different among 4 groups. Therefore, this study shows that the organism related to *K. takedai* infection could pass through all nets easily.

In the microsporidians *Amblyospora* and *Parathelohania* species which are infective to mosquitoes, a spore formed in a copepod interemediate host is responsible for horizontal transmission to the mosquito host (Micieli et al. 2000). Sweeney et al. (1985) demonstrated that uninucleate spores of *Amblyospora dyxenoides* from the mosquito (*Culex annulirostris*) are infective to the copepod (*Mesocyclops albicans*) and that there is a sequence of development and achievement different type of mature spores infective to mosquitoes. Similarly, Andreadis (1985) succeeded the transmission of *A. connecticus* to the copepod (*Acanthocyclops vernalis*). Though the report about the obvious existence of intermediate organisms in the fish microsporidians have not been published yet, some authors have suggested that intermediate or carrier hosts may be involved (McVicar 1975, Olson 1976). The present results also suggest the presence of unknown transmission stage of *K. takedai* to fish, whose size might be much smaller than 40 μm. Further studies related to the transmission stage to fish are needed for elucidation of the life cycle of *K. takedai*.

In 1999, due to the unusual increase of water temperature, heavy infection of *K. takedai* occurred among juvenile masu salmon in the Chitose Hatchery, and approximately 100,000 infected fish were disposed to prevent this parasite from spreading (Urawa 2001). Since it is difficult to cure the infected fish by temperature manipulations (Urawa 2001), the most important things is the prevention of *K. takedai*. The findings in the present study show a possibility that the unknown transmission stage of *K. takedai* may exists. For the establishment of the prevention method in the Chitose Hatchery, further studies should be performed to clarify the transmission stage and infective period to fish in the Chitose River.

Acknowledgements

The authors wish to express our appreciation to the staffs of National Salmon Resources Center and Chitose Hatchery for their kind help to provide fish and information for this study, and their generous hospitality. We also thank Yamanashi Prefectural Fisheries Technology Center for assistance with providing fish. Similarly we are grateful to Dr S.-J. Lee for her technical help.

References

Andreadis, T. G. 1985. Experimental transmission of a microsporidian pathogen from mosquitoes to an alternate copepod host. Proc. Natl. Acad. Sci., USA, 82: 5574-5577.

Awakura, T. 1974. Studies on the microsporidian infection in salmonid fishes. Sci. Rep. Hokkaido Fish Hatchery, 29: 1-95. (In Japanese with English summary.)

Awakura, T. 1978. A new epizootic of a microsporidiosis of salmonids in Hokkaido, Japan. Fish Pathol., 13: 17-18. (In Japanese with English summary.)

Awakura, T., and S. Kurahashi. 1967. Studies on the *Plistophora* disease of salmonid fishes-III. On prevention and control of the disease. Sci. Rep. Hokkaido Fish Hatchery, 22: 51-68. (In Japanese with English summary.)

Awakura, T., S. Kurahashi, and H. Matsumoto. 1966. Studies on the *Plistophora* disease of salmonid fishes-II. Occurrence of the microsporidian disease in a new district. Sci. Rep. Hokkaido Fish Hatchery, 21: 1-12. (In Japanese with English summary.)

Kent, M. L., S. C. Dawe, and D. J. Speare. 1995. Transmission of *Loma salmonae* (Microsporea) to chinook salmon in sea water. Can. Vet. J., 36: 98-101.

Lom, J., and I. Dyková. 1992. Protozoan parasites of fishes. Elsevier, Amsterdam. 315 p.

Micieli, M. V., J. J. Garcia, and J. J. Becnel. 2000. Horizontal transmission of *Amblyospora albifasciati* Garcia and Becnel, 1994 (Microsporidia: Amblyosporidae), to a copepod intermediate host and the neotropical mosquito, *Aedes albifasciatus* (Macquart, 1837). J. Invertebr. Pathol., 75: 76-83.

McVicar, A. H. 1975. Infection of plaice *Pleuronectes platessa* L. with *Glugea* (*Nosema*) *stephani* (Hagenmiiller 1899) (Protozoa: Microsporidia) in a fish farm and under experimental condition. J. Fish Biol., 7: 611-619.

Mizuno, T. 1991. Class Eurotatorea. In An illustrated guide to freshwater zooplankton in Japan (edited by T. Mizuno and E. Takahashi). Tokai University Press, Tokyo. pp. 181-305. (In Japanese.)

Olson, R. E. 1976. Laboratory and field studies on *Glugea stephani* (Hagenmuller), a micirosporidian parasite of pleuronectid flatfishes. J. Protozool., 23: 158-164.

Sweeney, A. W., E. I. Hazard, and M. F. Graham. 1985. Intermediate host for an *Amblyospora* sp. (Microspora) infecting the mosquito, *Culex annulitostris*. J. Invertebr. Pathol., 46: 98-102.

Takahashi, S. 1981. Studies on *Glugea* infection of ayu, *Plecoglossus altivelis*. Rep. Bull. Shiga Pref. Fish. Exp. Stn., 34: 1-81.

Takeda, S. 1933. A new disease of rainbow trout. Keison Iho, 5: 1-9. (In Japanese.)

Urawa, S. 1989. Seasonal occurrence of *Microsporidium takedai* (Microsporida) infection in masu salmon, *Oncorhynchus masou*, from the Chitose River. Physiol. Ecol. Japan, Spec.Vol., 1: 587-598.

Urawa, S. 2001. Occurrence of *Kabatana takedai* (Microspora) in juvenile masu salmon (*Oncorhynchus masou*) reared at varying water temperature in a hatchery. Bull. Natl. Salmon Resources Center, 4: 7-11.

Vyalova, G. P. 1999. Diseases of Sakhalin salmon (review). Sci. Rep. Hokkaido Fish. Exp. Stn., 54: 47-51.

Vyalova, G. P., and V. N. Voronin 1987. Microsporidiosis of Salmonidae from Sakhalin: distribution and dynamics of the infection. Parazitologiia, 21: 553-558. (In Russian with English summary.)

サケ科魚類に寄生する武田微胞子虫*Kabatana takedai*の感染源の推定

藤山　勲・浦和茂彦・横山　博・小川和夫

武田微胞子虫*Kabatana takedai*のサケ科魚類への感染経路を調べるため，感染魚から分離した成熟胞子を用いてニジマスに対する経口投与，腹腔内注射，浸漬による人工感染を試みた．その結果，いずれの方法によっても心臓および体側筋肉中にシストの形成はみられず，感染は不成立に終わった．次に，感染源の大きさを推定するため，3種のプランクトンネット（300 μm，100 μm，40 μm）で濾過した千歳川河川水でサクラマスを飼育したところ，いずれのネットを通過した河川水によっても感染が起きた．以上の結果より，魚への感染は胞子による直接伝播ではなく，40 μm以下の未知の感染ステージが寄与していることが示唆された．