

# 魚類から分離されたOchroconis humicolaに対するイトラコナゾールのin vitroとin vivoにおける効果

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著者名	Munchan,C. 畑井,喜司雄 高木,修作 山下,亜純
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## In Vitro and In Vivo Effectiveness of Itraconazole against *Ochroconis humicola* Isolated from Fish

Chutharat MUNCHAN<sup>1,2</sup>, Kishio HATAI<sup>1,\*</sup>, Shiyuusaku TAKAGI<sup>3</sup>  
and Azumi YAMASHITA<sup>3</sup>

**Abstract:** Antifungal activities of amphotericin B, fluconazole, 5-fluorocytosine, itraconazole, micafungin, miconazole, terbinafine and voriconazole against four strains of *Ochroconis humicola* isolated from fish were tested by the broth microdilution method. Three of these drugs (itraconazole, terbinafine and voriconazole) were effective against all isolates. The most active drug was terbinafine (for liniment) with a MIC (MFC) range of 0.06 to 0.13 (0.0625 to 0.125)  $\mu\text{g/ml}$ . Itraconazole (for oral administration), with a MIC (MFC) range of 0.5 to 2.0 (0.5 to 1.0)  $\mu\text{g/ml}$ , was chosen for in vivo treatment. In vivo treatment with itraconazole of striped jack *Pseudocaranx dentex* experimentally infected with *O. humicola* was conducted for 50 days. No fish died, but grey to white nodules were found in the visceral membrane, kidney, liver and spleen in the fish. Granulomatous inflammatory reactions were histopathologically found in all fish injected with conidia of *O. humicola* NJM 0472. Clinical signs and histopathological findings indicated that itraconazole showed no efficacy for curing the fish infected with *O. humicola*.

**Key words:** *Ochroconis humicola*; Itraconazole; Striped jack; Antifungal agent

Fungi of the genus *Ochroconis* are ubiquitously distributed in soil and plant roots, and occasionally cause disease in animals and humans. *Ochroconis humicola* is a member of dematiaceous fungi, and recognized as an etiological agent of fungal infection in aquatic animals, especially in fishes (Ross and Yusutake 1973; Ajello et al. 1977; Schaumann and Priebe 1994; Wada et al. 1995; Bowater et al. 2003; Wada et al. 2005; Munchan et al. 2006). Moreover, *Ochroconis gallopava* has been reported as a problematic infection in humans and animals. Many reports have been published on the in vitro and in vivo treatment of *O. gallopava* in humans, while such reports are rare for fishes. Munchan et al. (2006) reported systemic mycosis in cultured striped jack *Pseudocaranx dentex* causing a cumulative mortality of 25% in one month. The

aim of the present study is to investigate the in vitro efficacy of antifungal agents against *O. humicola* isolated from fish, and the in vivo activity of itraconazole against systemic infection of *O. humicola* NJM 0472 in striped jack.

### Materials and Methods

#### Fungal strains

Four strains of *O. humicola*, isolated from diseased fish, were used in this study. The source of each isolate is shown in Table 1. Strains cultured at 25°C for 4 weeks on potato dextrose agar (PDA) were used for the in vitro study of the antifungal susceptibility tests.

#### Antifungal agents

Eight antifungal agents, amphotericin B

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<sup>1</sup>Laboratory of Fish Diseases, Nippon Veterinary and Life Science University, Musashino, Tokyo 180-8602, Japan.

<sup>2</sup>Present Address: Faculty of Agricultural Technology, Rajabhat Mahasarakhum University, Mahasarakhum, 44000, Thailand.

<sup>3</sup>Ehime Prefecture Fish Disease Control Center, Uwajima, Ehime 798-0087, Japan.

\*Corresponding author: Tel: +81-0422-31-4151, E-mail: hatai@nvl.u.ac.jp

**Table 1.** Source of strains used in this study

Strains	Location	Host
Present isolate NJM 0472	Ehime Pref.	Striped jack <i>Pseudocaranx dentex</i>
<i>Ochroconis humicola</i> NJM 0565	Kagoshima Pref.	Red sea bream <i>Pagrus major</i>
<i>O. humicola</i> NJM 0663	Oita Pref.	Rockfish <i>Sebastes marmoratus</i>
<i>O. humicola</i> ATCC 24910	USA	Coho salmon <i>Oncorhynchus kisutch</i>

(AMPH) (Wako, Osaka, Japan), fluconazole (FLCZ) (Wako), 5-fluorocytosine (5-FC) (TCI®, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), itraconazole (ITCZ) (Janssen Pharmaceutical K.K., Tokyo, Japan), micafungin (MCFG) (Astellas, Tokyo, Japan), miconazole (MCZ) (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), terbinafine (TBN) (Novartis Pharma K.K., Tokyo, Japan) and voriconazole (VRCZ) (Pfizer, Tokyo, Japan) were used in this study. All agents, with the exception of FLCZ and 5-FC, were dissolved in dimethyl sulfoxide (DMSO) (Wako) at a concentration of 1,600  $\mu\text{g/ml}$  for AMPH, ITCZ, MCFG, MCZ, TBN and VRCZ. FLCZ was dissolved in methanol (Wako) at a concentration of 5,120  $\mu\text{g/ml}$ . 5-FC was dissolved in sterile distilled water at a concentration of 5,120  $\mu\text{g/ml}$ . Each agent was diluted with RPMI-1640 medium (with L-glutamine; without bicarbohydrate) (Nissui, Tokyo, Japan), buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma®, Louis, USA) according to the National Committee for Clinical Laboratory Standard (NCCLS) guideline M38-A (2002). Final concentrations ranged from 0.031 to 16  $\mu\text{g/ml}$  for AMPH, ITCZ, MCFG, MCZ, TBN and VRCZ, and from 0.031 to 64  $\mu\text{g/ml}$  for FLCZ and 5-FC.

#### *Antifungal susceptibility testing*

An inoculum preparation of each fungal strain was collected by adding 10 ml of sterile water to each fungal plate. Subsequently, the conidia mass was collected by scraping the surface of the fungal colony using a sterile loop. The number of conidia was determined under a microscope using a hemacytometer, and further diluted with RPIM-1640 to  $2.0 \times 10^4$  conidia/ml. A broth microdilution method was developed according to the NCCLS M38-A document. Tests were performed in 96-well

flat-bottom microtitration plates. Each well contained 100  $\mu\text{l}$  of the diluted drug and 100  $\mu\text{l}$  of the conidial suspensions. The dilution medium containing 0.01% DMSO served as the control in the case of drugs using DMSO as solvent. Similarly for the other drugs, a mixture of the corresponding solvent and dilution medium was used as growth control. Duplicate tests were done for each experiment. Microdilution trays were incubated at 25°C and MIC was determined after 6 days. The minimal inhibitory concentration (MIC) was determined visually as the lowest concentration showing visible growth inhibition when compared with the growth of the drug-free control. The minimal fungicidal concentration (MFC) was also determined. The MFC was the lowest drug concentration that resulted in either no growth or fewer than three colonies (99.9% killing) (Espinel-Ingroff 2001). The most potent drug (itraconazole) was selected for in vivo study of striped jack experimentally infected with *O. humicola* NJM 0472.

#### *Experimental fish*

One hundred striped jack were purchased from a private fish farm in Ehime Prefecture for use in this experiment. The fish were acclimatized at room temperature for one week before the experiment. The experiments were carried out at the Ehime Prefecture Fish Disease Central Center.

#### *Challenge protocol*

*O. humicola* NJM 0472 was incubated at 25°C for 28 days on PDA. The fungal conidia were collected by adding 10 ml of 0.85% NaCl (physiological saline) into each culture plate and scraping the surface of colonies with a sterile loop. The conidia suspension was then filtrated through medical gauze and transferred to a 30-ml

autoclaved tube. The conidial suspension was observed under a microscope, and the number of conidia was adjusted to  $1.0 \times 10^5$  conidia/ml using a hemacytometer. Experimental challenge was performed by injecting 0.1 ml of conidial suspension into the abdominal cavity. Fish of the negative control group were injected intraperitoneally with physiological saline.

### Treatment

In vivo treatment was carried out on fish experimentally infected with *O. humicola*. The antifungal drug itraconazole (ITRIZOLE®, Tokyo, Japan) was prepared by mixing the agent with commercial feed pellets (dosage 40 mg/kg). The feeding ratio of each fish was 2% of its body weight per day. The treatment group was divided into five groups consisting of 20 fish each, and the fish were fed on medicated food as follows: fish in group 1 were fed on medicated food for 7 days commencing 4 h after injection; fish in group 2 were fed on medicated food continuously for 7 days commencing 4 days after injection; fish in group 3 were fed on medicated food continuously for 7 days commencing 8 days after injection; fish in group 4 were challenged with fungal conidia to serve as the positive control, and were fed on untreated pellets during the period of the experiment; and fish in group 5 were injected with physiological saline to serve as the negative control, and were fed on untreated pellets for the positive control. All experiment groups were maintained in 200 l polyethylene plastic tanks containing approximately 160 l of aerated, continuously flowing seawater with a flow rate of 7 l/min. Water temperature ranged from 13 to 20°C during the period of the experiment. Mortality was observed daily for 50 days after injection. Fungi

were re-isolated from the kidneys of both dead and surviving fish to evaluate the etiological agent. Briefly, pieces of fish kidney were inoculated onto PYGS agar supplemented with small amounts of ampicillin and streptomycin. The plates were incubated at 25°C for 2–4 weeks.

### Histopathological findings

Histopathological examinations were conducted on dead fish during the experiment period and on surviving fish on the final day to confirm the presence of the inoculated fungus. Small pieces of fixed kidney, liver, spleen and pyloric caeca were embedded in paraffin and sectioned at 5 µm. The sections were stained with methenamine-silver nitrate Grocott's variation, and counter-stained with H&E (Grocott-HE).

## Results

### Susceptibility to antifungal agents

The MIC and MFC values for four isolates of *O. humicola* after 6 days incubation are summarized in Table 2. All isolates of *O. humicola* were susceptible to three drugs: ITCZ, TBN and VRCZ. The most active drug was TBN with MIC ranging from 0.06 to 0.13 µg/ml. ITCZ, for which MIC ranged from 0.5 to 2.0 µg/ml, was less potent than TBN, but more active than VRCZ. MIC values of VRCZ ranged from 1.0 to 8.0 µg/ml. The isolate NJM 0472 was mildly susceptible to 5-FC and the isolate ATCC 24901 was mildly susceptible to AMPH, while other isolates showed resistance. All isolates were resistant to FLCZ, MCFG and MCZ.

The MFC values demonstrated that TBN showed greater fungicidal activity than ITCZ. The MFC values ranged from 0.0625 to 0.125 µg/ml in TBN and from 0.5 to 1.0 µg/ml

**Table 2.** MICs and MFCs of *Ochroconis humicola* against eight antifungal drugs

<i>O. humicola</i>	MIC (MFC) (µg/ml)*							
	5-FC	FLCZ	MCFG	MCZ	TBN	AMPH	VRCZ	ITCZ
NJM 0472	8 (>16)	>16	>16	>16	0.0625 (0.0625)	>16	8 (8)	1 (1)
NJM 0565	16 (>16)	>16	>16	>16	0.0625 (0.0625)	>16	8 (8)	1 (1)
NJM 0663	16 (>16)	>16	>16	>16	0.0625 (0.0625)	>16	8 (8)	1 (1)
ATCC 24910	>16	>16	>16	>16	0.125 (0.125)	16 (>16)	1 (1)	0.5 (0.5)

5-FC: 5-fluorocytosine, FLCZ: fluconazole, MCFG: micafungin, TBN: terbinafine, AMPH: amphotericin B, VRCZ: voriconazole, ITCZ: itraconazole.

\*The fungi were incubated at 25°C for 6 days on PDA.

in ITCZ. VRCZ showed weak activity (MFC, 1.0 to 8.0  $\mu\text{g/ml}$ ), while 5-FC, AMPH, FLCZ, MCFG and MCZ did not show any antifungal activity against *O. humicola*.

#### Clinical signs and mortality

The percentage of fish presenting numerous white nodules, and re-isolation rates observed in surviving fish at day 50, are summarized in Fig. 1. Dead fish and fish infected with *O. humicola* typically presented grey to white nodules in parts of the visceral membrane, kidney,

liver, and spleen (Fig. 2). The kidney was swollen and accompanied with the presence of grey to white nodules. Some fish showed adhesion between the internal organs. These symptoms were similar to cases of natural and artificial infection described by Munchan et al. (2006) and Munchan et al. (in press). Under a microscope, many hyphae were found in the fresh lesions with nodules in all tissues.

The cumulative mortalities of fish experimentally infected with *O. humicola* NJM 0472 were 25% in group 1 (treated after 4 h), 5% in group 2

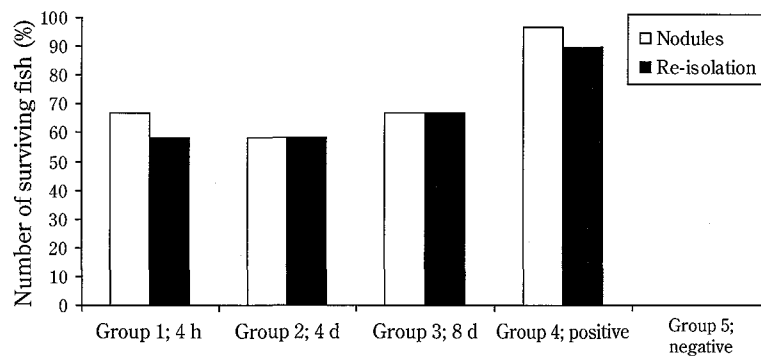


Fig. 1. The percentage of surviving fish displaying numerous white nodules, and re-isolation rate from kidney.

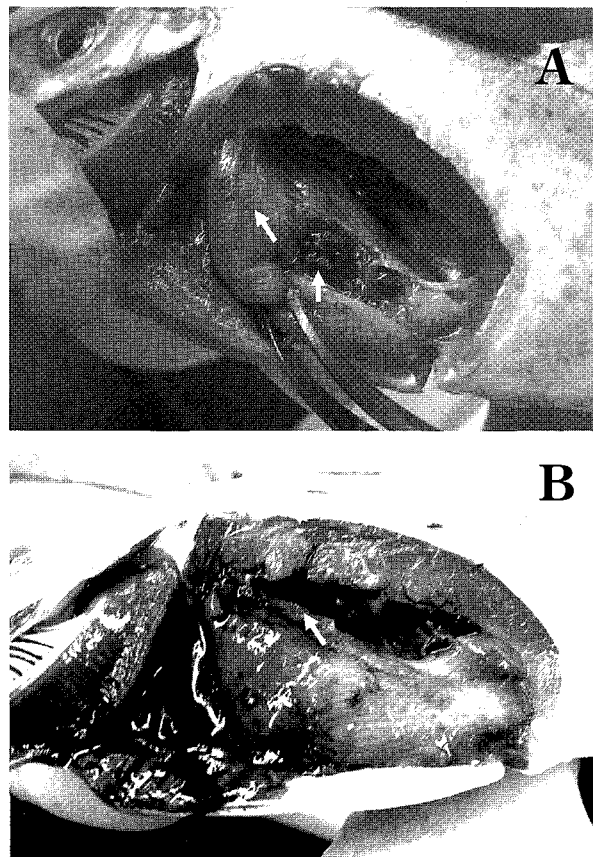


Fig. 2. Experimental fish 50 days post-inoculation showed abundant white nodules (white arrows) in internal organs (A) and kidney (B).

(treated after 4 d), 10% in group 3 (treated after 8 d), and 5% in group 4 (positive control). No mortalities were observed in group 5 (negative control group). The mortalities in this trial were variable among treatment groups and mortality was quite low, even in the positive control group.

Re-isolation rates of the fungus from surviving fish were 58.3%, 58.3%, 66.7%, and 89.7% in groups 1, 2, 3 and 4, respectively. Some experimental groups showed a higher percentage of individuals with white nodules than the re-isolation rate from the infected fish.

#### *Histopathological findings*

Histopathological sections of surviving fish showed granulomatous inflammatory reactions in all fish injected with fungal conidia. The current histopathological findings revealed similar pathogenicity to that in striped jack experimentally infected with high doses of *O. humicola* NJM 0472 conidia (Munchan et al. in press). Munchan found large granulomas with irregularly necrotic cores, which were considered to have resulted from the fusing of several caseous granulomas, with mats of fungal hyphae embedded in the center of the granuloma. Such mycotic features were found in all tissue samples that showed white nodules. No histopathological changes were observed in the negative control.

### **Discussion**

In this study the microdilution method was used according to the NCCLS M38-A approved standard for filamentous fungi, with a few modifications. Namely, MIC values are determined at 35°C for 74 h. However, the present fungal strains do not grow at 35°C. Hence the experiment was conducted at 25°C, which is the optimum temperature, and the MIC endpoint was observed at 144 h (6 days) post-inoculation. Efficacy of eight antifungal agents against four strains of *O. humicola*, isolated from diseased fish, revealed that three out of the eight drugs (TBN, ITCZ and VRCZ) showed both fungistatic and fungicidal activities. The most potent drug was TBN, but this drug has been reported for use in superficial mycosis. Thus, ITCZ was

chosen for the *in vivo* experiment to evaluate the activity of ITCZ against systemic mycosis caused by *O. humicola* NJM 0472. In the present study, the MIC endpoint of itraconazole was similar to that reported by Espinel-Ingroff (2001). The MIC endpoint of terbinafine corresponded with De Hoog et al. (2000). The MIC endpoint of voriconazole was similar to that reported by Espinel-Ingroff (2001), McGinnis and Pasarell (1998), and Meletiadiis et al. (1999). Several papers reported that amphotericin B was the most effective drug with the lowest MIC values (McGinnis and Pasarell 1998; Meletiadiis et al. 1999; Bowyer et al. 2000; Espinel-Ingroff 2001; Yarita et al. 2007), but in this study, MIC values of amphotericin B ranged from 16.0 to >16.0  $\mu\text{g/ml}$ . It is thought that this is due to the different sources of isolates and biological characteristics. Most of the previous papers examined strains isolated from humans or the environment.

Thus, we attempted an *in vivo* experiment to evaluate the effectiveness of itraconazole against *O. humicola* NJM 0472 infection. In this trial, fish did not eat much of the medicated food compared with the control groups. However, the necropsy findings on the final day revealed that the treatment groups developed fewer small white nodules than the non-treatment group. Mortalities were variable in the present study, and treatment groups showed a higher or equal level of mortalities when compared with the positive control group. Histopathological findings showed diffuse granulomatous inflammatory reactions in the fish with white nodules in internal organs. As mentioned previously, there were differences between the percentages of fish with white nodules and the re-isolation rates in some experimental groups, and there were large areas of giant granulomas with the very small fragments of fungal hyphae embedded inside. This indicated that fish were successful in preventing fungal invasion, because the fungal pathogen was not successfully isolated from the kidneys of some surviving fish. Nevertheless, in some fish showing severe clinical signs, abundant white nodules were observed in the visceral organs.

Histopathological findings in those fish suggest that the fish might have died if the experiment had continued for a longer period. From the clinical signs and histopathological findings, it can be stated that itraconazole shows no efficacy for curing fish infected with *O. humicola*.

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## 魚類から分離された *Ochroconis humicola* に対するイトラコナゾールの in vitro と in vivo における効果

Chutharat MUNCHAN · 畑井喜司雄 · 高木修作 · 山下亜純

魚類から分離された *Ochroconis humicola* 4 株に対するアンホテリシンB, フルコナゾール, 5-フルシトシン, イトラコナゾール, ミカファンギン, ミコナゾール, テルビナフィンおよびボリコナゾールの in vitro での効果を比率法で検討した。その結果, イトラコナゾール, テルビナフィンおよびボリコナゾールの 3 剤は供試全株に有効であった。各薬剤の MIC (最小発育阻止濃度) と MFC (最小殺真菌濃度) は, 最も効果的な薬剤であったテルビナフィンが, 0.06~0.13 と 0.0625~0.125  $\mu\text{g/ml}$  で, 次にイトラコナゾールの 0.5~2.0 と 0.5~1.0  $\mu\text{g/ml}$  であった。テルビナフィンは塗布剤であることから, 経口薬であるイトラコナゾールを選択して実験感染魚 (シマアジ) に人体と同様の投与量を与え, 50日間経過を観察した。死亡魚は認められなかったが, いずれの試験区でも内臓に結節が認められ, 病理組織学的にも対照区との差異は認められなかった。このことから, イトラコナゾールはオクロコニス症に対して効力がないと判断された。