Kasetsart J. (Nat. Sci.) 45 : 84 - 89 (2011)

Sensitivity Comparison of Pathogenic Aquatic Fungal Hyphae to Sodium Chloride, Hydrogen Peroxide, Acetic Acid and Povidone Iodine

Wichukarn Fuangsawat, Narong Abking and Ong-Ard Lawhavinit*

ABSTRACT

Nine isolates of pathogenic aquatic fungi: *Aphanomyces piscicida* NJM 9510, *Aphanomyces astaci* FDL 445, *Aphanomyces* sp. NJM 9406, *Aphanomyces* sp. NJM 9623, *Saprolegnia diclina* NJM 9219, *Achlya* sp. E. MCF 1-02, *Achlya* sp. T. MCF 1-02, *Achlya bisexualis* NJM 0611 and *Achlya* sp. NE 08, were tested for their sensitivity to four chemicals: sodium chloride, hydrogen peroxide, acetic acid and povidone iodine. Hyphal agents at the edge of the growing colonies were cut and immersed in various concentrations of the four chemicals for 60 min and then placed on glucose yeast extract agar. The results showed that the diameter of the fungal colonies increased when the chemical concentrations were decreased. The minimal inhibitory concentrations (MIC) of the nine isolates were: 5.00, 0.38, 0.75 and 0.31% for sodium chloride, hydrogen peroxide, acetic acid and povidone iodine, respectively. For the same four chemicals, the fungicidal concentrations of the nine isolates were: 20, 0.75, 1.60 and 0.63%, respectively.

Keywords: water mold, sodium chloride, hydrogen peroxide, acetic acid, povidone iodine

INTRODUCTION

Saprolegnia, *Achlya*, and *Aphanomyces* belong to the water molds in the family Saprolegniaceae (Willoughby, 1994). These fungi are found naturally in fresh water and soils with high moisture content. They reproduce both sexually and asexually and the hyphae are long and non-septate. They have been reported as causes of aquatic animal diseases that can be characterized by a growth of white cottony mycelia on the skin of infected animals. Some chemicals with antifungal activity have been used widely to control fungal infection in aquatic animals. Malachite green was one of the most effective antifungal agents used widely to control aquatic fungal growth and infection (Pottinger and Day, 1999). However, it is carcinogenic and remains in aquatic animals until they reach market size. Because of its toxicity, the use of malachite green in the aquatic animal industry has been prohibited in many countries (Stammati *et al*., 2005). The loss of this antifungal agent has driven scientists to look for an effective antifungal chemical that is less hazardous to animals and the environment and consequently, can be used instead of malachite green.

The present research studied the sensitivity of nine pathogenic aquatic fungi: *Aphanomyces piscicida* NJM 9510, *Aphanomyces astaci* FDL 445, *Aphanomyces* sp. NJM 9406, *Aphanomyces* sp. NJM 9623, *Saprolegnia diclina*

Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: fvetonl@ku.ac.th

Received date : 15/06/10 Accepted date : 13/09/10

NJM 9219, *Achlya* sp. E. MCF 1-02, *Achlya* sp. T. MCF 1-02, *Achlya bisexualis* NJM 0611 and *Achlya* sp. NE 08 to four chemicals: sodium chloride, hydrogen peroxide, acetic acid and povidone iodine, which have been considered as low priority regulatory drugs by the U.S. Food and Drug Administration (FDA, 2009).

MATERIALS AND METHODS

Fungal isolates

A total of nine isolates of aquatic fungi were used in the study. Six isolates consisted of *Aphanomyces piscicida* NJM 9510, *Aphanomyces astaci* FDL 445, *Aphanomyces* sp. NJM 9406, *Aphanomyces* sp. NJM 9623, *Saprolegnia diclina* NJM 9219 and *Achlya bisexualis* NJM 0611, which were obtained from Dr Kishio Hatai, Nippon Veterinary and Life Science University, Tokyo, Japan. The other three isolates, *Achlya* sp. E. MCF 1-02, *Achlya* sp. T. MCF 1-02 and *Achlya* sp. NE 08 were isolated from Mekong giant catfish eggs and Neon tetra fish collected at the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Thailand (Table 1). The fungi were grown on glucose yeast extract agar (1% glucose, 0.25% yeast extract, 1.5% agar; Hatai and Egusa, 1979) and incubated at 20°C.

Minimal inhibition concentration (MIC) and fungicidal concentration

The method used to study the minimal inhibition concentration (MIC) and fungicidal concentration was modified from Bailey (1983). The fungi were grown on Petri dishes containing 15 mL of glucose yeast extract agar and incubated at 20°C for 3-4 d. The hyphae at the edge of each growing colony sampled were cut using a cork borer 8 mm in diameter. The hyphae were immersed in Petri dishes containing 40% sodium chloride, 3% hydrogen peroxide, 3.2% acetic acid or 2.5% povidone iodine. The process using fresh samples of hyphae was repeated with twofold dilutions of the chemical concentrations down to 1.25% sodium chloride, 0.01% hydrogen peroxide, 0.006% acetic acid and 0.01% povidone iodine. The experiments were carried out in triplicate with sterilized distilled water as a control group. After immersion for 60 min, the hyphae were washed three times in sterilized distilled water and placed on glucose yeast extract agar and incubated at 20°C. The diameter of each fungal colony was measured daily for 5 d using vernier calipers.

Statistical analysis was carried out by one-way ANOVA to compare the average diameter of the fungal colonies at the 95% confidence level using the NCSS 2007 software. [Available from: www.ncss.com]. The MIC was defined as the minimal concentration of the chemical that produced an average diameter of the colony

Table 1 Fungal isolates used in the experiment.

Fungus	Strain	Host	Isolated year	Country
Achlya sp.	E. MCF 1-02	Mekong giant catfish egg	2008	Thailand
Achlya sp.	T. MCF 1-02	Mekong giant catfish egg	2008	Thailand
Achlya bisexualis	NJM 0611	Tilapia egg	2006	Thailand
Achlya sp.	NE 08	Neon tetra	2008	Thailand
Aphanomyces piscicida	NJM 9510	Spiny eel	1995	Thailand
Aphanomyces astaci	FDL445	European crayfish	1984	England
Aphanomyces sp.	NJM 9406	Archerfish	1994	Singapore
Aphanomyces sp.	NJM 9623	Soft shell turtle	1996	Japan
Saprolegnia diclina	NJM 9219	Rainbow trout	1992	Japan

significantly ($P<0.05$) smaller than the control group and the fungicidal concentration was defined as the concentration at which no growth of fungal hyphae was evident over a 5-day period.

RESULTS AND DISCUSSION

The results showed that the diameter of the fungal colonies increased when the chemical concentrations were decreased. The MICs of the four chemical agents for the nine fungi are shown in Table 2. The colonies of *Achlya* sp. E. MCF 1-02 treated with sodium chloride, hydrogen peroxide, acetic acid and povidone iodine at MIC are shown in Figure 1. The fungicidal concentrations of sodium chloride, hydrogen peroxide, acetic acid and povidone iodine against the nine fungi are shown in Table 3.

The results indicated that sodium chloride, hydrogen peroxide, acetic acid and

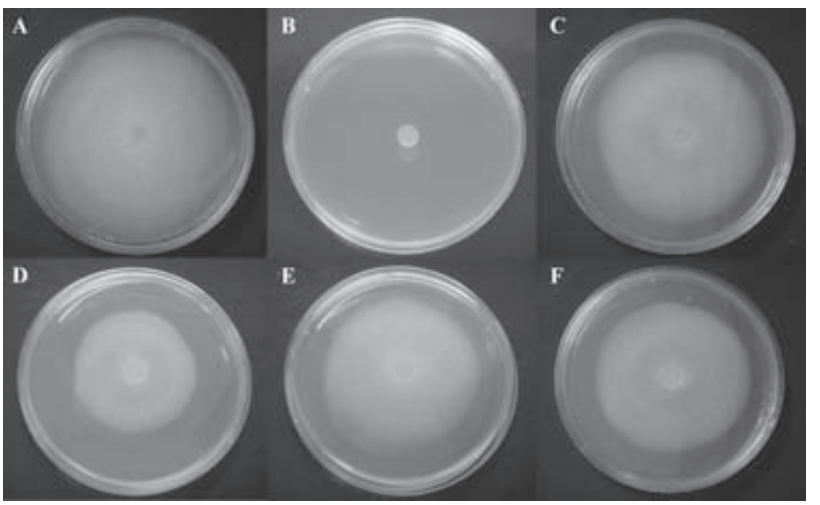

Figure 1 5-day colonies of *Achlya* sp. E. MCF 1-02 treated by the chemical agents at MIC.
A. Control group
B. 40% sodium chloride (fungicidal concentration)
C. 2.5% sodium chloride
D. 0.09% hydrogen peroxide
E. 0.40% acetic acid
F. 0.08% povidone iodine

Table 2 MIC of sodium chloride (NaCl), hydrogen peroxide (H_2O_2), acetic acid and povidone iodine for the fungi.

Fungus	MIC (%)			
	NaCl	H_2O_2	Acetic acid	Povidone iodine
Achlya sp. E. MCF 1-02	2.50	0.09	0.40	0.08
Achlya sp. T. MCF 1-02	2.50	0.09	0.75	0.04
Achlya bisexualis NJM 0611	5.00	0.09	0.40	0.08
Achlya sp. NE 08	1.25	0.09	0.40	0.08
Aphanomyces piscicida NJM 9510	5.00	0.01	0.10	0.16
Aphanomyces astaci FDL 445	1.25	0.02	0.01	0.16
Aphanomyces sp. NJM 9406	1.25	0.38	0.20	0.31
Aphanomyces sp. NJM 9623	5.00	0.19	0.02	0.16
Saprolegnia diclina NJM 9219	1.25	0.09	0.01	0.04

povidone iodine had fungistatic and fungicidal activity against all the fungi investigated.

The MICs of sodium chloride against the nine fungal isolates were different. This may have been due to the ability of the fungi to adapt to salty water (Hussein, 2001). Although growth of the fungi was inhibited at various concentrations of sodium chloride, all fungi were killed by 20% sodium chloride. *Aphanomyces astaci* FDL 445, *Aphanomyces* sp. NJM 9406, *Saprolegnia diclina* NJM 9219 and *Achlya* sp. NE 08 were the most sensitive fungi to sodium chloride. Their growths were inhibited at 1.25% sodium chloride, but *Aphanomyces piscicida* NJM 9510, *Aphanomyces* sp. NJM 9623 and *Achlya bisexualis* NJM 0611 survived in up to 2.50% sodium chloride. This showed that the strains in the present experiment exhibited greater resistance to sodium chloride than the strains reported in Hatai *et al*. (1994), who used sodium chloride at 1.5% concentration.

The most sensitive fungus to hydrogen peroxide was *Aphanomyces piscicida* NJM 9510, which was inhibited at 0.01% solution. *Aphanomyces* sp. NJM 9406, *Aphanomyces* sp. NJM 9623 and *Achlya* sp. T. MCF 1-02 were able to tolerate up to 0.38% hydrogen peroxide, but were killed at 0.75% hydrogen peroxide.

Achlya sp. E. MCF 1-02, *Achlya* sp. T. MCF 1-02 and *Achlya bisexualis* NJM 0611 were killed with 1.60% acetic acid, which was a higher fungicidal level than reported by Nilubol *et al*. (1997) who found that 0.15% was injurious to rainbow trout eggs, while an MIC of 0.05% for the zoosporic stage of aquatic fungi and 0.1% hydrogen peroxide was found to be the most effective to control fungal infection and increase the hatching rate of rainbow trout eggs.

The most sensitive fungi to povidone iodine were *Saprolegnia diclina* NJM 9219 and *Achlya* sp. T. MCF 1-02. They were inhibited after immersion in 0.04% povidone iodine. *Aphanomyces* sp. NJM 9406, *Achlya* sp. T. MCF 1-02 and *Achlya bisexualis* NJM 0611 survived at 0.31% povidone iodine, with the minimal fungicidal concentration (MFC) being 0.63%. The results of the present study produced higher fungicidal concentrations than Loan *et al*. (2006). However, the concentrations of the chemicals were different due to genetic changes occurring in the aquatic fungi strains used by Loan *et al*. (2006).

The chemicals used in the present experiment were effective against all the fungi studied. However, the MICs of all chemicals were higher than recommended by the U.S. Food and Drug Administration, who have published concentrations of 0.1-0.2, 3, 0.025 - 0.005 and 0.01% for acetic acid, NaCl, hydrogen peroxide and povidone iodine, respectively (US FDA,

Table 3 Minimal fungicidal concentrations of sodium chloride (NaCl), hydrogen peroxide (H_2O_2), acetic acid and povidone iodine for the nine fungi studied.

Fungus	Minimal fungicidal concentrations (%)			
	NaCl	H_2O_2	Acetic acid	Povidone iodine
Achlya sp. E. MCF 1-02	20	0.19	1.60	0.31
Achlya sp. T. MCF 1-02	20	0.75	1.60	0.63
Achlya bisexualis NJM 0611	20	0.38	1.60	0.63
Achlya sp. NE 08	20	0.38	0.80	0.16
Aphanomyces piscicida NJM 9510	20	0.05	0.40	0.31
Aphanomyces astaci FDL 445	20	0.09	0.10	0.31
Aphanomyces sp. NJM 9406	20	0.75	0.40	0.63
Aphanomyces sp. NJM 9623	20	0.75	0.10	0.31
Saprolegnia diclina NJM 9219	20	0.38	0.20	0.31

2009). However, Marking *et al*. (1994) reported that immersion of rainbow trout eggs in 3.00% sodium chloride for 15 min every 2 d could prevent fungal infection. Khodabandeh and Abtahi (2006) also reported that 3.5% sodium chloride effectively killed and prevented further fungal growth on treated common carp eggs. The results showed some of the MICs were higher than the recommended concentrations and perhaps may be injurious to aquatic biota, but the chemicals inhibited and killed the experimental fungi. Thus, there should be further studies to determine the appropriate concentration and immersion time and to focus on the toxicity of these chemicals to aquatic animals and their eggs for effective control of fungal infection in aquaculture. Without such investigations, the high MICs concentrations may affect the health of animals.

CONCLUSION

Following the prohibition of the use of malachite green in aquaculture, scientists have been searching for other chemicals that are effective in controlling fungal infections in aquatic animals. The present study compared the sensitivity of nine fungi to four chemicals: sodium chloride, hydrogen peroxide, acetic acid and povidone iodine. The results showed that all four chemicals had antifungal properties. The minimal inhibition concentrations of sodium chloride, hydrogen peroxide, acetic acid and povidone iodine were 5.00, 0.38, 0.75 and 0.31%, respectively, and the minimal fungicidal concentrations were 20, 0.75, 1.60 and 0.63%, respectively. Although the concentrations used in the experiment could inhibit fungal growth, they were higher than the levels recommended by the U.S. Food and Drug Administration and may cause adverse effects to animal health. Further studies should focus on the toxicity of these chemicals to aquatic animals and their eggs, to achieve effective control of fungal infection in aquaculture.

LTERATURE CITED

Bailey, T.A. 1983. Method for in vitro screening of aquatic fungicides. **Journal of Fish Diseases** 6: 91-100.

Food and Drug Administration. 2009. **Aquaculture Drugs.** Fish and fisheries products hazards and control guidance, [cited: 9 October 2009]. [Available from: http://www.fda.gov].

Hatai, K. and S. Egusa. 1979. Studies on pathogenic fungus of mycotic granulomatosis. III. Development of the medium for MG-fungus. **Fish Pathol.** 13: 147-152.

Hatai, K., K. Nakamura, S.A. Rha, K. Yuasa and S. Wada. 1994. *Aphanomyces* Infection in Dwarf Gourami (*Colisa latia*). **Fish Pathology** 29: 95-99.

Hussein, M.M.A., K. Hatai and T. Nomura. 2001. Saprolegniosis in salminids and their eggs in Japan. **Journal of Wildlife Diseases** 37: 204-207.

Khodabandeh, S. and B. Abtahi. 2006. Effects of sodium chloride, formalin and iodine on the hatching success of common carp, *Cyprinus carpio*, eggs. **J. Appl. Ichthyol**. 22: 54-56.

Loan, L.T.T., V.H. Phuong, T.P. Thanh and N.T. Huyen. 2006. Experimental screening some pharmaceutical chemistry compounds affecting *Achlya* sp. isolated from catfish eggs. In: **Proceedings of Biotechnology Workshop,** Nong Lam University HCMC, Vietnam, (in press).

Marking, L.L., J.J. Rach and T.M. Schreier. 1994. Evaluation of antifungal agents for fish culture. **Prog. Fish-Cult.** 56: 225-231.

Nilubol, K., A. Yamamoto and K. Hatai. 1997. Fungicidal effect of hydrogen peroxide on fungal infection of rainbow trout eggs. **Mycoscience** 38: 375-378.

Pottinger, T.G. and J.G. Day. 1999. A *Saprolegnia parasitica* challenge system for rainbow trout: Assessment of Pyceze as an anti-fungal

control agent for both fish and ova. **Diseases of Aquatic Organisms** 36: 120-141.

Stammati, A., C. Nebbia, I.D. Angelis, A.G. Albo, M. Carletti, C. Rebecchi, F. Zampaglioni and M. Dacasto. 2005. Effects of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in two human cell lines. **Toxicology in Vitro** 19: 853-858.

Willoughby, L.G. 1994. **Fungi and Fish Disease**. Pisces Press, Polton House Press, lasswade, Midlothian. 57 pp.