Infection Experiments with *Aphanomyces invadans* in Advanced Fingerlings of Four Different Carp Species

P. K.PRADHAN[1], C.V. MOHAN[2], K. M. SHANKAR[3] and B. MOHANA KUMAR[4]

[1]*College of Fisheries, Central Agricultural University, Lembucherra, Tripura-799210, India*
[2]*Network of Aquaculture Centers in Asia-Pacific (NACA) P.O.Box 1040, Kasetsart Post Office, Bangkok, Thailand*
[3]*Fish Pathology and Biotechnology Laboratory, Department of Aquaculture College of Fisheries, Karnataka Veterinary, Animal and Fisheries Science University Mangalore-575002, India*
[4]*Gyeongsang National University, Republic of Korea, 660701*

ABSTRACT

Using artificial infection tests with *Aphanomyces invadans*, the etiological agent of epizootic ulcerative syndrome (EUS), the present investigation examined the disease susceptibility and inflammatory response of advanced fingerlings of four different carp species, *i.e.*, three species of Indian major carps (catla, rohu and mrigal) and common carp averaging 12.1±1.8, 11.4±1.1, 12.7±1.5 and 10.2±0.96 cm in body length, respectively. The findings of disease susceptibility experiments indicated that over an experimental period of 12 days, there was 100% mortality with severe gross lesion development in Indian major carps, whereas in common carp, neither any mortality nor any gross visible lesions were observed. Inflammatory response studies demonstrated that the injected zoospores were able to germinate in the muscles of all the four experimentally infected carp species. Only in Indian major carps, the germinated hyphae were able to massively proliferate and induce extensive necrotic lesions in the large areas of myotome. In the common carp, the lesion area was confined to the line of injection and with time course the lesion area appeared to be healed with regenerated muscle fibres. Thus, the findings inferred that advanced fingerlings of Indian major carps are highly susceptible to EUS. Therefore, in the EUS season, the cultured populations of Indian major carps, which are in this age group, are likely to be at high risk.

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Corresponding author: P.K. Pradhan, caupkp@gmail.com

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is one of the most destructive disease of both fresh and brackish water farmed and wild fish which caused major fish losses in many countries for over last three decades (Baldock *et al*., 2005). The disease is caused by an oomycete fungus, *Aphanomyces invadans* (Mohan and Shankar, 1995; Lilley *et al*., 1998; Thompson *et al*., 1999; Johnson *et al*., 2004). More than 100 fish species are reported to be affected by it (Lilley *et al*., 1998) and until recently, EUS remains an important issue in the carp culture ponds (Ahmed and Hoque, 1999; Lilley *et al*., 2002; Khan and Lilley, 2002; Islam *et al*., 2003; Nandeesha and Karim, 2006) particularly during the winter months. Fingerlings of Indian major carps (IMC) suffering from heavy mortalities during natural outbreaks (Roberts *et al*., 1989; Chinabut and Roberts, 1999; Khan and Lilley, 2002) and artificial infection experiments (Mohan, 2002) have been reported. Interestingly, during EUS outbreaks in several southern (Vishwanath *et al*., 1997a, b, 1998; Jayaraman, 1991) and northeastern states of India (Kumar *et al*., 1991), IMC in many water bodies had been observed to be unaffected. High temperature in south India has been suggested as one possible factor responsible for the increased resistance of IMC to *A. invadans* infection (Roberts *et al*., 1994). However, the temperature theory alone may not support some of the observations made in northeastern states of India, where temperature was ideal for EUS outbreaks. The possibility of age or size influencing the susceptibility of IMC to EUS was suggested by Lilley *et al*., (1998) and Chinabut and Roberts (1999). However, no artificial infection studies have been undertaken on IMC to confirm this observation. Hence, using artificial infection tests with *A. invadans*, the present study examined the susceptibility of four-month old advanced fingerlings (higher age/size group of fingerlings) of IMC (catla, *Catla catla*; rohu, *Labeo rohita* and mrigal, *Cirrhinus cirrhosus)* to EUS in south India, and for comparison, corresponding age groups of EUS resistant common carp, *Cyprinus carpio* (Wada *et al*., 1996; Lilley *et al*., 1998; Kurata *et al*., 2000) was used in the artificial infection trials.

MATERIALS AND METHODS

Fish and experimental systems

Fifteen-day old IMC and common carp fry were procured from Karnataka State Government Bhadra reservoir project fish hatchery. Cement cisterns (50 m^2 area; 15 cm soil bed) were used to rear the fish in the College of Fisheries fish farm facilities, Mangalore, India, for four months. Prior to stocking, the cisterns were drained, dried for a week, filled with freshwater to a depth of 0.5 m, and fertilized with agricultural lime ($CaCO_3$) and cow dung, respectively. After one week, the water level was increased to 0.9 m, and each fish species was stocked separately at a density of 100/50 m^2. The fish were fed with rice bran and ground nut oil cake mixture (1:1) at the rate of 10% of their body weight. Fifty percent of the water was changed with open well water once in 15 days and re-fertilization was done with cow dung.

Preparation of fungal spores

Suspension of motile secondary zoospores of *A. invadans* (strain B99C provided by J.H. Lilley) were prepared as described by Lilley *et al*. (1998). Briefly, three agar blocks (3×3 mm in size) of actively growing mycelium were placed in a petri dish containing glucose-peptone-yeast (GPY) broth and incubated for four days at 20°C. After four days, the nutrient agar from the resulting fungal mat was washed out by sequential transfer through five petri dishes containing autoclaved pond water (APW) and mats were kept in a petri dish containing 25 ml of (APW) at 20°C. After about 12 hr, the motile secondary zoospores were collected and number of zoospores in the suspension was counted ($6x10^4$ spores per ml) using haemocytometer.

Challenge with *A. invadans* spore

Forty advanced fingerlings each of catla, rohu, mrigal and common carp (CC) (averaging 12.1±1.8, 11.4±1.1, 12.7±1.5 and 10.2±0.96 cm, respectively) were used for challenge test. All the fish species were divided into four groups such as disease susceptibility group, sequential inflammatory response study group and two control groups (one for each study) having equal numbers of fish. The experimental fish were injected intramuscularly (into the left flank of fish just below the middle of dorsal fin region) with 0.1 ml of spore suspension ($6x10^4$ spores per ml) of *A. invadans* (strain B99C) as described by Chinabut *et al*., (1995). The control fish groups were treated with 0.1 ml autoclaved pond water at the same time. After injection, each species of experimental and control groups were kept separately in 500 l capacity fiberglass tubs containing 400 l water. Aeration was maintained with replenishment of 50% of water daily, and water temperature of the experimental tanks ranged from 26 to 29°C as measured twice daily in the morning and evening. For disease susceptibility studies, the fish mortality pattern was recorded daily up to 12 days post challenge and specific causes of mortalities confirmed by histology and reisolation of *A. invadans* from muscle tissue as described by Lilley *et al*., (1998). For sequential inflammatory response studies, one fish each from experimental and control groups were sampled at every alternate day (till all the experimental fish had died or completion of experimental period of 12 days which ever was earlier).

Histopathological analysis

After gross (eye) observation, lesion area was excised and fixed in 10% neutral buffered formalin. All the histopathological analysis was carried out as described by Chinabut and Roberts (1999). Samples were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). Selected slides were stained with Grocott's methenamine silver nitrate for demonstration of fungal hyphae.

RESULTS

There was 100% mortality in the case of catla, rohu and mrigal and the mortality had started after 6 days in catla, 8 days in rohu and 9 days in mrigal. There was 100% mortality after 10 days in catla and rohu and 11 days in mrigal; there was no mortality in the case of common carp and also in control group of fish. The detailed cumulative mortality pattern of advanced fingerlings of catla, rohu and mrigal recorded over a period of 12 days is presented in Table 1. At the time of morbidity or mortality, 100% of the fish had severe swollen hemorrhagic areas. Histopathological observations of the moribund Indian major carps indicated massive proliferation of fungal hyphae in the lesion area (Figure 1), and severe myonecrosis in large areas of myotome and the severity was so high that virtually no normal muscle fibres were observed in the lesion area (Figure 2) and around most of the hyphae there were no inflammatory cells. On the other hand, none of the common carp had developed any gross visible lesion or mortality at the end of experimental period of 12 days, but mycotic granulomatous lesions were observed histologically (Figure 3). The sequential inflammatory response studies indicated that in the case of Indian major carps, the sequence of progression of infection was similar as demonstrated by Mohan (2002) for fingerlings. Briefly, after one day of injection of zoospores, few fungal hyphae penetrating the muscle fibers were observed in the lesion area but no inflammatory cellular responses were found around the hyphae. After two days of injection, many hyphae were observed in the lesion area of the injected side. Four days after injection, the mycotic lesion had occupied both injected and non-injected sides. Six to nine days after injection, injected and non -injected sides and almost all the internal organs were extensively occupied by the mycotic lesions and there was massive proliferation of hyphae in the lesion area and there was extensive myonecrosis in large areas of myotome. These extensive pathological changes were always associated with gross visible lesions (*i.e.* severe swollen hemorrhagic areas) and morbidity or mortality.

Table 1. Cumulative percentage mortality of advanced fingerlings of four different species of carp (catla, rohu, mrigal and common carp) injected with zoospores of Aphanomyces invadans over a period of 12 days.

Days post challenge	Cumulative percentage mortality of advanced fingerlings of four different species of carp				
	Catla	Rohu	Mrigal	Common carp	Control
4					
5	0				
6	10				
7	50	0			
8	80	40	0		
9	90	90	60		
10	100	100	90		
11	100	100	100		
12	100	100	100	0	0

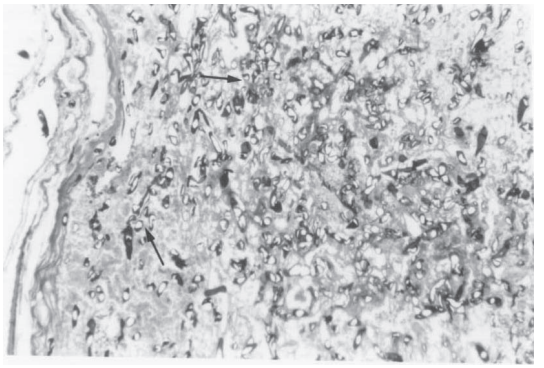

Figure 1. Mycotic lesion area in catla after 6 days of post injection (dpi) showing massive proliferation of hyphae (arrows) (Grocotts – H&E, x100).

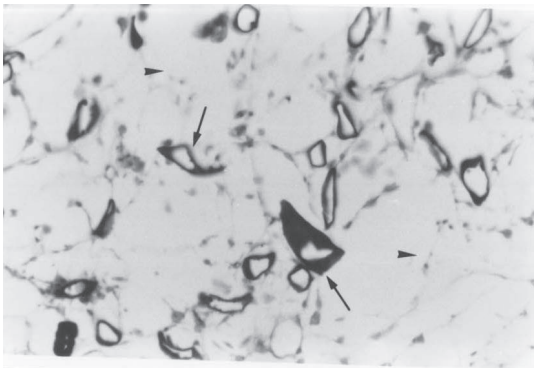

Figure 2. Extensive liquefaction of muscle fibers (arrow heads) and hyphae without any inflammatory cells around (arrow) in the lesion area of rohu at 8 dpi (Grocotts – H&E, x400).

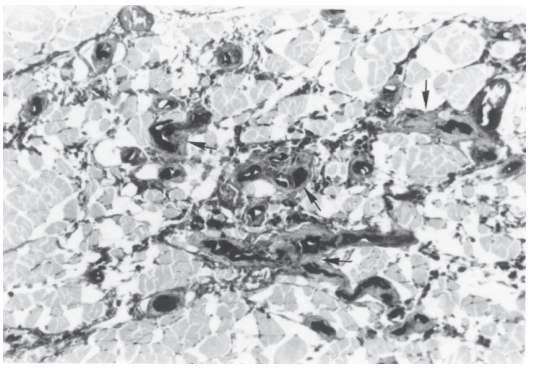

Figure 3. Well developed encapsulatory response by the epithelioid cells around the hyphae (arrows) and adjacent normal muscle fibers in the mycotic lesion area of common carp (CC) at 12 dpi (Grocotts – H&E, x200).

Between the three species of Indian major carps, the sequence of events (following challenge with zoospores of *A. invadans*), *i.e.*, penetration of muscle fibres by the germinated fungal hyphae, degeneration of those muscle fibres, infiltration of inflammatory cells to the lesion area, development of inflammatory foci of macrophages and/or epithelioid cell granulomata at the central part of lesion area, increase in fungal hyphae number in the lesion area and frequent penetration of fungal hyphae to the adjacent muscle fibres, necrosis of those muscle fibres and increase in overall mycotic lesion area with respect to days of post injection etc. were also similar. But, with respect to time course of these events, there was little difference between the species i.e. it was faster in catla and comparatively slower in rohu and mrigal. The mortality pattern of the susceptibility experiment was also reflected in the similar manner.

On the other hand in the case of common carp (CC), there was distinct difference in terms of inflammatory response when compared to IMC and their lower age groups, *i.e.*, fingerlings. In the case of CC, at 4 dpi, there was extensive infiltration of inflammatory cells into the lesion (Figure 4) and after 8 dpi, there were well developed epithelioid cell granulomata (Figure 5) and the lesion area appeared to be healed with regenerated muscle fibres. At the end of experimental period of 12 days, in the case of CC, the lesion area appeared completely healed (Figure 6) and the biological activity of the fungal hyphae appeared suppressed.

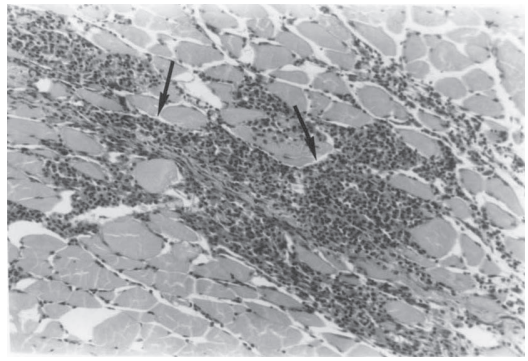

Figure 4. Mycotic lesion areas in CC at 4 dpi, showing extensive infiltration of inflammatory cells (H&E, x200).

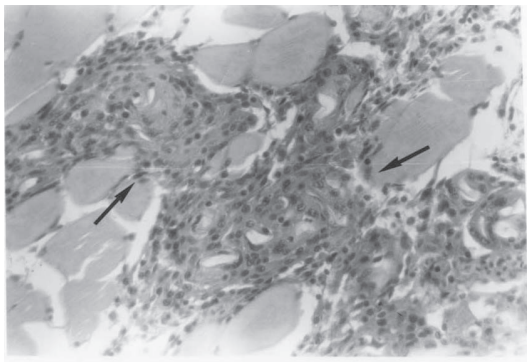

Figure 5. Well developed epithelioid cell granulomata consisting of several layers of epithelioid cells in CC at 8dpi (arrows) (H&E, x 400).

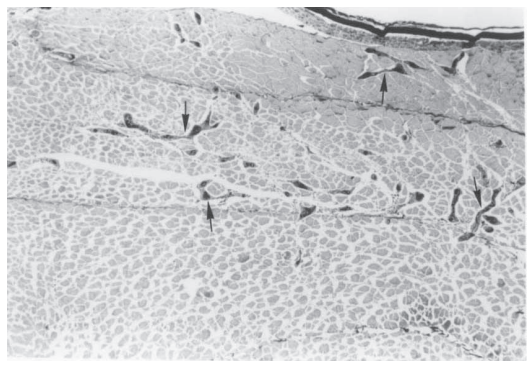

Figure 6. Lesion areas in CC at 12 dpi, appearing to be healed with well developed regenerated muscle fibers and injection site showing many fungal hyphae (arrows) (Grocotts–H&E, x40).

DISCUSSION

Mortalities due to artificial infection with *A. invadans*, in the case of advanced fingerlings of IMC have not been previously reported. However, in the present study, consistently in all of the three species of IMC, mortalities were observed in 100% of the fish and the gross observational results were supported by the histopathological observations. Therefore, it was considered that the advanced fingerlings of IMC could not resist against *A. invadans* infection whereas the corresponding age groups of common carp could do that.

However, in both groups of fish (*i.e.* IMC and CC), the injected spores had germinated but only in the case of IMC, the germinated hyphae were able to massively proliferate resulting in extensive necrotic pathology in large areas of myotome and almost all the internal organs and was reflected in the form of severe gross lesions, that in turn caused severe mortality. On the other hand, in the CC, the germinated hyphae were not able to proliferate and with time course the lesion area was healed. But, mycotic granulomatous lesions were observed histologically. Khan *et al*., (1998) had reported that injection of spores might circumvent the normal means of protection in some resistant fish and in their artificial infection studies, they had observed low mortality of tilapia, even though tilapia is considered as one of the resistant species to EUS (Lilley *et al*., 1998).

In the present study, since, same concentration of spores (from the same batch of spore suspension) were injected to all the fish, it was assumed that some of local and systemic factors might be providing an appropriate environment for multiplication of the hyphae in the IMC. While the effect in the case of CC, it would have been the opposite. Kurata *et al*., (2000) has reported that CC serum has fungicidal activities. Hatai (1980) opined that growth of *A. piscicida (=A.invadans)* would decrease if the fungus were exposed to an environment unconducive for its growth, which supports such an argument. Further, similar to our findings, Wada *et al*., (1996) reported that in ayu (a species susceptible to EUS), the number of hyphae were significantly more than that in CC (species resistant to EUS). In addition, the number of hyphae in ayu significantly increased as the infection progressed. Comparison of the degree of inflammatory cellular infiltration between IMC and CC indicated that in CC, there was very extensive infiltration of inflammatory cells in the lesion area and the inflammatory cells encapsulated almost all the hyphae. On the contrary, in the case of IMC, around most of the hyphae no inflammatory cells were found. Therefore, it was assumed that, the extensive inflammatory cells might be one of the factors in preventing the spread of hyphae to neighboring tissues and further proliferation, in the resistant fish group. Thompson *et al.,* (1999), through *in vitro* studies, have indicated that macrophages were getting clumped around the growing hyphal tips. Therefore, it appears that a similar phenomenon might be occurring in *in vivo* conditions to prevent the spread of hyphae in the case of CC. Since in IMC, around most of the hyphae, no inflammatory cells were observed, in those fish species, the hyphae might have migrated, unopposed, causing extensive myonecrosis in the myotome area due to release of proteolysins. Hence, it was considered that advanced fingerlings of IMC are highly susceptible to *A. invadans* infection. This was supported by field level studies (with a pathology-based diagnosis) in Bangladesh, where it has been reported that the major carps are the most significantly affected farmed fish and once an outbreak occurs in a carp pond, EUS can damage the entire crop (Khan and Lilley, 2002; Lilley *et al*., 2002).

However, in extensive (observational) studies, during EUS outbreaks, in Karnataka (South India), it was found that IMCs present in many water bodies were not affected (Vishwanath *et al*., 1997a, b, 1998). Similarly, in Tamil Nadu, IMCs present in culture ponds were also reported to be unaffected during EUS outbreaks (Jayaraman, 1991). Roberts *et al*., (1994) consider temperature in south India to be high enough for the IMCs to resist the fungus. Chinabut and Roberts (1999) feel that there is an anomaly in relation to geography in that in southern India, IMCs appear resistant but not in the north. Interestingly, in several northeastern states, even during low temperature periods, IMCs were found to be mildly affected or resistant during EUS outbreaks (Kumar *et al.*, 1991). Based on the present experimental study findings, it may be assumed that fish in those affected water bodies could be of still higher age groups. Therefore, artificial infection studies should be undertaken on still higher age groups of IMCs to confirm this hypothesis.

CONCLUSION

From the present study, it is clear that advanced fingerlings of IMCs are highly susceptible to *A. invadans* infection. Therefore, in the EUS season, the cultured population of IMCs, which are in this age group and/or lower age groups, are likely to be at high risk.

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