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Injection of Forty -Five Day Old Fingerlings of Common Carp with Zoospores of *Aphanomyces invadans* Leads to Histopathological Lesions Suggestive of Epizootic Ulcerative Syndrome (EUS)

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Abstract

Using artificial infection tests with *Aphanomyces invadans*, the etiological agent of Epizootic Ulcerative Syndrome (EUS), the present investigation examined the disease susceptibility of fingerlings (forty five days old and averaging 6.5±0.18 cm) of common carp (*Cyprinus carpio*, Cyprinidae), considered to be one of the resistant fish species to EUS. The study was carried out in two different experiments. The first experiment was carried out for a period of 12 days and the number of mortalities was recorded daily. The second experiment was carried out for a period of 10 days. Regular sampling was done to know the sequence of progression of infection. Results of the first experiment indicated that over an experimental period of 12 days, there was 50% mortality in the injected fish and the histopathological features of moribund and / or dead fish was typical of a disease condition of EUS.

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Results of second experiment indicted that the injected zoospores of *A invadans* were able to germinate in the muscle of all the experimentally infected common carp fingerlings. In due time, the germinated hyphae were able to proliferate massively, invade the neighbouring tissues and induce extensive necrotic lesions in large areas of myotome in about 60% of the fish. Among the remaining 40% of the fish, the lesion area was restricted to the injected side but within the injected side, large proliferative lesions were observed and within the lesion area a large number of fungal hyphae was observed. This is the first report where such extensive necrotic pathology and /or mortalities have been recorded in the common carp due to *A. invadans* infection.

Introduction

Epizootic Ulcerative Syndrome (EUS) is one of the most destructive diseases among fresh and brackish water farmed and wild fish and has caused major fish losses in many countries for over three decades (Baldock et al. 2005). This disease is caused by an oomycete fungus, *Aphanomyces invadans* (Thompson et al. 1999; Johnson et al. 2004; OIE 2006) and more than 100 fish species are reported to be affected by it (Lilley et al. 1998). However, some of the commercially important species like common carp have been reported to be resistant to EUS in natural outbreaks (Lilley et al. 1998; Kurata et al. 2000) and to artificial infection (Wada et al. 1996). Interestingly, in contrast to the reports of Lilley et al. (1998) and Kurata et al. (2000), several workers (Sinha 1991; Sanjeevaghosh 1991; Bhrusundi 1992; Das 1993; Das 1997) have reported common carp as being affected by EUS. However, the diagnostic features confirming the OIE recommended diagnostic features of EUS (OIE 2006), have not been demonstrated in their reports. The present investigation using artificial infection tests with *A. invadans*, examined the disease susceptibility of fingerlings common carp.

Materials and Methods

Forty-five day old fingerlings (averaging 6.5±0.18 cm) of common carp were used in the present investigation (in two different artificial infection experiments). In each experiment, 20 fish were used. Of the 20 fish, 10 were injected with zoospores of *A. invadans* (strain B99C) and were used as the experimental group while the remaining 10 numbers were injected with autoclaved pond water and were used as the control group. Each experimental fish was injected intramuscularly (into the left flank of fish just below the dorsal fin region) with 0.1 ml of spore

suspension ($6x10^4$ spores per ml) as described by Chinabut et al. (1995). Suspension of motile secondary zoospores of *A. invadans* was prepared as described by Lilley et al. (1998). The control group was injected with 0.1 ml autoclaved pond water.

After injection, the 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. Water temperature of the experimental tanks ranged from 26 to 29°C, measured twice daily (in the morning and evening). In the first experiment, (studying the mortality pattern), no intermediate sampling was done but the number of mortalities was recorded daily up to 12 days post challenge and specific mortalities were confirmed through demonstration of fungal hyphae in the histological sections and re-isolation of *A. invadans* from the muscle tissue as described by Lilley et al. (1998). No histological analysis was carried out for the control group as well as for rest of the surviving fish of the experimental group. In the second experiment, (studying the sequence of progression of infection), one fish was sampled daily and after gross observation, the lesion area was excised and fixed in 10% neutral buffered formalin. The histopathological analysis was carried out as described by Chinabut and Roberts (1999).

Results and Discussion

In the first experiment, over an experimental period of 12 days, mortality was observed in fifty percent (five out of ten fish) of the injected fish. Mortality started on day 6 and by 8, 9 and 11 days there were 30%, 40% and 50% mortalities, respectively. At the time of mortality, all the fish had gross visible lesions characterized by slight swelling and redness. Tissue pathology of all the moribund fish was typical of a disease condition of EUS (Fig. 1). The mycotic lesions extensively occupied both injected and non-injected sides and almost all the internal organs and there was massive proliferation of hyphae in the lesion area and it was possible to re-isolate the fungus from the muscle tissue of all the moribund fish. In the control group (injected with autoclaved pond water), no mortality was observed over the experimental period of 12 days.

In the second experiment, based on the gross visible lesion of the sampled fish, the fish were grouped into three categories i.e. (i) fish sampled at 6, 8 and 10 days post injection (dpi) had clear swollen hemorrhagic areas at the injection site and were moribund, (ii) fish sampled at 3, 5 and 7 dpi had slight swollen and hemorrhagic areas at the injection site, but were active and (iii) fish sampled at 1, 2, 4 and 9 dpi did not have any gross visible lesions and were active. Histopathological features of moribund fish (sampled at 6, 8 and 10 dpi) were typical of a disease condition of EUS and were exactly similar to that of the moribund fish of the first experiment.

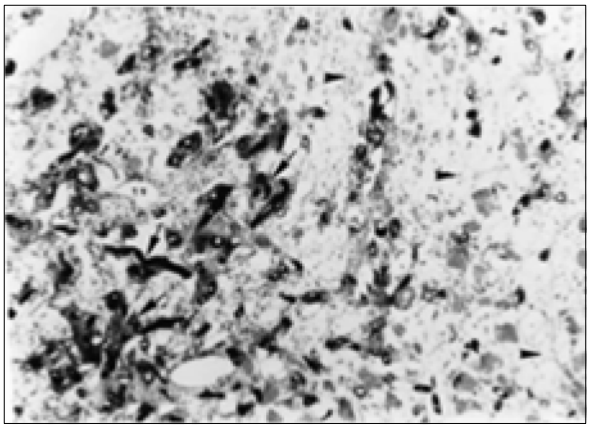

Fig. 1. Mycotic lesion area of moribund common carp fingerling at 6 days of post injection (dpi) showing extensive myonecrosis (arrowheads) and massive proliferation of fungal hyphae (arrows) (Grocott-H&E, x100).

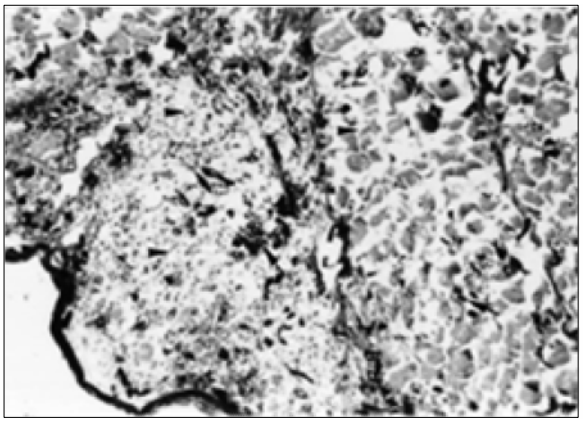

Fig. 2. Mycotic lesion area on the injected side at 3 dpi showing myonecrosis (arrow heads) and many fungal hyphae (arrows) (Grocott–H&E, x40).

Histopathological examination of fish (sampled at 3, 5 and 7 dpi) with gross visible lesions, indicated extensive mycotic lesions. At 3 dpi, the mycotic lesion had occupied most of the myotome area on the injected side (Fig. 2) and many of the hyphae had also migrated to the non-injected side. At 5 dpi, mycotic lesion had extended to both injected and non-injected sides and many of the hyphae had reached the internal organs and in the injected side, most of the muscle fibers were degenerated (Figs. 3 & 4). At 7 dpi, the histopathological features were typical of a disease condition of EUS as described earlier. Histopathological examination of the fish sampled without any gross visible lesion (sampled at 1, 2, 4 and 9 dpi) indicated that even though no gross visible lesions were observed in those fish, mycotic lesions were observed histologically. In the case of fish sampled at 1 and 2 dpi, the lesion was restricted to the line of injection. In the case of fish sampled at 4 and 9 dpi, the lesion had extended from the line of injection and large proliferative lesions were observed (Figs. 5 & 6) and within the proliferative lesion, there were large numbers of fungal hyphae (Fig. 6). But, the lesion was restricted to the injected side and there were extensive infiltration of inflammatory cells around the hyphae. In the control group of fish injected with autoclaved pond water, initially, tissue damage caused by the passage of the needle consisted of small areas of hemorrhage and few degenerated muscle fibers and in due course, the lesion appeared to be healing with numerous regenerated muscle fibers.

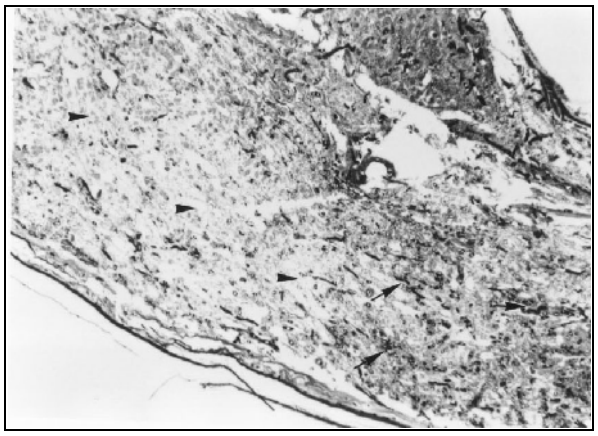

Fig. 3. Mycotic lesion area at 5 dpi showing extensive myonecrosis (arrow heads) in large areas of myotome and internal organ with large number of fungal hyphae (arrows) (Grocott – H&E, x 40).

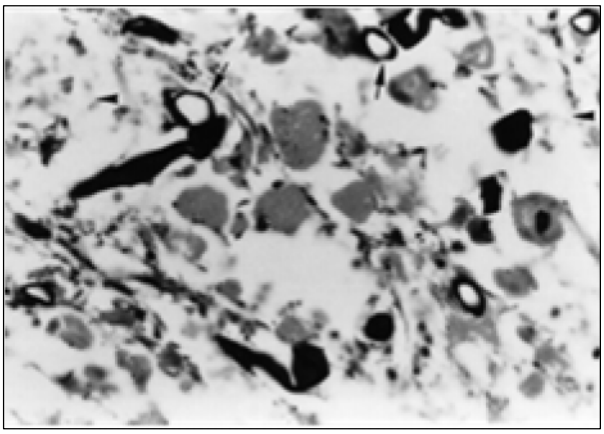

Fig. 4. Central part of mycotic lesion area showing extensive myonecrosis at 5 dpi (Grocott – H&E, x400).

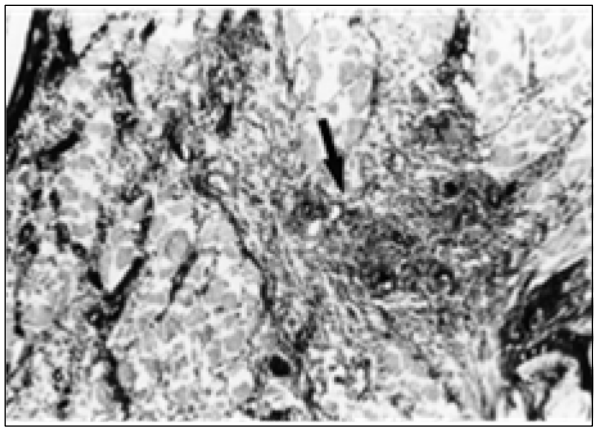

Fig. 5. Large proliferate lesion (arrow) at the center of mycotic lesion at 4dpi (Grocott – H&E, x100).

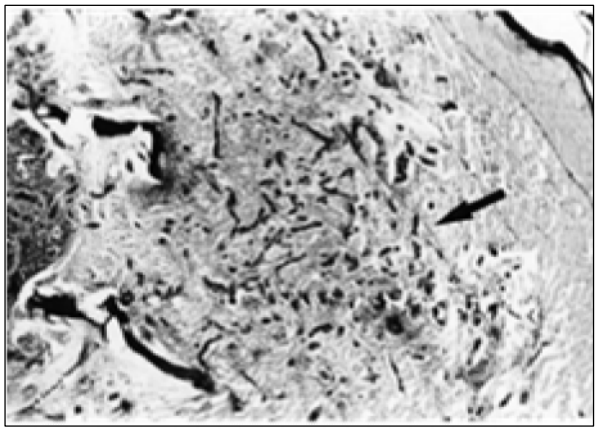

Fig. 6. Mycotic lesion area showing large proliferative lesion (arrow) at 9 dpi (Grocott – H& E, x40).

Histological examination of all the moribund fish in both experiments indicated severe myonecrosis of large areas of myotome and necrotic lesions in almost all internal organs due to the invading fungal hyphae. The massive internal necrotic pathology was reflected in the form of gross lesions and it was possible to re-isolate the fungus from all the moribund fish. Hence, it was concluded that mortalities and/or necrotic pathology were due to *A. invadans* infection.

Importantly, results of both experiments were consistent i.e., about 50% - 60% of the injected fish had extensive necrotic pathology typical of EUS.

For EUS to develop, the injected zoospores of *A. invadans* must be able to germinate in the muscle tissue. After germination, the hyphae must be able to proliferate and spread to the neighboring tissues and cause extensive necrotic pathology. From the present study, it was clear that the injected spores were able to germinate in the muscles of all the injected fish but in the case of the moribund fish and fish having gross visible lesions, the germinated hyphae were able to proliferate massively and spread to the neighboring tissues and cause extensive necrotic pathology. On the other hand, in the case of fish having restricted internal lesions and / or showing no gross visible lesions, the germinated hyphae were prevented from extending to the neighboring tissues.

Common carp is considered to be resistant to natural outbreaks of EUS (Lilley et al. 1998; Kurata et al. 2000). In artificial infection studies, Wada et al. (1996) have reported that in common carp the fungal growth gets suppressed due to stronger inflammatory response and the fish was able to resist the infection. Similar to the findings of Wada et al. (1996), Pradhan et al. (2008) also reported that the advanced fingerlings of common carp (four months old with an average size of 10.2±0.96 cm) were able to resist the infection against *A. invadans*.

The dose of spores injected in the present study was similar to that reported by Wada et al. (1996) and Pradhan et al. (2008). The fish used in the present artificial infection experiment were about 45 days old with an average size of 6.5 cm. Where as Wada et al. (1996) used fish having an average size of 11.1 cm but the age of the fish was not reported. The fish used by Pradhan et al. (2008) was four months old with an average size of 10.2 cm. Therefore, it appears, in the Wada et al. (1996) and Pradhan et al. (2008) studies, that due to higher age/size of fish, the immune system might have been more developed which might have played a role in resisting infection. In addition, the pathogen load with respect to size and/or age of fish could have also been a factor influencing the susceptibility.

It is also important to note that in the present investigation, fish were infected artificially through injection of zoospores, which is not a natural mode of challenge. Khan et al. (1998) in their artificial infection studies have reported low mortality in tilapia (considered as one of the resistant species to EUS) and they have suggested that injection of spores might circumvent the normal means of protection in some resistant fish. Hence, in the present experiment, similar phenomenon might have occurred. However, interestingly, several workers (Sinha 1991; Sanjeevaghosh 1991; Bhrusundi 1992; Das 1993; Das 1997) have reported common carp as being affected in the natural outbreaks of EUS. In view of the present findings, it will be

interesting to examine more closely the susceptibility of smaller sized common carp during natural outbreaks of EUS.

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