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Immunisation of *Labeo rohita* with antigenic preparation of *Aphanomyces invadans*

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ABSTRACT

Aphanomyces invadans is an important fungal pathogen infecting freshwater and brackishwater fishes. In the present study, an attempt has been made to determine the effect of immunisation in *Labeo rohita* advanced fingerlings against *A. invadans* infection. The efficacy of the immunisation was evaluated by challenge with *A. invadans* as well as quantification of antibody level by ELISA. Following an initial immunisation in conjunction with adjuvant, the fish were given a booster dose after 35 days. After 14 and 28 days of the booster injection, blood was collected from the immunised rohu for monitoring the antibody level. The fish were also challenged with *A. invadans* zoospores to determine the relative percent survival. The immunised fish had significantly higher antibody level after 14 days of booster injection as compared to the control fish. However, the antibody level after 28 days of booster injection was not significantly different from the control fish. More importantly, similar to control fish, 100% mortality was observed in the immunised fish challenged after 14 and 28 days of booster immunisation. Therefore, it can be concluded that it may not be suitable to induce protective immunity following immunisation with conventional antigenic preparations.

Keywords: *Aphanomyces invadans*, Immunisation, *Labeo rohita*

Infection with *Aphanomyces invadans* is a serious disease of freshwater and brackishwater fishes (Andrew *et al.*, 2008; Oidtmann *et al.*, 2008; Saylor *et al.*, 2010; Oidtmann, 2012). More than 130 fish species are reported to be naturally affected by this disease (Boys *et al.*, 2012; OIE, 2013; Kamilya and Baruah, 2014, Pradhan *et al.*, 2014) and the host range is increasing (Boys *et al.*, 2012, Songe *et al.*, 2012). In India, the disease was first reported in 1988 and even after more than two decades of its first occurrence, outbreaks resulting in large scale fish mortalities have been reported (Pradhan *et al.*, 2014). Till date, no effective treatment is available for this disease (OIE, 2013) and vaccination could be one of the health management strategies to counteract the disease. Although *A. invadans* invasion has been reported to elicit an antibody response, it is not clear whether the antibodies are protective (Thompson *et al.*, 1997, 1999). Recently, Saikia and Kamilya (2012), following single immunisation in *Catla catla* with different antigens of *A. invadans*, reported that amongst the antigen preparations, fungal extract mixed with Freund's incomplete adjuvant showed the highest antibody production. However, these antibodies did not offer significant protection against *A. invadans* challenge. The authors suggested that one of the reasons for not showing protection could be that booster immunisation was not given. In the present study, an attempt was made to evaluate if vaccination could induce protective

immunity against *A. invadans* infection, following a booster immunisation.

For the experiment, one hundred and fifty rohu (*Labeo rohita*) advanced fingerlings (54.63±1.79 g) were divided into five groups and stocked in fibre-reinforced plastic (FRP) tanks (@30 fish per tank) containing 1000 l water in each tank. Approximately 30% water was exchanged daily in the tanks. The fish were fed with a pellet diet @ 2% of their body weight per day and acclimatised for 7 days in the tanks prior to immunisation. Water quality parameters such as temperature, pH, dissolved oxygen, ammonia and nitrite were monitored in the experimental tanks and the mean values recorded during the study period were 16.0±2.1°C, 7.9±0.43, 5.45±0.68, 0.107±0.026 and 0.014±0.009 mg l⁻¹ respectively.

A. invadans strain INM20101, used for zoospore production, in the present study was isolated from *Cirrhinus mrigala* during a recent EUS outbreak (Pradhan *et al.*, 2014). Zoospores were prepared as described by Yadav *et al.* (2014). Briefly, four to five agar plugs (approx. 4 mm dia) of mycelium were put in a petri dish containing glucose peptone yeast (GPY) broth and cultured for 4 days at 20°C. Thereafter, the agar was washed out by transferring through petri dishes with autoclaved pond water (APW) and fungal mats were left overnight at 20°C in APW. After about approx. 12 h of incubation, the secondary zoospores were

harvested by centrifugation at 1000 g at 20°C. Fungal extract was prepared as described by Lilley *et al.* (1997), briefly, the zoospore suspensions were added to an equal volume of 2X GPY broth. The germlings were grown for 3 days at 20°C. Culture medium was decanted and the germlings were washed in 500 ml sterile distilled water. Thereafter, germlings were harvested on sterile filter paper (Whatman 541) and excess water was removed. The fungal mats were ground in a pestle and mortar using liquid nitrogen. The resulting powder was homogenised in 1 ml Wood's extraction buffer containing 1 mm MgCl₂, 85 mm Tris HCl, 10 mm KCl, 1 mm EDTA, 0.198 g l⁻¹ ascorbic acid and 1 g l⁻¹ glycerol at pH 7.5 (Wood, 1988), with the addition of 5 µM phenylmethylsulphonyl fluoride (Sigma-Aldrich, USA). Afterwards, the homogenate was centrifuged twice at 13000 g for 5 min and the concentration of protein in the extract was estimated as described by Bradford (1976) using a commercial kit (Fermentas Life Sciences, Vilnius, Lithuania). The protein concentration was adjusted to 5 mg ml⁻¹ with phosphate buffered saline (PBS) and the fungal extract was frozen at -80°C until further use.

For the immunisation trail, 90 fish from 3 FRP tanks were immunised individually with 200 µg fungal extract (in 100 µl), emulsified with Freund's incomplete adjuvant (1:1) by intraperitoneal route. The fish of the remaining two FRP tanks were intraperitoneally injected with 0.1 ml sterile PBS which served as control group. Similarly, a booster dose was given to the immunised group as well as control group, after 35 days of 1st immunisation. On 14 and 28 days of the booster immunisation, 5 fish from each tank were randomly sampled, anaesthetised with MS-222 (Sigma-Aldrich) and bled through caudal vein. Serum was separated from the collected blood samples after allowing to clot and by centrifuging the clotted blood at 5000 rpm for 5 min. The serum samples collected were stored in deep-freezer at -20°C for measuring antibody titre. For evaluation of protective immunity, on 14 and 28 days of booster dose, 10 fish from each immunised and control tanks were transferred to different tanks and challenged with 100 zoospores per fish by intramuscular injection below the dorsal fin. After challenge, mortality pattern of fish in both the groups was monitored and the relative percent survival (RPS) was calculated using the formula: $RPS \% = [1 - (\% \text{ test mortality} / \% \text{ control mortality})] \times 100$. The cause of mortality was further confirmed by histopathological analysis for presence of fungal hyphae.

For monitoring the antibody level, the reactivity of the collected sera of vaccinated and control group fish to the antigen (fungal extract) was assessed by indirect ELISA which was performed in a 96 well microtitre plate as described by Das *et al.* (2009) with a few modifications. Briefly, the wells of the ELISA plates were coated with 50 µl of the antigen (0.5 µg ml⁻¹), diluted in coating buffer (carbonate-bicarbonate

buffer, pH 9.6), whereas in antigen control wells, only coating buffer was added. The plates were incubated overnight at 4°C and subsequently, the wells were washed with washing buffer *i.e.* PBS containing 0.05% Tween-20 (PBS-T). Thereafter, 100 µl of 5% skim milk powder was added to the wells and the plates were incubated for 12 h at 4°C. The wells were again washed in PBS-T followed by addition of 50 µl of serially two fold diluted sera (1:50-1:200) and the plates were incubated overnight at 4°C. After washing with PBS-T, 50 µl of diluted 1:16000 anti-rohu IgM monoclonal antibody (Rathore *et al.*, 2008) was added to each well and left for 12 h at 4°C. After washing with PBS-T, 50 µl of 1:4000 anti-mouse HRP conjugate (Sigma-Aldrich) was added to each well and incubated overnight at 4°C. The plates were then washed and 50 µl of *ortho*-phenylenediamine containing hydrogen peroxide (H₂O₂) was added as substrate solutions and incubated at room temperature for 5 min in dark. The reaction was terminated by adding 50 µl 1.25 M H₂SO₄. The colour development was read at 492 nm using an ELISA reader (Tecan, Grodig, Austria).

All the experimental data were expressed as mean ± SE. Before analysis, data were checked for normality using Shapiro-Wilk test. Thereafter, one way analysis of variance (ANOVA) followed by the comparison of means as per Tukey's test available with SPSS (17.0 version, USA) were carried out. Finally, the level of significance was expressed as p-value less or greater than 0.05.

The antibody titre in both groups was assessed by indirect ELISA. Compared to the control group, the immunised group showed significantly ($p < 0.05$) higher antibody level on 14th day post-booster immunisation. However, at 28th day post-booster immunisation, the antibody level in immunised group declined and was comparable with the control group (Fig. 1). Interestingly, sera of control group fish showed cross-reactivity with the oomycete antigen.

Hundred percent mortality was recorded in both the immunised and control group following challenge of the fish after 14 and 28 days of booster immunisation and no

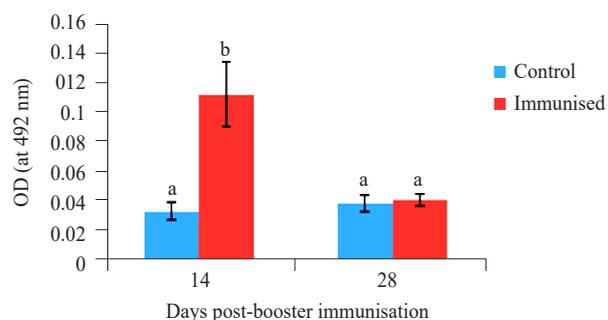


Fig. 1. Antibody levels of vaccinated and control *L. rohita* with 1/100 serum dilution

significant difference was observed in mortality pattern ($p>0.05$) (Figs. 2 and 3). In both the groups, gross lesions (slight swelling and redness) were observed at the injection site on 8 day post-injection (dpi) and by 12 dpi, clear swollen and reddish areas were developed in all the infected fish. Severely swollen haemorrhagic areas were observed on both sides of the body by 18 dpi. The mortality had started by the 20th day after challenge and by 25th day, there was 100% mortality in both the groups. Histopathological analysis indicated that in both groups, there was severe myonecrosis on both injected and non-injected sides and massive proliferation of hyphae was observed and there were no difference in the pathological features between control and immunised groups (Fig. 4).

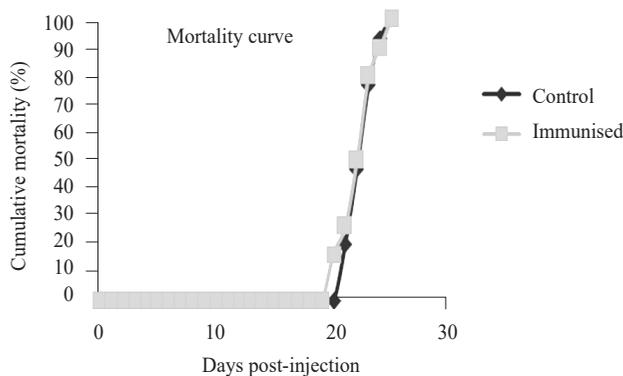


Fig. 2. Cumulative mortality of control and immunised groups of *L. rohita* challenged with *Aphanomyces invadans* on 14th day of booster dose (1:1)

et al. (2007) reported humoral antibody response against a pathogenic isolate of *Saprolegnia parasitica* following immunisation with antigenic extracts in brown trout *Salmo trutta*. Similarly, Saikia and Kamilya (2012) reported significant antibody response in *Catla catla* following single immunisation with fungal extract, fungal extract mixed with FIA and extracellular products. Previously, Thompson *et al.* (1999) also reported that immunised fish had significantly higher antibody level at 11th week of immunisation, compared to the control group. In our study, the antibody level in the immunised group was comparable to control group on day 28 post-booster immunisation. The results indicated that increase in antibody level was transient. The cross-reactivity of control sera as observed in the current study is

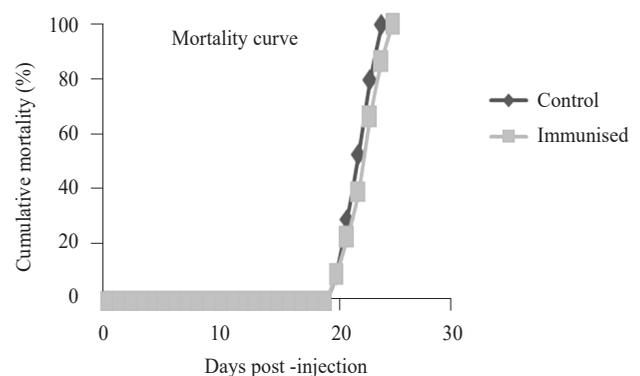


Fig. 3. Cumulative mortality of control and immunised group of *L. rohita* challenged with *Aphanomyces invadans* on 28th day of booster dose

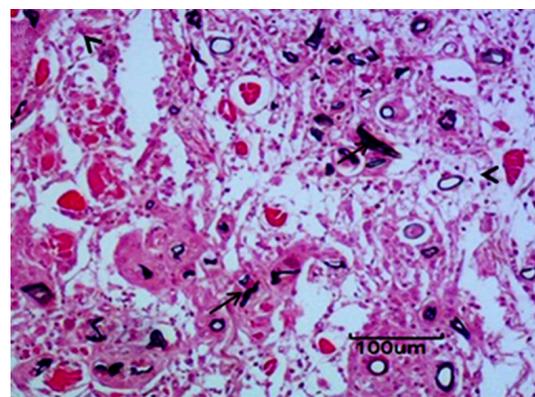
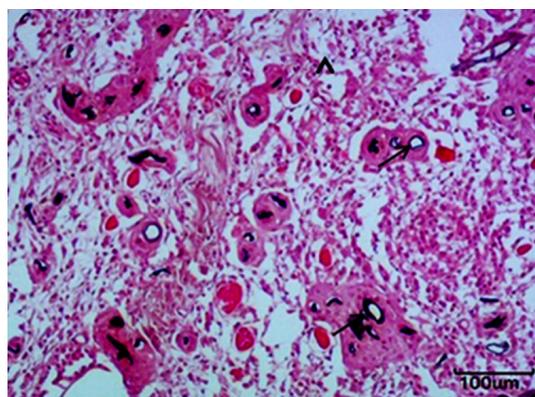


Fig. 4. Histopathology of control and immunised *L. rohita* challenged after booster immunisation with *A. invadans* at 24 dpi. a. control fish; b. Immunised fish. Lesion area showing extensive myonecrosis (arrowheads) and hyphae (arrows) (Grocott-Hand E)

As the immunised group of fish had significantly higher antibody level (detected by ELISA) compared to that of the control group at 14th day post-booster immunisation, it was assumed that the antigenic preparation of *A. invadans* was able to induce humoral immune response in immunised fish. In accordance with our results, Fregeneda-Grandes

in conformity with earlier report (Thompson *et al.*, 1999) in which cross-reactivity of the control sera from rainbow trout with antigenic preparation of *A. invadans* was observed. The authors hypothesised that control fish could have been exposed to environmental fungi, which might have antigens common to *A. invadans* as well as an unsuspected *Aphanomyces*.

The effectiveness of fish vaccination in inducing protective immunity can be evaluated by experimental challenge with the homologous pathogen. In the present study, challenge of both the immunised and control group of fish with *A. invadans* indicated that there was no difference in the mortality pattern. Importantly, 100% mortality was observed in both groups of fish. In contrast to our findings, Saikia and Kamilya (2012) reported that there was reduction in the mortality percentage of the immunised group of fish, but it was not statistically significant ($p > 0.05$). The authors suggested that one of the reasons for not showing significantly higher protection against *A. invadans* in the immunised groups could be that secondary immunisation was not performed in their experiment. However, in the present study, fish were given a booster dose following primary immunisation and were challenged with less number of zoospores *i.e.* 100 zoospores per fish compared to 20000 zoospores by Saikia and Kamilya (2012). Therefore, it can be concluded that it may not be easy to induce protective immunity following immunisation with conventional antigenic preparations *A. invadans*. These findings of the present study may be helpful for further investigations on development of a protective vaccine against EUS.

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