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FUNDAMENTAL STUDIES

Specific immune response in mucosal and systemic compartments of *Cirrhinus mrigala* vaccinated against *Edwardsiella tarda*: In vivo kinetics using different antigen delivery routes

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Mucosal immune barriers confer protection against invading fish pathogens. Here, we conducted an experiment for 60 days to assess the mucosal and systemic immune response in Mrigal (*Cirrhinus mrigala*), an Indian major carp. Fish were immunized with inactivated *Edwardsiella tarda* by four different routes, namely, oral, immersion, injection, and anal intubation. An indirect enzyme-linked immunosorbent assay (ELISA) was used to measure the specific immune response (antibody) in serum and mucus (collected from skin, gill, and gut) of the fish on 0, 15, 30, 45, and 60 days postimmunization. For specific immune response in the serum, significantly higher ($p < 0.05$) optical density (OD) values were obtained in the anal group (0.52 ± 0.03) and in the oral group (0.48 ± 0.03). In the skin mucus, significantly higher OD values were obtained in the oral group (0.48 ± 0.04) and immersion group (0.32 ± 0.03). In the gill mucus, significantly higher OD values were obtained in the oral group (0.82 ± 0.08) and the immersion group (0.73 ± 0.03). In the gut mucus, significantly higher OD values were obtained in the immersion group (0.080 ± 0.007) compared to the rest of the treatments. Fish from all the groups were challenged with LD_{50} dose of *E. tarda* at the end of the experiment. We conclude that oral and immersion immunization routes offer better protection of *C. mrigala* compared to other antigen delivery routes.

KEYWORDS

Cirrhinus mrigala, *Edawardsiella tarda*, ELISA, IgM, mucus, mucosal immunity

1 | INTRODUCTION

Mrigal (*Cirrhinus mrigala*) is a preferred table fish and one of the widely cultured Indian major carp (IMC) in Southeast Asian countries. In India, it is cultivated along with other IMCs and economically important exotic carps in a polyculture system. It is a comparatively hardy fish and, as such, also forms an important component in the sewage-fed carp culture system. Besides, this species has an omnivorous feeding habit and easily accepts artificial feed during cultural practices. These factors, together with its higher growth rate, have attracted fish farmers to culture this species on a large scale.

However, intensive culture practices of IMCs, including mrigal, have resulted in various disease outbreaks, particularly of bacterial origin. These outbreaks have substantially reduced its production, coupled with other health concerns. In most of the cases, bacteria are frequently isolated during a fish disease outbreak (Austin & Austin, 2012). One such serious bacterial fish pathogen is *Edwardsiella tarda*, which infects both farm-cultured and wild fish. This pathogen has a wide distribution and causes disease both in freshwater and marine environments. It also has a wide host range, and fish like tilapia, eel, catfish, mullet, salmon, trout, and flounder are frequently encountered by this pathogen (Mohanty & Sahoo, 2007). *E. tarda* causes edwardsiellosis, a septicemic disease characterized by lesions in skin, muscle, and internal organs. Externally, loss of skin pigmentation, exophthalmia, ascites, and petechial hemorrhages in fin and skin are frequently seen during gross examination.

Mucosal barriers play a pivotal role in the defense mechanism of fish. It is known that components of the fish mucosal immune system react instantly against the invading pathogenic organisms as a part of the first line of defense (Li et al., 2013). Furthermore, the role of mucosal barriers in fish defense is greater compared to terrestrial animals because the medium being water contains various pathogens and invading microorganisms that are in close contact with the fish surface (Rombout, Yang, & Kiron, 2014). The mucosa-associated lymphoid tissue, besides playing a role in the maintenance of mucosal homeostasis, also contains B cells and immunoglobulins, which form an important part of the specific immune system of fish (Brandtzaeg, 2009). The gut-associated lymphoid tissue, skin-associated lymphoid tissue, and gill-associated lymphoid tissue are three main mucosal immune compartments found in bony fish (Salinas, Zhang, & Sunyer, 2011).

Nowadays, focus has shifted from antibiotics to use of prophylactic measures like vaccination, which confers long-term protection on fish without any risk of bacterial resistance. Use of mucosal vaccines can be a quite promising health management strategy to effectively counter the foreign pathogens. Not much work has been conducted in the field of fish mucosal immunity except for some studies in the recent past, where some research has been carried out in this area to explore the role of the various immune components present in the mucus of fish. Recently, Makesh, Sudheesh, and Cain (2015) reported that IgM plays a key role in the systemic as well as mucosal immunity of teleosts. Interestingly, now, there has been an increasing trend to study the immune protective role of mucous in fish. In this context, the present work was carried out to explore antibody (IgM) response in mucosal and systemic compartments of economically important fish species, like *C. mrigala*, immunized via four different routes: oral, immersion, injection, and anal intubation.

2 | MATERIALS AND METHODS

2.1 | Fish

Fingerlings of *C. mrigala* were collected from a private fish farm located in Maharashtra, India. The average weight of the fish was 15 g. The fish were disinfected with 5 ppm $KMnO_4$ for 30 s prior to acclimatization in the fibreglass reinforced plastics (FRP) tanks (1,000 L) for 7 days. The physicochemical parameters like pH and dissolved oxygen were maintained at an optimum level. The fish were fed daily with pelleted feed at the rate of 2% of their body weight, divided into two equal doses. A total of 25% of water exchange was performed on every alternate day.

2.2 | Bacteria

E. tarda used in the study was obtained from American Type Culture Collection and maintained at the Aquatic Animal Health laboratory, Central Institute of Fisheries Education, Mumbai. *E. tarda* was revived in brain heart infusion (BHI) broth from glycerol stock stored at −80°C. The broth was incubated for 18–24 hr at 28°C. The bacterial culture was then streaked on Salmonella Shigella agar (SS agar) and incubated at 28°C.

2.3 | Antigen preparation

Bacterial cells were inactivated following the protocol of Hossain, Kawai, and Oshima (2009). Formalin (0.1%) combined with heat (70°C for 10 min) and citric acid (0.9%) was used to inactivate *E. tarda*. Briefly, 0.1% formalin and 0.9% citric acid was added to the bacterial cells. The cell suspension was mixed and heated at 70°C for 10 min. The bacterial cell suspension was then incubated at 30°C overnight. The following day, a small aliquot of the bacterial cell suspension was streaked on BHI agar plate, and another aliquot was inoculated in BHI broth to confirm complete inactivation of bacteria. The cells were then washed thrice with phosphate-buffered saline (PBS) by centrifuging at 5000*g* for 20 min at 4°C, and the pellet was resuspended in PBS.

2.4 | Experimental design

Fish were divided into five groups (control and four treatments), with 50 fish in each group. Circular tanks (300 L approx.) of a depth of about 55 cm were used for the experiment. The first group of fish did not receive any treatment (unimmunized) and was designated as the control (T1). In the second group (T2), fish were immunized orally with inactivated *E. tarda*. In the third group (T3), fish were immunized by subjecting them to immersion treatment in water containing inactivated *E. tarda* antigen. The fourth group (T4) of fish received an intraperitoneal injection of inactivated *E. tarda* antigen. The fish in the fifth group (T5) were immunized by anal intubation with inactivated *E. tarda* antigen.

2.5 | Immunization protocol

For immunization by the oral route (T2), inactivated *E. tarda* antigen (10 mL [1.0×10^9 cells/mL]/kg feed) was mixed with feed, and feeding of the antigen-mixed feed was carried out for 10 consecutive days. The antigen mixture was freshly prepared prior to feeding of the fish each day. The fish in the immersion group (T3) were transferred to another tank containing about 10 L of water containing 1.0×10^7 inactivated *E. tarda*/mL. Fish were placed in the bacterial suspension for about 60 min with vigorous aeration. After immunization, the fish were transferred back to the original FRP tank. In the injection group (T4), fish were injected intraperitoneally with 50 μL/fish of inactivated *E. tarda* (1.0×10^8 cells/mL) using a 1 mL tuberculin syringe, after which the fish were placed back in the original FRP tank. In the anal intubation group (T5), fish were administered with 50 μL of inactivated *E. tarda* (1.0×10^8 cells/mL) into the hindgut using a 200 μL microtip and pipette. After antigen loading, microtip was dipped in glycerol prior to immunization to prevent any injury or rupture. For efficient delivery of the antigen, the fish were starved for 2 days prior to immunization. Table 1 shows the different immunization routes and the doses used.

2.6 | Sample collection

Serum and mucus samples were collected on 0, 15, 30, 45, and 60 days postimmunization from five fish from each treatment at each time point. The fish were anesthetized with clove oil (50 μL/L of water) (NavNiketan pharmaceutical, Mumbai, India) before collecting the samples.

TABLE 1 Different immunization routes and the doses

Treatment	Route of immunization	Dose
T1	Control	None
T2	Oral	10 mL inactivated *Edwardsiella tarda* (1.0×10^9 cells/mL)/kg feed for 10 days (2% BW)
T3	Immersion	1.0×10^7 cells/mL of inactivated bacterial suspension for 60 min
T4	Injection (IP)	50 μL/fish of inactivated *E. tarda* (1.0×10^8 cells/mL)
T5	Anal	50 μL/fish of inactivated *E. tarda* (1.0×10^8 cells/mL)

IP: intra peritoneal.

2.6.1 | Collection of serum

Approximately 0.5 mL of blood was collected from the caudal vein using a 2-mL syringe with a 24-gauge needle. The blood was allowed to clot and stored at 4°C for 4 hr. The blood samples were centrifuged at 2,000*g* for 5 min, and the serum was separated and stored at −20°C for further analysis.

2.6.2 | Collection of skin mucus

Skin mucus samples were collected by placing the anesthetized fish in a ziplock bag and gently rubbing the skin surface of the fish. In order to collect the mucus effectively and adequately, 0.2 mL of sterile PBS was added to the ziplock bag prior to mucus collection. In addition, to prevent the degradation of samples by proteases, a protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) was added to PBS for a final concentration of 1 mM. After collecting mucus in the ziplock bags, the samples were then transferred to microcentrifuge tubes, vortexed for 1 min, and centrifuged at 2,000*g* for 15 min at 4°C, and the supernatant was stored at −20°C for further analysis.

2.6.3 | Collection of gill mucus

Gill mucus samples were collected by gently swabbing the gill surface of the anesthetized fish using a cotton swab. Subsequently, swabs saturated with gill mucus were placed in microcentrifuge tubes containing 0.2 mL of sterile PBS with PMSF for a final concentration of 1 mM. The samples were then vortexed for 1 min and centrifuged at 2,000*g* for 15 min at 4°C, and the supernatant was stored at −20°C for further analysis.

2.6.4 | Collection of gut mucus

After the collection of skin and gill mucus, the fish were killed with an overdose of clove oil. The fish were dissected, and the hindgut was excised. Prior to collection of gut mucus, care was taken to completely remove all the gut contents. Gut mucus was collected by gently squeezing the gut of the anesthetized fish, and the collected mucus was placed in a microcentrifuge tube containing 0.2 mL of sterile PBS with PMSF for a final concentration of 1 mM. The samples were then vortexed for 1 min and centrifuged at 2,000*g* for 15 min at 4°C, and the supernatant was stored at −20°C for further analysis.

2.7 | Revival of hybridoma

Hybridoma clones secreting monoclonal antibodies (MAbs) against mrigal IgM, developed at Aquatic Animal Health laboratory of Central Institute of Fisheries Education, were used for secondary antibody production (Mohanty, 2011). A cryopreserved hybridoma clone (Cm4A11) was thawed quickly in a water bath (37°C) and washed once with Iscove's Modified Dulbecco's Medium (IMDM) by centrifuging at 500*g* for 5 min. The cells were grown in 25 cm^2 flasks containing 7 mL IMDM with 10% fetal bovine serum in a CO_2 incubator at 37°C, 5% CO_2, and 90% relative humidity.

2.8 | Bulk production of monoclonal antibodies

After 24 hr, the medium was changed, and the cells were maintained by replacing 80% medium with fresh medium every 3 days. The culture medium was gradually replaced with a serum-free medium (CD Hybridoma medium; Invitrogen, Madison, WI) to culture the cells and expanded from a 25 cm^2 flask to 75 cm^2 and finally to a 185 cm^2 flask. Cell-free supernatant from the culture flasks was collected when cells showed optimum growth and was stored at 4°C for further use.

2.9 | Antibody detection by ELISA

An indirect ELISA was performed to detect antibody in fish sera and mucus (skin, gill, and gut) with modifications to the protocol of Swain, Nayak, Sahu, Mohapatra, and Meher (2002) using 96-well polystyrene ELISA plates (Himedia, Mumbai, India). Each well of the microtiter plate was coated with 100 μL of inactivated *E. tarda* containing 1×10^7 cells in carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed thrice with PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) and blocked with 100 μL of 3% BSA in PBS for 1 hr at room temperature (RT). The wells were further washed with PBS-T thrice. Each test serum/mucus sample of nonimmunized and immunized fish (collected on 0, 15, 30, 45, and 60 days postimmunization) was diluted (1:20 for serum, 1:1 for skin mucus, 1:7 for gill mucus, and 1:0 for gut mucus) with PBS (pH 7.2) and incubated for 30 min. A 100 μL of each preincubated test serum/mucus was added to the first well of each column of the ELISA plate and serially diluted two-fold in successive wells. The plates were incubated for 1 hr at RT and washed with PBS-T thrice. Hybridoma supernatant containing anti-mrigal Ig was added to each well at 100 μL per well. The plates were incubated for 1 hr at RT and further washed with PBS-T thrice. Furthermore, 100 μL of goat anti-mouse horseradish peroxidase (HRP) conjugate was added to each well at a dilution of 1:2,000 and incubated for 1 hr at RT. The plates were washed with PBS five times, and 100 μL of the freshly prepared TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was added to each well, and plates were then incubated for 5–10 min in a dark chamber. The reaction was stopped by adding 100 μL of stop solution (2 M H_2SO_4) to each well, and finally, the optical density (OD) was recorded at 450 nm in a microplate reader (Biotek, Winooski, VT).

2.10 | Estimation of lethal dose 50 (LD_{50})

Ten-fold serial dilutions of fresh broth culture of *E. tarda* were made in sterile PBS, and the bacterial concentration was estimated using the spread plate method. Fish were divided into six groups, with six fish in each group. A total of 50 μL of different dilutions of the bacteria, ranging from 1.3×10^3 to 1.3×10^8, were injected intraperitoneally into each fish using a 1 mL tuberculin syringe. Mortality was recorded daily for 10 days, and LD_{50} value was calculated by the method described by Reed and Muench (1938).

2.11 | Challenge study

After 60 days of the experiment, 10 fish from each treatment were challenged (in duplicate) with live *E. tarda*. Ten fish from the control group (T1) were kept unchallenged. *E. tarda* was cultured in nutrient broth at 28°C for 24 hr. The bacterial culture was centrifuged at 5000*g* for 20 min. The supernatant was discarded, and the pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4). The final bacterial concentration was adjusted to LD_{50} (1.3×10^6 CFU per mL) by serial dilution; 50 μL of bacterial suspension was injected intraperitoneally to each fish in all the treatments using a 1 mL tuberculin syringe. Care was taken during the entire process to avoid any undue stress and injury to the fish. All challenged fish were daily monitored, and mortality was observed for 10 days. Attempts were made to isolate and identify the causative agent from moribund fish. Survival was measured every day, and the cumulative number of dead fish was recorded each day. The relative percentages of survival (RPS%) in different treatment groups were calculated using the following formula (Amend, 1981):

$$RPS = [1 - (\%mortality/\%control\ mortality)] \times 100$$

2.12 | Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16, in which data were subjected to one-way ANOVA, and Duncan's multiple range test was used to determine the significant differences between the means. Comparisons were made at the 5% probability level.

3 | RESULTS

3.1 | Serum antibody levels

The mean ELISA OD values (±*SE*) of 1:20 diluted test sera collected at different time points postimmunization are given in Figure 1. The Day 0 samples had no significant difference in OD values between the treatments. However, there was significant difference ($p < 0.05$) between the treatments compared to the control, with the highest values observed in the anal intubation group (0.52 ± 0.03) on the 15th day postimmunization, followed by the oral group (0.48 ± 0.03) on the 60th day postimmunization.

3.2 | Skin mucus antibody levels

The mean ELISA OD values (±*SE*) of 1:1 diluted skin mucus collected at different time points postimmunization are given in Figure 1. The highest IgM level was observed in the orally immunized group (0.48 ± 0.04) on the 60th day postimmunization, followed by the immersion group (0.32 ± 0.03) on the 15th day postimmunization.

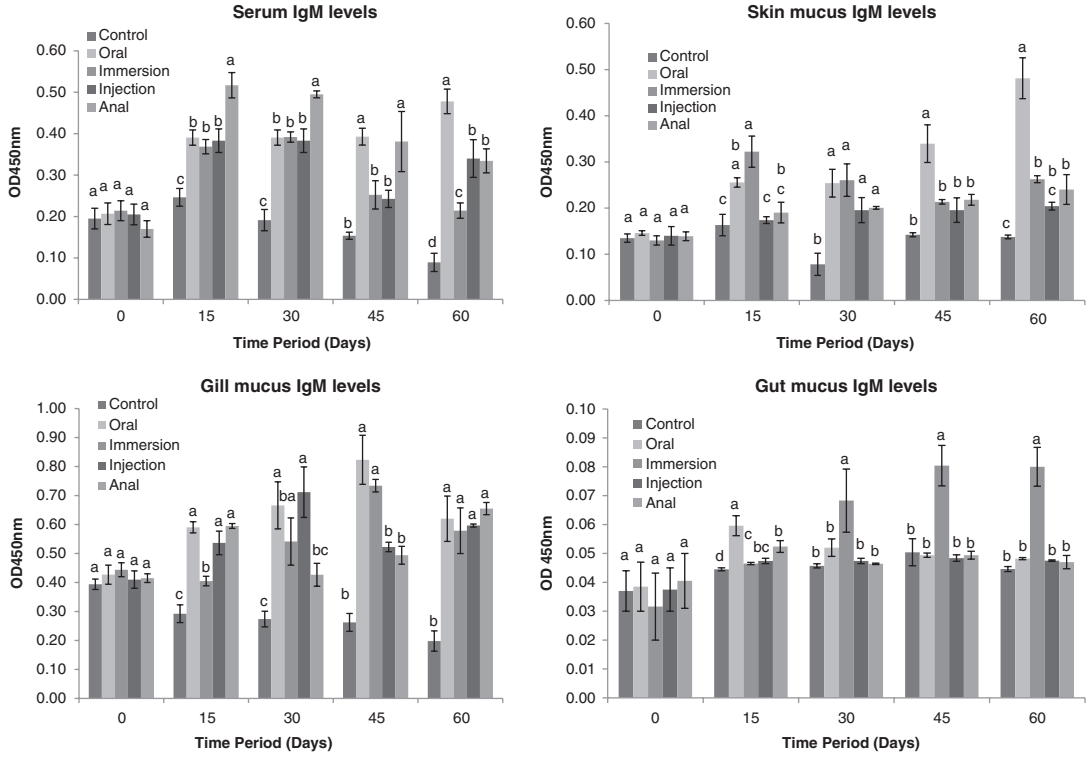

FIGURE 1 Mean IgM levels ±*SE* ($n = 5$) in serum (1:20), skin (1:1), gill (1:7), and gut (neat) mucus of *Cirrhinus mrigala* immunized with inactivated *Edwardsiella tarda* through different routes. The mean with different superscript letter per time point indicates significant difference ($p < 0.05$)

3.3 | Gill mucus antibody levels

The mean ELISA OD values (±*SE*) of 1:7 diluted gill mucus collected at different time points postimmunization are given in Figure 1. The highest IgM level was obtained in the oral group (0.82 ± 0.08) and the immersion group (0.73 ± 0.02) on the 45th day postimmunization, followed by the injection group (0.71 ± 0.09) on the 30th day postimmunization.

3.4 | Gut mucus antibody levels

The mean OD values (±*SE*) of 1:0 (undiluted) gut mucus collected at different time points postimmunization are given in Figure 1. The highest IgM levels were obtained in the immersion group (0.080 ± 0.007) on the 45th day postimmunization, followed by the oral group (0.060 ± 0.003) on the 15th day postimmunization.

3.5 | Challenge and relative percentage survival

The LD_{50} dose estimated was 1.3×10^6 CFU/fish. The immunized fish in each group were challenged with an LD_{50} dose of 1.3×10^6 CFU/fish by intraperitoneal injection. The percentage of mortality in the control (T1) was 80% after 10 days of challenge. Accordingly, RPS in different treatment groups postchallenge was calculated and is shown in Figure 2.

4 | DISCUSSION

The mucosal health of fish is of prime importance as mucosal surfaces constitute the first line of defense in fish to counter against invading pathogens. Over the last few decades, some efforts have been made to gain a better understanding of fish mucosal immune system in order to develop efficient vaccination strategies (Rombout et al., 2014). Mucosal vaccines have the potential to confer protection on fish against a vast number of infectious pathogens. Studies have shown that different routes of immunization confer different levels of immune protection in fish. Of the various host-related factors, antibody response (both in mucous and serum) is the main factor that reflects the protective efficacy following an immunization regime (Fouz et al., 2001; Palm, Landolt, & Busch, 1998). Valdenegro-Vega, Crosbie, Vincent, Cain, and Nowak (2013) reported that intra-peritoneal (IP) injection can induce a mucosal immune response to some extent. Immersion vaccination of fish leads to uptake of antigens through the skin surface, gills, and gut, which are continuously covered by mucus and are the first sites to interact with the invading pathogens (Wendelaar Bonga, 1997). On the other hand, oral antigens are uptaken and transported through the hindgut, after which it elicits a local and systemic antibody response (Rombout, Blok, Lamers, & Egberts, 1986). Anal immunization

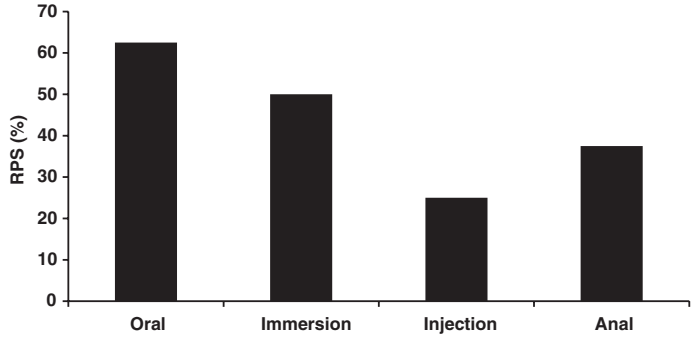

FIGURE 2 Relative percent survival of *Cirrhinus mrigala* after challenge with virulent *Edwardsiella tarda* through different routes

also results in bypass of the antigen through the hindgut, and in this case, even a small amount of antigen is sufficient to elicit a specific immune response (Rombout et al., 1986; Rombout, Van den Berg, Berg, Witte, & Egberts, 1989).

In our present study, oral immunization resulted in significant antibody production in skin mucus and gill mucus of the fish. This suggests that oral vaccine has a significant role to play in the activation and subsequent elicitation of immune components in the fish mucous. The higher antibody levels in the orally immunized group can be a result of the fact that the vaccine was administered for 10 days, while for other groups, the vaccine was administered as a single dose. The previous work by Vervarcke, Ollevier, Kinget, and Michoel (2004) supports our findings as their study reported increased antibody levels in the skin mucus of African catfish following oral vaccination in comparison to intraperitoneal immunization with *Vibrio anguillarum* O_2 antigens. Furthermore, Joosten, Tiemersma, Threels, Caumartin-Dhieux, and Rombout (1997) also reported higher immune response in gill mucus after oral immunization with encapsulated antigens.

The immersion route of immunization showed significant antibody production in gill mucus, which is expected as the gill is primarily involved in respiration, and there is a continuous influx of surrounding water. Furthermore, it has been reported by Santos et al. (2001) that locally stimulated antibody-secreting cells present in the gills play a role in their immune function. However, our study showed that the immersion route of immunization resulted in significant antibody production in gut mucus than the rest of the treatments, which is in contrast to some previous studies (Jenkins, Wrathmell, Harris, & Pulsford, 1994; McLean & Donaldson, 1990) that have reported increased immune response in gut only after oral immunization and anal intubation. The lower immune response produced in the gut after oral immunization can be because of degradation of the vaccine by the digestive enzymes and, hence, a low amount of antigen reaching the immune system of fish (Bøgwald, Stensvåg, Stuge, & Jørgensen, 1994; Hatten, Fredriksen, Hordvik, & Endresen, 2001; Johnson & Amend, 1983; Lillehaug, 1989). This suggests the use of encapsulated feed to prevent the proteolytic degradation of the antigen in the gut; however, on the other hand, it should be ensured that antigen degradation does not take place during feed preparation. The degradation of the antigen by the digestive enzymes is also the reason for low OD values obtained in gut compared to skin mucus, gill mucus, and serum. The lower antibody production in the gut after anal immunization can be attributed to the inefficacy of formalin-killed antigen to elicit a significant immune response in the hindgut of the fish, coupled with the lower efficiency of host fish to take up whole-cell antigen in contrast to lipopolysaccharide (LPS) and vaccine supernatant, which contains the active immunogenic part of the bacterin (Joosten, Kruijer, & Rombout, 1996). However, our findings are supported by the recent study of Liu et al. (2014), where mucosal immunity in the intestine of zebrafish (*Danio rerio*) was induced by bath vaccination with a live attenuated *V. anguillarum* vaccine.

The anal route of immunization showed significant antibody production in the serum of the fish. Villumsen et al. (2014) also reported that oral and anal vaccination gave full protection to rainbow trout against enteric redmouth disease. Although several workers (Badran et al., 2000; Lida et al., 1982; Pasnik et al., 2005) have reported increased levels of antibody in fish serum using injection as an immunization route, but in our study, injection did not produce significant immune response in the serum, and as such, the role of a booster dose against formalin-killed antigens appears to play an important role in eliciting sufficient immune response in fish serum. Thuvander et al. (1993) also reported that a booster dose of formalin-killed *Aeromonas saominicida* induced significant protection in brown trout, which signifies the fact that the level of protection is increased with further immunizations.

With regard to the correlation between the immune response and subsequent protection, the present study showed high antibody titer in serum, skin mucus, and gill mucus following oral and immersion immunization, which correlates with the RPS result suggesting that antibodies developed against *E. tarda* efficiently neutralized the antigen and thereby conferred protection on the host. Intriguingly, anal intubation and injection vaccination resulted in higher antibody titer in the serum and gill mucus of fish, respectively, but failed to confer protection, as is evident by the RPS result suggesting that, even though antibodies were produced against the antigen, they lacked the neutralizing property or were unable to initiate opsonization, and as such, fish succumbed to death irrespective of the high antibody titer. Although disparity in the neutralizing property of the antibody does exist, the extent to which antibodies formed at mucosal surfaces following vaccination do protect against infection is not fully understood

(Evensen, 2016). Nonetheless, such differences in conferring protection warrants further study to better elucidate the host immune response against different antigen delivery routes.

In conclusion, our study showed that the oral route of immunization offers better protection to *C. mrigala*, followed by immersion, compared to the rest of the immunization routes. Both oral and immersion vaccination are preferred methods for fish vaccination as these are nonstressful, user friendly, less time consuming, cost effective, and can be easily administered to large numbers of fish, even smaller ones. However, the problem arises from antigen degradation by gastric acidity and proteolytic enzymes in the intestinal lumen. In the present study, we mixed the required concentration of antigen with the commercially available feed. The mixture was freshly made prior to feeding of the fish, and this procedure was preferred over adding the antigen before feed preparation because of the greater risk of antigen degradation during feed preparation. Future studies should be conducted to improve the effectiveness of oral vaccination and develop an efficient delivery method, which protects the antigen against digestive degradation in the anterior part of the digestive tract of the fish.

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