Aquaculture 314 (2011) 18–23

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Effect of parasitism on vaccine efficacy against *Streptococcus iniae* in Nile tilapia

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ARTICLE INFO

Article history:
Received 9 December 2010
Received in revised form 6 January 2011
Accepted 19 January 2011
Available online 27 January 2011

Keywords:
Tilapia
Vaccine
Streptococcus
Parasitism Ichthyophthirius
Trichodina
Gyrodactylus

ABSTRACT

Limited information is available on vaccine performance in parasitized fish. The objective of this study was to determine if parasitism of fish affected vaccine efficacy. Antibody level, hematology and survival of Nile tilapia vaccinated with a modified *S. iniae* bacterin were compared among non-parasitized fish, fish parasitized by *Trichodina heterodentata* and *Gyrodactylus cichlidarum*, and fish parasitized by *T. heterodentata*, *G. cichlidarum* and *Ichthyophthirius multifiliis* (Ich). Among vaccinated fish, fish free from parasites (*Trichodina*, *Gyrodactylus* and Ich) had the highest antibody level (0.43, SE = 0.14). Significantly ($p<0.05$) lower anti-*S. iniae* antibody was noted in parasitized vaccinated fish (0.30, SE = 0.08). Among the vaccinated treatments, fish parasitized by *Trichodina*, *Gyrodactylus* and Ich showed the lowest survival (80.0%, SE = 10.0), significantly ($p<0.05$) lower than vaccinated fish free from parasites (97.5%, SE = 2.5) or parasitized by *Trichodina* and *Gyrodactylus* (95.0%, SE = 5.0). Following challenge with *S. iniae*, non-vaccinated fish free from parasites showed the higher survival (47.5%, SE = 2.5) than non-vaccinated fish parasitized by *Trichodina* and *Gyrodactylus* (37.5%, SE = 2.5). Non-vaccinated fish parasitized by all 3 parasites showed the lowest survival (27.5%, SE = 2.5) post challenge. Relative percent survival (RPS) demonstrated a decrease in vaccine performance for the group of fish that were parasitized with *Trichodina* and *Gyrodactylus* and Ich. RPS was 72% compared to 95 and 92%, respectively, in the other vaccinated treatments following challenge. This study demonstrated a reduction in vaccine performance in parasitized tilapia and highlights the importance of monitoring or controlling parasite levels in the aquaculture setting to optimize vaccine efficacy.

1. Introduction

Species of the ciliated protozoan *Trichodina* and monogenoidean platyhelminths are common parasites of cultured fish (Martins et al., 2008; Ghiraldelli et al., 2006; Thoney and Hargis, 1991). These parasites usually do not cause fish mortality except in the case of heavy infestations (Madsen et al., 2000; Huh et al., 2005; Martins et al., 2010a). However, parasitism may facilitate the development of systemic bacterial infections (Busch et al., 2003; Pylkko et al., 2006; Xu et al., 2009a). Rainbow trout (*Oncorhynchus mykiss*) parasitized by *Gyrodactylus derjavini* were more likely to develop bacterial infection (Busch et al., 2003). Tilapia (*Oreochromis niloticus*) coinfected with *Streptococcus iniae* and *G. niloticus* had a higher mortality rate than tilapia infected with *S. iniae* alone (Xu et al., 2007). Evans et al. (2007) infected two groups of channel catfish (*Ictalurus punctatus*) fingerlings, parasitized and non-parasitized by *Trichodina* sp. with *S. iniae* and *S. agalactiae*. The mortality rate was significantly higher in fish that were coinfected. Increased mortality of tilapia to *S. iniae* infection was also observed after experimental parasitism with *Ichthyophthirius multifiliis* (Xu et al., 2009a).

Host–parasite interactions and the fish immune system may be affected by inadequate handling, suboptimal water quality, inadequate nutrition, stress, and parasite infection (Boshra et al., 2006). Therefore, it is necessary to understand host–parasite relationships and the mechanisms whereby parasites influence the fish immune system (Sitja-Bobadilla, 2008). Fish possess innate immunity that is modulated by pathogen recognizing receptors found on the skin and gills that can limit the parasite load (Alvarez-Pellitero, 2008). Adaptive immunity has been demonstrated by the presence of T and B cells in teleosts (Alvarez-Pellitero, 2008). Leukocyte migration in response to *I. multifiliis* parasitism and induction of interleukine-1 expression during the primary monogenoidean parasitism has been demonstrated (Alvarez-Pellitero, 2008).

Non-specific response of fish to *I. multifiliis* parasitism is mediated by cytotoxic cells (NCC) analogous to NK cells in mammals (Buchmann et al., 2001). However, there is also evidence for the production of specific antibodies to *I. multifiliis* (Xu et al., 2009b). Sigh et al. (2004) also demonstrated an increase in the anti-*I. multifiliis* IgM on kidney and skin of rainbow trout. Buchmann and Lindestrom (2002) suggest several mechanisms of host recognition in response to monogenoidean platyhelminths. Among them are the presence of lectins, complement system and antibody production. Sea bass

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doi:10.1016/j.aquaculture.2011.01.022

(*Dicentrarchus labrax*) experimentally parasitized with the monogenoidean *Diplectanum aequans* showed an increase in the interleukine-1 in the spleen and gills (Faliex et al., 2008). These authors found that low level parasitism did not induce adaptive immunity but rather this parasite load may have elicited an innate immune response.

The effects of parasitism on vaccination efficacy in fish are little studied. In commercial aquaculture fish often harbor parasites, bacteria and/or viral pathogens concurrently. Anecdotal information from a commercial tilapia farm suggested that heavy parasite loads of trichodinids and dactylogyrids negatively affected the immune response and decreased effectiveness of a killed *S. iniae* vaccine (Shoemaker and Klesius, personal communication). Treatment and subsequent reduction of the parasite load restored vaccine performance. In mammals, vaccine efficacy is affected by a chronic concurrent infection and subsequent treatment to decrease the parasite load has been shown to improve vaccine performance (Borkow and Bentwich, 2008). Urban et al. (2007) discussed the negative effect of protective immunity in mice experimentally parasitized with *Heligmosomoides polygyrus* to model the condition of gastrointestinal parasitism in mammalian populations targeted for vaccination. Recent work demonstrated that parasitism with *Ascaris suum* reduced the efficacy of a *Mycoplasma hyopneumoniae* vaccine in co-infected pigs that resulted in vaccine failure (Steenhard et al., 2009).

The objective of this work was to determine if parasitism of fish affected vaccine efficacy. Antibody level, hematology and survival of Nile tilapia vaccinated with a modified *S. iniae* bacterin were compared among non-parasitized fish, fish infected by *T. heterodentata* and *G. cichlidarum*, and fish infected by *T. heterodentata*, *G. cichlidarum* and *I. multifiliis*.

2. Material and methods

2.1. Fish and water quality

Tilapia fry from the same spawning pond mean of 8.4 cm (SE = 0.9) total length and mean of 11.4 g (SE = 3.2) body weight were kept in a holding tank using filter-recirculated water at the US Department of Agriculture-Agriculture Research Service (USDA-ARS), Aquatic Animal Health Research Unit (AAHRU), Auburn, Alabama. Prior to the trial, tilapia moved to the experimental tanks with flowing water were moderately to heavily infested with *G. cichlidarum* and *T. heterodentata*. The prevalence of *Trichodina* parasitism was 100%, with a mean abundance of 858.8 (SE = 233.5). The prevalence of *Gyrodactylus* infestation was 70%, with a mean abundance of 30.6 (SE = 10.6). Fish were divided into two groups. One group was treated twice with potassium permanganate (5 ppm bath treatment) for 1 h on two consecutive days, followed by a 150 ppm formalin bath treatment for 1 h on the third day. After treatment, no parasites were recovered from biopsies of the skin and gills of the treated fish. Fish were acclimated for 7 days post treatment and fed ~3–4% body weight daily (Aquamax Grower 400, *PMI Nutrition International, LLC*., Brentwood, MO). The number of parasites from ten fish in each group was estimated just before the trial.

During the experiment total ammonia nitrogen, nitrite-nitrogen, alkalinity, pH and dissolved oxygen were measured once a week in random tanks. Water temperature was measured every day. The means and standard error (SE) of pH was 6.39 (SE = 0.03), ammonia 0.26 (SE = 0.02) $mg \cdot L^{-1}$, nitrite 0.13 (SE = 0.04) $mg \cdot L^{-1}$, alkalinity 58.14 (SE = 6.9) mg $CaCO_3 \cdot L^{-1}$, dissolved oxygen 6.44 (SE = 0.11) mg·L and water temperature 25.5 (SE = 0.14) °C.

2.2. Experimental design

A total of 320 fish with 12.5 (SE = 2.0) cm in length and 20.2 (SE = 0.6) g in weight was distributed in 16 glass aquaria divided in 8 treatments with 2 replicates in each, as follows (see Table 1): 1) fish not parasitized with *Trichodina*, *Gyrodactylus* (No TRICH/GYRO) and *I. multifiliis* (NoICH), not vaccinated (NoVAC), not exposed to *S. iniae* (No *S. iniae*); 2) no parasites or vaccination, but exposed to *S. iniae* (No TRICH/GYRO-NoICH-NoVAC-*S. iniae*); 3) no TRICH/GYRO-NoICH-VAC-*S. iniae*; 4) TRICH/GYRO-NoICH-No VAC-*S. iniae*); 5) TRICH/GYRO-NoICH-VAC-*S. iniae*; 6) TRICH/GYRO-ICH-NoVAC-*S. iniae*; 7) TRICH/GYRO-ICH-VAC-No *S. iniae*; and 8) TRICH/GYRO-ICH-VAC-*S. iniae*.

2.3. Parasites

Trophonts of *I. multifiliis* were originally isolated from a parasitized channel catfish from the USDA-ARS, AAHRU, Auburn, Alabama. Infected channel catfish were kept in 50 L glass aquaria and the theronts obtained as described by Xu et al. (2000). Briefly, fish were humanely euthanized by immersion in 300 mg/L tricaine methane sulfonate (MS-222), mature trophonts were gently scraped to dislodge the parasites, transferred to a 10 L glass aquarium with aeration, and incubated for 24 h at 24 °C. Theront concentrations were quantified with the aid of a Sedgewick–Rafter chamber. Fish were exposed to 40,000 theronts per fish (Xu et al., 2009b).

Prior to the experiment, 10 fish from the holding tank were examined for the parasites *Trichodina* and *Gyrodactylus*. After that, parasite samples were collected from 6 fish of each treatment at day 7 and day 21 post vaccination and 10 fish of each treatment at the end of the trial. For parasite quantification, body surface mucus was scraped into a Falcon tube and fixed in a 5% formalin solution for counting in a Sedgewick–Rafter chamber according to Ghiraldelli et al. (2006). Twenty percent of the sample was counted and estimated from the total volume. The mean abundance of parasites was calculated according to Bush et al. (1997).

2.4. Vaccination

Non-vaccinated fish were intraperitoneally (i.p.) injected with 100 μL of sterile trypic soy broth (TSB). Vaccinated fish were

Table 1
Mean abundance (mean ± standard error) of parasites 7 days post vaccination with *Streptococcus iniae* vaccine. Within a column, means followed by the different lower case letter are significantly different ($p<0.05$).

Treatments	*I. multifillis*	*T. heterodentata*	*G. cichlidarum*	Total*
NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae*	0^a	0^a	0^a	0^a
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*	0^a	0^a	0^a	0^a
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	0^a	0^a	0^a	0^a
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	0^a	644.7 ± 254.0^b	47.6 ± 13.8^b	692.3 ± 256.3^b
TRICH/GYRO-NoICH-VAC-*S. iniae*	0^a	142.7 ± 80.6^c	39.2 ± 7.6^b	182.0 ± 84.3^c
TRICH/GYRO-ICH-NoVAC-*S. iniae*	14.5 ± 5.4^b	680.5 ± 238.5^b	26.2 ± 11.8^c	721.2 ± 250.4^b
TRICH/GYRO-ICH-VAC-No *S. iniae*	12.7 ± 4.1^b	696.7 ± 83.7^b	28.8 ± 7.3^c	738.2 ± 85.8^b
TRICH/GYRO-ICH-VAC-*S. iniae*	4.0 ± 4.0^a	720.3 ± 305.1^b	9.8 ± 4.5^d	734.2 ± 306.8^b

* Total count of parasites, including *T. heterodentata*, *G. cichlidarum* and *I. multifillis*.

Table 2
Mean abundance (±standard error) of parasites between vaccinated and non-vaccinated tilapia 21 days post vaccination (prior to challenge) with *Streptococcus iniae*. Different letters indicate significant difference ($P<0.05$) among the treatments.

Treatments	*T. heterodentata*	*G. cichlidarum*	Total*
NoTRICH/GYRO-NoICH-NoVAC-No *S. niae*	0^{a}	0^{a}	0^{a}
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*	0^{a}	0^{a}	0^{a}
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	0^{a}	0^{a}	0^{a}
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	52.0 ± 31.7^{b}	3.0 ± 2.0^{a}	55.0 ± 33.5^{b}
TRICH/GYRO-NoICH-VAC-*S. iniae*	22.0 ± 6.9^{a}	14.7 ± 7.7^{b}	36.7 ± 7.8^{c}
TRICH/GYRO-ICH-NoVAC-*S. iniae*	13.3 ± 8.9^{a}	6.7 ± 1.7^{c}	20.0 ± 9.2^{c}
TRICH/GYRO-ICH-VAC-No *S. iniae*	6.7 ± 3.3^{a}	5.0 ± 1.8^{a}	11.7 ± 2.8^{d}
TRICH/GYRO-ICH-VAC-*S. iniae*	6.0 ± 2.1^{a}	2.5 ± 1.7^{a}	8.5 ± 2.2^{d}

* Total count of parasites, including *Trichodina heterodentata* and *Gyrodactylus cichlidarum*. No *Ichthyophthirius multifiliis* was observed in fish sampled at this time.

injected i.p. with killed *S. iniae* vaccine. To prepare vaccine, *S. iniae* (ARS-98-60) was cultured in TSB for 72 h at 28 °C. The culture was then treated for 24 h with 10% neutral buffered formalin to give a final concentration of 3%. The formalin-treated culture was centrifuged at 7000 *g* for 30 min, and the cell pellet and supernatant were separated. The cell-free supernatant was concentrated 20-fold using a 2 kDa concentrator (Amicon, Inc., Beverly, MA, USA), filter sterilized (0.2 μm), and used to resuspend the cell pellet at a ratio of 10:1 (v/v) (Klesius et al., 1999, 2000). The final vaccine cell concentration was 4×10^{9} CFU·mL^{-1}.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Antibody level (optical density—OD) against *S. iniae* was determined 21 days after immunization and 18 days after challenge by indirect enzyme-linked immunosorbent assay (ELISA) (Shelby et al., 2002). ELISA plates were coated for 1 h at 25 °C with 100 μL *S. iniae* (ARS-98-60) antigen in carbonate buffer (CB). The antigen was obtained following sonication and size-exclusion chromatography of *S. iniae* (a 1:10 dilution in CB of the initial fraction, which represented the highest molecular weight fraction, was used). Plates were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and then blocked with 3% bovine serum albumin in PBS for 1 h. Following blocking, plates were washed three times with PBS-T. Tilapia serum was added to the well of an ELISA plate at a 1:20 dilution in PBS. Serum was incubated for 30 min at 25 °C and then plates were washed three times with PBS-T. Monoclonal anti-tilapia immunoglobulin (1 H1; Shelby et al., 2002) was diluted 1:1000 in PBS-T and then added to all wells (100 μL per well) for 30 min. Following washing three times with PBS-T, 100 μL of sheep anti-mouse IgG peroxidase conjugate (1:5000 in PBS-T) was added and incubated for 15 min. Plates were washed as described earlier and 100 μL substrate was added (tetramethylbenzidine; Pierce). The reaction was stopped after 15 min by adding 50 μL of 2 M sulfuric acid to each well and the OD was read at 450 nm using a spectrophotometer. *S. iniae*-positive and *S. iniae*-negative sera were included on each plate as assay controls.

2.6. Exposure to S. iniae

Twenty one days after vaccination, fish were exposed to *S. iniae* (ARS-98-60) (Shoemaker et al., 2010). A fresh isolate obtained following passage through a tilapia with subsequent isolation on a blood agar plate was used to inoculate 250 mL tryptic soy broth (TSB). The culture was incubated at 28 °C (±2) for 24 h to an optical density of 1.2 at 540 nm. Plate counts determined the concentration of *S. iniae* to be 1.55×10^{8} colony forming units (CFU)·ml^{-1}. This culture was diluted 1:10 and 100 μl was injected intraperitoneally (i.p.). The challenge dose was 1.55×10^{6} CFU fish^{-1}. Fish were observed until 18 days after challenge until the mortality had stabilized.

2.7. Hematological analysis

Blood samples were collected 21 days after vaccination (from 6 fish per treatment) and 18 days after challenge (from 10 fish per treatment). After fish were anesthetized by immersion in 100 mg/L MS-222 solution, the blood was withdrawn from the caudal vein using a 1.0 mL syringe with a drop of 10% EDTA to make duplicate blood smears. The smears were stained with Giemsa/MayGrunwald (Rosenfeld, 1947) for differential counting of leukocytes and total counting of thrombocytes and leukocytes. One aliquot was used to determine hematocrit (Goldenfarb et al., 1971) and the rest was stored in tubes on ice to quantify the total number of red blood cells in a hemocytometer. The total number of thrombocytes and leukocytes were counted in blood extension by the indirect method (Martins et al., 2008).

2.8. Statistical analysis

SAS software was used to analyze the data. Parasite abundance, serum antibody level, survival and hematological parameters of different treatment groups were analyzed with Duncan's multiple range test of the general linear model (GLM) procedure (SAS Institute, 1989). P-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Parasitological analysis

No *Trichodina*, *Gyrodactylus* or Ich were found in non-parasitized fish 7 days post vaccination or prior to challenge (21 days post vaccination) with *S. iniae* (Tables 1, 2). Seven days after vaccination *Trichodina* (143–720) and *Gyrodactylus* (10–48) were found in all groups of parasitized fish (Table 1). There was a mean of 4 to 15 Ich parasites per host in groups of fish infected by Ich. No Ich was found in groups of fish not exposed to the parasite. No trophonts or theronts of Ich were observed in any fish prior to challenge (Table 2) suggesting the fish cleared the Ich infection. Except for the group TRICH/GYRO-NoICH-VAC-*S. iniae*, mean abundance of *Trichodina* at 7 days post vaccination did not vary among the treatments. Fish from the groups TRICH/GYRO-NoICH-NoVAC-*S. iniae* and TRICH/GYRO-NoICH-VAC-*S. iniae* showed higher mean abundance of *Gyrodactylus* than fish of Ich-inoculated (TRICH/GYRO-ICH-VAC-*S. iniae*). Total mean abundance of parasites was lower ($p<0.05$) in TRICH/GYRO-NoICH-VAC-*S. iniae* fish compared to the other treatments.

Table 3
Serum antibody level (Elisa OD) (±standard error) between vaccinated and non-vaccinated tilapia parasitized or not by *Trichodina heterodentata*, *Gyrodactylus cichlidarum* or *Ichthyophthirius multifiliis* 21 days after vaccination (pre-challenge) and 18 days after challenge with *Streptococcus iniae*. Different letters indicate significant difference ($P<0.05$) among the treatments.

Treatments	Pre-challenge	Post-challenge
NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae*	$0.18\pm0.03^{a,*}$	0.16 ± 0.02^{d}
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*	$0.18\pm0.03^{a,*}$	0.93 ± 0.20^{a}
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	0.43 ± 0.14^{b}	1.39 ± 0.15^{b}
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	0.21 ± 0.05^{c}	1.08 ± 0.11^{a}
TRICH/GYRO-NoICH-VAC-*S. iniae*	0.29 ± 0.08^{c}	0.91 ± 0.13^{c}
TRICH/GYRO-ICH-NoVAC-*S. iniae*	0.18 ± 0.04^{a}	1.11 ± 0.20^{a}
TRICH/GYRO-ICH-VAC-*S. iniae*	$0.30\pm0.08^{c,*}$	1.06 ± 0.17^{a}
TRICH/GYRO-ICH-VAC-No *S. iniae*	$0.30\pm0.08^{c,*}$	0.29 ± 0.04^{d}

* These values were analyzed together pre-challenge.

Table 4
Percent survival (±standard error) and relative percent survival (RPS, Amend 1981) between vaccinated and or non-vaccinated tilapia parasitized or not by *Trichodina heterodentata*, *Gyrodactylus cichlidarum* or *Ichthyophthirius multifiliis* 18 days after challenge with *Streptococcus iniae*. Different letters indicate significant difference ($P<0.05$) among the treatments.

Treatments	DF*/TF	Survival (%)	RPS
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*	21/40	47.5 ± 2.5^{a}	–
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	1/40	97.5 ± 2.5^{b}	95[1]
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	25/40	37.5 ± 2.5^{c}	–
TRICH/GYRO-NoICH-VAC-*S. iniae*	2/40	95.0 ± 5.0^{b}	92[2]
TRICH/GYRO-ICH-NoVAC-*S. iniae*	29/40	27.5 ± 2.5^{d}	–
TRICH/GYRO-ICH-VAC-*S. iniae*	8/40	80.0 ± 10.0^{e}	72[3]
TRICH/GYRO-ICH-VAC-No *S. iniae*	0/40	100^{b}	–
NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae*	0/40	100^{b}	–

* DF: dead fish, TF: total fish.
[1] Calculated using the mean percent mortality of the NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*.
[2] Calculated using the mean percent mortality of the TRICH/GYRO-NoICH-NoVAC-*S. iniae*.
[3] Calculated using the mean percent mortality of the TRICH/GYRO-ICH-NoVAC-*S. iniae*.

3.2. Enzyme-linked immunosorbent assay (ELISA)

Fish showed significantly ($p<0.05$) higher serum anti-*S. iniae* antibody in vaccinated groups compared to non-vaccinated fish (Table 3). Among vaccinated fish, fish free from infection by *Trichodina*, *Gyrodactylus* and Ich had the highest antibody level (0.43, SE = 0.14). Serum anti-*S. iniae* antibody was significantly ($p<0.05$) lower in the vaccinated fish when infected by *Trichodina* and *Gyrodactylus*(0.29, SE = 0.08) or infected by all three parasites together (0.30, SE = 0.08). After challenge with *S. iniae*, all surviving fish showed significantly ($p<0.05$) high antibody level compared to those not challenged with *S. iniae*.

3.3. Clinical signs and survival after challenge with S. iniae

The day after challenge non-vaccinated fish showed the first signs of streptococcal disease including erratic swimming, lethargy and darkened skin color as described in previous studies (McNulty et al., 2003; Russo et al., 2006). The fish were frequently located to the bottom of aquarium. From the fourth day after challenge fish showed no acceptance of food, eye opacity and body curvature that persisted in non-vaccinated fish during the whole trial period. In fresh dead fish hemorrhages were observed on liver, spleen and kidney. Pale heart, congested gills and increased mucus production was also noted.

All vaccinated fish showed significantly higher survival (80–97.5%) than non-vaccinated fish (27.5–47.5%) after challenged with *S. iniae* (Table 4). Among vaccinated fish, fish free from parasitism by *Trichodina*, *Gyrodactylus* and Ich had the highest survival (97.5%, SE = 2.5). The vaccinated fish parasitized by 3 parasites (*Trichodina*, *Gyrodactylus* and Ich) showed the lowest survival (80.0%, SE = 10.0), significantly ($p<0.05$) lower than vaccinated fish free from parasite or infected by *Trichodina* and *Gyrodactylus* (95.0%, SE = 5.0). When challenged with *S. iniae*, non-vaccinated fish free from parasitism showed higher survival (47.5% SE = 2.5) than non-vaccinated fish with *Trichodina* and *Gyrodactylus* parasitism (37.5%, SE = 2.5). Non-vaccinated fish parasitized by all 3 parasites showed the lowest survival (27.5%, SE = 2.5) after challenge. No fish died in non-challenged controls and fish showed 100% survival in groups of TRICH/GYRO-ICH-VAC-No *S. iniae* and NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae* (Table 4).

Relative percent survival (RPS) showed a decrease in vaccine effectiveness for the group of fish that were parasitized with *Trichodina* and *Gyrodactylus* and exposed to Ich at time of vaccination. In this treatment, RPS was 72% compared to 95% and 92%, respectively, in the other vaccinated treatments (Table 4).

3.4. Hematological analysis

Twenty one days after vaccination, some differences were noted between the treatments with regard to hematological parameters measured (Table 5). The most significant ($p>0.05$) was the decrease in red blood cell count and lymphocyte numbers observed in the fish parasitized by all three parasites but not vaccinated (Table 5).

After challenge red blood cell count of control fish (NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae*) was higher ($p<0.05$) than that observed in the other treatments (Table 6). Fish from the group NoTRICH/GYRO-NoICH-NoVAC-*S. iniae* showed high hematocrit percentage when compared to TRICH/GYRO-NoICH-VAC-*S. iniae*. The highest number of white blood cell was noted in TRICH/GYRO-ICH-NoVAC-*S. iniae* fish. After challenge with *S. iniae*, a reduced number of lymphocytes was observed in all groups of non-vaccinated fish challenged with *S. iniae* regardless of parasite status. Vaccination appeared to maintain the lymphocyte numbers. A significantly increased ($p<0.05$) number of circulating neutrophils was seen in the TRICH/GYRO-ICH-NoVAC-*S. iniae* treatment.

4. Discussion

Parasitism influences immune protection and vaccine efficacy in human and animal models (Urban et al., 2007; Borkow and Bentwich, 2008; Steenhard et al., 2009). This study demonstrated a decrease in vaccine performance in fish parasitized by *Trichodina*, *Gyrodactylus* and Ich. Antibody response was also decreased in parasitized fish. Interestingly, parasitism with the levels of *Trichodina* and *Gyrodactylus* in our study resulted in a decrease in antibody level as compared to non-parasitized fish but not a decrease in vaccine performance. This result could be a reflection of the parasite abundance (182, *Trichodina* and *Gyrodactylus*) in this group 7 days post vaccination, which was significantly ($p<0.05$) lower than in the other parasitized groups (total abundance was about 700 parasites). The decrease in vaccine performance was only observed in fish parasitized by all three

Table 5
Hematological parameter between vaccinated and non-vaccinated tilapia (means ± standard error) 21 days after vaccination with killed *Streptococcus iniae* vaccine. Different letters indicate significant difference ($P<0.05$) among the treatments. RBC: red blood cells, HTC: hematocrit, WBC: white blood cells, Thro: thrombocytes, Lymp: lymphocytes, Mono: monocytes, Neut: neutrophils.

Treatments (after vaccination)	RBC × 10^6	HTC %	WBC × 10^3	Thro × 10^3	Lymp × 10^3	Mono × 10^3	Neut × 10^3
NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae**	1.45 ± 0.8^{a}	31.8 ± 1.1^{a}	16.9 ± 1.5^{a}	48.5 ± 5.8	$14.1 \pm 1.4^{a,b}$	$2.8 \pm 0.4^{a,b}$	0.02 ± 0
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae**	1.45 ± 0.8^{a}	31.8 ± 1.1^{a}	16.9 ± 1.5^{a}	48.5 ± 5.8	$14.1 \pm 1.4^{a,b}$	$2.8 \pm 0.4^{a,b}$	0.02 ± 0
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	$1.30 \pm 0.3^{a,b}$	47.8 ± 2.1^{b}	$12.9 \pm 1.7^{a,b}$	53.2 ± 18.2	$11.0 \pm 1.9^{a,b}$	$1.3 \pm 0.1^{a,b,c}$	0.6 ± 0.4
TRICH/GYRO-ICH-NoVAC-*S. iniae*	1.00 ± 0.2^{b}	25.7 ± 1.4^{a}	8.3 ± 1.5^{b}	33.6 ± 10.3	7.2 ± 1.4^{b}	0.9 ± 0.5^{c}	0.2 ± 0.2
TRICH/GYRO-ICH-VAC-No *S. iniae**	$1.40 \pm 0.1^{a,b}$	$40.9 \pm 2.0^{b,c}$	19.7 ± 2.7^{a}	44.8 ± 3.9	17.0 ± 2.5^{a}	$2.0 \pm 0.4^{a,b,c}$	0.05 ± 0.05
TRICH/GYRO-ICH-VAC-*S. iniae**	$1.40 \pm 0.1^{a,b}$	$40.9 \pm 2.0^{b,c}$	19.7 ± 2.7^{a}	44.8 ± 3.9	17.0 ± 2.5^{a}	$2.0 \pm 0.4^{a,b,c}$	0.05 ± 0.05
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	1.53 ± 0.1^{a}	39.5 ± 4.7^{c}	18.0 ± 3.1^{a}	27.1 ± 4.4	16.2 ± 2.9^{a}	$1.1 \pm 0.4^{b,c}$	0.6 ± 0.3
TRICH/GYRO-NoICH-VAC-*S. iniae*	1.53 ± 0.1^{a}	$41.3 \pm 2.8^{b,c}$	16.3 ± 2.5^{a}	28.3 ± 2.8	$13.0 \pm 2.2^{a,b}$	2.9 ± 1.0^{a}	0.4 ± 0.4

* These values were analyzed together prior to challenge.

Table 6
Hematological parameter between vaccinated and non-vaccinated tilapia (means ± standard error) 18 days after challenge with *Streptococcus iniae*. Different letters indicate significant difference ($P<0.05$) among the treatments. RBC: red blood cells, HTC: hematocrit, WBC: white blood cells, Thro: thrombocytes, Lymp: lymphocytes, Mono: monocytes, Neut: neutrophils.

Treatments (after challenge)	RBC × 10^6	HTC %	WBC × 10^3	Thro × 10^3	Lymp × 10^3	Mono × 10^3	Neut × 10^3
NoTRICH/GYRO-NoICH-NoVAC-No *S. iniae*	1.75 ± 0.1^{a}	$35.3 \pm 2.4^{a,b}$	16.0 ± 2.0^{b}	71.6 ± 8.0^{a}	7.9 ± 1.4^{a}	5.5 ± 0.8^{a}	4.1 ± 1.9^{b}
NoTRICH/GYRO-NoICH-NoVAC-*S. iniae*	$1.01 \pm 0.1^{b,c,d}$	38.4 ± 3.2^{a}	10.0 ± 1.9^{b}	60.9 ± 14.3^{a}	2.2 ± 0.5^{d}	5.1 ± 1.4^{a}	2.3 ± 0.7^{b}
NoTRICH/GYRO-NoICH-VAC-*S. iniae*	$1.28 \pm 0.02^{b,c}$	$37.1 \pm 4.5^{a,b}$	8.9 ± 0.7^{b}	$45.8 \pm 5.8^{a,b}$	$5.4 \pm 0.6^{a,b}$	1.1 ± 0.3^{b}	2.3 ± 0.2^{b}
TRICH/GYRO-ICH-NoVAC-*S. iniae*	$1.0 \pm 0.2^{c,d}$	$33.0 \pm 4.9^{a,b}$	29.1 ± 15.4^{a}	$53.9 \pm 12.9^{a,b}$	2.4 ± 1.0^{d}	5.3 ± 1.6^{a}	21.4 ± 13.3^{a}
TRICH/GYRO-ICH-VAC-No *S. iniae*	1.31 ± 0.03^{b}	$34.0 \pm 2.0^{a,b}$	12.4 ± 0.9^{b}	67.4 ± 8.7^{a}	$2.9 \pm 0.5^{c,d}$	6.0 ± 0.7^{a}	3.2 ± 0.7^{b}
TRICH/GYRO-ICH-VAC-*S. iniae*	1.31 ± 0.1^{b}	$34.4 \pm 1.7^{a,b}$	7.9 ± 1.1^{b}	$53.3 \pm 7.7^{a,b}$	$5.2 \pm 0.7^{b,c}$	0.6 ± 0.2^{b}	1.9 ± 0.5^{b}
TRICH/GYRO-NoICH-NoVAC-*S. iniae*	0.83 ± 0.1^{d}	$31.9 \pm 2.9^{a,b}$	17.4 ± 6.1^{b}	30.1 ± 5.4^{b}	2.0 ± 0.5^{d}	5.3 ± 1.7^{a}	9.3 ± 4.3^{b}
TRICH/GYRO-NoICH-VAC-*S. iniae*	1.32 ± 0.1^{b}	27.9 ± 2.7^{b}	9.1 ± 0.9^{b}	$43.1 \pm 5.8^{a,b}$	$7.5 \pm 0.8^{a,b}$	0.3 ± 0.1^{b}	1.2 ± 0.2^{b}

parasites and may be due to Ich influencing the overall immune response due to the administration of Ich at time of vaccination. Ich may have resulted in a local immune response with increased immune gene expression in the skin of tilapia as has been demonstrated in the skin of rainbow trout (Sigh et al., 2004) and carp (*Cyprinus carpio*) (Gonzalez et al., 2007a,b), that may have negatively influenced the humoral immune response to the killed *S. iniae* vaccine. Alternatively, the stress of multiple parasites (Bowers et al., 2000; Tully and Nolan, 2002) negatively influenced the immune response so that vaccine performance was hindered.

The results of this study also support earlier work that concurrent parasitic and bacterial infection reduces the probability of survival (Bandilla et al., 2006; Busch et al., 2003; Evans et al., 2007; Xu et al., 2007, 2009a). Compared with non-parasitized, *S. iniae* challenged fish, chronic infection with *Trichodina* and *Gyrodactylus* followed by *S. iniae* challenge as well as parasitism by all three parasites resulted in increased mortality. The possibility of enhanced mortality is likely a result of the potential for increased bacterial transmission as suggested by Cusack and Cone (1986) or alternatively due to the stress of parasitism (Bowers et al., 2000; Tully and Nolan, 2002).

Few studies have examined the influence of parasitism, vaccination and challenge on hematological parameters. At 21 days post vaccination, although differences were observed, the values were within the "normal" range for tilapia (Martins et al., 2008, 2010b; Silva et al., 2009; Jerônimo et al., 2011). Compared with non-parasitized and not vaccinated fish, the fish parasitized by all three parasites and not vaccinated showed lower red blood cell count, white blood cell count and lymphocyte count. This decrease may be due to parasitism reducing these values. Interestingly, this group of fish post challenge showed the lowest survival rate and may be a reflection of the low hematological values recorded for these fish prior to challenge that ultimately influenced innate resistance.

After challenge, red blood cell count (RBC) was decreased in fish from all treatments except for non-parasitized no challenge control treatment. This decrease has been reported in Indian major carp, *Labeo rohita* (Misra et al., 2006), carp (Harikrishnan et al., 2003) and goldfish, *Carassius auratus* (Harikrishnan et al., 2010) following challenge with *A. hydrophila*. According to Misra et al. (2006) decreased number of RBC may be related to anemia. In the present work the number of RBC may characterize a slight anemia, but a severe reduction on these values may affect the oxygen transportation to tissues. An increase in RBC number reported by Garcia et al. (2007) in pacu (*Piaractus mesopotamicus*) after challenge with *A. hydrophila* was contrary to our results. An increased number of lymphocytes in TRICH/GYRO-ICH-VAC-*S. iniae* fish is in agreement with the observations in goldfish (Shao et al., 2004), pacu (Garcia et al., 2007) and tilapia (Balfry et al., 1997; Martins et al., 2008). Our results indicate that these cells were maintained by vaccination. In contrast, a decrease in the number of monocytes observed in NoTRICH/GYRO-NoICH-VAC-*S. iniae*, TRICH/GYRO-ICH-VAC-*S. iniae* and TRICH/GYRO-NoICH-VAC-*S. iniae* fish was certainly influenced by vaccination. It indicates that these cells could be migrating to the lesion site as reported by Silva et al. (2009) in tilapia intraperitoneally vaccinated. Neutrophilia observed in TRICH/GYRO-ICH-NoVAC-*S. iniae* fish after challenge was also reported in cichlid fish *Etroplus suratensis* infected by Epizootic Ulcerative Syndrome (Pathiratne and Rajapakshe, 1998), LPS-injected carp (Selvaraj et al., 2004), pacu challenged with *A. hydrophila* (Garcia et al., 2007), and tilapia infected with *Enterococcus* sp. (Martins et al., 2008). Seven days after intraperitoneal injection with a polyvalent vaccine in tilapia, Silva et al. (2009) observed neutrophilia in the circulating blood. Interestingly, they found that 21 days after vaccination no difference was seen when compared with control fish. Neutrophils in fish are involved in cellular immune response and phagocytosis (Iwama and Nakanishi, 1996). Increased population of neutrophils in the circulating blood can indicate an inflammatory response (Martins et al., 2006; Overland et al., 2010). Our results are in agreement, in part, with the results of Silva et al. (2009). The post challenge collection occurred 18 days after challenge. Results suggest that in this period, except for TRICH/GYRO-ICH-NoVAC-*S. iniae*, the neutrophil population did return to a normal level.

5. Conclusion

Until recently, no work has tested the influence of parasitic infection on vaccine efficacy in fish. This is especially important when applying not only the preventive measures but also vaccine strategies. The present study demonstrated a reduction in vaccine performance in tilapia parasitized by *Trichodina*; *Gyrodactylus* and Ich at the level of infection recorded for this study. We also observed a decrease in antibody levels, RBC, WBC count and lymphocyte number in the parasitized vaccinated fish. Further studies are warranted, likely with another *I. multifiliis* strain or exposure to a higher number of theronts to induce a greater level of parasitism while examining vaccine response.

Acknowledgements

The authors thank Jana Mladek and Paige Mumma for technical contributions and CNPq (no. 201485/2009-5) for financial support to M.L. Martins. We also thank Drs. Julie Bebak and Benjamin La Frentz for critical review prior to submission. This work was supported by USDA-ARS CRIS no. 6420-32000-024D.

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