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Immunization of Basa fish (*Pangasius bocourti*) against *Ichthyophthirius multifiliis* with live and sonicated trophonts

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Abstract

The high density of Basa fish (*Pangasius bocourti*) culture leads to outbreaks of *Ichthyophthirius multifiliis* (Ich), also knows as white spot disease. In this research, immunization of Basa fish against Ich with live and sonicated trophonts by intraperitoneal (IP) injection was investigated. Anti-Ich antibody titer was determined using ELISA and Western immunoblotting 21 days post immunization. The results revealed that pre-immunized fish, non-immunized fish and fish immunized with bovine serum albumin (BSA) at a concentration 65 µg g^{-1} fish did not show specific antibody against Ich. 21 days post immunization, fish immunized with live trophonts exhibited higher anti-Ich antibody titer than fish immunized with sonicated trophonts at the same antigen concentration. Fish immunized with 65 µg trophonts protein/g fish live trophonts showed the highest titer 1:1,000 (p<0.05). The results from Western immunoblotting showed two parasite protein bands of 66 kDa and <14 kDa, which reacted with antibodies from serum of immune fish. No fish in the non-immunized group survived. At the same concentration of antigen (65 µg g^{-1}), fish immunized with live trophonts exhibited the highest survival rate, 63.33±5.77% (p<0.05). Therefore, these results are the Basa fish immunizing procedure will be the way to conduct immunization against Ich to prevent disease outbreaks in aquaculture.

Keywords: *Pangasius bocourti*, *Ichthyophthirius multifiliis*, Immunization

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Introduction

Basa fish or Asian catfish (*Pangasius bocourti*) is a freshwater fish in the family Pangasiidae found in Southeast Asia, especially in the Mekong and Chao Phraya Rivers. This fish has an important economic impact in Nakhon Phanom Province, Thailand. It is cultured in cages in the Mekong basin and exported to the other Southeast Asian countries as well as to Europe and Russia. It is prized for its white meat, tender texture, good taste, low fat and easily digestible protein (Hung *et al*., 2002; Jiwyam, 2010; Sriphairoj *et al*., 2010). Increasing consumer demand has encouraged rapid extension of Basa fish farming. This form of aquaculture involves high density fish culture which unfortunately has led to disease outbreaks (Dickerson and Findly, 2014). Antibiotics as oxytetracycline are used to control and treat this disease. Their dosages are usually high which promotes antibiotic resistance in microorganisms, low meat quality and environmental pollution (Saini *et al*., 2014). White spot disease is caused by *Ichthyophthirius multifiliis* (Ich) and is commonly found in native and farmed freshwater fish. Diseased native fish can spread Ich to farmed fish (Xu *et al*., 2013). Many infections have been reported in fish cultures, especially in tropical and subtropical regions (Scholz, 1999). Ich can spread rapidly in poor quality water or unsuitable culture conditions (Osman *et al*., 2009). In Ich infected fish skin and gills are damaged leading to fish mortality, especially in fingerlings. This represents a significant economic loss in aquaculture (Martins *et al*., 2011). Currently, chemicals such as formalin, methylene blue, copper sulfate, potassium permanganate, hydrogen peroxide and acetic acid are used to treat fish against this parasite (Xiao-Feng *et al*., 2014; Song *et al*., 2015). The most effective chemical used for treatment is malachite green, which was banned due to its toxicity (Song *et al*., 2015). The chemicals presently used are applied for treatment only after the presence of parasite has been detected on fish skin. Thus, the treatment is not effective enough (Xu *et al*., 2008a, b). Moreover, Ich treatment is more complicated due to its life cycle. This parasite has a temperature dependent life cycle that includes three stages, theront, trophont and tomont (Shinn *et al*., 2009). Tomont referred as cyst of Ich in a free form living in an aquatic environment. Cyst walls block chemical penetration making treatment difficult.

Infected fish may acquire systemic and mucosal immunity against Ich (Swennes *et al*., 2007; Alvarez-Pellitero, 2008). Fish vaccination is an alternative choice for parasite prevention that is harmless to the environment. Several studies reported successful fish immunization against Ich. Dalgaard *et al*. (2002) reported protection against Ich in rainbow trout (*Oncorhynchus mykiss*) after immunization with sonicated formalin killed trophonts. Heidarieh *et al*. (2014) reported an immune response against Ich in rainbow trout immunized with irradiated trophonts. Xu *et al*. (2008a) reported immunization of Nile tilapia (*Oreochromis niloticus*) against Ich by

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

immersing them in water containing live theronts and sonicated trophonts as well as by intraperitoneal (IP) injection at levels of 20,000 theronts per fish and 65 µg trophont protein g^{-1} fish, respectively. The results showed that anti-Ich antibody of fish immunized by immersion in water containing live theronts and by IP injection of live theronts and sonicated trophonts had significantly higher anti-Ich antibodies than fish immunized with sonicated trophonts by immersion and non-immunized fish. Moreover, Xu *et al*. (2008b) reported that the channel catfish immunized with formalin killed and freeze-thawed trophonts did not produced anti-Ich antibodies and were infected by Ich. Osman *et al*. (2009) immunized Goldfish (*Carassius auratus*) against Ich at levels 20,000 live theronts per fish, 2,000 trophonts per fish and 65 µg sonicated trophonts/ g fish by immersion and IP injection. Fish immunized with theronts by immersion and IP injection showed higher anti-Ich antibodies and survival rates than fish immunized with trophonts by immersion and IP injection. Martins *et al*. (2011) evaluated temperature effects on *Ictalurus punctatus* immune response against live theronts of Ich and found that immunized fish at low temperature had high mortality and no anti-Ich antibody production. However, immunity of Basa fish against Ich is not clear. Therefore, this study aims to investigate immune response of Basa fish against Ich after immunization with live and sonicated trophonts.

Materials and methods

Fish, parasite and water quality

Basa fish, having a mean length of 8.9±0.7 cm mean, mean weight of 11.30±1.93 g and no previous infection with Ich were cultured in glass tanks at a density of 30 fish 100 L^{-1} of dechlorinated tap water. Fish were distributed among 8 treatments with 2 replicates and were fed 40% crude protein at a level of 5% of their body weight daily using commercial fish feed. Infected Basa fish were obtained from Nakhon Phanom Inland Fisheries Research and Development Center. These infected fish were rinsed with distilled water and fish skin was gently scraped to isolate Ich. Water quality measurement and water exchange were performed every three days. Water temperature, pH and dissolve oxygen (DO) were determined using a pH meter (HI-98127, Hanna, UK) and DO meter (HI9147, Hanna, UK), respectively. Ammonia and nitrite concentrations were measured using ammonia and nitrite test kits (Advance Pharma, Thailand). During the experiment, water temperature, pH, DO, ammonia and nitrite concentrations were 31.3±2.4 °C, 7.13±0.30, 6.8±0.5 mg L^{-1}, 0.2±0.1 mg L^{-1}, 0.2±0.2 mg L^{-1}, respectively.

Antigen preparation

Antigen preparation was modified from Xu *et al*. (2008a). Trophonts were separated from fish skin and mucus then pooled in a plastic tube. The collected trophonts were counted with Sedgewick Rafter counting chamber (SPI Supplies, USA) and concentrated

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

by centrifugation at 1,000 rpm for 5 min. Then the supernatant was discarded and the pellet resuspended in PBS buffer, pH 7.4. The trophont suspension was used as the first antigen (live trophonts). Another antigen (sonicated trophonts) was prepared by sonicating a trophont suspension for 1 min on ice. The protein concentration in live and sonicated trophonts was measured using a spectrophotometric method (HALO RB-10, Dynamica, Australia) (Bradford, 1976).

Immunization

Fish were divided into 8 treatments and immunized by IP injection with 100 µL of antigen. Antigen was mixed with Freund's adjuvant before injection. The first treatment was non-immunized fish. The second treatment was immunized with bovine serum albumin (BSA) at 65 µg protein g^{-1} fish. First and second treatments were used as negative controls. Fish in treatments 3-5 were immunized with live trophonts at 45, 55 and 65 µg protein g^{-1} fish, while fish in treatments 6-8 were immunized with sonicated trophonts at same respective protein concentrations.

Serum sampling

Before immunization, fish serum was kept as pre-immunized. Twenty one days post immunization, five fish in each of treatments 1-8 were randomly selected for serum sampling. Fish blood was sampled from the base of the tail. Blood coagulation was allowed at 4 °C overnight and then the samples were centrifuged at 10,000 rpm at 4 °C for 10 min (Z236K, Hermle, Germany). The antibody titer against Ich in the collected serum was determined using ELISA and Western immune blotting.

ELISA

ELISA was performed according to Kuendee *et al*. (2015) with slight modification. Ich was diluted in a carbonate buffer, pH 9.5 to 20 µg mL^{-1}. Each well of 96-well polystyrene plates (Nunc, Denmark) was coated with 50 µL of the diluted Ich solution. The coated plate was washed 3 times with TBST and blocked with 5% skim milk (Scharalau, Spain). Then, the wells were added with various serial dilutions of fish serum and incubated at 37 °C for 1 hr. After that, it was washed 3 times with TBST and incubated with mouse anti-Basa fish IgM antiserum ($1:10^5$) at 37 °C for 1 hr, followed by washing 3 times with TBST again. Afterward, the plate was incubated with conjugated anti-mouse IgG linked with alkaline phosphatase ($1:5\times10^3$, Zymed, USA) at 37 °C for 1 hr. Subsequently, it was washed 3 times with TBST and 3 times with TBS. 100 µL of 1 mg mL^{-1} ρ-Nitrophenyl phosphate (Amersham, Canada) was added as substrate. Next, the absorbance was determined at 405 nm using a Microplate Reader (Bio-Rad, USA). The titer in this study was defined as the highest dilution that still showed a positive result.

Western immunoblotting

Ich proteins were separated with 12% separing gel and 4% stacking gel with constant voltage at 150 V. Then, the proteins were transferred from SDS-

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

PAGE gel onto a nitrocellulose membrane. After this transfer, the membrane was divided as a part was allocated for protein molecular weight estimation and a part of the membrane was used for samples immunoblotting. The marker part of the membrane part stained with 0.1% Amido Black, whereas the sample part of the membrane was incubated in 5% skim milk. After incubation, the sample part of the membrane was further incubated with a various serial dilution of fish serum as 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$ at room temperature for1 hr and washed 3 times with TBST. Next, it was incubated with anti-Basa fish serum (1: 10^4) at room temperature for1 hr. After that, the membrane was washed 3 times with TBST and incubated with anti-mouse IgG linked with alkaline phosphatase (1: 5×10^3) at room temperature for1 hr. Subsequently, it was washed 3 times with TBST and 3 times with TBS. Then, the membrane was soaked twice in a substrate buffer, pH 9.5 and added with a NBT/BCIP substrate solution (Bio-Rad, USA). Finally, this membrane was agitated in a substrate solution until the protein band could be observed.

Challenge with Ich

Twenty one days post immunization, 10 fish from each group were transferred to new glass tank containing with 100 L of dechlorinated tap water. These fish were challenged with 10^4 theronts $fish^{-1}$ for 2 hr. White spots on the fish skin and scratching behavior against the walls of the tank were observed and recorded. Two days post challenge, three fish form each group were random selected for estimated the number of parasites as described previously by Osman *et al*. (2009). The parasites infection level was assessed by scoring, no infection = 0, <50 trophonts $fish^{-1}$= 1, 50-100 trophonts $fish^{-1}$ =2 and >100 trophonts $fish^{-1}$=3. One week after challenge, the survival rate of fish in each tank was determined. The experiment was done in duplicate.

Statistical analysis

The data was analyzed using SPSS for Windows Version 17.0. Parameter values were reported as mean±standard deviation. The differences in parameter values of each treatment were examined using one-way ANOVA with Scheffé post hoc tests (p<0.05).

Results

Anti-Ich antibody titer

Anti-Ich antibody was not detected in pre-immunized fish whereas it was detected in fish serum that was sampled from treatments 3-8 (Table 1). Non-immunized fish and fish immunized with BSA did not produce antibody against Ich (Table 1). Fish immunized with live trophonts revealed higher antibody titers against Ich than those immunized with sonicated trophonts. Fish immunized with live trophonts at a protein concentration 65 µg g^{-1} fish showed the highest antibody titer against Ich at a dilution 1:1,000 (p<0.05). These fish had significantly higher anti-Ich antibody titers than fish immunized with sonicated trophonts at the same protein concentration.

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

Moreover, they had significantly higher antibody titer against Ich than fish immunized with live or sonicated trophonts at 45 and 55 µg protein g^{-1} fish. Fish immunized with sonicated trophonts at 65 µg protein g^{-1} fish showed antibody titers at a 1:100 dilution similar to fish immunized with live trophonts at 45 and 55 µg protein g^{-1} fish. Fish immunized with sonicated trophonts at 45 and 55 µg protein g^{-1} fish showed antibody titers against Ich at a 1:10 dilution. However, no statistical difference was found between antibody titers from fish immunized with live trophonts at 45 and 55 µg protein g^{-1} fish including fish immunized with sonicated trophonts at 45 and 55 µg protein g^{-1} fish.

Table 1: ELISA absorbance at 405 nm of non-immunized and immunized Basa fish (*Pangasius bocourti*) serum 21 days post immunization.

Treatment	Dilution	A405nm
Pre-immunized	1:10	0.000±0.000[a]*
Non-immunized	1:10	0.032±0.005[a]
Immunized with BSA at 65 µg g^{-1} fish	1:10	0.031±0.006[a]
	1:10	0.302±0.032[b]
	$1:10^2$	0.147±0.016[c]
Immunized with live trophonts at 45 µg g^{-1} fish	$1:10^3$	0.043±0.008[a]
	$1:10^4$	0.009±0.006[a]
	$1:10^5$	0.000±0.000[a]
	1:10	0.334±0.077[b]
	$1:10^2$	0.196±0.045[c]
Immunized with live trophonts at 55 µg g^{-1} fish	$1:10^3$	0.077±0.019[a]
	$1:10^4$	0.017±0.005[a]
	$1:10^5$	0.000±0.000[a]
	1:10	0.369±0.060[b]
	$1:10^2$	0.201±0.051[b]
Immunized with live trophonts at 65 µg g^{-1} fish	$1:10^3$	0.114±0.015[c]
	$1:10^4$	0.033±0.002[a]
	$1:10^5$	0.000±0.000[a]
	1:10	0.183±0.013[c]
	$1:10^2$	0.049±0.010[a]
Immunized with sonicated trophonts at 45 µg g^{-1} fish	$1:10^3$	0.006±0.003[a]
	$1:10^4$	0.000±0.000[a]
	$1:10^5$	0.000±0.000[a]
	1:10	0.218±0.008[c]
	$1:10^2$	0.081±0.010[a]
Immunized with sonicated trophonts at 55 µg g^{-1} fish	$1:10^3$	0.024±0.004[a]
	$1:10^4$	0.005±0.004[a]
	$1:10^5$	0.000±0.000[a]
	1:10	0.256±0.038[b]
	$1:10^2$	0.173±0.021[c]
Immunized with sonicated trophonts at 65 µg g^{-1} fish	$1:10^3$	0.092±0.016[a]
	$1:10^4$	0.006±0.004[a]
	$1:10^5$	0.000±0.000[a]

*Different superscripts indicate statistically significant differences.

The results determined using western immunoblotting were similar to ELISA. Anti-Ich antibody was not detected in pre-immunized fish, non-immunized fish and fish immunized with BSA. Fish immunized with live or sonicated trophonts showed specific protein bands at approximately 66 kDa and below 14

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

kDa (Fig.1A and 1B). Fish immunized with live and sonicated trophonts at a concentration 65 µg protein g^{-1} fish indicated the highest titer, 1:100. Both of fish immunized with live and sonicated trophonts at a concentration 45 and 55 µg protein g^{-1} fish revealed a titer at a 1:10 dilution. However, protein band intensity of fish immunized with live trophonts was higher than that of fish immunized with sonicated trophonts.

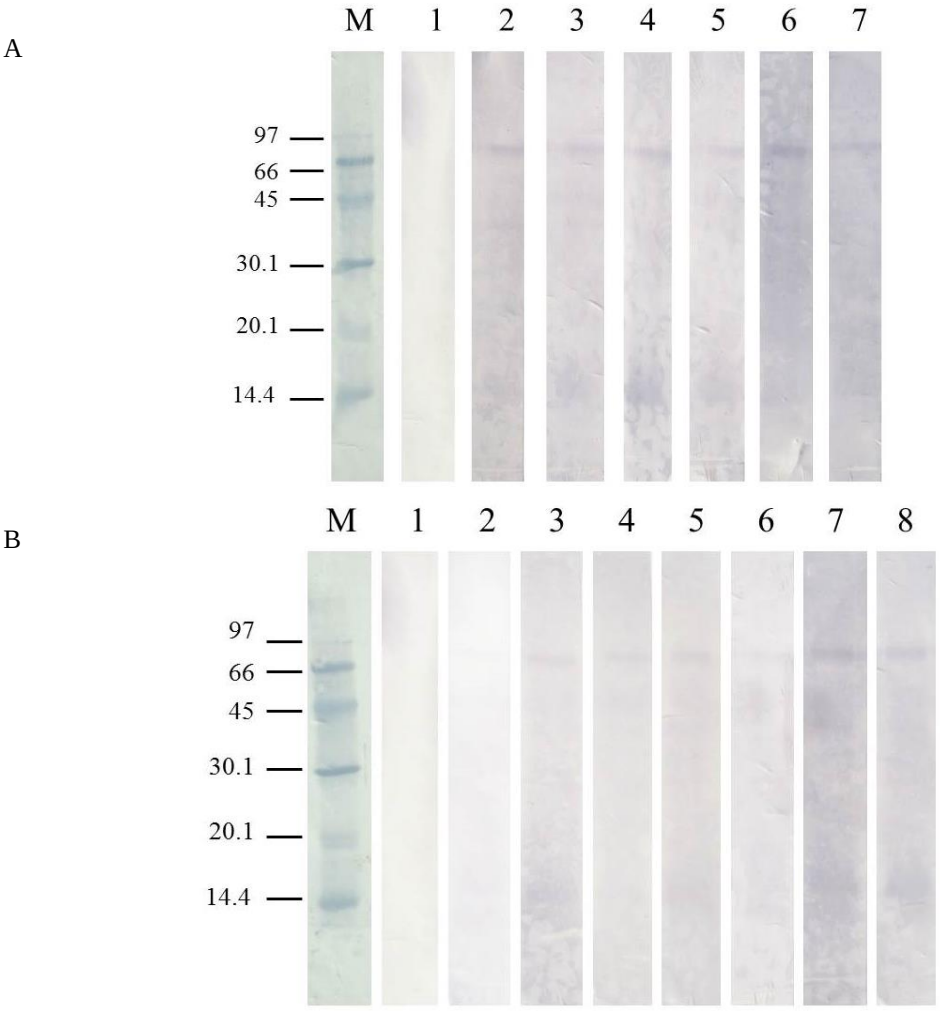

Figure 1: Western immunoblotting of immunized Basa fish (*Pangasius bocourti*) serum 21 days post immunization. (A) Fish immunized with live trophonts, lane M: marker, lane 1: pre-immunized fish at dilution 1:10, lanes 2 and 3: immunized with 45 µg protein g^{-1} fish at dilution 1:10 and 1:100, lanes 4 and 5: immunized with 55 µg protein g^{-1} fish at dilution 1:10 and 1:100 and lanes 6 and 7: immunized with 65 µg protein/g fish at dilution 1:10 and 1:100. (B) fish immunized with sonicated trophonts lane M: marker, lane 1: non-immunized serum at dilution 1:10, lane 2 immunized with BSA 65 µg protein g^{-1} fish, lanes 3 and 4: immunized with 45 µg protein g^{-1} fish at dilutions 1:10 and 1:100, lanes 5 and 6: immunized with 55 µg protein g^{-1} fish at dilution 1:10 and 1:100 and lanes 7 and 8: immunized with 65 µg protein g^{-1} fish at dilution 1:10 and 1:100.

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

Survival rate of fish with Ich

After challenge with Ich for two days, the number of parasites on the fish was estimated. The results showed that Non-immunized fish and fish immunized with BSA had similar higher parasites infection level per fish than fish immunized with live and sonicated trophonts. Fish immunized with live trophonts at a level of 65 µg g^{-1} fish had the lowest number of parasites per fish (Table 2). Non-immunized fish and fish immunized with BSA showed heavy infection, whereas fish immunized with live or sonicated trophonts revealed light infection. First dead fish in Non-immunized group was found three days post challenge while fish immunized with live trophonts was found dead four days post challenge. After challenge with Ich for one week, Non-immunized fish showed the highest mortality, 100% (Table 2). All non-immunized fish has been found dead four days after challenge. Fish immunized with BSA had a low survival rate of 3.33% (Table 2). The survival rate of fish immunized with live trophonts at a level of 65 µg g^{-1} fish had the highest and most statistically significant difference (Table 2) (p<0.05). There were no statistically significant differences between fish immunized with live and sonicated trophonts at lower protein concentrations. Two weeks post challenge, all survive fish that immunized with live and sonicated trophonts at a level of 45, 55 and 65 µg g^{-1} fish were still survive while all survive fish that immunized with BSA was mortal (data not shown).

Table 2: Parasites infection level per fish two days post challenge and survival rate one week post challenge of non-immunized and immunized Basa fish (*Pangasius bocourti*).

Treatment	parasites infection level	Survival rate (%)
Non-immunized	3	0.00 ± 0.00^{a}*
Immunized with BSA at 65 µg g^{-1} fish	3	3.33 ± 5.77^{a}
Immunized with live trophonts at 45 µg g^{-1} fish	2	33.33 ± 5.77^{b}
Immunized with live trophonts at 55 µg g^{-1} fish	2	40.00 ± 10.00^{b}
Immunized with live trophonts at 65 µg g^{-1} fish	1	63.33 ± 5.77^{c}
Immunized with sonicated trophonts at 45 µg g^{-1} fish	2	30.00 ± 10.00^{b}
Immunized with sonicated trophonts at 55 µg g^{-1} fish	1	33.33 ± 15.28^{b}
Immunized with sonicated trophonts at 65 µg g^{-1} fish	1	43.33 ± 5.77^{b}

*Different superscripts indicate the statistical significant differences.

Discussion

The anti-Ich antibody titers observed in this study were similar to other fish immunization using the same method of antigen injection (Dalgaard *et al*., 2002; Xu *et al*., 2004; Xu *et al*., 2008a; Xu *et al*., 2008b; Osman *et al*., 2009; Martins *et al*., 2011). Intraperitoneal injection was potential method for fish immunization (Burk *et al*., 1990). Non-immunized fish and fish immunized with BSA did not produce anti-Ich antibody according to a previous report (Xu and Klesius, 2013). Fish immunized with live trophonts showed higher antibody titers than that of fish immunized with sonicated trophonts. These results indicated that

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

live trophonts exhibited better antigenicity than sonicated trophonts. Therefore, fish immunized with live trophonts produced significantly higher antibody titers than fish immunized with inactivated trophonts. These results are in accordance with previous studies of immunization with inactivated antigens as formalin killed and freeze-thawed trophonts that reported lower antibody production than when immunized with live antigens (Xu *et al*., 2008a, b). However, live trophonts that used as antigen has the advantage when compare with live theronts that used as antigen in the previous study because antigen preparation is less complicated. Moreover, the antigen dose had an effect on antibody production. The antibody of fish immunized with 65 µg of live and sonicated trophont protein g^{-1} fish revealed significantly higher antibody than that of fish immunized with 45 and 55 µg trophont protein g^{-1} fish. However, no statistical difference of antibody titer was found in fish immunized with live and sonicated trophonts at concentrations of 45 and 55 µg g^{-1} fish. These results agree with a previous study of antibody production for immune protection, which found that a suitable concentration of antigen was required for immune protection (Xu *et al*., 2008b). In previous studies, fish immunoglobulin (Ig) as IgM and IgD were produced in immunized channel catfish, while IgM and IgT were detected in rainbow trout (Olsen *et al*., 2011; Heinecke and Buchman, 2013; Xu *et al*., 2016). From this, we hypothesized that IgM should be produced in Basa fish as a serum antibody. Moreover, another immune response against Ich, increased white blood cell counts, was also found in fish (Abdel-Hafez *et al*., 2014; Tancredo *et al*., 2015). We suggest a study of immunological parameter responses as a future study. Furthermore, multiple serotypes immobilization antigen of Ich are found and have been classified into five serotypes referred to serotypes A, B, C, D and E (Dickerson and Clark, 1996). There are the differences in virulence and protein molecular weight between serotypes. For example, serotypes A infected fish at a lower level than serotypes D but all infected fish with serotypes A died. In the previous study, cross-reactivity of anti-Ich antibody with different serotypes of immobilization antigen was found (Swennes *et al*., 2007). Probably, anti-Ich antibody from immunized fish with trophonts in this study may be reacting with multiple serotypes immobilization antigen.

The results obtained from western immunoblotting revealed that fish antibody could react with Ich antigen protein at approximately 66 kDa and below 14 kDa. In contrast with a previous report, a protein approximately 55 kDa acting as a surface immobilization antigen of Ich was not found in this research (Wang and Dickerson, 2002; Maki and Dickerson, 2003). Moreover, proteins at approximately 34, 39, 45 and 46 kDa acting as antigens against Ich membrane proteins play an important role in fish immunization (Wang *et al*., 2002). These proteins were not found in

[DOR: 20.1001.1.15622916.2018.17.4.9.3]

the current study. We theorize that the proteins at approximately 66 kDa and below 14 kDa should be important Ich antigens in Basa fish. We also hypothesize that the proteins at approximately 66 kDa may promote a surface immobilization antigen against Ich. However, the proteins below 14 kDa should be the interaction of antibody with the reduced parts of proteins as break down products from the immobilization antigens (i-antigens; ranging in size from 40 to 70 kDa) (Lin *et al*., 1996; Swennes *et al*., 2006).

In this study, non-immunized fish and fish immunized with BSA showed heavy infection and died after few days. This was similar to results of a study of infected Nile Tilapia that exhibited a mean of five days to death (Xu *et al*., 2014). The immunized fish could produce protective antibody against Ich. They had high anti-Ich antibody titers and exhibited light infections with high survival rates. Fish immunized with high level of antigen had the lowest number of parasites infection per fish that exhibit the quantitative exact result of protection. The results showed that the immune response and immune protection were proportional to anti-Ich antibody. However, the fish were not fully protected and this may be due to an in-sufficient level of anti-Ich antibodies or other factors, as was observed in other studies (Xu *et al*., 2008a; Osman *et al*., 2009). We proposed that Ich successfully penetrate the epithelium of immune fish but are forced to exit due to cutaneous antibody attachment.

In conclusion, Basa fish immunized with live and sonicated trophonts by IP injection showed an immune response against Ich. Basa fish immunized with live trophonts produced higher anti-Ich antibody titers and had greater survival rates than those of fish immunized with sonicated trophonts. Therefore, immunization of Basa fish with live and sonicated Ich by IP injection enhance protection against this parasite.

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