

# A modified enzyme-linked immunosorbent assay for detection of cutaneous antibody against *Ichthyophthirius multifiliis*

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## Abstract

Fish surviving *Ichthyophthirius multifiliis* (Ich) infection develop an immune response against this parasite. Enzyme-linked immunosorbent assays (ELISA) currently used to determine the cutaneous anti-Ich antibody response lack sensitivity. This study modified and optimized an ELISA assay for detection of low levels of cutaneous antibody against Ich in channel catfish and in water. The modified ELISA used tetramethylbenzidine (TMB) as substrate for horseradish peroxidase (HRP). The modified ELISA required less theront coating protein, shorter coating time for the plate, and a lower concentration of conjugate. The optical density using modified ELISA was approximately 4 fold higher than the ELISA using o-phenylenediamine (OPD) when measuring anti-Ich antibody in the culture fluid from immune skin. The modified ELISA was able to detect the antibody in water samples collected from tanks with Ich immune fish. The ELISA by using OPD failed to detect the antibody even though the water samples were concentrated 40 fold. The results of this study showed that the modified ELISA using TMB as a HRP substrate was a sensitive, quantitative and time saving assay to measure low concentrations of anti-Ich cutaneous antibody excreted from skin of channel catfish immune to Ich.

## Introduction

*Ichthyophthirius multifiliis* (Ich) is a parasitic ciliate that infects most species of fresh water fish world wide. The disease is highly infective and spreads rapidly from fish to fish, especially fish in a high population density. The parasite damages gills and skin of fish, leads to high mortality, and causes heavy economic loss in aquaculture (Jessop, 1995; Traxler et al., 1998).

The fish that survived a sublethal *Ichthyophthirius* infection develop an immune response against Ich re-infection (Becker and Allison, 1964; Hines and Spira, 1974; Dickerson and Clark, 1998). Anti-Ich antibody

was commonly measured with an immobilization assay or enzyme-linked immunosorbent assay (ELISA) (Hines and Spira, 1974; Clark et al., 1988; Lin et al., 1996; Sigh and Buchmann, 2001; Xu et al., 2002; Wang and Dickerson, 2002). Immobilization assays are direct, rapid, and simple assays. Some factors, however, may influence the measurement of anti-Ich antibody, including components from fish, diluents used in the assay and quality of theronts (Clark et al., 1987; Sigh and Buchmann, 2001; Xu and Klesius, 2002). The ELISA is a more specific, reliable, and sensitive method for measuring anti-Ich antibody concentration (Sigh and Buchmann, 2001).

Cutaneous antibody was found in mucus or skin culture fluid of fish immune to Ich. However, the concentration of cutaneous antibody is usually low. The ELISA currently used to determine the cutaneous anti-Ich antibody provided low optical density reading (Xu and Klesius, 2002; Wang and Dickerson, 2002). There is a need for a sensitive ELISA which can detect low level of cutaneous antibody. The purpose of this study is to modify and optimize an ELISA assay for detection of low levels of cutaneous antibody against Ich in channel catfish and in water.

## Materials and methods

### *Fish and parasite*

Fingerling channel catfish were used as host fish and reared at the United States Department of Agriculture, Aquatic Animal Health Research Laboratory, Auburn, Alabama. Fish were kept in tanks supplied with flowing dechlorinated water. *Ichthyophthirius multifiliis* was isolated from an infected fish obtained from a local pet shop. It was routinely maintained by exposing catfish to infected fish in 50-l glass aquaria. Trophonts were collected and theronts were harvested as described previously (Xu et al., 2002).

### *Immune catfish and skin culture fluid*

Skin culture fluid from Ich immune catfish ( $28.6 \pm 1.4$  cm in length and  $191.8 \pm 13.5$  g in weight) was obtained as described by Xu et al. (2002). Briefly, the infected fish were treated with formalin to prevent reinfection after the fish showed a heavy load of Ich trophonts. The formalin treatment was repeated daily for 5-7 days until no trophonts were seen on the surface of infected fish. The treated fish were then kept and fed in the

aquaria with flowing water for about 3 weeks. To test the immunity, four immunized fish that had survived from Ich infection and four control fish were exposed to 30,000 theronts/fish overnight and kept in tanks for 5 days. Control fish developed heavy infection whereas immunized fish had no trophonts on the body surface, confirming protective immunity. Skin from seven Ich immune and seven susceptible fish was collected from the lateral body wall with sterile instruments and cultured in the supplementing Medium 199 (Sigma Chemical Co., St.Louis, Missouri) with heat inactivated fetal bovine serum at 5%, 100 IU of penicillin/ml, and 100  $\mu$ g of streptomycin/ml (Sigma). The culture fluid was harvested after 24 h incubation and centrifuged at  $228 \times g$  for 10 min. The supernatant was collected and stored at  $-80^\circ\text{C}$ .

### *Water samples containing cutaneous anti-Ich antibody*

Four 60-l tanks were set up to collect water samples containing cutaneous anti-Ich antibody excreted from fish immune to Ich. Two tanks were used for Ich immune fish and two were used for non-immune control fish. Two immune or non-immune fish were added to each tank. The water in each tank was drained and replaced with flowing water for one hour at the rate of two liters per minute to remove mucus excreted from fish after handling. Following, water was turned off and water volume was adjusted to 20 l per tank. After two hours, two 50-ml water samples were collected from each tank. Water samples were centrifuged at  $910 \times g$  for 10 min, 5, 10, and 20-ml supernatant from each sample was concentrated to 0.5 ml with Millipore 10 KDD cutoff centrifugal filter device (Millipore

Corporation, Bedford, Massachusetts) at 2060 x g for 30 min. The anti-Ich antibody in the water samples was determined in triplicate using ELISA.

#### *Modified and optimized ELISA*

The ELISA described by Xu and Klesius (2002) was modified and optimized to measure cutaneous antibody in skin culture fluid and in water. The following factors were determined for specific steps of the assay: 1) concentration of coating protein and coating time, 2) suitable substrate, 3) conjugate concentration and 4) incubation time of substrates. The coating proteins of Ich theronts at the concentration of 2.5, 5, and 10  $\mu\text{g}/\text{ml}$  were used to coat the ELISA plate for 1 h or 2 h at 22 °C. The optimal combination of coating protein and coating time should give a high OD reading for anti-Ich positive samples and low background for the negative controls. Tetramethylbenzidine (TMB) and o-phenylenediamine (OPD) are two commonly used substrates for horseradish peroxidase (HRP). To find out the suitable substrate for HRP and its optimum conjugate concentration, TMB and OPD were tested using dilution 1:2000 and 1:5000 of goat anti-mouse IgG conjugated with HRP (GAM-HRP)(Pierce, Rockford, Illinois). To determine the effect of substrate incubation time, the substrates were incubated at 22 °C for 15, 30, and 60 min, respectively. The test assays were repeated 5 times for each optimal factors. The other steps of ELISA are similar to that described previously. Briefly, each well of 96-well microtiter plates was coated with 100- $\mu\text{l}$  coating buffer (pH 9.6) containing sonicated Ich theront Ag at 22 °C. The wells of the plate were emptied and washed five times with PBS (pH 7.2) contain-

ing 0.05% Tween 20 (PBS-T) using automatic plate washer (Tecan US Inc., Durham, North Carolina). Nonspecific protein binding was blocked by adding 100- $\mu\text{l}$  1% BSA in PBS-T to each well for 15 min. A two fold series dilution was made for each culture fluid sample with PBS-T and incubated for one hour. After washing as described above, 100- $\mu\text{l}$  murine anti-catfish Ig heavy chain Monoclonal antibody at the dilution of 1:100 was added to each well and incubated for 30 min. One hundred  $\mu\text{l}$  of GAM-HRP was added to each well of the plate and incubated for 30 min. Then 100  $\mu\text{l}$  of substrate was added to each well of the plate. The TMB substrate is a 1-Step™ Ultra TMB (Pierce), which contains 3,3',5,5'-tetramethylbenzidine plus dilute hydrogen peroxide in a complete substrate solution for ELISA. The reactions for OPD and TMB were stopped with 50- $\mu\text{l}$  3 M  $\text{H}_2\text{SO}_4$  and 2 M  $\text{H}_2\text{SO}_4$ , respectively, after incubation. The plate was read at 450 nm using an ELISA-reader (Dynatech, Chantilly, Virginia).

#### *Precision of ELISA and limit of detection*

The skin culture fluids from six channel catfish immune to Ich were pooled and two samples from the pooled fluid were assayed with the ELISA. The assay was repeated 7 times within 2 months. The variability of repetitive measurements for each of the substrates was calculated as coefficient of variation (CV). The skin culture fluids from six catfish naive to Ich was pooled as negative control. Two-fold dilutions of culture fluids were made with PBS. The lowest detectable concentration of antibody in the culture fluid was determined for two substrates. Optical density in immune fluid two times greater than the OD readings in control fluid were considered as quantified

Coating protein ( $\mu\text{g/ml}$ )	Modified ELISA			ELISA using OPD		
	10	5	2.5	10	5	2.5
Immune fluid						
1:2	0.789	0.620	0.616	0.182	0.180	0.155*
1:16	0.306	0.257	0.244	0.143	0.136	0.129
Control fluid						
1:2	0.257	0.115	0.109	0.099	0.095	0.089
1:16	0.113	0.107	0.105	0.092	0.093	0.090

Table 1. Effect of the coating protein concentration ( $\mu\text{g/ml}$ ) on ELISA optical density for the anti-Ich antibody in the culture fluid from channel catfish immune to *Ichthyophthirius*, assayed with a modified ELISA by using tetramethylbenzidine (TMB) as substrate. The culture fluid from catfish naive to *Ichthyophthirius* was used as control. The ELISA using substrate o-phenylenediamine (OPD) is listed here for comparison. The plates were coated with the coating protein for one hour. The dilution of goat anti-mouse IgG conjugated with HRP (GAM-HRP) and substrate incubation time used in the assay were 1:5000 and 15 min, respectively. \*Each value is the mean with N = 5.

positive. The ELISA titer was the highest dilution in which immune sample showed quantified positive.

#### Statistics

The OD readings by ELISA for anti-Ich antibody in the immune skin culture fluid were analyzed with Duncan's multiple range test (SAS Institute 1989). Probabilities of 0.05 or less were considered statistically significant.

### Results and discussions

The optimal coating protein concentrations were from 2.5 to 5  $\mu\text{g/ml}$  for the modified ELISA when the plates were coated at 22 °C for one hour. These concentrations produced a high OD reading for the positive sample and low background for the negative control (Table 1). With the increment of coating protein concentration to 10  $\mu\text{g/ml}$ , it increased both OD reading of the positive sample and unwanted background of the negative control. The modified ELISA through the use of TMB substrate required less coating protein com-

pared to the ELISA using OPD. A coating protein at the concentration of 5 to 10  $\mu\text{g/ml}$  was needed in order to get a good OD reading when using substrate OPD. When the plate coating time was extended to 2 hours with 5  $\mu\text{g/ml}$  coating protein, the mean OD readings of the modified ELISA were 0.741 and 0.358 (N=5) for positive and negative samples at the 1:2 dilution, respectively. The prolonging of coating time greatly increased

Conjugate dilution	Modified ELISA		ELISA using OPD	
	1:2000	1:5000	1:2000	1:5000
Immune fluid				
1:2	0.785	0.661	0.214	0.160*
1:16	0.273	0.264	0.125	0.117
Control fluid				
1:2	0.258	0.112	0.097	0.095
1:16	0.117	0.104	0.094	0.093

Table 2. Effect of the dilution of goat anti-mouse IgG conjugated with HRP on ELISA optical density for the anti-Ich antibody in the culture fluid from channel catfish immune to *Ichthyophthirius*. The plates were coated with the coating protein at the concentration of 5  $\mu\text{g/ml}$  for one hour and the substrate incubation time was 15 min. \*Each value is the mean with N = 5.

Incubation time (min)	Modified ELISA			ELISA using OPD		
	15	30	60	15	30	60
Immune fluid						
1:2	0.687	0.734	0.725	0.180	0.273	0.322*
1:16	0.372	0.543	0.540	0.118	0.174	0.187
Control fluid						
1:2	0.116	0.349	0.302	0.095	0.144	0.199
1:16	0.108	0.147	0.130	0.059	0.071	0.095

Table 3. Effect of the substrate incubation time on ELISA optical density for the anti-Ich antibody in the culture fluid from channel catfish immune to *Ichthyophthirius*. The modified ELISA used tetramethylbenzidine (TMB) as substrate and the other ELISA used o-phenylenediamine (OPD) for comparison. The plates were coated with the coating protein at the concentration of 5  $\mu\text{g}/\text{ml}$  for one hour and the assay used GAM-HRP conjugate at the dilution of 1:5000. \*Each value is the mean with N = 5.

the background. The 1:5000 dilution of GAM-HRP conjugate provided a high OD reading for the positive sample with an acceptable background for the negative control when assayed with the modified ELISA (Table 2). The ELISA using OPD required a higher concentration of GAM-HRP conjugate (1:2000 dilution) than the modified ELISA. A 15-min incubation time with TMB substrate was adequate for the modified ELISA. The prolonged incubation with TMB increased the background of the negative control (Table 3). Based on the results for specific factor determination, the modified ELISA could be conducted optimally when plates were coated with the coating protein from 2.5 to 5  $\mu\text{g}/\text{ml}$  at 22 °C for one hour, using GAM-HRP conjugate at 1:5000 dilution and incubating with TMB substrate for 15 min. Compared to the modified ELISA, the ELISA using OPD provided low OD reading at the same assay conditions. The ELISA using OPD can be improved by 1) increasing the concentration of coating protein to 10  $\mu\text{g}/\text{ml}$  or higher, 2) ex-

tending plate coating time to 2 hours and 3) using higher concentration of conjugate. However, such improvement not only requires more costly reagents and longer assay time, but also the sensitivity of the OPD based assay is less than that of the modified ELISA.

The OD readings using TMB were approximately 4 fold higher than using OPD when measuring anti-Ich antibody in the immune skin culture fluid at the dilution from 1:2 to 1:16 (Table 4). The ELISA titer were 1:64 and 1:8, assayed with the modified ELISA and the OPD based ELISA, respectively. Water samples collected from the tanks with Ich immune fish contained a low concentration of cutaneous antibody. The modified ELISA was able to detect the antibody after samples were concentrated 20 fold. The ELISA by using OPD failed to detect the antibody even though the water samples were concentrated 40 fold (Table 5). Our result confirmed the previous finding that HRP based ELISA assay through the use of TMB substrate was sensitive in detection of antibody at low concentrations due to

Dilution	Modified ELISA		ELISA using OPD	
	Immune fluid	Control fluid	Immune fluid	Control fluid
1:2	0.724 ± 0.112 <sup>aA</sup>	0.103 ± 0.145 <sup>aB</sup>	0.195 ± 0.088 <sup>aC</sup>	0.063 ± 0.099 <sup>aB</sup>
1:4	0.587 ± 0.102 <sup>bA</sup>	0.096 ± 0.161 <sup>aB</sup>	0.146 ± 0.108 <sup>bC</sup>	0.059 ± 0.140 <sup>aB</sup>
1:8	0.440 ± 0.115 <sup>cA</sup>	0.087 ± 0.078 <sup>aB</sup>	0.107 ± 0.107 <sup>cB</sup>	0.056 ± 0.090 <sup>aB</sup>
1:16	0.319 ± 0.095 <sup>cA</sup>	0.073 ± 0.064 <sup>aB</sup>	0.084 ± 0.130 <sup>cB</sup>	0.055 ± 0.076 <sup>aB</sup>
1:32	0.134 ± 0.150 <sup>dA</sup>	0.065 ± 0.127 <sup>aB</sup>	0.075 ± 0.118 <sup>cB</sup>	0.054 ± 0.143 <sup>aB</sup>
1:64	0.124 ± 0.143 <sup>dA</sup>	0.061 ± 0.100 <sup>aB</sup>	0.061 ± 0.119 <sup>cB</sup>	0.051 ± 0.092 <sup>aB</sup>
Mean CV (%)	12.0	11.3	11.2	10.7
ELISA titre	1:64		1:8	

Table 4 Comparison of optical density measured by modified ELISA and ELISA using OPD for anti-Ich cutaneous antibody in the culture fluid of excised skin from channel catfish immune to *Ichthyophthirius* (Ich). The culture fluid of skin culture from catfish naive to Ich was used as a control. Each value is the mean ± coefficient of variation with N = 14. Within a column, means followed by the same lower case letter are not significantly different ( $P > 0.05$ ). Within a row, means followed by the same upper case letter are not significantly different.

the rapid reaction rate of TMB (Goka and Farthing, 1987; Volpe et al., 1998).

The modified ELISA provided acceptable reproducibility and the average CV was 12.0% for detecting anti-Ich cutaneous antibody in the immune skin culture fluid (Table 4). The precision for the ELISA assays was a between-run precision, which measured the ability of the assay to reproduce a result on the same sample from assay to assay and from day to day. The assay was repeated 7 times within 2

months. The between-run precision is a reliable indicator of reproducibility because samples are analyzed not only from run to run but also from day to day. In summary, this study modified and optimized an ELISA assay. The ELISA by using TMB as a HRP substrate was a sensitive, quantitative and time saving assay to measure low concentrations of anti-Ich cutaneous antibody excreted from skin of channel catfish immune to Ich.

Concentration (fold)	Modified ELISA			ELISA using OPD		
	immune	control	E-titer	immune	control	E-titer
40	0.161	0.064	+	0.072	0.059*	-
20	0.147	0.063	+	0.064	0.055	-
10	0.094	0.062	-	0.057	0.054	-

Table 5 Comparison of optical density measured by modified ELISA and ELISA using OPD for anti-Ich cutaneous antibody in water with channel catfish immune to *Ichthyophthirius* (Ich). The water with catfish naive to Ich was used as a control. Water samples were concentrated with centrifugal filter device before ELISA assay. The ELISA titer (E-titer) here was the lowest concentration in which immune samples showed OD readings two times greater than the OD readings in control samples. \*Each value is the mean with N = 12.

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