Vol. 77: 143–147, 2007
doi: 10.3354/dao01828

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 14

Passive immunization of channel catfish *Ictalurus punctatus* with anti-*Flavobacterium columnare* sera

R. A. Shelby*, C. A. Shoemaker, P. H. Klesius

Aquatic Animal Health Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 990 Wire Road, Auburn, Alabama 36832, USA

ABSTRACT: Passive immunization of channel catfish *Ictalurus punctatus* (Rafinesque) was conducted to determine if anti-*Flavobacterium columnare* serum was protective when injected intraperitoneally (i.p.) into channel catfish. The anti-*F. columnare* serum was produced by actively immunizing (i.p. injection) channel catfish with sonicated whole cells or purified lipopolysaccharide (LPS) of *F. columnare* in Freund's adjuvant. Serum anti-*F. columnare* activity was verified by Western blotting and ELISA of serum. Normal serum and sterile culture broth were used as controls. Complement was inactivated in all sera by heating. After 48 h, passively immunized fish were challenged with virulent *F. columnare* by i.p. injection. A group of unchallenged fish served as controls. The immune response of catfish to the antigenic fractions was different when examined by Western blotting. Antibody produced with whole-cell antigen responded to a broad range of molecular weight components, while LPS antigens were restricted to a pair of bands near 20 kDa. Control fish injected with culture medium experienced 100% mortality 14 d post-challenge. Relative percent survival was 77 and 73 for catfish passively immunized with anti-LPS and anti-whole-cell serum, respectively. Results suggest that antibodies in the serum are involved in the protective immune response against columnaris disease in channel catfish.

KEY WORDS: *Flavobacterium columnare* · Passive immunization

INTRODUCTION

Flavobacterium columnare (formerly *Flexibacter columnaris*) is the causative agent of columnaris disease, and is the second most important pathogen in the cultured channel catfish industry in the United States (Wagner et al. 2002). *F. columnare* exists in fresh and brackish water throughout the world (Plumb 1999) and affects >36 fish species, including eels, salmonids, centrarchids, carps, minnows, perchids and aquarium fishes (Plumb 1999). Our previous research to develop immunoassays for this disease (Shoemaker et al. 2003, 2005b) showed that catfish anti-*F. columnare* immunoglobulin levels increased in convalescent or vaccinated fish.

Experimental passive immunization has been attempted with varying results in fish, including attempts by interspecific passive transfer of immunity. Akhlaghi et al. (1996) successfully used sheep and rabbit antibody against streptococcal infection in rainbow trout *Oncorhynchus mykiss*, but mortalities were not reduced after passive immunization with rainbow trout antibodies against *Streptococcus* sp. Akhlaghi (1999) later used sheep, rabbit and trout anti-*Vibrio anguillarum* antibodies and was able to reduce mortality in rainbow trout due to vibriosis. Passive immunization has also been attempted in other fish species. LaFrentz et al. (2003) demonstrated protection from coldwater disease caused by *Flavobacterium psychrophilum* in rainbow trout using serum from convales-

*Email: rshelby@ars.usda.gov

cent trout, but was unable to produce the same effect using goat anti-*F. psychrophilum* serum. Lin et al. (1996) induced immunity against *Ichthyophthirius multifilis* (Ich) in channel catfish using murine monoclonal antibodies. Even within the same species, passive immunization has not always proven to be successful. Klesius & Sealey (1995) were unable to reduce mortality of catfish to *Edwardsiella ictaluri* using sera of fish that had survived infection, even though antibody titers were significantly elevated in passively immunized animals.

In our earlier experiments with anti-*Streptococcus iniae* antibody from Nile tialpia (Shelby et al. 2002), we demonstrated the role of immunoglobulin and protection against this pathogen by passive immunization. From these previous studies on passive immunization with whole sera from immune and normal fish, it was shown that the role of antibody in immunity against fish pathogens can be elucidated. The objective of the present study was to determine if passive immunization of channel catfish with catfish anti-*Flavobacterium columnare* antiserum could provide protection against columnaris disease. We included different antigen preparations in order to identify the potential antigenic determinants of *F. columnare.*

MATERIALS AND METHODS

Bacterium. *Flavobacterium columnare* isolate ALG-00-530 was originally isolated from a diseased channel catfish *Ictalurus punctatus* from a commercial pond in Greensboro, Alabama, USA, and maintained in pure culture at the USDA-ARS Aquatic Animal Health Research Laboratory, Auburn, Alabama. Isolate ALG-00-530 was grown for 24 h in modified Shieh broth at 28°C with shaking at 100 rpm (Shoemaker et al. 2005a). This isolate was grown under similar conditions and used for antibody production as well as subsequent disease challenge.

Fish. Channel catfish (NWAC 103 strain) reared at our laboratory from fry to juveniles were used as experimental animals. Prior to initiation of the study, fish were housed for 14 d in 55 l glass aquaria using flow-through water (0.5 l min^{-1}), and a photoperiod was maintained on a 12 h light:12 h dark schedule. Daily water temperature averaged 25 ± 1°C, and mean daily dissolved oxygen was 5.5 ± 0.7 mg l^{-1}. Fish were fed daily (3 to 4% body weight) with commercial trout chow (No. 5104, Purina Mills). Prior to initiation of this study, fish were tested by ELISA (Shoemaker et al. 2003, 2005b) and determined to be negative for anti-*Flavobacterium columnare* antibody. The ELISA test was conducted on plastic microtiter plates (Nunc maxisorp, Nalge Nunc International) coated with 100 µl of a solution of 4 µg ml^{-1} of *F. columnare* antigen in 0.05 M sodium carbonate buffer (pH 9.6) for 1 h at 25°C. Antigen for ELISA coating was from the same batch used for the whole-cell sonicate described in detail below. Plates were then rinsed 5 times with PBST (phosphate-buffered saline, pH 7.4) + 0.05% Tween-20. Then, 100 µl of fish sera (diluted 1:800 in PBST) was placed in the antigen-coated ELISA wells, and incubated at room temperature for 30 min. Plates were rinsed as above, and mouse anti-catfish immunoglobulin monoclonal Antibody E8 (Klesius 1990, Shoemaker et al. 2003) was added at a concentration of 400 ng ml^{-1} in PBST. After 30 min of incubation at 25°C, plates were washed with PBST. Sheep anti-mouse IgG peroxidase conjugate (A-5906, Sigma) was diluted 1:5000 in PBST; 100 µl was added to each well and incubated for 15 min at 25°C. Plates were rinsed with PBST and 50 µl of 3,3′,5,5′-tetramethylbenzidine (TMB). The peroxidase reaction was stopped after 15 min with 50 µl of 3 M H_2SO_4 and read spectrophotometrically at 450 nm.

Columnaris antigen production. Different *Flavobacterium columnare* antigens were used to immunize channel catfish to produce antibody for passive transfer experiments. Briefly, *F. columnare* ALG-00-530 cells were grown as described above. The broth culture was centrifuged (4000 × *g* for 15 min), and the cells were washed in sterile PBS. Whole-cell sonicates were produced by sonicating cells 30 s on ice and adjusting to 1 mg ml^{-1} of protein as determined by the BCA protein assay (No. 23225, Pierce Biotechnology). The lipopolysaccharide (LPS) fraction of *F. columnare* ALG-00-530 was also used to immunize channel catfish. ALG-00-530 was grown in 300 ml broth as before, and the LPS was purified by the hot phenol extraction method (Maclean et al. 2001). Antigens and antibodies were examined by Western blotting. Electrophoresis was on 4 to 20% gradient acrylamide gels (NH-21-420, Life Theraputics). Antigen samples were denatured by heating to 100°C for 5 min in sample buffer containing sodium dodecyl sulfate and mercaptoethanol (BG-165, Life Theraputics). Gels were electrophoretically transferred to polydivinylidene fluoride (PVDF) membranes (Biotrace, Gellman Laboratory) at 30 V constant voltage, at 5°C, overnight. Membranes were probed with catfish antiserum diluted 1:1000, followed by monoclonal Antibody E8 at a concentration of 2 µg ml^{-1} for the catfish immunoglobulin (Shoemaker et al. 2003). The appropriate peroxidase conjugate (A9044 anti-mouse, Sigma-Aldrich) was used at 1:1000 and visualized with 4-chloronapthol.

Donor antibody production. The sonicated whole-cell preparation was mixed 1:1 with Freund's complete adjuvant (FCA), and 100 µl was injected i.p. into 10 channel catfish (~15 g). Immunization was repeated

14 d later with Freund's incomplete adjuvant (FIA), and blood was sampled from the caudal vasculature after an additional 14 d. Anti-whole-cell catfish serum (WCC) was isolated by centrifugation at 500 × *g* after clotting at room temperature for 1 h and stored frozen for testing and passive immunization. All sera were heated at 56°C for 1 h in a water bath to denature complement (Drevets & Campbell 1991). Sera were tested for anti-*Flavobacterium columnare* antibody by ELISA (Shoemaker et al. 2003), prior to passive immunization. The LPS fraction was lyophilized and dissolved in sterile PBS (1 mg ml^{-1} w/v), mixed 1:1 with FCA, and 100 µl was injected into 5 channel catfish (~15 g). Booster immunization was with FIA and anti-LPS catfish serum (LPSC) purification and conducted as described above for the anti-sonicated cell serum preparation. An additional group of 10 fish that were not immunized provided control serum (NC).

Passive immunization. Twelve channel catfish, mean weight 4.4 g (range 3.8 to 5.6 g), were placed in 3 replicate aquaria (36 fish per treatment; 180 fish total) as previously described, and allowed to adjust for 1 wk prior to passive immunization. Then, 100 µl of each of the 3 anti-*Flavobacterium columnare* antisera (WCC, LPSC, NC) were injected i.p. into each fish. Three control tanks of fish were similarly injected with sterile, modified Shieh broth, which served as a sham injection control to determine any mortality resulting from handling and injection and to serve as non-immunized challenged controls. Three additional tanks of fish were used as non-injected, non-challenged controls. Forty-eight hours after passive immunization with anti-*F. columnare* antisera or injection with control serum and/or sterile culture media, the fish were bled to confirm the presence or absence of anti-*F. columnare* antibody by ELISA.

Disease challenge. Virulent *Flavobacterium columnare* ALG-00-530 was grown in broth culture for 24 h, as described previously, and adjusted to an optical density of 0.7 at 540 nm with sterile PBS. Samples were spiral plated on modified Shieh agar to determine colony-forming units (CFU) per milliliter. All fish, with the exception of non-challenged controls, were injected i.p. with 8.79×10^6 CFU $fish^{-1}$ 48 h after passive immunization (Klesius et al. 1999). Fish were fed twice daily to satiation and monitored for disease signs for 14 d. Mortalities were recorded, and dead fish were removed twice daily.

Statistical analysis. Statistical differences were analyzed by SAS (SAS Institute). Significant differences in mean ELISA values and mean cumulative mortality between treatments were determined by 1-way analysis of variance (ANOVA) using the least significant difference test to determine significant differences at $p < 0.05$.

RESULTS AND DISCUSSION

Immune sera

Immunization of channel catfish *Ictalurus punctatus* with whole-cell sonicates and with phenol-extracted LPS produced a measurable humoral immune response compared to naive fish (Table 1), with cellular antigens producing a higher measurable ELISA response then LPS antigens. Western blotting of whole cells and LPS fractions with these sera revealed distinctly different immune responses (Fig. 1). Blotting whole-cell antigen with WCC antiserum revealed a

Table 1. ELISA absorbance (450 nm) of catfish *Ictalurus punctatus* sera used for passive immunization. Each value represents the mean of 10 individual sera diluted 1:3200. Values with different letters are significantly different ($p < 0.05$). LPS: lipopolysaccharide

Antigen source	Mean absorbance	SD
Cells	0.639a	0.194
LPS	0.478b	0.179
Control	0.069c	0.020

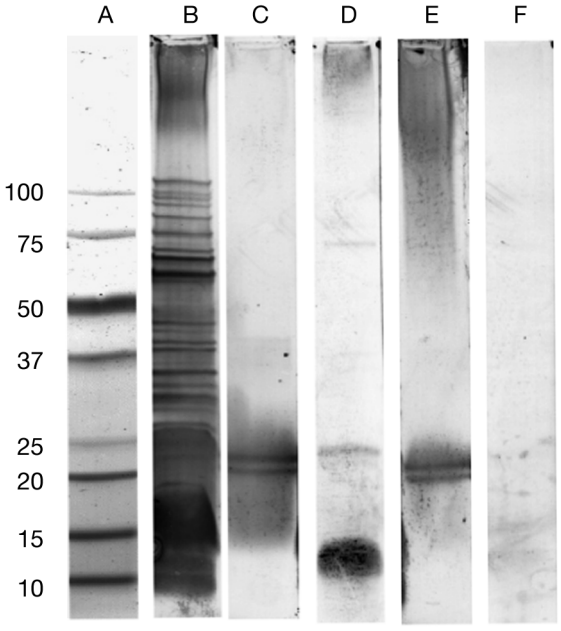

Fig. 1. Western blotting analysis of cellular (Lanes B, D, F) and lipopolysaccharide (LPS) (Lanes C, E) antigens used to produce antiserum for passive immunization. Lane A: prestained molecular weight standards; Lanes B, C: Western blot with channel catfish *Ictalurus punctatus* anti-*Flavobacterium columnare* cell antiserum (WCC); Lanes D, E: Western blot with channel catfish anti-*F. columnare* LPS antiserum (LPSC); Lane F: Western blot with normal channel catfish serum (NC)

broad size range of putative protein antigens in the whole-cell preparation. The LPS fraction probed with WCC antiserum appears to show only a pair of antigenic bands at about 20 kDa. We believe that this represents the core polysaccharide. There does not appear to be a 'ladder-like' profile in the antibody response to the O-antigen as found in *Flavobacterium psychrophilum* (Maclean et al. 2001) and in Western blots with other gram-negative fish pathogens (Newton & Triche 1993).

Recently, LaFrentz et al. (2007) demonstrated that the previously described high-molecular size o-polysaccharide of *Flavobacterium psychrophilum* is actually extracellular polysaccharide glycocalyx. Using LPS antiserum, the whole-cell antigen showed a prominent low-molecular weight band around 15 kDa, which was lacking in the LPS preparation. This component is obviously lost in the hot phenol extraction procedure. Also evident are the pair of bands at 20 kDa, and polydisperse components (glycocalyx) in the higher molecular weight range. There was no antibody response detected in naive (NC) serum by Western blotting.

Passive immunization

The passively immunized catfish showed measurable levels of anti-*Flavobacterium columnare* antibody 48 h after administration of the immune sera (Fig. 2). Catfish WCC and LPSC sera had ELISA optical density (OD) higher than fish administered normal serum, broth, or no serum. Levels of antigen-specific antibody at 48 h were generally reflective of the antibody measured in the immune sera.

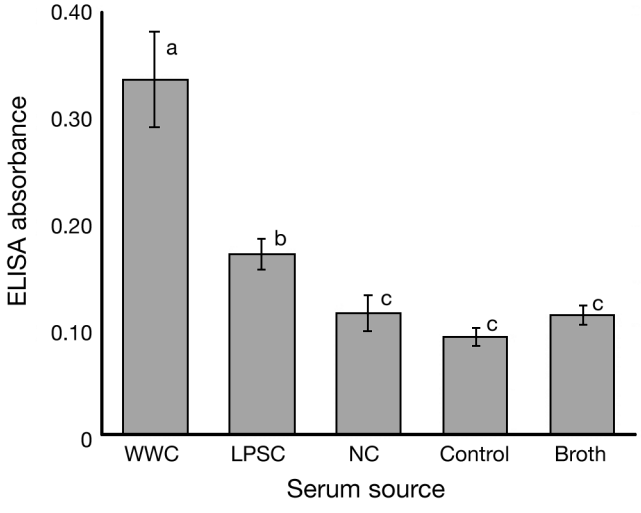

Fig. 2. Specific anti-*Flavobacterium columnare* antibody level (optical density) measured by ELISA in sera from channel catfish *Ictalurus punctatus* 48 h after passive immunization with heat-inactivated anti-*F. columnare* whole-cell catfish (WCC), lipopolysaccharide catfish (LPSC), or normal catfish (NC) antisera. Sterile broth and non-immunized controls were also included. SD indicated by bars. Values with different letters are significantly different ($p < 0.05$)

Disease challenge

Passive immunization with catfish anti-LPS or anti-WC serum resulted in significantly reduced ($p < 0.002$) mortalities compared to broth-injected or normal-serum-injected controls (Table 2). Relative percent survival (RPS) was 73 and 77 for catfish passively immunized with anti-LPS and anti-WC serum, respectively, following challenge with the homologous *Flavobacterium columnare* isolate. Catfish administered normal catfish serum had an RPS of 40. This value is below that estimated to afford protection (i.e. 60; Amend 1981). We also did not detect anti-*F. columnare* antibody in these fish 48 h following injection of the serum (i.e. prior to challenge). LaFrentz et al. (2003) demonstrated passive transfer of anti-*F. psychrophilum* serum in rainbow trout *Oncorhynchus mykiss*, which resulted in protection against challenge with a virulent isolate of *F. psychrophilum*. Other studies have similarly demonstrated by passive immunization the importance of antibody in protection against other fish pathogens (Akhlaghi 1999, Shelby et al. 2002).

When the LPS fraction of *Flavobacterium columnare* was tested by Western blotting, catfish showed an antigenic response to a single low-molecular weight fraction typical of the core polysaccharide antigen. The administration of complement-free immune serum from heat-inactivated channel catfish was protective against the homologous challenge by *F. columnare*, suggesting that antibody is important in columnaris disease protection.

Table 2. *Ictalurus punctatus*. Cumulative percentage mortality and relative percent survival following challenge with *Flavobacterium columnare* of channel catfish passively immunized with sterile broth, normal catfish serum (NC), anti-*F. columnare* whole-cell catfish antiserum (WCC), anti-*F. columnare* lipopolysaccharide catfish antiserum (LPSC), and non-challenged controls. Values with different letters are significantly different ($p < 0.05$)

Treatment	Cumulative percent mortality (±SE)
Shieh broth	100 (±0.0)a
Normal whole serum	60 (±5.8)b
Anti-LPSC	27 (±14.5)c
Anti-WCC	23 (±12.0)c
Controls (non-challenged)	0 (±0.0)

Acknowledgements. The authors thank P. Mumma and N. Qin and the staff of the AAHRU aquatic facility for technological assistance and animal care. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply endorsement by the United States Department of Agriculture.

LITERATURE CITED

Akhlaghi M (1999) Passive immunization of fish against vibriosis, comparison of intraperitoneal, oral and immersion routes. Aquaculture 180:191–205

Akhlaghi M, Munday BL, Whittington RJ (1996) Comparison of passive and active immunization of fish against streptococcosis (enterococcosis). J Fish Dis 19:251–258

Amend DF (1981) Potency testing of fish vaccines. Dev Biol Stand 49:447–454

Drevets DA, Campbell PA (1991) Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. Infect Immun 59:2645–2652

Klesius PH (1990) Effect of size and temperature on the quantity of immunoglobulin in channel catfish, *Ictalurus punctatus.* Vet Immunol Immunopathol 24:187–195

Klesius PH, Sealey WM (1995) Characteristics of serum antibody in enteric septicemia of catfish. J Aquat Anim Health 7:205–210

Klesius PH, Lim C, Shoemaker C (1999) Effect of innate resistance and antibody response to *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus.* Bull Eur Assoc Fish Pathol 19:156–158

LaFrentz BR, LaPatra SE, Jones GR, Cain KD (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. J Fish Dis 26:377–384

LaFrentz BR, Lindstrom NM, LaPatra SE, Call DR, Cain KD (2007) Electrophoretic and western blot analyses of the lipopolysaccharide and glycocalyx of *Flavobacterium psychrophilum.* Fish Shellfish Immunol 23:770–780

Lin TL, Clark TG, Dickerson H (1996) Passive immunization of channel catfish (*Ictalurus punctatus*) against the ciliated protozoan parasite (*Ichthyophthirius multifiliis*) by use of murine monoclonal antibodies. Infect Immun 64: 4085–4090

Maclean LL, Vinogradov E, Crump EM, Perry MB, Kay WW (2001) The structure of the lipopolysaccharide o-antigen produced by *Flavobacterium psychrophilum.* Eur J Biochem 268:2710–2716

Newton J, Triche PL (1993) Electrophoretic and immunochemical characterization of lipopolysaccharide of *Edwardsiella ictaluri* of channel catfish. J Aquat Anim Health 5:246–253

Plumb JA (1999) Health maintenance and microbial diseases of cultured fishes. Iowa State University Press, Ames, IA

Shelby RA, Shoemaker CA, Klesius PH (2002) Passive immunization of tilapia (*Oreochromis niloticus*) with anti-*Streptococcus iniae* whole serum. J Fish Dis 25:1–6

Shoemaker CA, Shelby RA, Klesius PH (2003) Development of an indirect ELISA to detect humoral response to *Flavobacterium columnare* infection of channel catfish (*Ictalurus punctatus*). J Appl Aquacult 14:43–52

Shoemaker CA, Arias CR, Klesius PH, Welker TL (2005a) Technique for identifying *Flavobacterium columnare* using whole cell fatty acid profiles. J Aquat Anim Health 18:267–274

Shoemaker CA, Xu DH, Shelby RA, Klesius PH (2005b) Detection of cutaneous antibodies against *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque). Aquac Res 36:813–818

Wagner BA, Wise DJ, Khoo LH, Terhune JS (2002) The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health 14:263–272

Editorial responsibility: Donald Evans, Athens, Georgia, USA

Submitted: July 11, 2006; Accepted: May 24, 2007
Proofs received from author(s): August 17, 2007