# CHAPTER 2

## Efficacy of a Recombinant Vaccine Expressing a Mammalian *Mycobacterium* sp. Antigen Against Acute Mycobacteriosis in Fish

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A Recombinant Vaccine Expressing a Mammalian *Mycobacterium* sp. Antigen is Immunostimulatory but Not Protective in Striped Bass
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**Abstract**

A recombinant vaccine was constructed for piscine mycobacteriosis utilizing a *Brucella abortus* strain RB51 vector expressing a mammalian *Mycobacterium* sp. 85A antigen. Juvenile striped bass were vaccinated with the resulting construct at doses equivalent to $10^6$, $10^7$, $10^8$, $10^9$, and $10^{10}$ colony-forming units/fish. Blood and tissue samples from these fish demonstrated significant specific humoral and cell-mediated immune responses towards the 85A antigen in a dose-dependant manner. However, survival studies determined that vaccinated fish failed to demonstrate cross-protective responses against acute disease after live *Mycobacterium marinum* challenge 70 days post-vaccination.
*Keywords*: *Mycobacterium*sp.; Fish; Vaccination; Immunity

**Introduction**

Piscine mycobacteriosis is an economically important bacterial disease of virtually all species of freshwater and marine fish (Plumb, 1994; Chinabut, 1999). This chronic progressive disease, commonly caused by *Mycobacterium marinum*, *M. fortuitum*, or *M. chelonae*, usually leads to systemic infections and often death (Austin and Austin, 1993; Smith, 1997). Perhaps most importantly, piscine mycobacteriosis has been reported to cause massive mortalities among fish grown in intensive aquaculture systems (Bruno et al., 1998; Diamant et al., 2000). Because of the difficult management of fish mycobacteriosis and due to its zoonotic potential, development of an effective vaccine against these *Mycobacterium* spp. is vital.

*Mycobacterium* spp., whether infectious to fish or mammals, are facultative intracellular bacterial pathogens. They cause severe, progressive, systemic disease, in part because they are able to evade the immune system by residing and replicating inside of host macrophages. Because of the intracellular location, effective immune responses against *Mycobacterium* spp. require cell-mediated responses as well as humoral responses. While piscine cell-mediated immune responses have not been fully characterized, they are known to include functional similarities to mammalian cell-mediated responses. For example, fish possess MHC and T cell receptors and also have

specific helper T lymphocyte activity and T cell-mediated cytotoxicity (Nakanishi et al., 2002). Unfortunately, assays to further test piscine cell-mediated immunity and cytokine activity are not widely available.

A number of vaccines and compounds have been studied that induce mammalian cell-mediated immune responses. Researchers have found that the RB51 strain of *Brucella abortus* could be used as a vector to deliver protective antigens from other intracellular pathogens for which cell-mediated immune responses are important. Vemulapalli et al. (2000) determined that mice injected with a recombinant RB51 strain containing a protective antigen from *M. bovis* developed strong cell-mediated immune responses against both *B. abortus* and *M. bovis*. Furthermore, their findings also suggested that cell-mediated immunity produced by one intracellular pathogen can synergistically upregulate the cell-mediated immunity for another intercellular pathogen (Vemulapalli et al., 2002).

It is known that the various species of mycobacteria have antigens with common epitopes: Silva and Lowrie (1994) found that conserved proteins of one *Mycobacterium* spp. can provide cross-protection against other *Mycobacterium* spp. One such cross-protective protein is the antigen 85A (Ag85A), which is a fibronectin-binding protein conserved across all tested *Mycobacterium* spp. (Ohara et al., 1997). Conserved proteins like the Ag85A can have high sequence similarity among the Ag85A of different species; for example, the Ag85A of *M. tuberculosis* and *M. bovis* BCG have been determined to have 100% similarity (Tanghe et al., 2001). Given the complete similarity between the Ag85A of these two *Mycobacterium* spp. and given that the aquatic pathogen *M. marinum* is closely related to *M. tuberculosis* (Barker et al., 1997), it is likely that a cross-reactive relationship would exist between *M. marinum* and *M. bovis* BCG. Therefore, it was postulated that the M. bovis BCG Ag85A may induce cross-protective immunity.

A recombinant RB51 strain expressing the *M. bovis* BCG Ag85A was readily available in our laboratories following mammalian vaccine research (Vemulapalli et al, 2002), and the study presented here was undertaken to evaluate if this strain could be used to vaccinate fish against *Mycobacterium marinum*. First, the entire mature protein sequence of *M. marinum* Ag85A was determined and compared to the known *M. bovis* BCG Ag85A sequence to assess the degree of similarity and predict possible cross-

protection. Then we assessed the ability of fish to mount immune responses to the *M. bovis* BCG 85A by ELISA and lymphoproliferative assay. Finally, fish were challenged with *M. marinum* to examine the cross-protective effects of the Ag85A vaccine.

## Materials and methods

### *Fish*

Because of their proven susceptibility to *Mycobacterium* spp. (Wolf and Smith, 1999), striped bass (*Morone saxatilis*) were used as the model to assess the efficacy of the vaccine. Young-of-year striped bass were obtained from a local state fish hatchery and housed at the Aquatic Medicine Laboratory of the VMRCVM, in Blacksburg, Virginia. Fish weighed approximately 20-40 grams and were maintained in a 2272 liter recirculation aquaculture system with appropriate biological filtration and aeration. Water temperature was maintained between 23-26°C, and the fluorescent light photoperiod consisted of 16 hours light/8 hours dark. Fish were fed daily a commercial pelleted feed (Rangen, Inc., Buhl, ID) at 3-5% body weight, and water quality parameters (ammonia, nitrite, nitrate, pH, and dissolved oxygen) were monitored daily using a water analysis kit (HACH Co., Loveland, CO). Fish were acclimated for two weeks before initiation of experiments.

### *Amplification of the Mycobacterium marinum antigen 85A gene (fbpA)*

The gene for the Ag85A (*fbpA*) was amplified by PCR from the *M. marinum* genomic DNA. *Mycobacterium marinum* (ATCC no. 927) was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) for 9 days at 28°C (Wolf and Smith, 1999). Genomic DNA was isolated from *M. marinum* based on the methods of Whipple et al. (1987). A primer pair consisting of a forward primer (5' AAC CGG ATG CGC CTA AAT GAA TGA GG 3') and a reverse primer (5' CGC CGC CGT TAA TCG CTG TTC TGT TA 3') were selected such that the amplified fragment would contain the ribosomal binding site, open reading frame, and stop codon of the *fbpA*. Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used for PCR. Amplification was performed using an Omni Gene thermocycler (Hybaid Limited, Franklin, MA) at 95°C

for 5 minutes, followed by 30 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 60°C, and 1 minute of extension at 72°C. Amplification products were analyzed on 1.5% agarose gels in 1x TBE buffer, and products with the predicted product size were submitted for DNA sequencing.

*Sequencing of the Mycobacterium marinum fbpA*

Nucleotide sequencing of the *M. marinum* Ag85A gene was determined at the Core Laboratory Facility, Virginia Bioinformatics Institute, VPI&SU, using an Applied Biosystems Inc. model 377 automated DNA sequencer (ABI, Foster City, CA). Cycle sequencing reactions were performed using the pCMV-85A plasmid template, T7 sequencing primer (Invitrogen, San Diego, CA), and ABI Big Dye Terminator ready reaction kit (ABI). Reactions were purified using the Millipore Multiscreen HV plates (Millipore, Bedford, MA) and dried and resuspended as per manufacturer's protocols for loading on the automated sequencer. LaserGene sequence analysis software (DNASTAR Inc., Madison, WI) was used for nucleotide sequence analysis. The determined *M. marinum* Ag85A gene sequence was assigned GenBank accession number AY225215.

*Vaccine construction*

The vaccine was constructed using methods described by Vemulapalli et al. (2000; 2002). Briefly, the *fbpA* gene from *M. bovis* BCG was amplified by PCR and subcloned into a pBBgroE vector, creating pBBgroE85A. The *Brucella abortus groE* promoter and *M. bovis* BCG *fbpA* gene were excised and subcloned into pBBSOD to generate pBBSOD/85A, and this plasmid was then electroporated into *B. abortus* strain RB51. The resulting recombinant strain was designated RB51SOD/85A, and the expression of *fbpA* was driven by the *B. abortus groE* heat shock promoter. The presence of the Ag85A in strain RB51SOD/85A was confirmed by Western blot analysis (Laemmli, 1970). Because *B. abortus* is a zoonotic pathogen, it was rendered non-replicative with radiation treatment. After treatment with a dose of 300 krad, *B. abortus* strain RB51 is non-infectious but remains able to induce protective immunity (Garcia et al., 1987; Schurig and Vemulapalli, unpublished data); indeed, inactivated and killed *B. abortus* has been shown to promote cell-mediated immune responses (Zaitseva et al.,

1995; Agranovich et al., 1999). Thus, the vaccine in this study was rendered completely non-infectious without changing its immunomodulatory properties.

*Vaccination*

On Days 0, 14, and 35, fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO) and vaccinated i.p. using a 1 ml syringe with a 27 guage needle. The i.p. route was chosen for the study because it previously stimulated protective immunity with recombinant RB51 vaccines (Vemulapalli et al., 2000; Vemulapalli et al., 2002). This route is also generally considered the most effective way to induce long-term protection in fish (Palm et al., 1998; Vinitnantharat et al., 1999) and is an accepted and established vaccination method in the aquaculture industry (Gudding et al., 1999). Furthermore, a pilot study using water immersion as the route of vaccination failed to elicit significant immune responses.

Fish were distributed into six groups, with each group receiving a different vaccine accordingly: one group was sham-innoculated with sterile saline, while the other five groups received the test vaccine at a dose equivalent to $10^6$, $10^7$, $10^8$, $10^9$ and $10^{10}$colony-forming units/fish, respectively. All doses were contained in 0.20 ml of volume. A subsample of fish from each group were then anesthetized (for bleeding) or euthanized (for harvesting of lymphocytes) with MS-222 on Days 0, 14, 35, and 49.

*Production of recombinant Mycobacterium sp. Ag85A fusion protein*

Recombinant Ag85A was generated for ELISA and lymphoproliferative assay using the methods of Vemulapalli et al. (2002). Briefly, PCR was utilized to amplify the open reading frame of the *M. bovis* BCG *fbp*A using the forward primer (5' TTT TCC CGG CCG GGC TTG 3') and reverse primer (5' TCT GTT CGG AGC TAG GCG 3'). The resulting fragment was cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and then subcloned into in the *Eco*RI and *Xba*I sites of the pMalC2 vector (New England Biolabs, Beverly, MA). The Ag85A was expressed in *E. coli* DH5 (GIBCO-BRL, Carlsbad, CA) as a fusion with maltose-binding protein, and the resulting fusion protein was purified by affinity chromatography (New England Biolabs). After removal of lipopolysaccharide using polymyxin B beads (Affi-Prep Polymyxin support; BioRad

Laboratories, Hercules, CA) and dialysis with PBS, the protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). Aliquots of the purified recombinant protein were stored at –70°C until use.

*Enzyme-linked immunosorbent assay (ELISA)*

The humoral antibody responses of the fish were evaluated for the presence of specific immunoglobulin against Ag85A using an indirect ELISA (Vemulapalli et al., 2000). Recombinant Ag85A was diluted to a 1 µg/50 µl concentration in bicarbonate coating buffer (pH 9.6) and the solution used to coat the polystyrene plates (Corning Glass, Corning, NY) with 50 µl/well. The plates were incubated at 4°C overnight, washed four times with wash buffer (Tris-buffered saline [TBS] at pH 7.4, 0.05% Tween 20), and blocked with 2% BSA in TBS for 2 hours at 21°C. The blocking solution was then removed and diluted fish serum samples (1:100 dilution in blocking solution) were added to individual triplicate wells at 100 µl/well. A diluent only sample (negative control) was tested in the same manner. The plates were incubated for 4 hours at 21°C and washed four times with wash buffer. The secondary antibody solution, the protein A-peroxidase conjugate (Sigma Chemical Co.), was added at 100 µl/well at a 1:500 dilution. After 1 hour incubation at room temperature, the plates were washed four times and 100µl of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) added to each well. After 20 minutes incubation at room temperature, 100 µl of stop solution (0.185 M sulfuric acid) was added. The absorbance at 450 nm was then recorded with a microplate reader (Molecular Devices, Menlo Park, CA) and the sample well abosrbance compared against negative control well absorbance.

*Lymphoproliferative assay*

Anterior kidney lymphocytes from fish in each control and vaccination group were cultured in triplicate in 96-well plates (Corning) in the presence of 5 µg Ag85A, 2.5 µg ConA (positive control), or no additives (negative control) (Ahmed et al., 1994; Vemulapalli et al., 2002). The cells were cultured at 28°C for 24 hours with RPMI 1640 medium supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum,

penicillin, and streptomycin. After 24 hours, 20 µl Alamar Blue (Trek Diagnostic, Westlake, OH) was added to each well. The cells were then incubated for 48 hours, and then absorbance was measured at 570 nm and 600 nm with a kinetic microplate reader (Molecular Devices).

The Alamar Blue assay was developed as a non-radioactive lymphocyte proliferation assay that indirectly measures cell proliferation (Ahmed et al., 1994, Gogal et al., 1999). The dye is added in an oxidized form (blue color) and is reduced (red color) with cell proliferation. The level of proliferation is indicated by the difference between the specific absorbance of the oxidized form (570 nm) and the reduced form (600 nm). The specific absorbance of the unstimulated cells (negative control) is subtracted from the specific absorbance of the cells to yield a delta-specific absorbance.

*Live bacterial challenge*

On Day 70, twenty fish from each vaccinated group as well as 20 sham-vaccinated control fish were challenged with live *M. marinum* (ATCC no. 927). Previous study by Wolf and Smith (1999) determined the $LD_{50}$ of this *M. marinum* isolate for striped bass at 8 days to be approximately $1.6 \times 10^8$ CFU/g body weight of fish IM. The isolate was grown in Middlebrook 7H9 broth at 25°C for 9 days. Bacteria were washed and suspended in PBS and the concentration of bacterial cells estimated by absorption spectrophotometry and confirmed by plate counts. The fish were injected with the solution i.m. in the dorsal musculature just ventral to the dorsal fin at the concentration stated previously. During the study, any morbid or dead fish were removed immediately. All surviving fish were euthanized 30 days post-challenge, and swab samples from the posterior kidney were plated on Middlebrook 7H9 agar and incubated at 25°C for 9 days. This was used to document *M. marinum* infections in the fish.

*Statistical analysis*

The ELISA data was subjected to a mixed model repeated measures analysis of variance, and a Bonferroni correction was performed to compare the various experimental dose groups within each sampling day. The lymphocyte proliferation and live bacterial challenge data were subjected to one-way analysis of variance, while only

the proliferative responses were subjected to Bonferroni correction. The data was reported as means with SEM determined from pooled means. An overall significance level of $p < 0.05$ was accepted.

**Results**

*Cloning and nucleotide sequence analysis of the M. marinum Ag85A gene*

After PCR amplification using the described primers, a fragment of approximately 1,100 bp was visualized on agarose gels and sequencing of this product revealed a gene with a 1,014 bp open reading frame. When compared to the published Ag85A sequences of *M. tuberculosis* and *M. bovis* BCG (Borremans et al., 1989; Huygen et al., 1994), the conserved mature protein encoded by this gene started with the same FSRPGL amino acid sequence. Furthermore, the entire amino acid sequence of the mature *M. marinum* protein exhibited a similarity of 84% to that of *M. tuberculosis* and *M. bovis* BCG Ag85A (Fig. 1). Therefore, the gene isolated in this study was the Ag85A homologue of *M. marinum*.

*Specific antibody production*

Serum samples were analyzed by ELISA for the presence of specific antibody responses against the Ag85A. The $10^9$ and $10^{10}$ vaccine concentration groups exhibited the most robust specific antibody responses to the Ag85A, peaking on Days 14 and 35, respectively (Fig. 2). By far, the most significant antibody production was exhibited by the $10^{10}$ group, with considerable induction seen after only the first vaccination. The other vaccination groups developed moderate specific responses that were usually not significantly higher than those of the saline vaccination group.

*Lymphocyte stimulation*

Anterior kidney lymphocytes were cultured *in vitro* in the presence of Ag85A or ConA. After Ag85A stimulation, the saline vaccination group failed to generate antigen-specific lymphoproliferation. However, both the $10^9$ and $10^{10}$ groups demonstrated significant lymphocyte responses beyond that of the saline vaccination group (Fig. 3).

These responses were approximately five- and eleven-fold higher, respectively, than that of the saline control group. Furthermore, the $10^{10}$ group exhibited significantly higher proliferation when compared to the $10^{9}$ group. The remaining RB51SOD/85A-vaccinated groups failed to generate significant responses.

A similar pattern was demonstrated with ConA stimulation. While all of the vaccination groups exhibited proliferative responses with mitogen stimulation, the $10^{9}$ and $10^{10}$ groups still demonstrated significant lymphocyte responses beyond that of the saline vaccination group. This may signify a higher percentage of activated T cells in the RB51SOD/85A vaccine groups. In addition, the $10^{10}$ group was again significantly higher than the $10^{9}$ group. The remaining vaccination groups failed to generate significant responses.

*Live bacterial challenge*

Vaccinated fish were exposed to live *M. marinum* at a high dose designed to cause the onset of mortality at approximately 8 days post-challenge. No significant differences ($p = 0.5654$) were seen among the groups in days to death, and the mean days to death for all fish in all groups were 13.57 +/- 2.05 days. Furthermore, there were no significant differences in onset of mortality or time to reach 100% mortalities (Fig. 4).

**Discussion**

The potential for a vaccine against piscine mycobacteriosis was suggested by Bartos and Sommer (1981). They were able to elicit cell-mediated immune responses in rainbow trout, *Salmo gairdneri*, from intraperitoneal immunization with *M. salmoniphilum* mixed with Freund's complete adjuvant. Later, Chen et al. (1996) vaccinated rainbow trout with i.p. injections of extracellular products from various aquatic *Mycobacterium* spp. mixed with Freund's incomplete adjuvant. This resulted in enhanced levels of reactive oxygen species, phagocytes, lysozyme, and antibodies produced against the mycobacterial organism. However, neither study reported whether these immune responses were sufficient for protection against disease.

Multiple vaccines have been developed against mammalian mycobacteriosis using the Ag85A. This antigen is utilized, because it is one of the major secreted fibronectin binding proteins for all mycobacterial species, including *M. bovis* BCG and *M. marinum* (Ohara et al., 1997; Stinear et al., 2000). Previous studies have established that the Ag85A induces immune responses and effective protection against infections of several mycobacterial species (Denis et al., 1998; Velaz-Faircloth et al., 1999; Naito et al., 2000). Furthermore, because of the high homology between the Ag85A of various mycobacterial species, immune responses to the Ag85A of one species can be cross-protective against infections by other mycobacterial species. This makes the Ag85A a prime candidate for cross-protection vaccine studies such as the one presented here.

Here, we report the sequencing of the Ag85A gene of *M. marinum*. This gene exhibited high similarity to the Ag85A homologue of *M. bovis* BCG and thus had the potential to induce cross-protection after vaccination. Striped bass vaccinated with the RB51 vaccine expressing the Ag85A exhibited antibody and lymphocyte responses that were specific and dose-dependant. This suggests that a sufficient concentration of protein is vital for the induction of a detectable immune response. The data also showed a trend towards decreased or only slightly higher antibody concentrations at Day 35 compared to Day 14 or at Day 49 compared to Day 35, despite the administration of booster vaccinations of Days 14 and 35. This contradicts the normal booster effect and secondary antibody response pattern typically seen in fish and mammals (Ellis, 1988). However, strong cell-mediated immune responses are known to subdue the humoral response; thus it is possible that because the RB51 strain and/or the 85A antigen induced strong cell-mediated responses, antibody formation was partially suppressed (Vemulapalli et al., 2000). This would indicate that the antibody response may be transient and the vaccine could confer long-term protection by cell-mediated immune responses. It is also possible that the antibody concentration on Day 35 was decreasing after the peak of the secondary antibody response. If this was indeed the case, serum from Day 28 might have given a better indication of the peak.

However, the vaccine failed to produce protective effects following live bacterial challenge with *M. marinum*. Mortality data showed little delay in onset of mortality or length of survival. All groups received a dose of live *M. marinum* that causes onset of

mortalities in approximately 8 days. Generally, all groups in this study reached 50% mortalities within 8 to 13 days and 100% mortalities in 21 to 26 days. Furthermore, no relationship between delayed mortality and vaccination dose or degree of elicited immune responses could be found. This indicates that the immune responses generated were not cross-protective, not sufficiently significant, or both. Despite the similarity between the mature proteins of *M. bovis* BCG and *M. marinum*, there still may be significant differences in the epitopes that affect immunoregulation and thus cross-protection.

Tanghe et al. (2001) found that cross-protective effects were elicited against *M. ulcerans* after vaccination of mice with a *M. bovis* BCG Ag85A vaccine. However, similar effects were not seen in our study, despite the fact that the mature Ag85A protein of *M. ulcerans* and *M. marinum* showed 98% sequence identity (data not shown) and that one of the putative immunodominant peptide regions of the gene was almost completely identical (Huygen et al., 1994; Tanghe et al., 2001). Therefore, we should consider the possibility that the immune responses were not sufficient enough to protect against acute disease, though protective effects may be conferred against a lower challenge dose or chronic disease.

The investigation presented here evaluated the use of a mammalian vaccine in fish. This is a valid area of study, because the use of mammalian vaccines in fish could help expand the vaccine repertoire available to fish-rearing facilities. Biomedical companies may be hesitant to develop fish vaccines that serve a limited market, thus hindering the growth of aquaculture. Yet, the prospect of marketing a specific vaccine for both mammalian and aquatic species is more financially appealing and would be widely beneficial. However, the findings in this study may preclude the use of mammalian *Mycobacterium* spp. Ag85A as protective antigens for aquatic mycobacteriosis vaccines and indicate aquatic *Mycobacterium* spp. Ag85A may be more appropriate antigens.

## Objectives Met

A previously described vaccine (RB51SOD/85A) was determined to stimulate humoral and cellular immune responses that were significantly upregulated at higher vaccine doses. However, the vaccine failed to provide detectable cross-protection against high-dose *M. marinum* challenge.

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FSRPGLPVEYLQVPSAAMGRNIKVQFQSGGANSPALYLLDGMRAQDDFSGRDIN
. . . . . .P S . . . D . . . . . . . . . . . . . . . . . . . . . L . . . . . . . . W. . .

TPAFEWYYRSG ISVAMPVGGQSSFYSDWYNPACGKAGCTTYKWETFLTSELPQ
. . . . . . . DQ . . L. .V . . . . . . . . . . . . . . . Q. . . . . . . .Q . . . . . . . . . . . . . . G

YL SANKGVKPTGSGVVGLSMAGSSALI LAAYHPDQFVYSGS L SALLDPSQGM
W. Q . . RH . . . . . A . . . . . . . A. . . . T . . I . . . Q . . . .A . AM .G . . . . . .A .

GPSLIGLAMGDAGGYKASDMWGPKDDPAWARNDPMLQVGKLVANNTRI WVY
. . T . . . . . . . . . . . . . . . . . . . . . . . E . . . . Q . . . . L ..N . . . . I . . . . . V . . .

CGNGKPSDLGGDNLPAKFLEGFVRTSNMKFQAAYNAAGGHNAVWNFDDNGTH
. . . . . . . . . . . N . . . . . . . . . . . . . . . . . I . . . D . . . . G . . . . G . F D . P . S . . .

SWEYWGAQLNAMKPDLQHTLGATPNTGDT-QGA.
. . . . . . . . . . . . . . . . . .RA . . . . . . . . PAP . . . .

Key to amino acid sequences: Top – *M. marinum*
Bottom – *M. bovis* and *M. tuberculosis*

Fig. 1. Percent identity of *M. marinum* Ag85A amino acid sequence (248/295 = 84%) to that of *M. bovis* and *M. tuberculosis*. Amino acid differences are represented by letters.

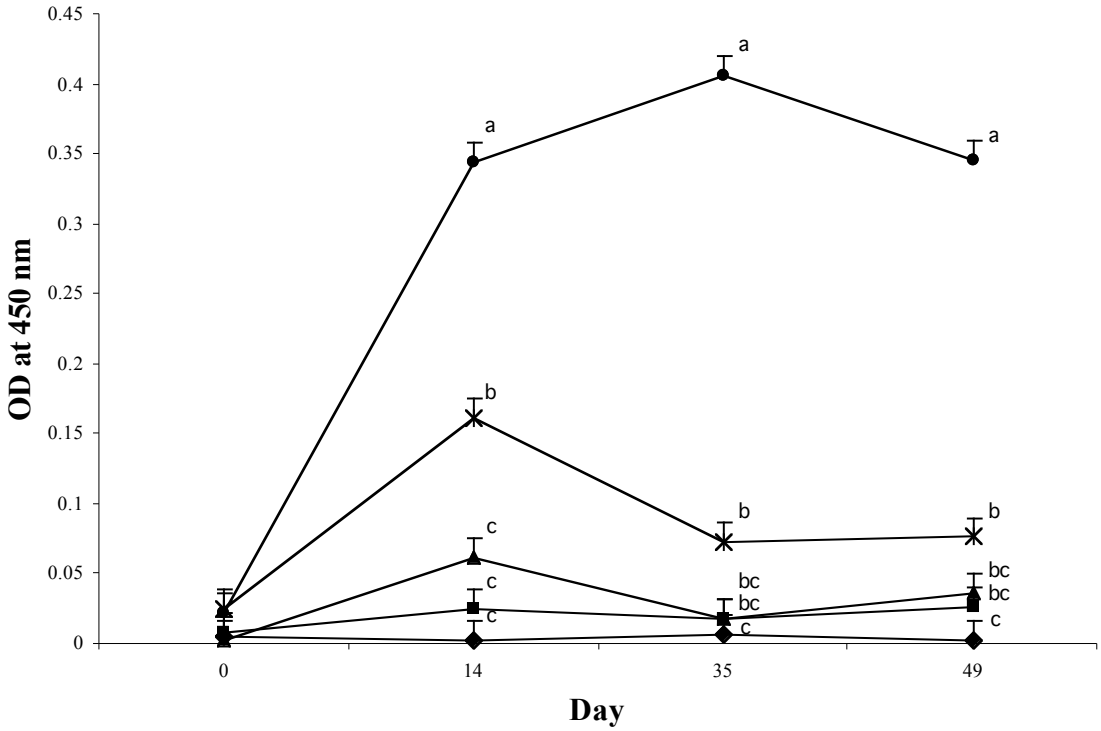


Fig. 2. ELISA detection of Ag85A-specific antibodies in serum of striped bass (*Morone saxatilis*) vaccinated with the RB51SOD/85A. Sera were collected on Days 0, 14, 35, and 49 post-vaccination, diluted 1:100, and assayed for the presence of antibodies to *M. bovis* Ag85A using absorption spectrophotometry. Vaccination groups included were groups injected with $10^{10}$ (closed circle), $10^{9}$ (asterik), $10^{8}$ (triangle), and $10^{7}$ (square) colony-forming units of RB51SOD/85A. The Saline control (diamond) group was also included. The $10^{6}$ vaccination group exhibited responses similar to that of the saline group. Results are shown as the mean + SEM of $OD_{450}$ of the developed color. Significant differences ($p < 0.05$) among vaccination groups within each sampling day were determined according to Bonferroni corrected data and are indicated on the graph by different alphanumeric letters.

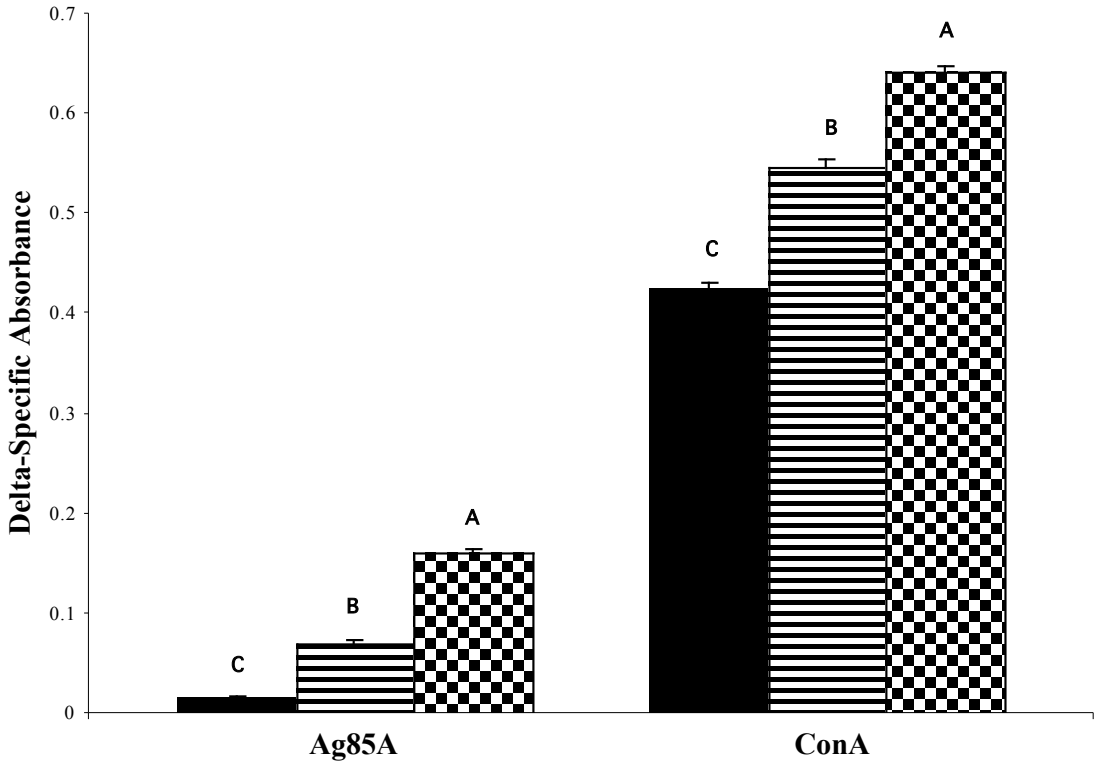


Fig. 3. Proliferation of anterior kidney lymphocytes from striped bass (*Morone saxatilis*) vaccinated with RB51SOD/85A after *in vitro* stimulation with saline, Ag85A, or ConA. Cells were harvested on Day 56, cultured for 3 days, and lymphocyte proliferation determined by Alamar blue assay. Vaccination groups included were groups injected with $10^{10}$ (checkered), $10^{9}$ (horizontal stripes) colony-forming units of RB51SOD/85A. The Saline control (solid black) group was also included. The remaining vaccination groups ($10^{6}$,$10^{7}$, and $10^{8}$ groups) exhibited responses similar to that of the saline control group. Results are shown as the mean + SEM of the delta-specific absorbance of the developed color. Significant differences ($p < 0.05$) among vaccination groups were determined according to Bonferroni corrected data and are indicated on the graph by different alphanumeric letters.

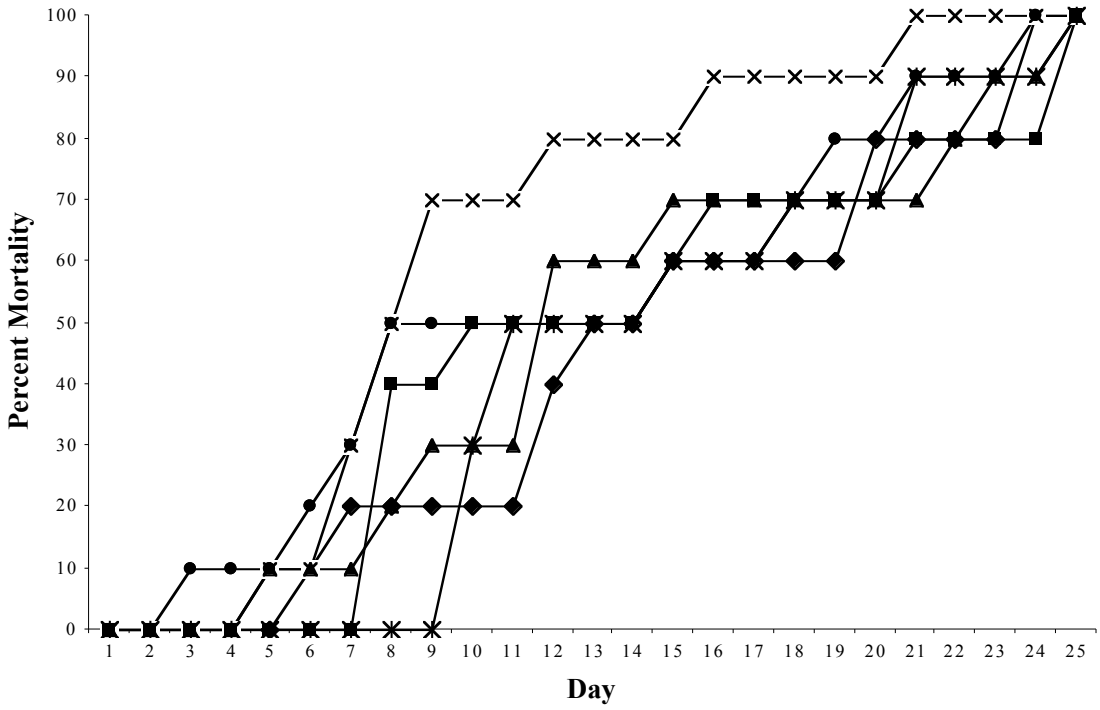


Fig. 4. Cumulative mortality curve for RB51SOD/85A-vaccinated striped bass (*Morone saxatilis*) following challenge with approximately 1.6 x $10^8$ CFU/g body weight of fish on Day 70 post-vaccination. Vaccination groups included were groups injected with $10^{10}$ (circle), $10^9$ (asterik), $10^8$ (triangle) $10^7$ (square), $10^6$ (letter X) colony-forming units of RB51SOD/85A. The Saline control (diamond) group was also included.