SUMMARY OF ACTIVITIES INVOLVING THE DEVELOPMENT OF ANTIBODY-BASED FIELD IDENTIFICATION KITS FOR ATLANTIC BLUE MARLIN, WHITE MARLIN, AND ATLANTIC SAILFISH, 1988-1992

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SUMMARY

Over the last four years the authors have worked towards developing antibody-based field tests to quickly and accurately identify processed Atlantic billfishes. Attempts to produce species-specific polyclonal antisera were not successful, as the antibodies cross-reacted between the target species. Thus, a rapidly evolving protein, serum albumin, was purified from representative billfish species and used for the production of monoclonal antibodies. Subsequent research has shown that serum albumin varies antigenically between billfish species. Species-specific monoclonal antibodies have been produced against Atlantic sailfish and incorporated into an enzyme-linked immunosorbent assay for identification of this species. Monoclonal antibodies showing a preliminary specificity to white marlin have been produced and hybridomas are currently being produced against blue marlin. The present assay is highly specific and allows the testing of 88 samples in a multiwell plate over a two hour period. A more rapid assay combining the use of species-specific and istiophorid-specific monoclonal antibodies is being developed.

RESUME

Depuis quatre ans, les auteurs ont travaillé à l'élaboration de tests de terrrain basés sur les anti-corps, afin d'identifier rapidement et de façon précise les istiophoridés de l'Atlantique manipulés. Les tentatives visant à produire des anti-sérums polyclonaux spécifiques n'ont pas eu de succès, du fait que la réacion des anti-corps présentait des recoupements entre les espèces visées. Ainsi, une protéine à évolution rapide, la séro-albumine, a été purifiée à partir d'espèces représentatives d'istiophoridés et utilisée pour la production d'anti-corps monoclonaux. Des recherches ultérieures ont montré que la séro-albumine présente des variations antigéniques entre les espèces d'istiophoridés. Des anti-corps monoclonaux spécifiques ont été préparés pour le voilier de l'Atlantique et incorporés dans un test avec enzymes de liaison inmuno-absorbants pour l'identification de cette espèce. Des anti-corps monoclonaux montrant une spécificité préliminaire pour le makaire blanc ont été élaborés, et des hybridomes sont actuellement préparés pour le makaire bleu. Le présent test est hautement spécifique, et permet de tester 88 échantillons sur plaque multiple en deux heures. Un test plus rapide est en cours d'élaboration, qui combine l'emploi d'anticorps monoclonaux spécifiques des espèces et d'autres spécifiques des istiophoridés.

RESUMEN

Durante los últimos cuatro años, los autores han trabajado en el desarrollo de pruebas de campo, basadas en anticuerpos, para identificar con rapidez y precisión los marlines atlánticos manipulados. Los intentos de producir antisueros policlonales específicos no tuvieron éxito, ya que los anticuerpos provocaron reacciones cruzadas entre las especies-objetivo. Por tanto, se purificó una proteína de evolución rápida, la sero albúmina, procedente de especies representativas de marlines, y se usó para producción de anticuerpos

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monoclonales. Investigaciones posteriores han revelado que la sero albúmina tiene variaciones antigénicas en los marlines, de una especie a otra. Se han producido anticuerpos monoclonales específicos para el pez vela atlántico y se han incorporado a un ensayo con enzimas de enlace para la identificación de esta especie. Se han producido anticuerpos monoclonales, que mostraban una especificidad preliminar para la aguja blanca, y actualmente se producen hibridomas para la aguja azul. El presente ensayo es altamente específico y permite ensayar 88 muestras en una bandeja múltiple durante dos horas. Se está trabajando en un ensayo mas rápido que combina el uso de anticuerpos monoclonales específicos de especies y otros específicos de istiofóridos.

1. INTRODUCTION

Billfishes are commonly targeted by foreign longline vessels, and may be a by-catch aboard U.S.-based boats. It is currently illegal to sell, or transport for sale, Atlantic sailfish (*Istiophorus albicans*), Atlantic blue marlin (*Makaira nigricans*), and white marlin (*Tetrapturus albidus*) in most of the United States and its possessions (1988 addition to the 1972 Magnuson Act). However, following processing, it is difficult to identify a carcass, much less its ocean of origin. Thus, enforcement agencies need an accurate, rapid means of identifying carcasses in the field.

Fisheries biologists require accurate statistics on the numbers of individuals of each billfish species being landed. Farber and Conser (1983) and Conser (1985) have suggested that Atlantic blue marlin and white marlin are currently fully exploited or overexploited. Conser (1980) and Limouzy and Cayre (1981) state that data on billfishes such as sailfish are difficult to interpret, in part due to the combining of statistics from different species. This problem results from the inability to differentiate and accurately identify billfish species following processing. Conser (1985) states that the future of billfish stocks may depend largely on our ability to determine the status of stocks with greater precision and accuracy.

In 1988 our laboratory began work on developing identification assays utilizing antibodies produced against sarcoplasmic biomarker proteins from Atlantic blue marlin, white marlin, and Atlantic sailfish. Supported by funding from The Billfish Foundation through the International Commission for the Conservation of Atlantic Tunas, Hartmann and Waldner (1988, 1990c) isolated biomarker proteins from these species using isoelectric focusing. The initial intent was to immunize rabbits with the biomarker proteins and harvest the polyclonal antisera for incorporation into portable, field-usable kits. A high level of cross-reactivity was displayed by the antibodies in the rabbits' sera. This prompted attempts to remove or reduce cross-reactivity by 1) tolerizing neonatal rabbits and juvenile mice with proteins from heterologous billfish species prior to immunization with the target species' biomarker protein(s), and 2) removal of unwanted antibodies by affinity chromatography (Hartmann and Waldner, 1989). However, cross-reactivity remained a problem. We therefore searched for a single immunogenic protein which could be readily purified and would be antigenically unique in each billfish species. Ray Simon (pers. comm.) suggested we investigate serum albumin. This rapidly evolving, highly immunogenic protein has been used by a number of researchers in evolutionary, systematic, and taxonomic studies (Lint *et al.*, 1990; Lowenstein, 1985; Sarich, 1969; Schill and Dorazio, 1990). After immunization with sailfish serum albumin, the antisera of most rabbits again demonstrated cross-reactivity to Atlantic blue marlin and white marlin (Hartmann *et al.*, 1990b). We attempted to reduce or remove the cross-reactivity by passing rabbit anti-sailfish serum through affinity columns of red muscle proteins from the heterologous species. This procedure strengthened the reaction to sailfish but did not remove cross-reactivity (Hartmann *et al.*, 1990b). The serum from a single albumin-immunized rabbit was specific for Atlantic sailfish (Hartmann *et al.*, 1990a); unfortunately, this animal subsequently died.

As a result of the problems encountered while working with polyclonal antisera, a decision was made to produce monoclonal antibodies against the three target species, using serum albumin as an immunogen. Rossi (1992) and Rossi *et al.* (1991, 1992) reported on the successful production of a hybridoma cell line (M2D1) that secretes monoclonal antibodies (MAbs) with specificity for Atlantic sailfish. The M2D1 antibody has been incorporated into an ELISA (enzyme linked immunosorbent assay) which allows up to 88 tissue samples to be tested simultaneously. Although the current ELISA is highly specific for sailfish, it requires two hours to complete. A more rapid immunoassay is being developed.

This paper describes the protocol used to develop the M2D1 hybridoma, the specificity and sensitivity of the M2D1 antibody, and the current state of development of monoclonal antibodies produced against Atlantic blue marlin and white marlin.

2. MATERIALS AND METHODS

2.1 Albumin Purification

Albumins from sailfish (SFA), Atlantic blue marlin (BMA), and white marlin (WMA) were purified from serum following the methods of Hoch and Chanutin (1954), with adjustments recommended by Ray Simon (pers. comm.). Sera were diluted 1:2 with distilled water, and caprylic acid was added to 0.2M. The solution was incubated for 90 minutes at 63^{0}C, and then clarified by centrifugation at 10,000 X g (4^{0}C). An equal volume of saturated ammonium sulfate was added to the supernatant while maintaining a pH of 7.4. The solution was again centrifuged at 10,000 X g following a one hour incubation at 4^{0}C. The supernatant was dialyzed against three changes of distilled water and then lyophilized.

2.2 Media

1. Macsfactor - The P-388 macrophage tumor cell line was grown to confluency in Hybrimax HY medium (HY) (Sigma Chemical Co., St. Louis, MO), supplemented with 2% fetal bovine serum (FBS) (Sigma). The supernatant fluid was replaced with HY supplemented with 2% FBS and 5ì g/ml lipopolysaccharide (*E. coli* endotoxin). After two days the macrophage-conditioned medium (macsfactor) was collected.

2. HAT - HY supplemented with 20% FBS, 5% macsfactor, AB-AM (100U/ml penicillin G, 100ì g/ml streptomycin, 0.25ì g/ml amphoterin B), 50ì g/ml gentamicin sulfate, 55ì M Ã -2-mercaptoethanol, 100ì M hypoxanthine, 0.4ì M aminopterin, and 16ì M thymidine

3. HT - HAT without aminopterin

4. Cloning Medium - HT supplemented with 20% FBS, 10% macsfactor, AB-AM, and gentamicin

5. Growth Medium - HY supplemented with 10% FBS, 5% macsfactor, AB-AM, and gentamicin

6. Freeze Medium - 90% FBS and 10% dimethylsulfoxide

2.3 Immunization Schedule

Female Balb/c mice were immunized intraperitoneally with 100ì g purified SFA emulsified in 500ì l Dulbecco's phosphate buffered saline (PBS) (Grand Island Biological Company, Grand Island, NY) and 500ì l Freund's complete adjuvant (Sigma). On days 14 and 28 post immunization, mice were boosted by intraperitoneal injection with 100ì g purified SFA emulsified in 500ì l PBS and 500ì l Freund's incomplete adjuvant.

On day 30 post immunization, sera were assayed for anti-SFA titre by ELISA. All procedures were conducted at room temperature (23^{0}C). In each well of a high binding ELISA plate (Corning Glass Works, Corning, NY), 100ì l aliquots of a solution consisting of 10ì g/ml SFA dissolved in 20mM Tris-buffered saline, pH 7.4 (TBS) were incubated for one hour. Unbound SFA was removed by a 3X wash with TBS. The wells were filled (300ì l) with 1% bovine serum albumin (BSA) in TBS for 30 min to block unbound sites. The plate was washed 3X with a 0.05% Tween-20/TBS solution (TTBS). Serial two-fold dilutions with antibody buffer (TTBS with 0.5% BSA) were performed on the sera of two SFA-immunized mice and a control mouse, and 100ì l aliquots were incubated for one hour in designated wells. Following a 3X TTBS wash, 100ì l of antibody buffer with goat anti-mouse alkaline phosphatase conjugate (GAM-AP) (Bio-Rad Laboratories, Richmond, CA) were incubated in each well for one hour. Following a 5X TTBS wash, 100ì l aliquots of a solution consisting of the alkaline phosphatase substrate (P-nitrophenyl phosphate) in diethanolamine buffer, pH 9.0, were added to each well. After one hour, the plate was read

spectrophotometrically at 405nm with a Bio-Tek microplate autoreader (Bio-Tek Instruments, Winooski, VT).

When an anti-SFA antibody titre of greater than 5000 was achieved, the mouse was given a final boost via the tail vein with 50µg of SFA in 80µl PBS.

2.4 Fusion

Three days after the final boost the activated splenocytes were fused to X-63 myelomas using the procedures outlined by Harlow and Lane (1988). The spleen was aseptically removed from the mouse and the splenocytes were teased out into 50ml of sterile PBS using a cell sieve dissociator (Sigma). The cells were pelleted by centrifugation at 2000 X g and washed with PBS. One ml of 50% polyethylene glycol 1500 (PEG) at 37^0C was slowly added to the combined cell pellet and the slurry was held in a 37^0C water bath for three minutes. The PEG was slowly diluted with PBS (37^0C) to 50ml over 15 minutes. The cells were pelleted and resuspended in 100ml of HAT medium. The undiluted cell suspension, a 1:2 dilution, and a 1:4 dilution were plated at 100µl/well into either seven or eleven 96-well tissue culture plates, respectively. The plates were kept in a water jacketed CO_2 incubator set at 37^0C and 7.5% CO_2.

2.5 Post Fusion

Five days after fusion 100µl of HAT was added to each well of the tissue culture plates. Nine days after fusion 100µl of supernatant fluid was removed from each well and replaced with HT.

2.6 Screening

Ten days after fusion the supernatant fluid of each well was screened for anti-SFA activity using the Bio-Rad Clone Selector Mouse Monoclonal Antibody Screening Kit (Bio-Rad Laboratories). Purified SFA (10µg/ml in TBS) was allowed to bind to the wells (100µl/well) of 25 high binding ELISA plates for one hour. Following a 3X TBS wash, the wells were filled with a 1% BSA/TBS solution to block unbound sites. Following a 3X TTBS wash, 50µl of the supernatant fluid of each tissue culture well was incubated for one hour in the corresponding well of the ELISA plates. Following a 3X TTBS, 100µl GAM-AP was added to each well. After one hour, the plates were washed 5X with TTBS. The p-nitrophenyl phosphate substrate solution was added to each well and the plates were read at 405nm after one hour.

Samples that tested positive for anti-SFA activity (O.D. > 0.1) were screened for cross-reactivity with blue marlin albumin (BMA) and white marlin albumin (WMA) by the above procedure. ELISA plates were coated with a solution consisting of 10µg/ml BMA and 10µg/ml WMA for this purpose.

2.7 Cloning

Hybridomas that were specific for SFA were cloned by limiting dilution. The hybridomas that displayed a high optical density (O.D.) value for SFA and a minimal O.D. for BMA and WMA were transferred to 24 well tissue culture plates with 1ml of HT in each well. After two days the cell concentration was calculated using a hemocytometer. A sample of the cell suspension was diluted with cloning medium to 3 cells/ml. This dilution was plated at 100µl/well into five 96-well tissue culture plates. After five days, 100µl of cloning medium was added to each well. The hybridomas were screened for both anti-SFA activity and cross-reactivity on the ninth day after plating.

Rapidly growing clones with specificity for SFA were expanded to several $75cm^2$ tissue culture flasks. These hybridomas were cultivated for three weeks in growth medium which was changed every two days. The antibody-containing supernatant fluids were stored at 4^0C. Important clones and their corresponding parent populations were preserved in liquid N_2.

Selected hybridomas were grown as ascites tumors in Balb/c mice. Mice were primed by intraperitoneal injection of 0.5ml of pristane (2,6,10,14-Tetramethylpentadecane) 10 days before innoculation with 10^6 cells.

2.8 Purification of MAbs

An equal volume of saturated ammonium sulfate was added to the antibody-laden supernate and the solution was maintained at 4^0C overnight.The precipitated proteins (globulin fraction) were pelleted by centrifugation at 10,000 X g for 20 minutes.The pellet was dissolved in 50ml of TBS and then 50ml of saturated ammonium sulfate was added to the solution. After several hours at 4^0C the precipitate was pelleted and then dissolved in 2ml of TBS. This solution was passed through a Sephadex G-25 desalting column equilibrated with TBS, and a final volume of 3.5ml of concentrated and crudely purified MAb was collected.

The concentrated MAbs were purified with a goat anti-mouse IgG agarose (GAMA) affinity column (Sigma), as follows. A 10ml GAMA column was equilibrated with 200ml of PB (0.01M $NaPO_4$, 0.5M NaCl, pH 7.2). The concentrated MAb solution was diluted in 200ml of PB and passed through the column which was then washed with 300ml of PB. The MAbs were eluted with 100ml of stripping buffer (0.1M glycine, 0.15M NaCl, pH 2.5) and collected in a vessel containing 10ml of 0.5M Tris, pH 8.0. The purified MAb was concentrated by 30,000 molecular weight cut-off ultrafiltration and suspended in 1ml of conjugation buffer (1M $NaHCO_3$, 0.9M NaCl, pH 9.5).

2.9 Isotyping of MAbs

MAb isotypes were ascertained with the Sigma Immunotype Mouse MAb Isotyping Kit. A 10ìl aliquot of purified MAb was diluted in 3ml of PBS and poured into a 12 X 75mm test tube containing an immunotype strip. After 30 minutes the strip was washed 3X with a PBS solution containing 0.05% Tween 20 and 1% BSA (PBS-T-BSA). Two drops of the anti-mouse Ig's-biotin conjugate in 4ml of PBS-T-BSA were added to the immunotype tube. After 15 minutes the strip was washed 3X with PBS-T-BSA and then once with PBS, after which the substrate solution was poured into the tube.

2.10 Conjugation of MAbs to Activated Peroxidase

A 60ìl aliquot (6mg/ml) of a purified MAb, designated M2D1, in conjugation buffer, was added to 1mg of Immunopure Activated Peroxidase (Pierce Chemical Co., Rockford, IL) that was reconstituted in 50ìl of distilled water. The solution was incubated for 15 hours at 4^0C. The reaction was quenched for two hours at 23^0C with 30ìl of 0.2M glycine. The antibody-enzyme conjugate (M2D1-HRP) solution was diluted to 7ml (1:50) with TBS and stored at
-70^0C.

2.11 Serum Dilution Optimization

Sera of seven Atlantic sailfish were diluted 1:100, 1:1000, 1:5000, and 1:10,000 in TBS. Each dilution was adsorbed to the wells of an ELISA plate (100ìl/well) for one hour. Following a 3X TBS wash, a 30 minute blocking step with BSA-TBS, and a 3X TTBS wash, 100ìl of a 1:40 dilution of the M2D1-HRP stock was added to each well. After one hour the plate was washed 5X with TTBS and 100ìl of the peroxidase substrate solution, TMB (3,3',5,5' tetramethyl benzidine) (Pierce), was added to each well. After five minutes the reaction was stopped with 1M H_3PO_4 and the plate was read spectrophotometrically at 450nm.

2.12 Assay of Billfish Sera with M2D1-HRP

Sera of 29 Atlantic sailfish, 26 Atlantic blue marlin, 10 white marlin, one longbill spearfish (*Tetrapturus pfluegeri*), three wahoo (*Acanthocybium solanderi*), one blackfin tuna (*Thunnus atlanticus*), one yellowfin tuna (*Thunnus albacares*), one king mackerel (*Scomberomonus cavalla*), and one common dolphin (*Coryphaena hippurus*) were diluted 1:5000 in TBS. Three 100ìl aliquots of each sample were adsorbed to wells of an ELISA plate for one hour. The ELISA procedure was completed as above, using the TMB substrate.

2.13 Western Blot Analysis of Sailfish Serum with M2D1-HRP

A Western blot was performed using a Pharmacia PhastSystem electrophoretic unit and a Pharmacia PhastTransfer Semi-Dry electroblotting unit. Two native 8-25 gradient polyacrylamide gels were

electrophoresed. Gel 1 contained sailfish serum (SFS), SFS 1:2, SFS 1:4, SFS 1:8, SFS 1:16, SFS 1:32, purified SFA 10mg/ml, and SFA 5mg/ml in lanes 1-7, respectively. Gel 2 contained SFS 1:2, SFS 1:16, BSA 10mg/ml, BSA 5mg/ml, BSA 1 mg/ml, SFA 10 mg/ml, and SFA 5mg/ml in lanes 1-7, respectively. Proteins from gel 1 were transferred (blotted) onto a nitrocellulose membrane (0.45µm porosity) following the PhastTransfer Semi-Dry manual (Pharmacia, Piscataway NJ). Post-blotting, the membrane was air-dried and then incubated in a 3% gelatin solution for 45 minutes, to block unbound sites. After washing the membrane 5X with TTBS, it was incubated in a solution containing M2D1-HRP conjugate for 2 hours. The membrane was then washed 3X with TTBS and developed with the HRP substrate 4-chloro-1-napthol (4-C1N). Gel 2 was developed with Coomassie brillant blue R-250 for comparison.

2.14 White Marlin Hybridoma Production

Hybridomas with specificity for white marlin were produced and screened following the procedures described for sailfish, with the exception that albumin was purified from the pooled sera of five white marlin.

2.15 Assay of White Marlin Sera

Ten white marlin serum samples were clarified by centrifugation at 10,000 X g for 30 minutes. The supernates were diluted 1:100 in TBS. A two-fold serial dilution from 1:100 to 1:1280 was made in an ELISA plate. A standard peroxidase-based ELISA protocol was followed a MAb that showed no cross-reactivity upon initial screening (E8).

2.16 Assay of Fish Sera Heterologous to White Marlin

Five parent hybridoma lines (not cloned) produced from the white marlin fusion, were shown to react with white marlin, Atlantic blue marlin, and sailfish during the primary screening. These were tested by ELISA against sera from the following fishes: sailfish, longbill spearfish, gag grouper (*Myctoperca microlepis*), wahoo, king mackerel, blackfin tuna, and common dolphin.

2.17 Western Blot Analysis of Sera with A6

Antibody purified from cell culture fluid of the cloned A6 hybridoma was analyzed by Western blot. The protocol outlined above was followed, except a second antibody conjugated to HRP was used in conjunction with 4-C1N to develop the blot. Samples tested included sera from white marlin, blue marlin, gag grouper, wahoo, sailfish, longbill spearfish, all diluted 1:2, and BSA at 10mg/ml in lanes 1-7, respectively.

2.18 Blue Marlin Hybridoma Production

Two attempts to produce anti-BMA hybridomas were unsuccessful, the first due to bacterial contamination, the second produced no viable hybridomas. Recently, a successful blue marlin fusion occured but it is too early to report results at this time.

3. RESULTS

3.1 Albumin Purification

Billfish serum yielded 0.5mg/ml of albumin using the purification method. Hydroxyapatite-HPLC analysis of the chemically purified albumin revealed one major and two minor peaks. The major peak was identified as serum albumin by comparison of its retention time with that of a bovine serum albumin standard (BSA). Additionally, SDS-PAGE analysis showed the major peak to have a relative mobility (M_r) analogous to that of BSA.

3.2 Immunization and Hybridoma Production

After immunization and two boosts, the antisera of two mice were shown to have a titre of 10,000. One of the mice was given a final boost and its splenocytes were used to produce hybridomas. Of the 18 hybridoma-containing wells that demonstrated anti-SFA activity, three did not exhibit cross-reactivity with BMA or WMA. One of the three, 21G12, appeared to consist of a single colony and demonstrated a high level of reactivity to SFA. Limiting dilution of 21G12 yielded the clone M2D1. MAbs produced by M2D1 were used in the following experiments.

3.3 Purification, Isotyping and Conjugation of M2D1 MAbs

Affinity purified MAbs from clone M2D1, at 6mg/ml, maintained anti-SFA activity (O.D. > 0.100) up to a 5 x 10^4 dilution. The M2D1 MAbs are of the mouse IgG1 isotype. The M2D1-HRP conjugate retained its activity toward SFA at a 2 x 10^3 dilution (1:40 dilution of stock).

3.4 Assay of Billfish Sera

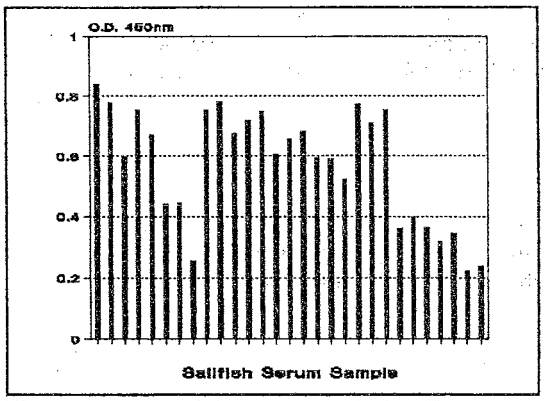

Figure 1. Assay of sailfish serum with M2D1-HRP Samples were diluted 1:5000 and assayed in triplicate.

The optimum serum dilution was determined to be 1:5000. Figure 1 shows that serum samples from all of the 29 Atlantic sailfish assayed yielded strong positive readings when tested with M2D1-HRP. Serum samples from all 48 heterologous individuals tested negative with M2D1-HRP. Serum samples were tested in triplicate, with O.D. (450nm) readings ranging from 0.222 to 0.847 for the sailfish samples. The replicate readings for each sample differed by no more than 0.040 O.D. units. The largest value recorded for any heterologous serum sample replicate was 0.008.

3.5 White Marlin Hybridoma Production

Of approximately 1000 hybridomas produced from a single fusion, 34 displayed strong activity (O.D. > 0.500) towards WMA. Seven of these hybridomas showed no cross-reactivity (O.D. < 0.015) with BMA/SFA. The three hybridomas with O.D. readings of 0.000 for BMA/SFA are currently being cloned by limiting dilution. The hybridoma E8 has been successfully cloned; data to date show that it recognizes all 10 of the white marlin samples currently available to us. However, the optical densities vary at specific dilutions, presumably due to disparities in serum quality.

3.6 Western Blot Analysis of Sailfish Serum

Figure 2 shows that the M2D1-HRP conjugate specifically reacts with albumin (left) when tested against the mixture of proteins in serum (right).

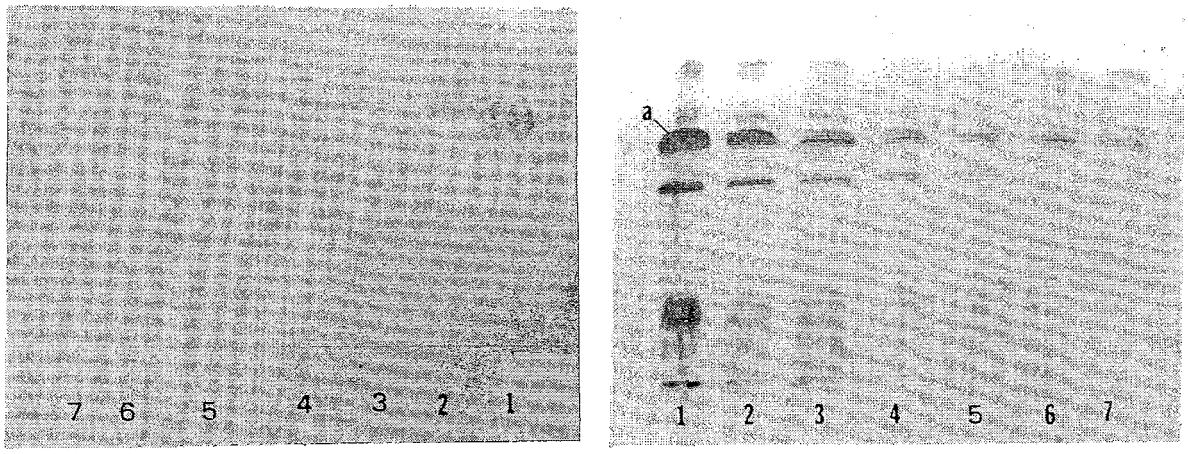

Figure 2 Western blot analysis of sailfish serum with M2D1-HRP. (left) Western blot with M2D1-HRP. Lanes 1-5 = sailfish serum; lanes 6,7 = purified SFA. (right) Native molecular weight PAGE gel. Lanes 1-5 = sailfish serum; lanes 6,7 = purified SFA. a = Sailfish albumin band as identified by previous comparisons to bovine serum albumin.

3.7 Assay of White Marlin Serum with E8

Figure 3 shows that E8 MAbs recognize each of the 10 white marlin samples. Lower O.D. values correlated to samples of poor quality.

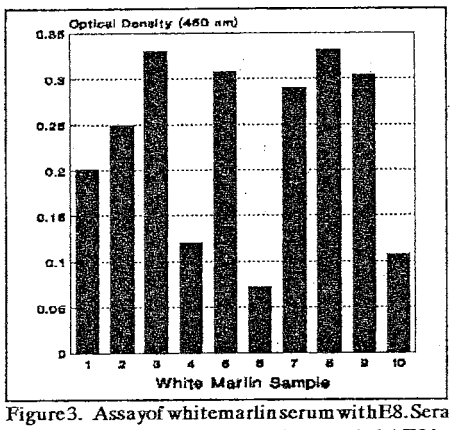

Figure 3. Assay of white marlin serum with E8. Sera was diluted 1:3200 and assayed with E8 in a peroxidase-based ELISA.

3.8 Assay of Heterologous Fish Species

As shown in Table 1, two classes of cross-reacting MAbs were generated from the white marlin fusion: one class (F9, E3, A6) is istiophorid specific (recognizing Atlantic blue marlin, white marlin, Atlantic sailfish and longbill spearfish); the other class (D3 + D4) does not recognize longbill spearfish. These hybridomas recognize only istiophorids.

Table 1. Reactivity of hybridomas with fish sera. Values are optical density readings from a peroxidase-based ELISA.

Clone	D4	D3	E3	F9	A6
Fish					
sailfish	1.5	0.5	1.6	1.1	1.5
spearfish	0.0	0.0	1.3	1.2	1.7
gag grouper	0.0	0.0	0.0	0.0	0.0
wahoo	0.0	0.0	0.0	0.0	0.0
king mackerel	0.0	0.0	0.0	0.0	0.0
blackfin tuna	0.0	0.0	0.0	0.0	0.0
dolphin	0.0	0.0	0.0	0.0	0.0

3.9 Western Blot Analysis of Fish Sera with A6

Figure 4 shows that A6 recognizes all four Atlantic istiophorids but none of the species outside of this family. When the blot (left) is compared to its corresponding gel (right) and to figure 2, it is evident that A6 recognizes an antigen other than albumin. The relative mobility of this antigen corresponds to that of transferrin (Sober, 1968) which is a likely contaminant in the purified albumin.

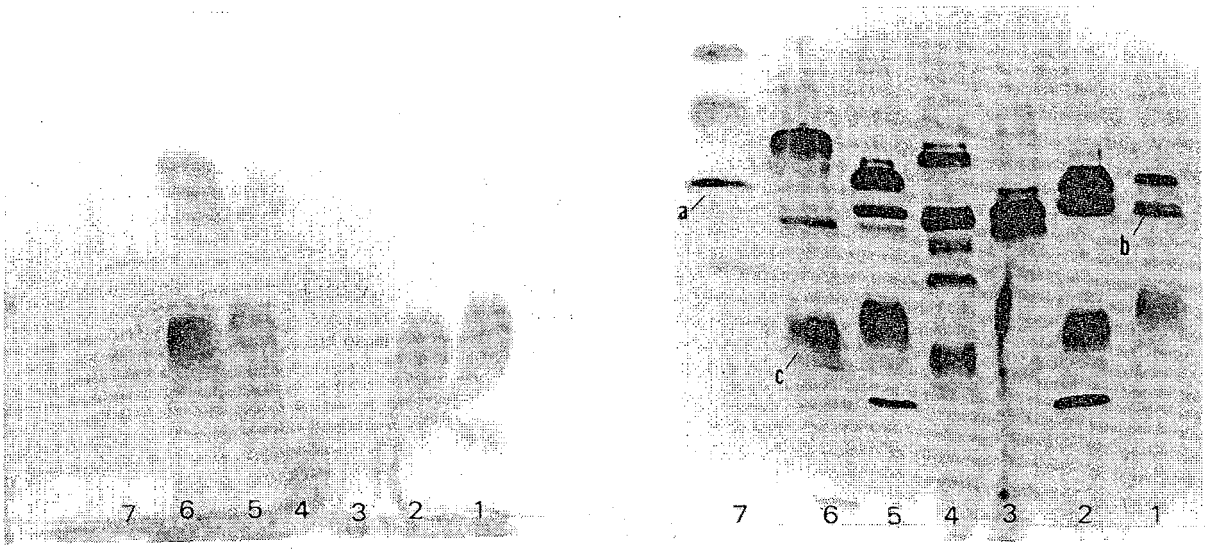

Figure 4. **Western blot analysis of fish sera with A6. (left) Western blot. (right) PAGE gel. For both left and right: lane 1 = wm serum; 2 = bm serum; 3 = gag grouper serum; 4 = wahoo serum; 5 = sf serum; 6 = longbill spearfish serum;7 = BSA; a = BSA; b = wm albumin; c = transferrin.**

4. DISCUSSION

Our research demonstrates that serum albumin can be used as a biomarker protein to identify billfish to the rank of species using an immunoassay procedure. The monoclonal antibody M2D1, conjugated to the enzyme horseradish peroxidase, recognizes an antigenic determinant present on sailfish albumin that is not evident on albumin from blue marlin, white marlin, longbill spearfish, or other closely related pelagic fishes. M2D1-HRP has been reported as the first use of monoclonal antibodies to identify fish to the rank of species.

The specificity of the M2D1-HRP conjugate is exquisite in that it has thus far correctly identified 29 sailfish from a total of 70 billfish serum samples, and it has shown no reactivity towards non-istiophorid species. The monoclonal antibody-enzyme conjugate has been incorporated into a simple immunoassay format that yields an easily detected colored product in a positive test. Recently, Finnerty and Block (1992) have quantified intraspecific variation in billfish species by sequencing a PCR fragment from the mitochondrial gene cytochrome b. Although this technique is important in elucidating genetic diversity and gene flow among billfishes, its use is limited to a laboratory setting and requires considerable time and expertise.

A main consideration in conducting the species immunoassay, as currently developed, is that the sera from billfish must be diluted approximately 1:5,000. It is surmised that more concentrated solutions of sera contain an excess of albumin molecules which desorb from the plastic surface of the assay plate and thus block adsorption of the monoclonal antibody-enzyme conjugate. Alternatively, proteins and/or lipids present in the more concentrated sera may interfere with the immunoassay reactions.

Although the current assay requires two hours to perform, the time can be shortened dramatically. Rather than a non-specific adsorption of the serum sample over a one hour period, capture antibodies

can be employed. Their use would also eliminate the 30 minute blocking step, thus shortening the assay period to 20-30 minutes. Lastly, the use of capture antibodies would greatly improve the sensitivity of the assay and possibly allow identification from a red muscle drip if blood is not available.

Since the monoclonal antibody M2D1 was produced against chemically purified albumin, it could be argued that it reacts with a contaminant rather than albumin. However, we have demonstrated by Western blot analysis that M2D1 reacts only with albumin and lacks reactivity against all other serum proteins.

A hybridoma (termed E8) isolated from an anti-white marlin fusion appears to be specific to white marlin albumin. Although it specifically recognized all available white marlin samples in an indirect immunoassay, it has yet to be conjugated to horseradish peroxidase and used in a direct immunoassay. Testing of an E8-HRP conjugate against additional white marlin samples, and various heterologous species, may prove its usefulness in identification of this species.

In the search for a white marlin specific clone, we have made an ancillary but potentially important discovery. One clone, A6, identifies serum from all istiophorids but does not react with sera from five heterologous species representing such diverse genera as *Mycteroperca* (gag grouper), *Acanthocybium* (wahoo), *Scomberomorus* (king mackerel), *Thunnus* (blackfin tuna) and *Coryphaena* (common dolphin). This clone may be useful in determining that a processed carcass represents a billfish, regardless of the species. We hypothesized that the antibodies from this clone could be used to capture albumin from extremely dilute solutions, thus shortening the assay. However, we determined by Western blot analysis that the antibody from clone A6 reacts to a 94,000 dalton protein, most probably transferrin, rather than albumin. The transferrin was a contaminant present in the chemically purified albumin preparation used to immunize the mice for hybridoma production. It is noteworthy that this is the first demonstration that a group of organisms can be identified to the rank of family by using transferrin (or another serum protein of similar charge, size, and/or conformation). We have immunized our current set of mice with HPLC-purified albumin to prevent isolation of hybridomas reactive to proteins other than albumin. We are pursuing the use of polyclonal antisera for use in capturing and concentrating albumin from dilute solutions.

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