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14. ABSTRACT Reagents and methods/procedures were developed to permit evaluation of general humoral immune status and immune status relative to specific pathogens in cetaceans marine mammals. Immunoglobulin isotypes IgG1, IgG2, IgA, and IgM were purified and poly- or monoclonal antibodies specific for these isotypes were developed. ELISA assays for immunodiagnosis of past exposure to Erysipelothrix rhusiopathiae, a bacterial pathogen of cetaceans, were developed. Tissue culture lines from three species of cetaceans were established and evaluated for virus susceptibility and culture characteristics.

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FINAL REPORT

GRANT #: N00014-98-1-0602

PRINCIPAL INVESTIGATOR: Dr. B. L. Middlebrooks

INSTITUTION: The University of Southern Mississippi

GRANT TITLE: Marine Mammal Health: Development of Immunodiagnostic and Viral Diagnostic Methodologies and Reagents

AWARD PERIOD: 1 MAY 98 - 13 NOV 00

OBJECTIVE: To develop reagents and methods/procedures to permit evaluation of general humoral immune status and immune status relative to specific pathogens in marine mammals (specifically bottlenose dolphins); to develop marine mammal tissue culture lines for study of viruses of marine mammals.

APPROACH: The approach to the overall objective included the following sub-objectives: (1) Development of diagnostic antisera for major immunoglobulin classes of three cetacean species, the bottlenose dolphin (*Tursiops truncatus*), the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and the Beluga whale (*Delphinapterus leuca*); (2) application of these diagnostic antisera in ELISA's developed for monitoring immune status with respect to one of the most important pathogens of captive dolphins, *Erysipelothrix rhusiopathiae*,.(3) development and characterization of cetacean tissue culture lines for use in isolation and characterization of viruses of cetaceans and for diagnostic purposes.

ACCOMPLISHMENTS: Sub-objective 1. Methodology for the purification of three immunoglobulin classes/subclasses (IgG, including subclasses IgG1 and IgG2, and IgA) of *Tursiops truncatus*, was developed. Protein G, Jacalin, and T-gel (thiophilic) affinity chromatography matrices were successfully used to purify IgG1, IgA, and IgG2 respectively. The methods were also demonstrated to be effective for purification of these immunoglobulin isotypes from *Lagenorhynchus obliquidens* and *Delphinapterus leuca*. Since no affinity column matrix (including a mannan binding protein affinity column) was found which selectively bound IgM, size exclusion chromatography or extraction of IgM from polyacrylamide gel electrophoresis bands were found to be the most effective methods at present for purification of cetacean IgM. For all three species of cetaceans New Zealand white rabbits were used to produce polyclonal antisera specific for (a) whole serum, (b) precipitated serum, (c) IgG whole molecule, and (d) gamma heavy chain. Separate mouse hybridomas were developed which produce monoclonal antibodies against (a) the gamma heavy chain of IgG (reactive with both IgG1 and IgG2) and (b)the alpha heavy chain of IgA of *T. truncatus* (insufficient amounts of available serum prevented production of monoclonal antibodies specific for *D. leuca* and *L. obliquidens* isotypes. Both the polyclonal and monoclonal antibodies were successfully used in enzyme linked immunosorbent assays (ELISA) to quantitate IgG and IgA

levels in a group of wild dolphins and four groups of captive dolphins.

Sub-objective 2.Seven wild isolates of *E. rhusiopathiae*, including four isolates from infected cetaceans at different marine mammal facilities and three cultures obtained from the American Type Culture Collection plus one culture of *E. tonsillarum* were employed. A 64 kDa protein which is highly conserved in all known strains of *E. rhusiopathiae* and which is reported to be the protective antigen was selected for use in an ELISA developed for monitoring antibody titers against *E. rhusiopathiae*. The 64 kDa protein was extracted and purified (on virtually a continuous basis) from cultured *E. rhusiopathiae*. Rabbit or mouse antisera were produced against heat killed cultures of the various *E. rhusiopathiae* strains and the extracted 64 kDa protein.

An ELISA was developed using a mixture of surface antigens extracted from the *E. rhusiopathiae* as the ELISA capture antigen and an indicator system consisting of biotin labeled rabbit anti-Atlantic bottlenose dolphin IgG, alkaline phosphatase labeled avidin and *p*-nitrophenyl phosphate as the chomagenic substrate. The ELISA was used to evaluate anti-*E. rhusiopathiae* titers in a series of serum samples from captive Atlantic bottlenose dolphins. Titers were compared to direct agglutination results using whole *E. rhusiopathiae cells*. No direct correlation was seen between the results of the two assays except that higher titers were seen for animals housed in open ocean pens (as opposed to closed water systems). Assays performed using mouse and rabbit antisera as positive controls showed that the extracted *E. rhusiopathiae* antigens were successful in detecting immunoglobulins specific for several isolates of *Erysipelothrix rhusiopathiae* and even an isolate of *E. tonsillarum*. It was also shown that the capture antigen was specific for capturing anti-*Erysipelothrix* immunoglobulins and not immunoglobulins produced against other closely related bacteria.

A second ELISA was developed using only the purified 64 kDa antigen as capture antigen. Results of this ELISA showed a smaller percent deviation within the assay and titers comparable to those obtained using the extracted antigen mixture. The results of this assay correlated with results of agglutination assays (using latex beads coated with *E. rhusiopathiae* antigen).

Sub-objective 3. Six cell culture lines from three cetacean species were developed and characterized. Cell lines derived from *Tursiops truncatus* tissue include a line developed from kidney, designated TurTruK, and lung tissue samples of two stillborn *T. truncatus* calves, designated TurTruLu1 and TurTruLu2.. Cell lines derived from beluga whale (*Delphinapterus leuca*) tissue include lines derived from the testis, designated DeLeuT, and a line derived from brain tissue, designated DeLeuBr. Cell lines derived from the pantropical spotted dolphin (*Stenella attenuata*) include a line derived from the lung, designated SaL5, and a line derived from the skin, designated SaSk Each of the lines proved to be culturable through at least 125 subcultures. Chromosome analysis indicated that all lines are polyploid/aneuploid (ploidy as high as tetraploid was observed). The lines were developed and maintained on L-15 basal medium supplemented with 10% fetal bovine serum plus MEM non-essential amino acids, BME vitamins, L-glutamine, and antibiotic/antimycotic mix. They can readily be subcultured at split ratios from 1/2 to 1/10. The lines have to date been tested for susceptibility to one virus of marine mammal origin, specifically the seal herpesvirus (SEHV). All lines proved to be

susceptible to SEHV, with cytopathic effect (CPE) being produced most rapidly and most extensively in the lines from *Stenella attenuata*. The CPE produced consisted of focal lesions in the monolayer having the appearance of grape-like clusters. A similar CPE has been reported in other mammalian cell lines susceptible to SEHV (e.g. Vero). The lines have also demonstrated broad susceptibility to human viruses representing the viral families of Herpesviridae, Poxviridae, Rhabdoviridae, Picornaviridae, Paramyxoviridae, Reoviridae, Togaviridae, and Bunyaviridae. Specific viruses tested included herpes simplex virus-1, vaccinia virus, vesicular stomatitis virus, echovirus-11, subacute sclerosing panencephalitis virus, measles virus, respiratory synctial virus, parainfluenza virus-3, and reovirus-3. The broad viral susceptibilities of the four cell lines are a favorable indication of their potential for use as viral diagnostic tools for marine mammals.

CONCLUSIONS: The studies have demonstrated the presence of four of the major mammalian immunoglobulin isotypes in cetaceans, and have resulted in the development of techniques for purifying these isotypes. The physical and physiological characteristics of the isotypes identified and purified to date are similar to corresponding isotypes in humans and other animals. ELISA's developed using whole killed *E. rhusiopathiae* or the 64 kDa "protective" antigen from *E. rhusiopathiae* permitted rapid and sensitive quantitation of levels of anti-*E. rhusiopathiae* in cetaceans. Application of these ELISA's to a series of dolphin serum samples indicated evidence of more exposure to *E. rhusiopathiae* in open ocean pens than in captive environments with closed systems. The broad viral susceptibilities of the six cetacean lines developed and characterized indicate excellent potential for their use in detection and isolation of cetacean viruses already identified as well as viruses not yet isolated. The lines are subcultured with ease in a standard media formulation, all lines show rapid growth at normal incubator temperatures and all have relatively high plating efficiencies

SIGNIFICANCE: The cetacean isotypes may be purified for use in isotype-specfic immunoassays or for comparative immunology studies. The immunodiagnostic (ELISA) assays which were developed to provide a rapid and sensitive means to monitor exposure to a major bacterial pathogen of cetaceans, *E. rhusiopathiae*. The tissue culture lines which have been developed and characterized are being distributed to interested laboratories as tools for isolation, growth and study of marine mammal viruses. They also provide an *in vitro* tool for physiological or biochemical studies of cetaceans.

PATENT INFORMATION: No applications for patents resulted from this project

AWARD INFORMATION: None

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Jones, J.C., R. A. Patterson, and B. L. Middlebrooks (2001) Evaluation and refinement of an ELISA assay designed to detect antibodies against *Erysipelothrix rhusiopathiae* in cetaceans. Infect. Dis. Rev. 2: 218-222

2. Patterson, R.A. and B.L. Middlebrooks. (2002) Methods for purification and study of cetacean immunoglobulins, pp193-204 *in* Cell and Molecular Biology of Marine Mammals , Krieger Publishing Company, Melbourne, Florida.

3. Middlebrooks, B. L., J,C, Jones, and R. A. Patterson (2002). Application of ELISA methodology for detection of *Erysipelothrix rhusiopathiae* antibody titers in cetaceans, pp245-252 *in* Cell and Molecular Biology of Marine Mammals, Krieger Publishing Company, Melbourne, Florida.

4. Jones, J.C., R.A. Patterson, and B.L. Middlebrooks. (Manuscript in preparation) The comparison of two capture antigen preparations for use in an ELISA for the measurement of *Erysipelothrix rhusiopathiae* specific antibodies.

5. Middlebrooks, B.L. and R.A. Patterson (Manuscript in preparation) Characteristics of six cell lines developed from tissues of three cetacean species, the bottlenose dolphin (*Tursiops truncatus*), the Beluga whale (*Delphinapterus leucas*) and the pantropical spotted dolphin (*Stenella attenuata*).

ABSTRACTS (A total of 11 abstracts were produced. Because of space limitations, only four are presented here.)

1. Patterson, R., and B. Middlebrooks (1999) Preparation of Antisera Specific for Major Immunoglobulin Isotypes (IgG, IgA, and IgM) of Three Species of Cetacean: Atlantic Bottlenose Dolphin (*Tursiops truncatus*), Pacific White-Sided Dolphin (*Lagenorhynchus obliquidens*), and the Beluga Whale (*Delphinapterus leucas*). Abstracts of the 13th Biennial Conference on the Biology of Marine Mammals, p 146.

2. Middlebrooks, B., L., J. Colvocoresses, and R. A. Patterson (2000) Susceptibility of Four Developmental Cell Lines from the Beluga Whale (*Delphinapterus leucas*) and the Pantropical Spotted Dolphin (*Stenella attenuata*) to Seal Herpes Virus and a Range of Mammalian Viruses from Several Families. Proceedings of the Joint Conference of the American Association of Zoo Veterinarians and the International Association for Aquatic Animal Medicine, p. 526

3. Jones, J.C., B. L. Middlebrooks, and R. A. Patterson (2001) Comparison of a developmental ELISA for anti-*Erysipelothrix rhusiopathiae* antibodies with a passive agglutination assay using latex beads coated with a purified 64 kDa *E. rhusiopathiae* specific protein. Proceedings of the 32th Annual Conference of the International Association for Aquatic Animal Medicine, 32:108.

4. Osgood, Robert C., Bobby L. Middlebrooks, and Rhonda A. Patterson (2001) The Use of Molecular, Immunological and Microbiological Methods to Evaluate Similarities and Differences Between ATCC Reference Strains of *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* and Environmental Isolates of *Erysipelothrix rhusiopathiae* Implicated in the Death of Marine Mammals. Proceedings of the 32nd Annual Conference of the International Association for Aquatic Animal Medicine, 32: 109.