DEVELOPMENT OF MOLECULAR DIAGNOSTIC TESTS FOR INCLUSION BODY DISEASE IN BOID SNAKES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2012

To researchers who are supportive and truly passionate in the study of Inclusion Body Disease, and individuals who inspired me to pursue my graduate degree

ACKNOWLEDGMENTS

This project would not have been successful without support, advice, and expertise provided by many collaborative institutes and individuals. Most importantly, I am grateful for the guidance and encouragement provided by my committee members: Elliott Jacobson, Ayalew Mergia, Jeffrey Abbott, Sixue Chen and William Farmerie.

I am grateful for my sponsor, Ministry of Education, Republic of China (Taiwan) that supported me for the first three years of graduate study at University of Florida.

A major portion of my PhD research was funded by the Morris Animal Foundation, without which this work would not have been possible. This funding was used to complete the following: 1) antibody validation; 2) development of the blood screening tests; and 3) and sequencing the Inclusion Body Disease Protein (IBDP).

This study was extra challenging since the causative agent of Inclusion Body Disease was unknown. Additionally, initial attempts at sequencing IBDP were unsuccessful due to the absence of homologous sequences in the National Center for Biotechnology Information protein database. This study would not have been successful without the tremendous support I received from several core laboratories within the Interdisciplinary Center for Biotechnology Research, University of Florida. This included the individuals within the Proteomic Core (Marjorie Chow, Cecilia Silva-Sanchez, Ran Zheng, Carolyn Diaz and Sixue Chen), the Cellomic Core (Linda Green and Diane Duke of the Hybridoma Laboratory), and the Electron Microscopy Laboratory (Karen Kelley). Significant help was also provided by laboratories within College of Medicine, University of Florida. This included the individuals within the Molecular Pathology Core (Ann Fu, Lynda Schneider and Martha Campbell-Thompson), and McKnight Brain Institute (Joy Guingab and Firas Kobeissy).

I am thankful to several collaborated institutes outside of University of Florida. Joseph DeRisi and Mark Stenglein of the DeRisi Lab at University of California San Francesco, helped with virus identification using deep sequencing techniques. Also kind support from Freeland Dunker (California Academy of Sciences, San Francisco, California, USA), Drury Reavill (Zoo/Exotic Pathology Services, West Sacramento, California, USA), Gregory Rich (The West Esplanade Veterinary Clinic, Metairie, LA, USA), Thomas Boyer (Pet Hospital of Penasquitos in San Diego, San Diego, CA, USA), Amy Wells (Avian and Exotic Clinic of Monterey, Del Rey Oaks, CA), Lauren Powers (Carolina Veterinary Specialists, Huntersville, NC, USA), Michael Garner (Northwest ZooPath, Monroe, WA, USA), and many other veterinarians provided samples used in my project.

I am grateful to the Anatomic Pathology Service (Patrick Knisley, Micaela Barter and Jeffery Abbott) of the Veterinary Medical Hospitals, University of Florida. I am also grateful to the following individuals for their technical advice and encouragement: James Coleman, Francesco Origgi, Edward Wozniak, Jorge Hernandez, Rick Alleman, Nicole Stacy, David Allred, and Lynn Herrman. My graduate work would not have progressed smoothly without assistance and encouragement from Sally O'connell, Barbara Dupont and April Childress. I deeply appreciate their help. The following colleagues shared their friendship while in the graduate program, College of Veterinary Medicine: Astrid Grosche, Patricia Dingman, Claudio Verdugo, Takashi Uemura, Cruz Fan, Penni Chao, Ronald Koh, Alice Chen, Galaxia Cortes, Katherine Saylor, Liliana Crosby, Alexa McDermott and Sylvia Tucker.

I am extremely thankful for unlimited support by my family. Especially my parents, who deeply believed in my potential, provided financial and spiritual support throughout my academic studies.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-ME	Beta-mercaptoethanol
2-D	Two dimensional
a.a.	Amino acids
AB	Ammonium bicarbonate
ACN	Acetonitrile
AR	Antigen retrieval
BF	Blood film
BSA	Bovine serum albumin
CASV	California Academy of Sciences virus
CI	Confident intervals
CID	Collision induced dissociation
CNS	Central nervous system
Co	Collection
CVV	Collierville virus
DAB	Diaminobenzidine
DDM	Dodecyl maltoside
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enyzyme linked immunosorbent assay
EtOH	Ethanol
FA	Formic acid
g	gram
GGV	Golden Gate virus

Gu-HCl	Guanidine hydrochloride
H&E	Hematoxylin and eosin
HB	Homogenization buffer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IB prep	Inclusion body preperation; semi-purified inclusion bodies
IBD	Inclusion body disease
IBD-	IBD negative
IBD+	IBD positive
IBDP	Inclusion body disease protein
ICBR	Interdisciplinary Center for Biotechnology Research
IDA	Information-dependent acquisition
IgG	Immunoglobulin G
IHC	Immunohistochemical/immunohistochemistry
IHC-	IHC stained negative
IHC+	IHC stained positive
Immuno-	Immuno-tested negative
Immuno+	Immuno-tested positive
IP	Immunoprecipitation
KDa	Kilodalton
L	Liter
Lab1	Laboratory of Anatomic Pathology Service, UF Veterinary Hospital
Lab2	Laboratory of Molecular Pathology Core, College of Medicine, UF

LB	Lysing buffer
LC	Liquid chromatography
LDS	Lithium dodecyl-sulfate
LSM	Lymphocyte separtation media
MAB	Monoclonal antibody
mL	Mililiter
mM	Milimolar
mm	Milimeter
MS/MS	Tandem mass spectrometry
n	Sample size
N/A	Not available/ not applicable
NBF	Neutral buffered formalin
NCBI	National Center for Biotechnology Information
Neg	Negative
nm	nanometer
NP	Nucleoprotein
nr	Non-redundant database, currently known as default
OBG	Octyl beta-glucoside
OD	Optical density
PAS	Periodic acid–Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
PCR	Polymerase chain reaction
PF	Paraformaldehyde

PnPP	para-nitrophenyl phosphate substrate
Pos	Positive
PTAH	phosphotungstic acid hematoxylin
PWBC	Peripheral white blood cells
PyB1RV	*Python curtus* endogenous retrovirus
PyT2RV	*Python molurus* endogenous retrovirus
rpm	Revolutions per minute
RT	Room temperature
RV	Retrovirus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-Blue	Toluidine blue
TBS	Tris-buffered saline
UF	University of Florida
µg	microgram
µL	microliter
µm	micrometer
VH2	Viper (*Vipera russelli*) heart cell line
WB	Western blots

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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December 2012

Chair: Elliott R Jacobson
Major: Veterinary Medical Sciences

Inclusion Body Disease (IBD) is commonly seen in captive boa constrictors (*Boa constrictor*) and occasionally in other boid snakes. This disease is characterized by abnormal accumulation of an insoluble intracytoplasmic 68 KDa protein (IBDP). The exact causative agent(s) and pathogenesis remain unknown. Currently, diagnosis of IBD is based on the light microscopic identification of eosinophilic intracytoplasmic inclusion bodies in hematoxylin and eosin (H&E) stained tissues or blood smears. Better ante-mortem diagnostic tests are needed for screening IBD affected captive snakes, and preventing the introduction of IBD into wild populations.

In this study, a mouse anti-IBDP monoclonal antibody (MAB) was produced against semi-purified IBD inclusion bodies. Using immunohistochemical (IHC) staining, the anti-IBDP MAB was validated by testing on a repository of IBD positive and negative paraffin embedded tissues collected from 1990 to 2011. In boa constrictors, the anti-IBDP MAB had a sensitivity of 80% and specificity of 100% in detecting IBD. The antibody also cross-reacted with IBD inclusion bodies in carpet pythons (*Morelia spilota*) and a ball python (*Python regius*).

An improved H&E staining method was developed, and was used to stain the isolated peripheral white blood cells (PWBC) on microscopic slides. Using anti-IBDP MAB, antigen detection of the isolated PWBC samples were performed by IHC staining and western blots. The presences of antibody against IBDP in the plasma of boa constrictors were also evaluated by utilizing a previously developed anti-snake IgG antibody on western blots. A total of 78 blood samples from boa constrictors, rainbow boas (*Epicrates cenchria*) and ball pythons were evaluated for the presence of IBDP using both H&E staining and IHC staining. The presence of IBDP antigen showed excellent agreement with the diagnosis of IBD. Plasma samples from IBD positive boa constrictors were negative for anti-IBDP antibody.

Finally, the IBD specific protein IBDP was sequenced using tandem mass spectrometry (MS/MS). The peptides of IBDP detected by MS/MS had a 90.5% amino acid coverage of the predicted nucleoprotein of a recently discovered arena-like virus (NCBI Gene ID: 13466438) that was identified in IBD positive boa constrictors.

CHAPTER 1
INTRODUCTION: INCLUSION BODY DISEASE IN BOID SNAKE*

Review of the Literature

Background

Snakes make up approximately 19% of all reptiles maintained as pets. Of these, various boid snakes (members of the families Boidae and Pythonidae) are bred in large numbers for the pet trade. Although accurate numbers are not available, it is believed that several million of these snakes are classified as pets or maintained in breeding operations within the United States. Of illnesses affecting boid snakes, inclusion body disease (IBD) has surfaced as the most important worldwide disease, a condition characterized by the formation of intracytoplasmic inclusion bodies. In Australia, where IBD has been identified in captive pythons and in other countries where boid snakes are being bred for release to the wild, there is concern that this disease will become established in native wild populations. [1]

History, Hosts, and Geographic Range

In the 1970s, IBD was first identified in the United States, where it affected multiple species of boid snakes in private and zoological collections. When first recognized, Burmese pythons (*Python bivittatus*) were the most common boid snake diagnosed with IBD. In 1998, IBD was reported in captive native carpet (*Morelia spilota variegata*) and diamond pythons (*M. spilota spilota*) in Australia, in captive boa constrictors in the Canary Islands, Spain, and subsequently in Belgium. Beginning in the early 1990s,

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more cases of IBD were diagnosed in boa constrictors than pythons, but the cause of this epidemiologic shift is unknown. Additional species diagnosed with IBD include the green anaconda (*Eunectes murinus*), yellow anaconda (*Eunectes notaeus*), rainbow boa (*Epicrates cenchris*), Haitian boa (*Epicrates striatus*), Madagascan boa (*Acranthophis madagascariensis*), Indian python (*P. molurus molurus*), reticulated python (*P. reticulatus*), and ball python (*P. regius*)(Table 1-1). In addition, a disease resembling IBD was diagnosed in an eastern king snake (*Lampropeltis getula*) that was housed with boa constrictors, and in a zoological collection of palm vipers (*Bothriechis marchi*). However, the correlation of the inclusion bodies in the king snake and viper cases to IBD has not been confirmed with molecular techniques or immunological reagents.[1]

Clinical Signs

Central nervous system (CNS) abnormalities. From the late 1970s and extending into the mid-1980s, Burmese pythons were the most common boid snake seen with IBD. Clinical signs of the disease in Burmese pythons primarily involved CNS abnormalities.[1] Signs of CNS disease observed in the IBD affected snakes were incoordination, disorientation,[2,3] head tremors, stargazing,[2-5] and opisthotonus (Figure1-1 and 1-2).[1,2,4,5] The affected pythons with CNS disease often become anorectic, showing progressive loss of their motor function, posterior paresis and some had developed flaccid paralysis.[2,3] The neurologic disorders have been reported in Burmese pythons, green tree pythons (*Morelia viridis*), African rock pythons (*P. sebae*),[2] carpet python, diamond pythons,[3] boa constrictors,[2,4,5,6] and Haitian boas.[2] The sign of neurologic disorders were thought to be more pronounced in Burmese pythons than in boas.[2]

Non-neurologic signs. Beginning in the early 1990s, more cases were diagnosed in boa constrictors in relation to Burmese and other pythons. Boa constrictors affected by IBD also regurgitated food items within several days of feeding, in addition to the CNS disease signs described for pythons.[1] In boas, the signs of CNS disease can be observed in some cases, but chronic intermittened regurgitation was the common first sign to be observed often before showing signs of the neurological disorder.[2,4,6] However, regurgitation was not a disease sign identified in Burmese pythons.[1] Some IBD affected boas progressively become more lethargic, and developed anorexia, chronic weight loss, pneumonia, sever stomatitis, and lymphoproliferative disorders.[2,4,6]

Subclinical infection. Although some snakes die within several weeks of first manifesting illness, others may survive for months.[1] Affected snakes may also appear healthy, and not showing any clinical signs.[4,5] Four experimentally infected boa constrictors that were observed up to 52 weeks developed inclusion bodies within 10 weeks post infection, and none of infected boas showed clinical signs of IBD.[4] Those that showed clinical signs may survive from several weeks to more than a year.[2] It is not known what percentage of IBD infected snakes will develop clinical signs, and how many of them will remain clinically normal.[1]

Pathology and Disease

Visceral tissues. The gross tissues of affected snakes may appear normal without observable lesions, or in some cases organs including spleen, pancreas, and thymus may atrophy.[2] Using light microscopy, eosinophilic to amphophilic intracytoplasmic inclusion bodies in hematoxylin and eosin (H&E) stain were identified in one or more visceral epithelial cells.[2] The inclusion bodies were commonly seen in pancreatic acinar cells (Figure 1-3), renal proximal and distal tubular epithelial cells, and

hepatocytes (Figure 1-4).[1,2,3,5] Inclusion bodies were also observed in spleen, cardiomyocytes,[6] thyroid follicular cells, pars intermedia of adenohypophysis, thymic epithelial cells, thymocytes, gastric epithelial cells,[2] lining epithelial cells of the air passage, lymphoid cells in the esophageal tonsils and circulating lymphocytes.[1-3] The size of inclusion bodies can vary from 1-4 µm,[2] 2-10 µm,[3] 5-20 µm [5] in diameter. Some affected snakes may have numerous large inclusion bodies found in almost all organs, others may have fewer or smaller inclusion bodies only in focal areas,[2] such as the CNS.[1] The inclusion body bearing cells often have accompanying cytoplasmic vocuolation.[2,4,6] Lymphoid depletion [2,3] and fibrosis were also commonly seen in IBD affected snakes. In some chronically infected boas, there were decreased numbers of lymphoreticular cells and loss of splenic follicles.[2]

Nervous system. Eosinophilic to amphophilic intracytoplasmic inclusion bodies were most commonly observed in neurons (Figure 1-5 and 1-6), especially in the hindbrain, and the ependymal cells of infected snakes.[1-3] Inclusion bodies were also found in the spinal cord of infected snakes, within the degenerating neurons of the grey matter.[2] Segmental swelling of myelin sheaths and axons were observed in the proximal spinal cord of an affected carpet python.[3] Other lesions found in the CNS of affected snakes including, neuronal degeneration, gliosis, and demyelinization.[2,3] Of IBD infected snakes with signs of CNS disease, nonsuppurative meningoencephalitis was commonly found with a corresponding infiltrate of lymphocytes.[2] In one IBD case of a carpet python, spongiform change of grey matter with occasional mild multifocal gliosis and perivescular lymphoplasmacytic cuffing were described.[3] Whereas a more pronounced inflammatory response is often seen in the CNS of Burmese pythons, the response is

only minimal to mild in boa constrictors.[2] However, the inclusion bodies were more numerous in the nervous tissue of boa constrictors than in pythons, although the inflammatory response in pythons were greater.[2]

Blood. Myeloid cells in the bone marrow of IBD infected snake often contain the cytoplasmic inclusion bodies.[5] The inclusion bodies have also been found in lymphocytes,[1,4] erythrocytes, and heterophils of IBD infected snakes.[1]In a survey of 13 IBD infected boa constrictors, the boas that showed signs of IBD for less than 2 months had lymphocytosis, whereas boas that showed signs over 2 months had lymphopenia.[2] Hematological and selected biochemical analyte values of acutely affected boa constrictors diagnosed with IBD included leukocytosis, relative lymphocytosis, lower total protein and globulin values, and significantly higher aspartate transaminase values compared with those of chronically affected snakes.[1]

Current Diagnostic Methods

Postmortem diagnosis. A postmortem diagnosis of IBD is based on the light microscopic identification of variably sized eosinophilic to amphophilic intracytoplasmic inclusion bodies in H&E stained tissue sections. The tinctorial characteristics of the inclusion bodies may vary with the type of hematoxylin used and differences in staining methods.[1] Brain, liver, pancreas, and kidneys were the most commonly collected tissues for making a diagnosis of IBD.[1-6] Among snakes with IBD, the number of inclusion bodies seen in tissue may be quite variable. Whereas, some may have inclusion bodies in most epithelial cells, others may have very few inclusion bodies.[6] In some snakes, inclusion bodies were only found in the brain.[1] A diagnosis of IBD is made when the characteristic inclusion bodies are detected. However, absence of inclusion bodies may not indicate that the snake is free of IBD.[5, 6]

Antemortem diagnosis. An antemortem diagnosis is made by demonstrating eosinophilic to amphophilic intracytoplasmic inclusion bodies in histologically processed and H&E-stained biopsyspecimens. Boid snakes have well-developed esophageal tonsils (Figure 1-7), and in snakes with IBD the tonsils may contain lymphoid cells or mucous epithelial cells with intracytoplasmic inclusion bodies (Figure 1-8). Using a flexible endoscope with a biopsy device, esophageal tonsils are easily biopsied, fixed, and routinely processed for light microscopy. Liver and kidney biopsy specimens can also be obtained for histological evaluation. For a more rapid diagnosis, cytological impression smears of the liver and renal biopsy samples can be stained with H&E (Figure 1-9) and/or Wright-Giemsa (Figure 1-10).[1] The inclusion bodies are easier to identify in H&E stained preparations.[1] Some snakes may have very few inclusion bodies that may be missed in a biopsy specimen.[1] Thus, failure to identify inclusion bodies in the sampled tissue cannot completely rule out the snake as IBD positive.

Inclusion bodies may be seen in erythrocytes (Figure 1-11), lymphocytes (Figure 1-11 and 1-12), and heterophils (Figure 1-13) in peripheral blood films of snakes with IBD.[1] Preparation of blood smears or buffy coats stained with H&E stain or Wright–Geimsa stain had been used for inclusion body identification.[1,5] The inclusion bodies are more easily identified on blood films using H&E stain, compared to the Wright–Geimsa stained inclusion bodies which is stain basophilic (light blue).[1] Using blood samples for examination is a relatively inexpensive method in making an antemortem diagnosis of IBD.[1] However, there is a lack of sufficient data to know whether all IBD snakes will develop inclusion bodies in peripheral blood cells,[1] or how long after infection will the inclusion bodies appear in the peripheral blood cells. Therefore, the

absence of inclusion bodies in blood cells does not necessarily indicate that the snake is IBD negative.

IBD Antigenic Protein

Wozniak et al. demonstrated that IBD inclusion bodies were phosphotungstic acid hematoxylin (PTAH) positive, orthochromatic with toludine blue (T-blue), periodic acid–Schiff (PAS) negative, and eosinophilic in H&E stained paraffin embedded tissue sections.[4] These profiles suggested that the materials within the inclusion bodies are proteinaceous.[4]

The inclusion bodies were found to consist of an antigenically distinct 68KDa protein named IBD inclusion protein (IBDP). [4] The protein was semi-purified by protein electrophoresis of a liver homogenate, and electro-eluted from the gel bands. [4] Monoclonal antibodies (MAB) against IBDP have been produced that recognize an IBDP band in a western blot and IBDP antigen in frozen tissue sections using immunohistochemical (IHC) staining.[1]

Using transmission electron microscopy, intracytoplasmic inclusion bodies identified within nerve cells in the CNS and visceral epithelial cells begin as polyribosome-derived clusters of small round subunits (Figure 1-13). Inclusion bodies enlarge as additional subunits are deposited on the periphery of individual inclusion bodies (Figure 1-14). In some sections the inclusion bodies have concentric profiles, with subunits observed on the surface.[1] Wozinak et al. demonstrated the smaller inclusion bodies (< 2 µm in diameter) were composed of none membrane limited intracytoplasmic aggregates of electron-dense materials. The larger inclusion bodies (3-6 µm in diameter) appeared as membrane-bound electron dense aggregates mixed with membrane-like fragments.[4]

Possible Causative Agents of IBD and Transmission

Retroviruses. In the early 1990s, using transmission electron microscopy, Schumacher et al. identified 110 nm enveloped viral-like particles in 2 of 17 boa constrictors having IBD affected snakes by transmission electron microscopic examination.[2] The infected organs included, brain, kidney, and pancreas.[2] In this study, the virus was successfully isolated in cultured primary kidney cells obtained from the IBD affected snakes.[2] Further, two healthy Burmese pythons inoculated with the cell free culture supernatant developed neurologic signs, nonsuppurative encephalitis, and small inclusion bodies in the brain and pancreas that were visible under electron microscopy at 4 to 10 week post-inoculation. [2] However, no viral particles were found in the inoculated snakes, and viral isolation attempt from the inoculated snakes were unsuccessful. Thus, Koch's postulates were only partially fulfilled.[2]

In the late 1990s, Wozniak et al. successfully infected healthy boas by inoculating them with 0.45 µm filtered liver homogenates obtained from an IBD affected boa constrictor. Viral-like particles that morphologically resembled the IBD associated retrovirus were observed in all IBD positive livers from inoculated snakes, but not in the IBD negative snakes.[4] Subsequently, in another study, three viral isolates were partially characterized from two IBD affected boa constrictors and one clinically healthy Madagascan ground boa (*Acranthophis dumereli*) that was housed with IBD infected snakes.[6] The viruses were isolated by co-culturing primary lymphocytes with commercial viper heart (VH2) cells (named RV1), or by culturing primary kidney cells (named RV2 and RV3).[6] Based on size (80-110 nm) and morphology, the virus resembled C-type retroviruses (Figure 1-15).[1] Reverse-transcriptase activity was

measured in infected cell cultures, and high activity levels were further evidence that the isolated viruses were retroviruses.[1] However, the VH-2 cells that were infected with isolated retrovirus did not form inclusion bodies.[1] The association between the identified retroviruses and IBD remained unclear, and the retroviruses were not sequenced.

In the early 2000s, Huder et al. completely sequenced an actively expressing endogenous retrovirus (named PyT2RV) from Burmese pythons.[7] Using polymerase chain reaction (PCR) techniques, a survey of the virus within peripheral blood mononuclear cells (PBMC) obtained from various snake species was conducted.[7] The viral sequence of PyT2RV was detected in all (18 out of 18) Burmese pythons whether or not they were showing signs of IBD.[7] A closely related endogenous retrovirus (named PyB1RV) was discovered in blood pythons (*P. curtus*), but not in five other species that were tested.[7] Interestingly, the presences of PyT2RV sequences were not correlated with the presences of IBD.[7] Boa constrictors having IBD were negative for PyT2RV, and the isolated lymphocytes from boa constrictors were not susceptible to PyT2RV infection in vitro.[7] Using transmission electron microscopy, all attempts to visualize PyT2RV were unsuccessful.[7] Therefore, there was no evidence that the sequenced endogenous retroviruses was the causative agents of IBD.[7]

Relationship between retroviruses and IBD. Although retroviruses have been observed in inclusion-bearing tissues by electron microscopy, many hours of searching are needed to locate mature retroviral particles.[1] For a number of IBD cases, neither viral particles nor reverse-transcriptase activity have been demonstrated in tissues of affected snakes.[1] In one study young Burmese pythons that were inoculated with the supernatant of primary cultured kidney cells taken from an infected boa constrictor,

developed clinical signs and microscopic lesions compatible with IBD.[2] In a second study, boa constrictors that were administered filtered liver homogenate obtained from an IBD-infected boa eventually developed intracytoplasmic inclusion bodies in hepatocytes.[4] Because purified virus was not used in these studies, it is impossible to implicate a retrovirus as the underlying etiology of IBD in the inoculated snakes.[1] Further, the presence of the inclusion bodies did not always co-exist with the presence of the retrovirus within affected cells.[4] IBD may represent a protein-storage disease induced by viral infection, or the protein itself may be behaving in a manner similar to that of a prion-like disease. The protein and the isolated viruses must be sequenced to gain a better understanding of a possible causal relationship.[1]

Transmission route and disease prevention. The route of transmission of IBD between snakes has not been determined, although it is believed that direct contact is involved. Because the snake mite (*Ophionyssus natricis*) (Figure 1-16) is present in many snake colonies experiencing an IBD outbreak, mites may be involved in the transmission of the infectious agent. Thus, preventing mites from entering a collection and eliminating established infestations are essential components of a preventive medicine program. It is also possible the causative agent is passed through vertical transmission from mother to young in both egg-laying and live-bearing snakes.[1]

Treatment and Prognosis

There are no effective treatments for snakes infected with IBD.[2,5] The infected snakes that are showing clinical illness are always fatal, although the general condition can sometimes improve with force-feeding and rehydration.[5] Euthanasia of the affected snake is recommended to reduce the risk of spreading the disease to other snakes in the collection.[2,5]

Recent Findings

Concurrent to this study, Stenglein et al. discovered three different arenavirus-like viruses within IBD infected snakes by using deep sequencing methodology and the results were published in 2012.[8] The virus found in annulated tree boas (*Corallus annulatus*) was named California Academy of Sciences virus (CASV), the other two viruses found in boa constrictors were named Golden Gate virus (GGV) and Colliervillle virus (CVV).[8] Using fluorescence IHC staining, a polyclonal antibody produced against the predicted peptide at the C-terminal of GGV nucleoprotein detected IBD inclusion bodies within the paraffin embedded tissue sections.[8] The arenavirus-like viruses were considered the candidate etiological agents for IBD.[8] However, under electron microscopic examination of tissues from numerous IBD snakes, the mature arenavirus-like virus were not seen in the inclusion body bearing tissues. The morphology of the arenavirus-like viruses remained unknown. It is unclear whether IBD is caused by the arenavirus-like viruses alone? Or are other factors involved in the development of IBD?

Research Goals

The Needs of Molecular Diagnostic Tests

Currently, a diagnosis of IBD is made by identifying H&E stained characteristic eosinophilic to amphophilic intracytoplasmic inclusion bodies in the histologically processed tissues.[1] However, some IBD cases have only very few inclusion bodies or the inclusion bodies are very small and easily overlooked during microscopic examination.[1,2] Additionally, it may be histologically difficult to differentiate IBD inclusion bodies from other intracytoplasmic protein accumulations. Therefore, a more sensitive and more specific molecular diagnostic test is needed for a more specific diagnosis of IBD.

Since infected snake may remain subclinical for long periods of times,[4] a more specific and sensitive test is needed to screen snakes for IBD. A screening test is especially needed for the large collections in zoos, breeding collections, and those boid snakes kept as pets. Furthermore, a screening test is also needed for assessing the disease prevalence, or for determining pathogenesis in transmission studies. Therefore, a molecular diagnostic test using blood samples will be the ideal format for screening IBD affected snakes.

A MAB was produced against IBDP and was capable of detecting IBD inclusion bodies in frozen tissue sections using IHC staining.[4] Unfortunately, the original MAB producing clone was lost.[1] It is possible that a IBDP specific MAB can be reproduced and utilized in developing immuno-based diagnostic tests.

Better Understand the Relationship Between IBD and IBDP

The key to understand the relationship between IBD and IBDP resides in the sequence of IBDP. By obtaining the amino acid sequence of IBDP which can be back translated into mRNA sequences and DNA genomic sequences. Using the genomic sequence codes for IBDP, which will allowed veterinarians and researchers to understand the origin of this protein: whether it is a protein derived from foreign organism? Or it is an overproduced self-derivate-protein being induced by an infection? Or the protein itself may be an infectious agent?

In addition, synthetic peptides of IBDP can be produced for developing immunoassays that requires large amount of consistent antigens, or for developing a better monoclonal antibody. Thus, sequencing IBDP is the key toward better understanding the disease mechanism and transmission of IBD. This will also provide better diagnostic tests and preventive programs for IBD.

Hypothesis and Objectives

Hypothesis

The overall goal of this research was to develop better diagnostic tests for identifying IBD in boid snakes, and to further characterize the relationship between IBDP and IBD. The central hypothesis for this research was: Based on the presence of antigenic novel protein (IBDP) that is present in IBD-positive snakes, a monoclonal antibody can be produced against IBDP and be used for immunodiagnostic assay developments. To better understand the nature of IBDP, this protein needs to be sequenced. In order to accomplish these goals, four specific research objectives were established: Objective 1. Produce MAB against semi-purified IBDP. Objective 2. Validate the mouse MAB against IBDP for immunodiagnostic assay development. Objective 3. Assess whether MAB can detect circulating IBDP in the peripheral white blood cells (PWBC) and if measurable circulating antibody against IBDP can be detected in plasma of IBD infected snakes. Objective 4. Partially sequence the 68 KDa IBDP.

Objectives

1. **Produce MAB against semi-purified IBDP.** Isolation of the intracytoplasmic inclusion bodies from liver homogenates obtained from an IBD positive snake. Production of the anti-IBDP antibodies by immunizing 2 mice with the semi-purified preparation, and screen the monoclonal antibodies with the semi-purified preparations. Eventually, develop a monoclonal antibody that recognizes the intracytoplasmic inclusion bodies under IHC staining, enyzyme linked immunosorbent assay (ELISA), and the 68 KDa IBDP band on western blots.

2. Validate the mouse MAB against IBDP for immunodiagnostic assay development. Paraffin embedded tissues from a repository of IBD positive or negative cases dated from 1990 to 2011 were tested using IHC staining. The performances of the MAB were evaluated in the following categories: a. Effects of prolonged formalin fixation; b. Effects of storage time in paraffin. c. Species cross-reactivity; d. Antigen cross-reactivity; e. IHC test diagnostic performance.

3. Assess whether MAB detects circulating IBDP in the peripheral white blood cells (PWBC) and if measurable circulating antibody against IBDP can be detected in plasma of IBD infected snakes. Whole blood samples were obtained from three species of snakes including, boa constrictor, rainbow boa, and ball python. The snakes came from private collections with unknown prevalence of IBD. The PWBC were separated from the plasma, and were tested with H&E stain, IHC stain, and western blots respectively, to determine whether IBDP can be detected by anti-IBDP MAB in circulating PWBC. The plasma of each samples were subjected for antibody detection using western blot to determine whether there are measurable antibodies produced against IBDP by the IBD infected snakes.

4. Sequencing IBDP. The IBDP was purified by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Digest the collected 68 KDa protein bands with trypsin, chymotrypsin, or Asp-N. Analyze digested peptides by tendon mass spectrometry (MS/MS), and search the MS/MS raw spectrums against known protein databases.

Table 1-1. List of snake species IBD had been reported

Family	Scientific names	Species common names
Boidae	*Acrantophis madagascariensis*	Madagascan ground boa [2]
	Boa constrictor	Boa constrictor [2,4,5,6,8]
	Corallus annulatus	Annulated tree boa [8]
	Epicrates cenchris	Rainbow boa [2]
	Epicrates striatus	Haitian boa [2]
	Eunectes murinus	Green anaconda [2]
	Eunectes notaeus	Yellow anaconda [2]
Pythoninae	*Morelia spilota spilota*	Diamond pythons [3]
	Morelia spilota variegata	Carpet pythons [3]
	Morelia viridis	Green tree python [2]
	Python bivittatus	Burmese python [2]
	Python curtus	Blood python [2]
	Python molurus molurus	Indian python [2]
	Python reticulatus	Reticulated python [2]
	Python regius	Ball python [2]
	Python sebae	Afican rock python [2]
Colubridae	*Lampropeltis getula*	Eastern king snake [1]
Viperidae	*Bothriechis marchi*	Palm vipers [1]

Figure 1-1. An IBD affected boa constrictor showing abnormal posture and sign of CNS disease.[1]

Figure 1-2. An IBD affected boa constrictor unable to right itself when placed in dorsal recumbency. Courtesy of CRC Press.[1]

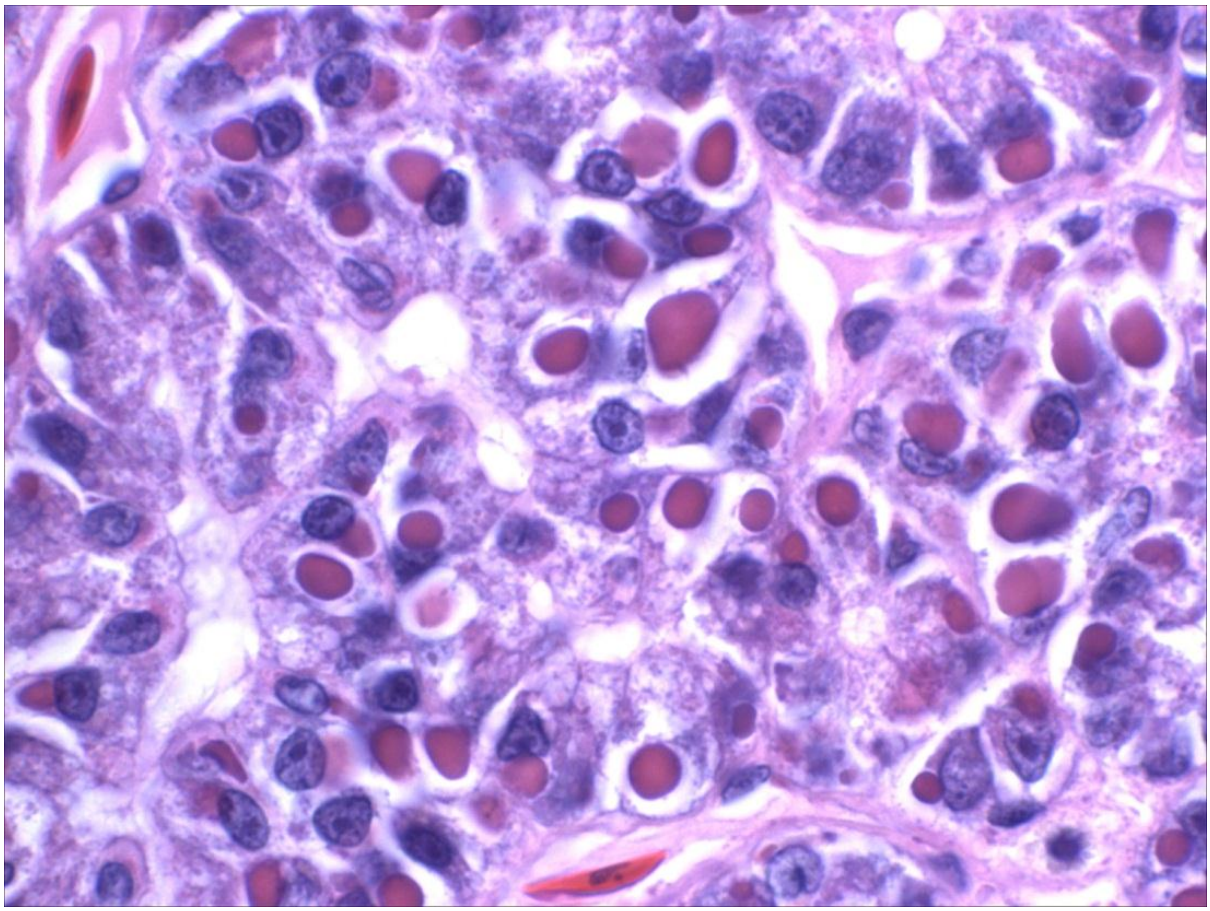

Figure 1-3. Photomicrograph of the H&E stained pancreas from an IBD affected boa constrictor with acinar cells containing eosinophilic intracytoplasmic inclusion bodies. Courtesy of CRC Press.[1]

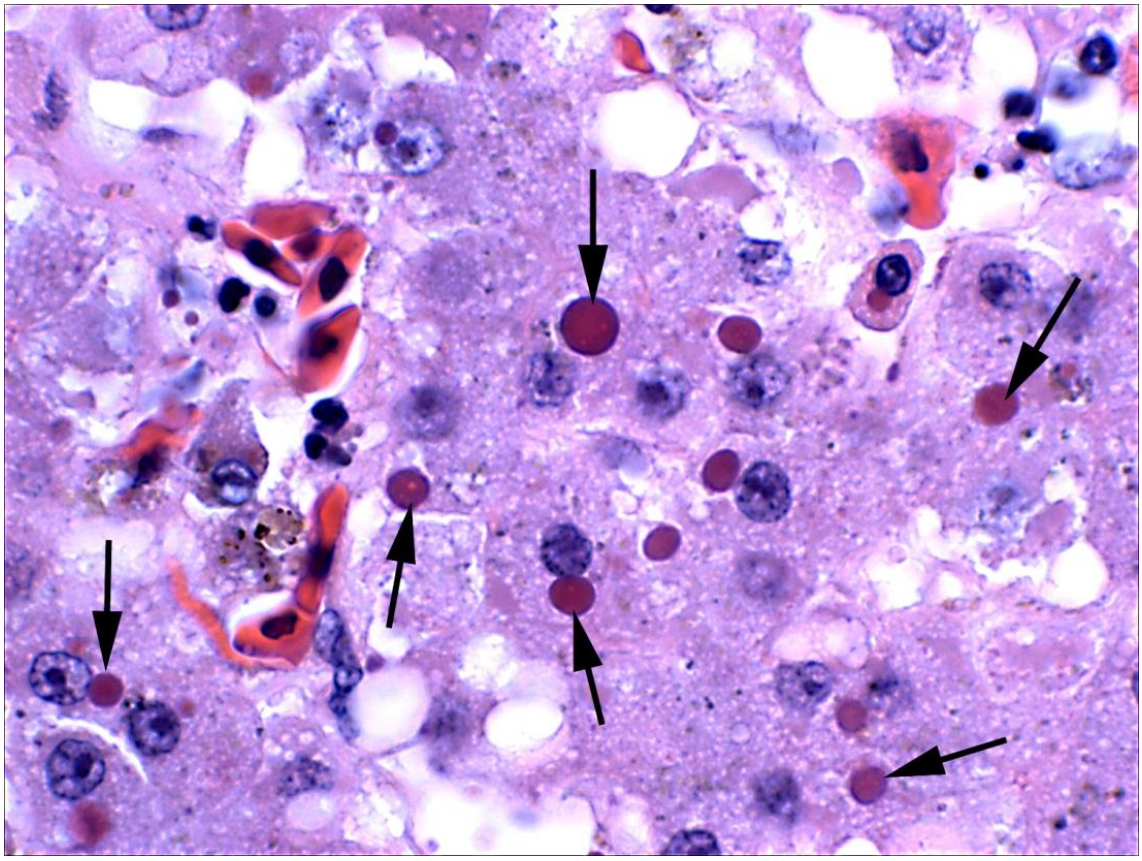

Figure 1-4. Photomicrograph of the H&E stained liver from an IBD affected boa constrictor with hepatocytes containing eosinophilic intracytoplasmic inclusion bodies (arrows). Courtesy of CRC Press.[1]

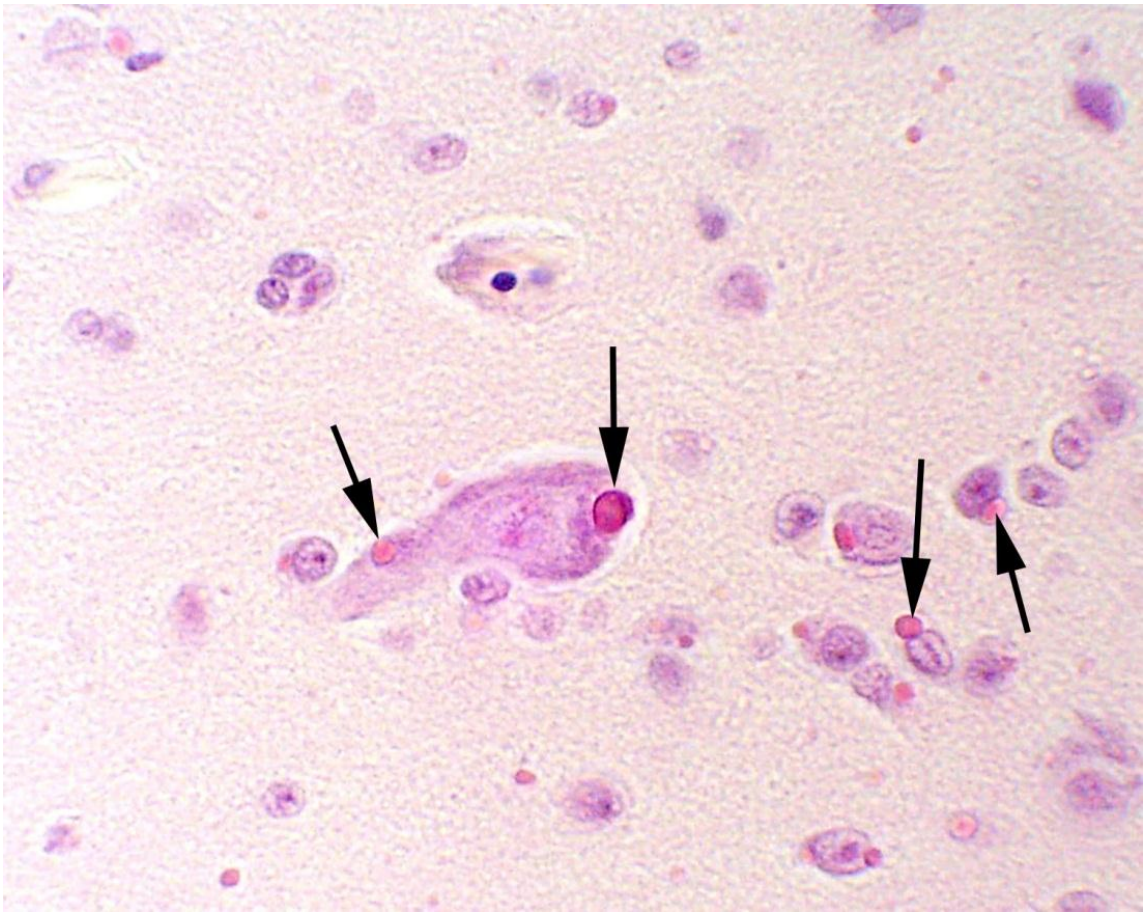

Figure 1-5. Photomicrograph of eosinophilic intracytoplasmic inclusion bodies (arrows) in neurons and glial cells in the H&E stained brain of an IBD affected boa constrictor.[1]

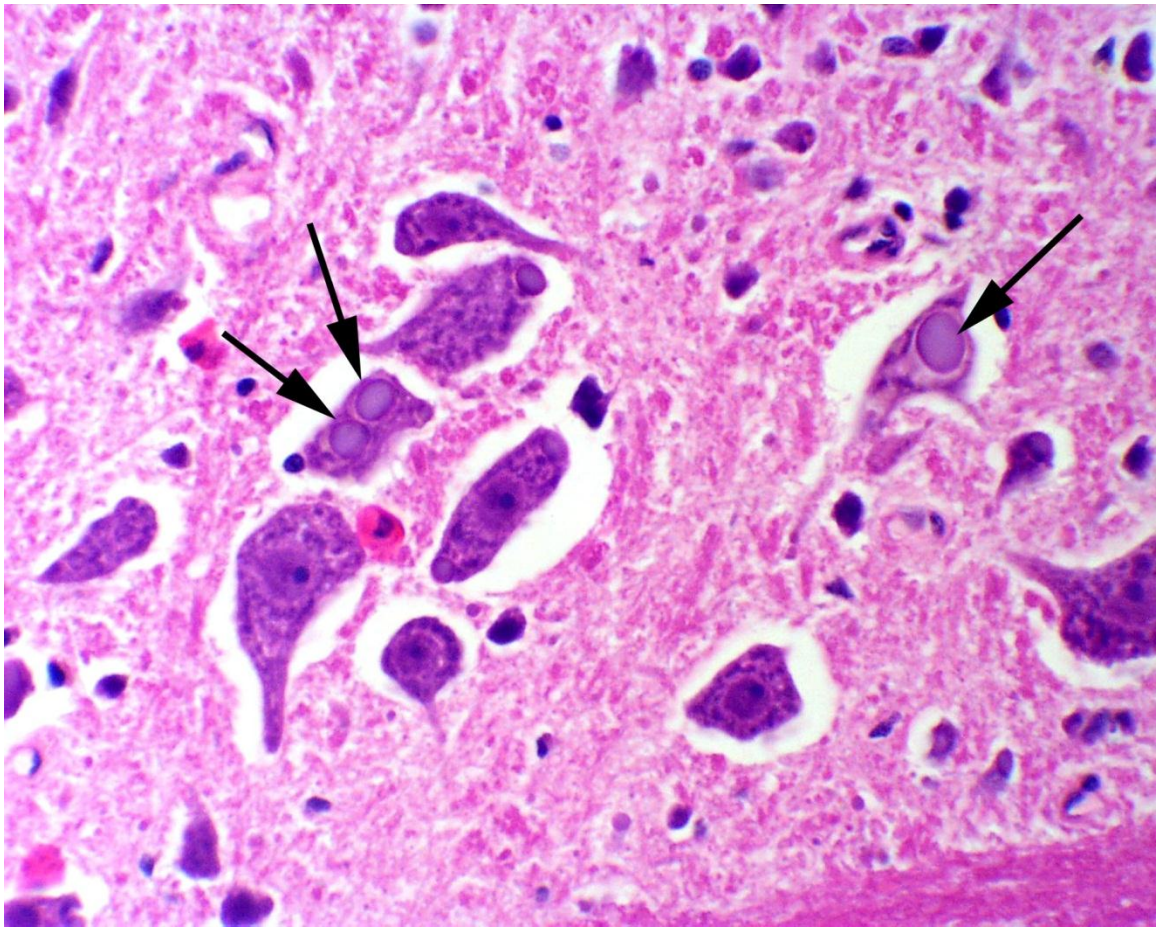

Figure 1-6. Photomicrograph of amphophilic intracytoplasmic inclusion bodies in neurons of the H&E stained brain of an IBD affected boa constrictor. Courtesy of Nikos Gurfield and CRC Press.[1]

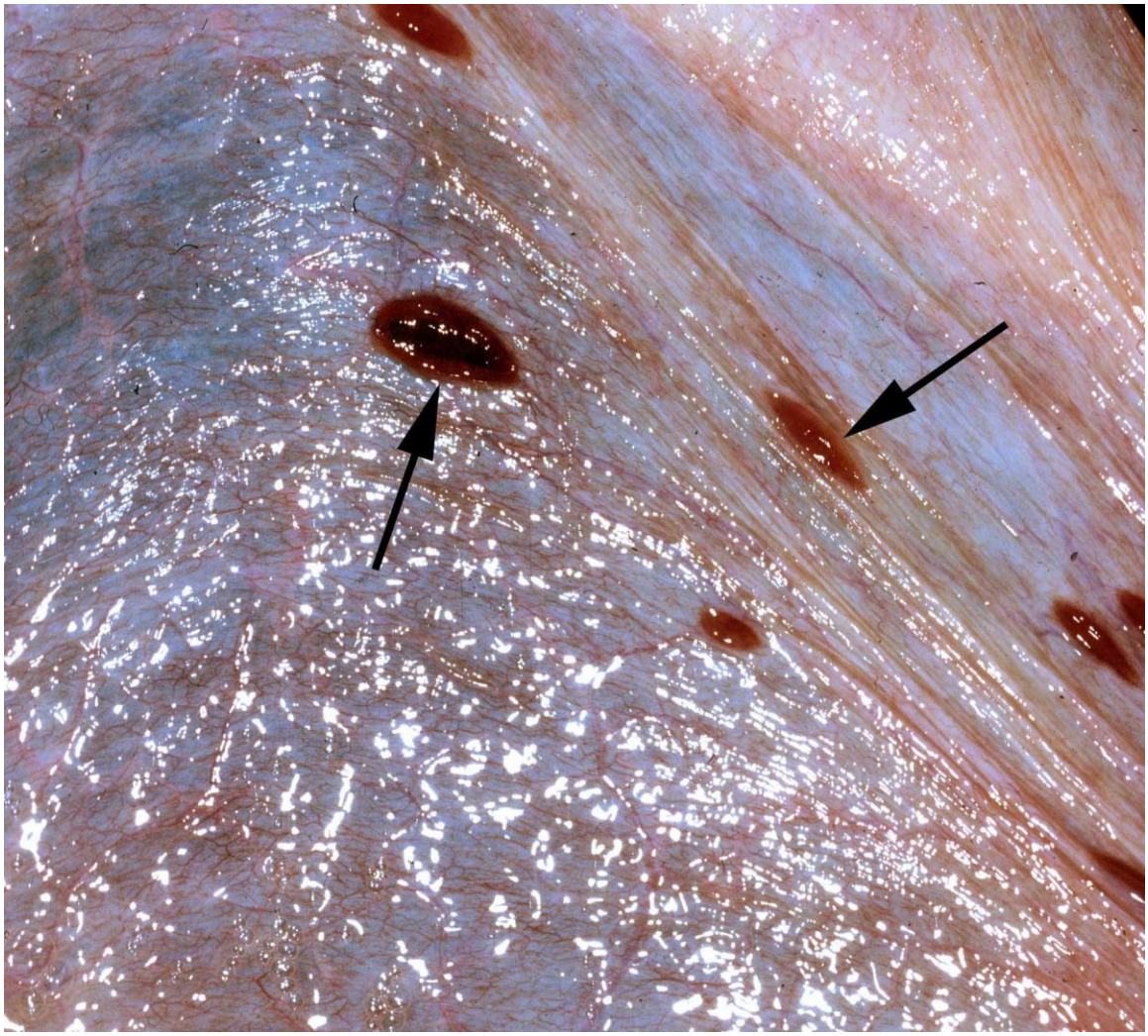

Figure 1-7. Esophageal tonsils of a reticulated python. Esophageal tonsils (arrows) are raised ovoid structures with a central cleft and covered by a mucous epithelium. Courtesy of CRC Press.[1]

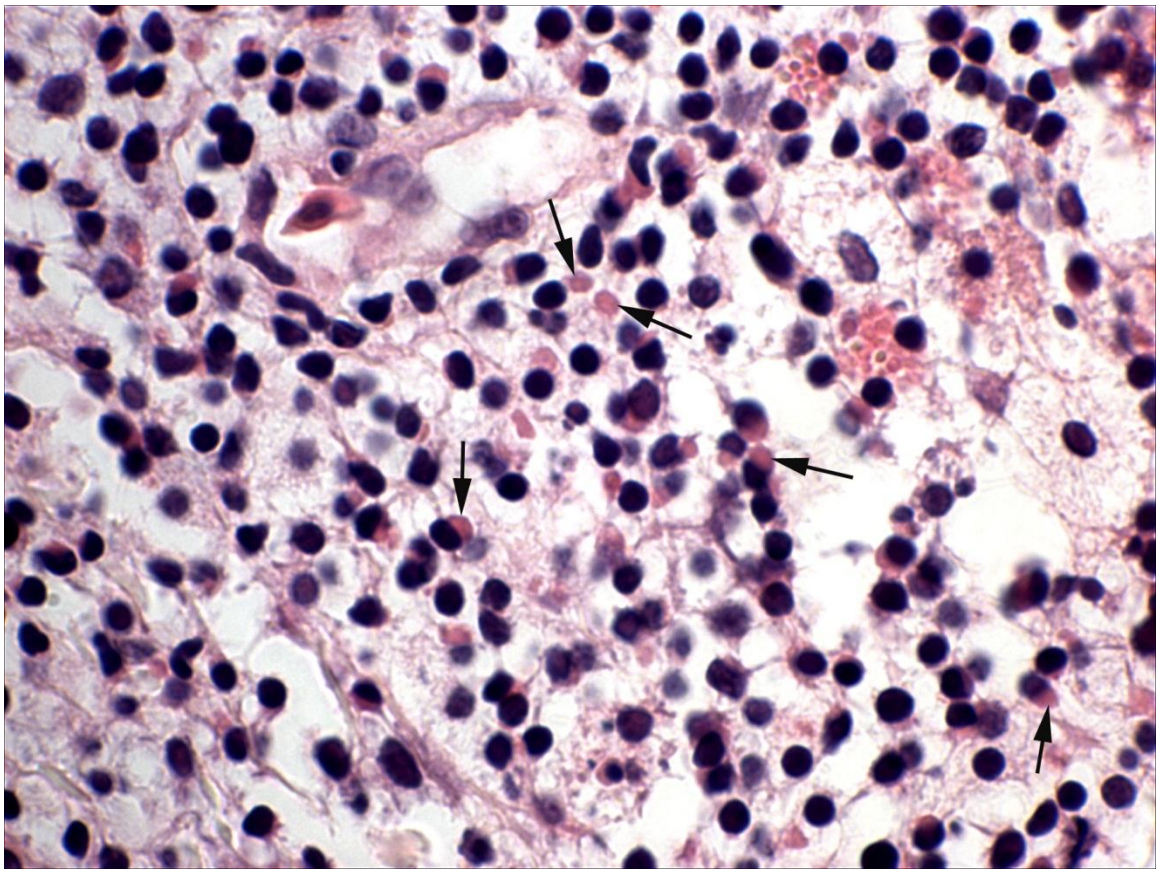

Figure 1-8. Photomicrograph of H&E stained esophageal tonsil from an IBD affected boa constrictor, with numerous eosinophilic intracytoplasmic inclusion bodies (arrows) within submucosal lymphoid cells. Courtesy of CRC Press.[1]

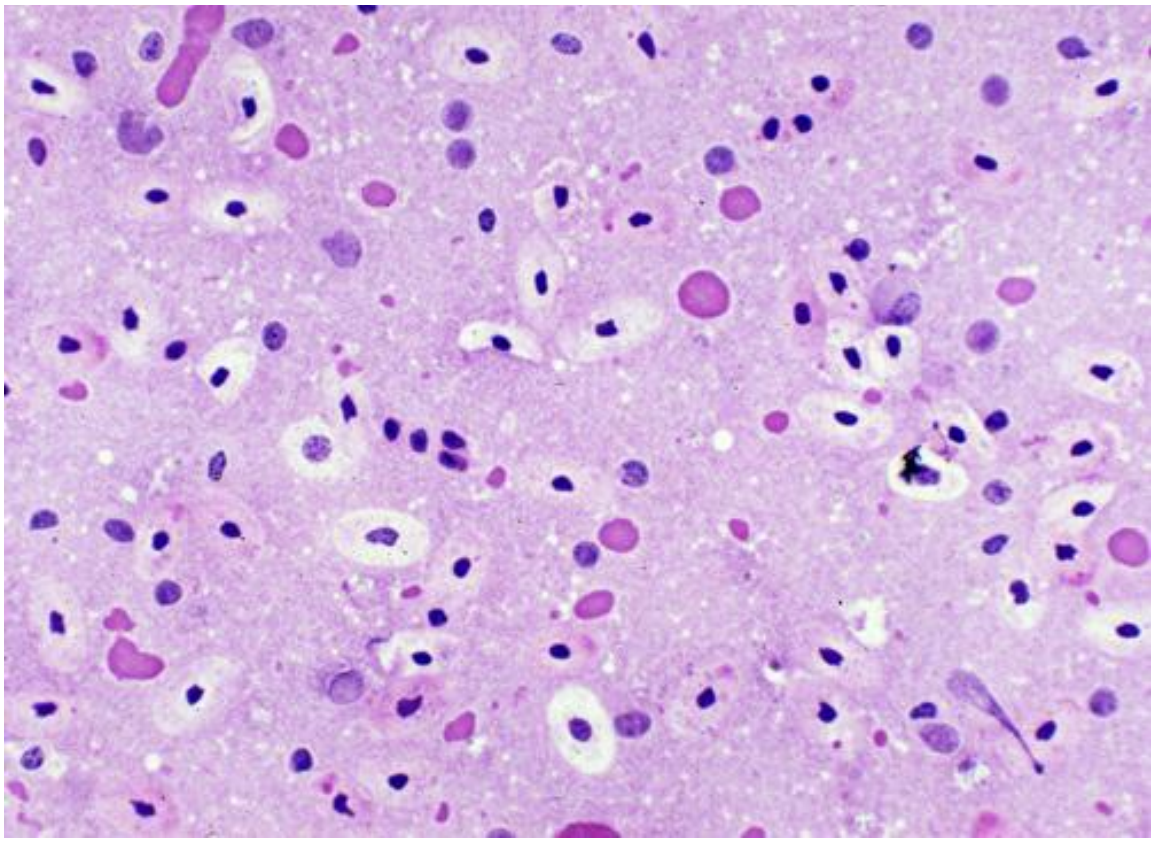

Figure 1-9. Photomicrograph of a cytological impression of liver from an IBD affected boa constrictor. Numerous eosinophilic intracytoplasmic inclusion bodies can be seen in H&E stain. Courtesy of CRC Press.[1]

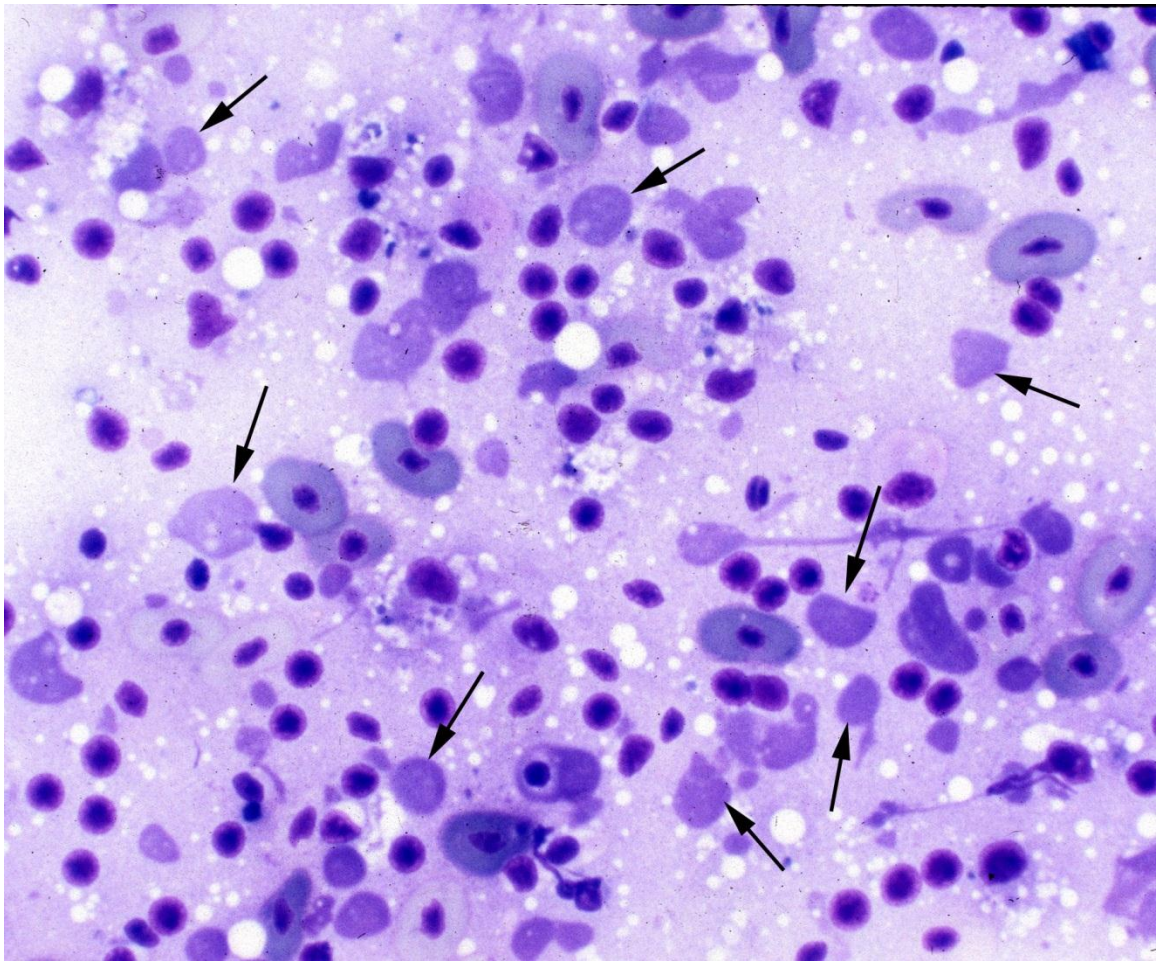

Figure 1-10. Photomicrograph of cytological impression of liver from an IBD affected boa constrictor. Numerous basophilic intracytoplasmic inclusion bodies (arrows) can be seen in Wright-Giemsa stain.[1]

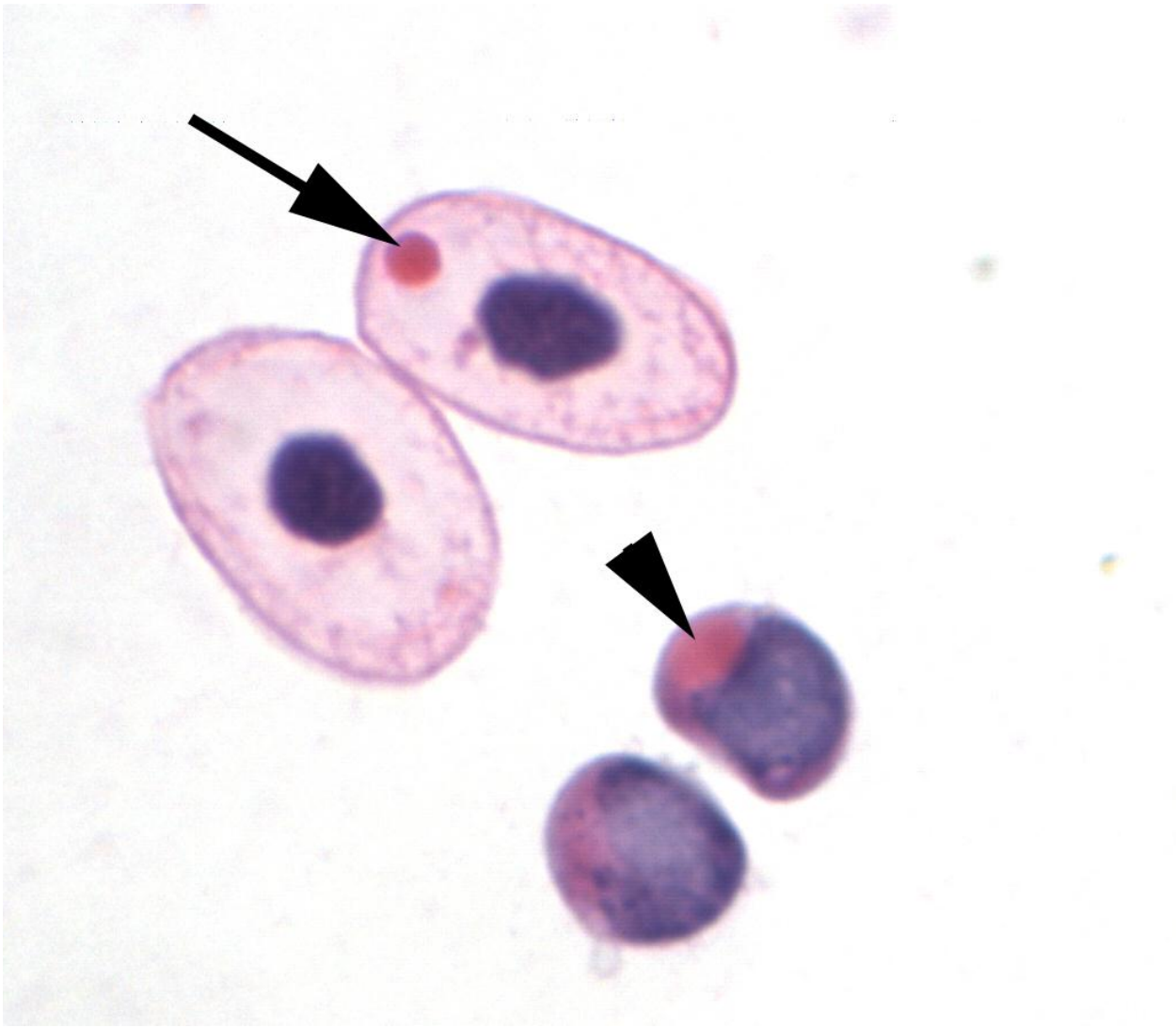

Figure 1-11. A H&E stained peripheral blood film obtained from an IBD affected boa constrictor with an erythrocyte (arrow) and lymphocyte (arrowhead) containing eosinophilic-staining inclusion bodies.[1]

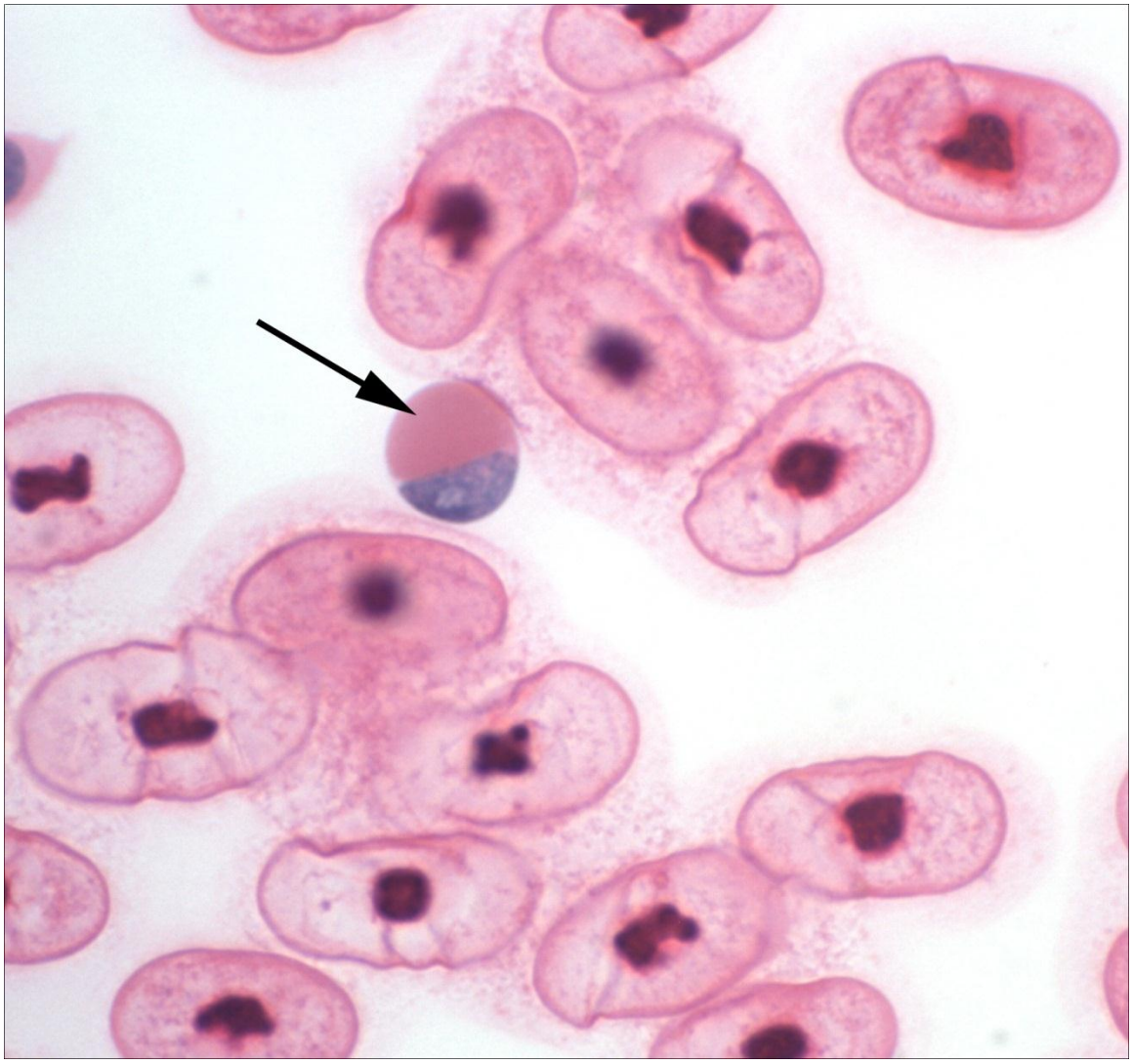

Figure 1-12. A H&E stained peripheral blood film obtained from an IBD affected boa constrictor with a lymphocyte containing an eosinophilic-staining inclusion body (arrow).[1]

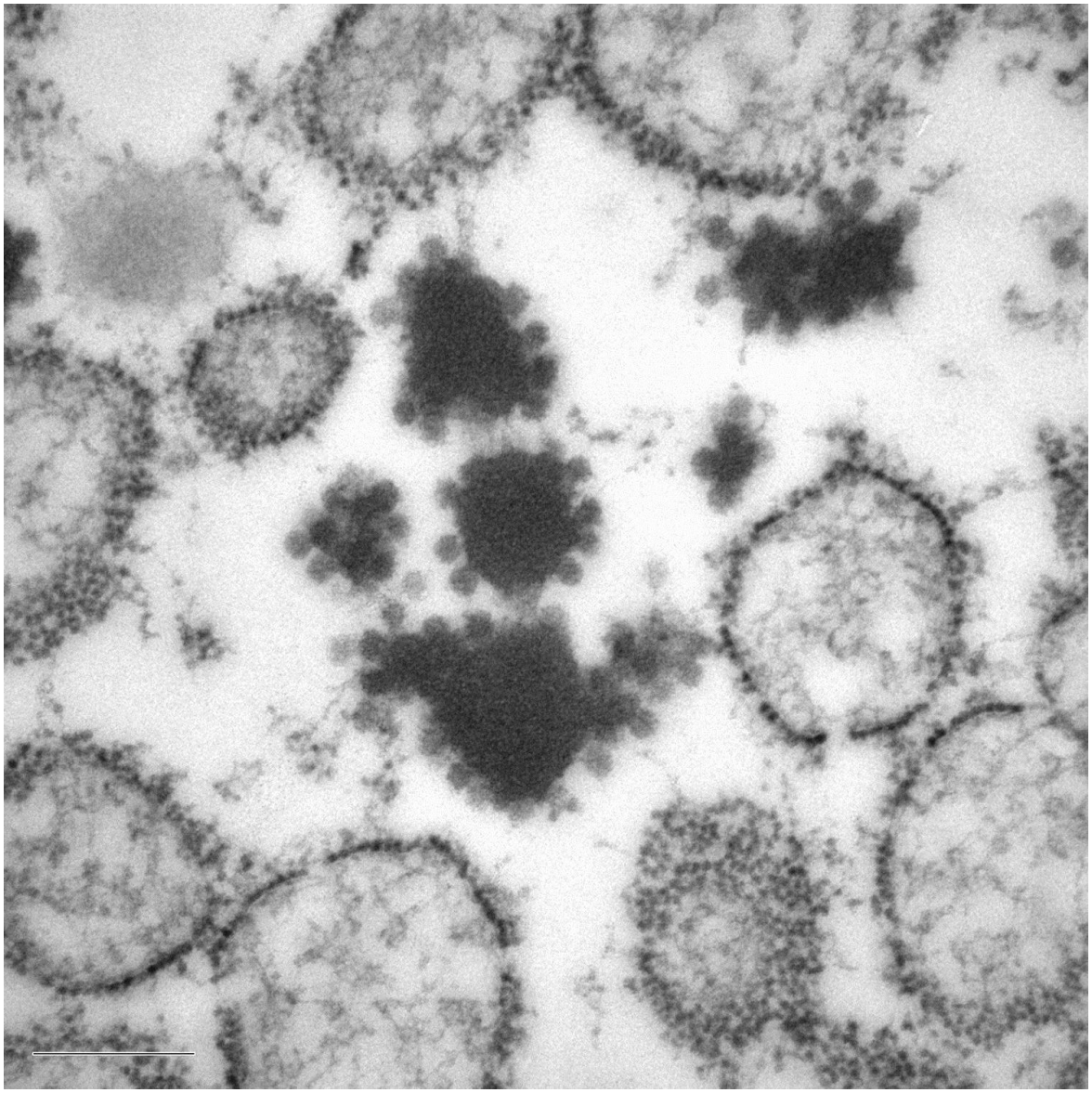

Figure 1-13. Transmission electron photomicrograph of an enterocyte in the small intestine of an IBD affected boa constrictor. During the initial stage of inclusion body formation, protein subunits from polyribosomes start accumulating in the adjacent cytoplasm. Uranyl acetate and lead citrate stain. Courtesy of CRC Press.[1]

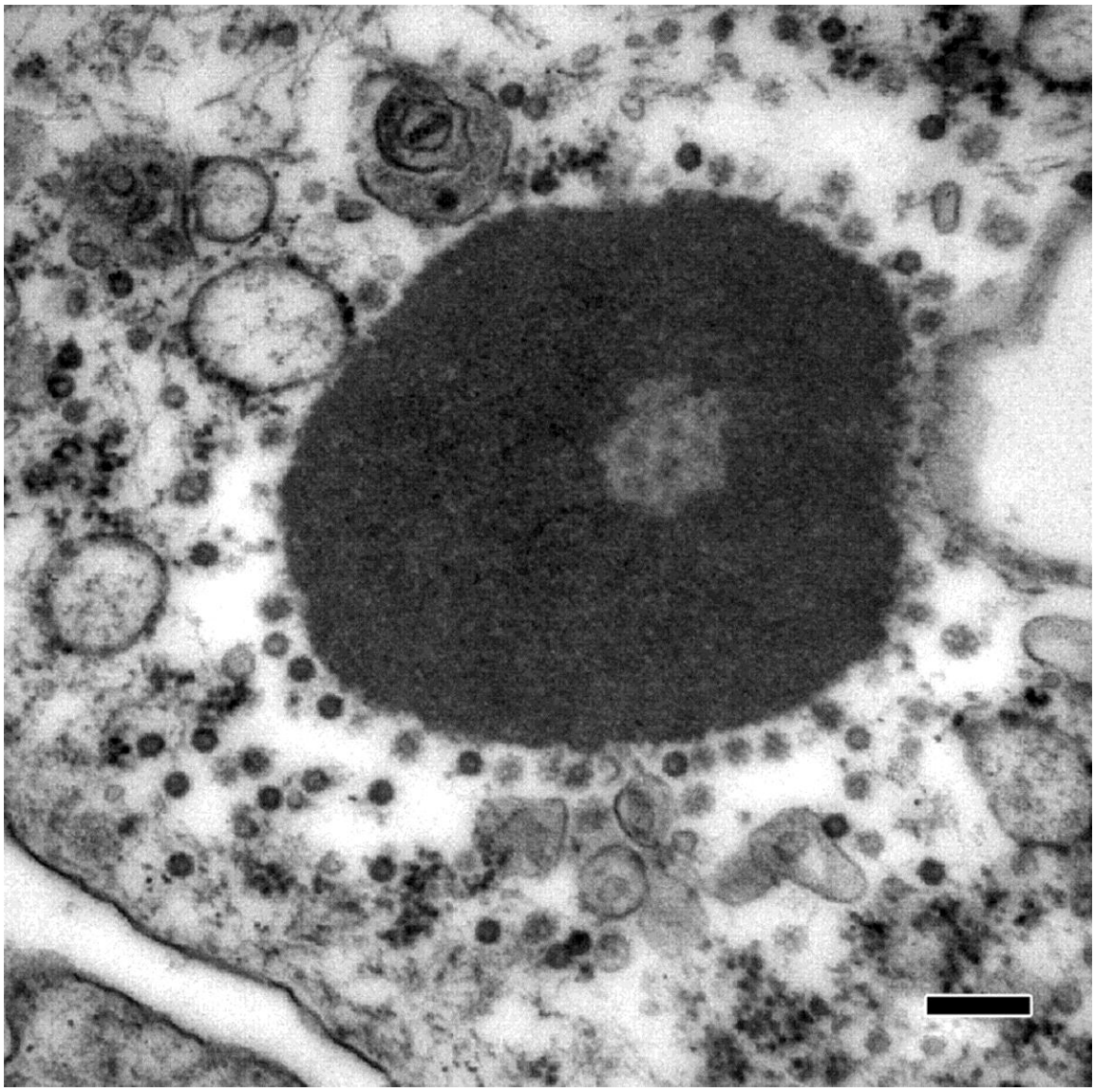

Figure 1-14. Transmission electron photomicrograph of an inclusion body within an enterocyte. Deposited protein subunits have a virus-like appearance. Tissue obtained from an IBD affected boa constrictor with uranyl acetate and lead citrate stain. Courtesy of CRC Press.[1]

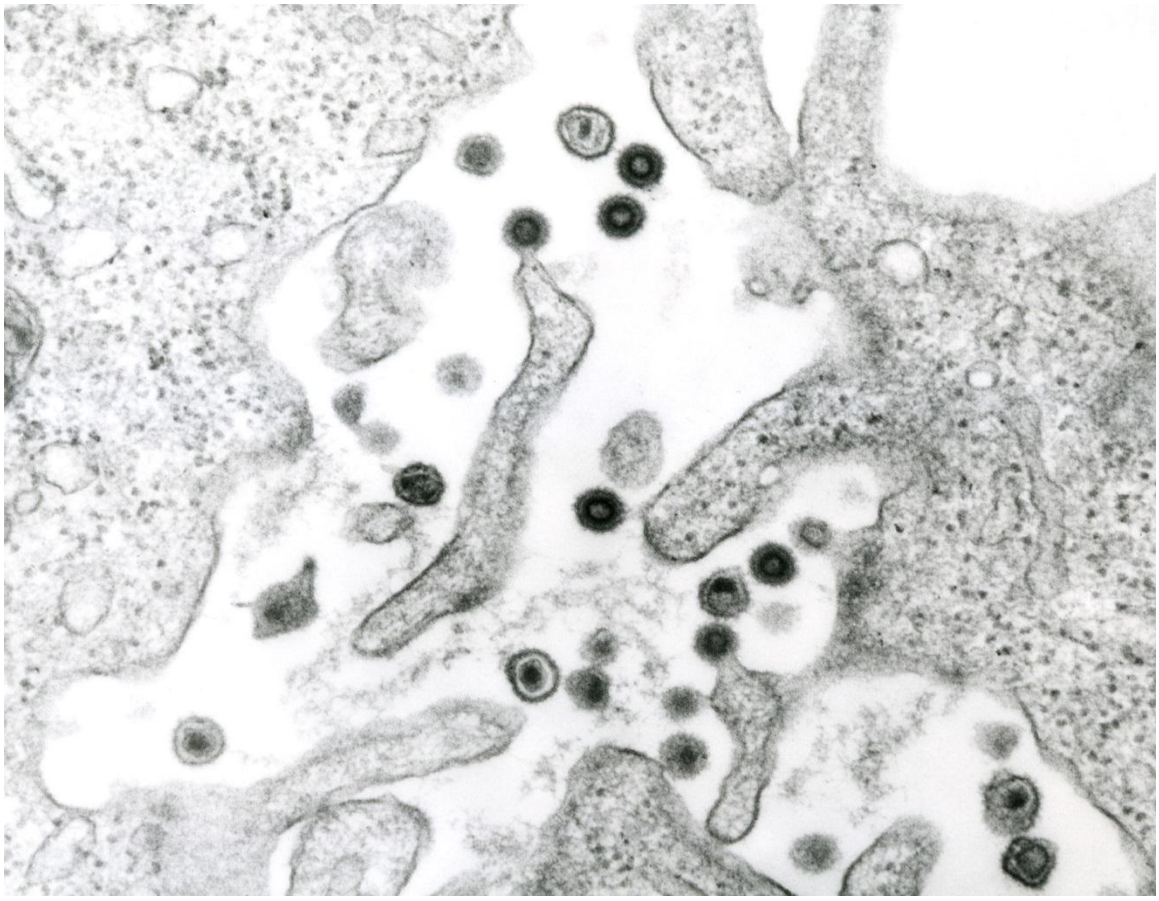

Figure 1-15. Transmission electron photomicrograph of extracellular retroviral particles on the surface of primary kidney cells obtained from an IBD affected boa constrictor. Uranyl acetate and lead citrate. Courtesy of CRC Press.[1]

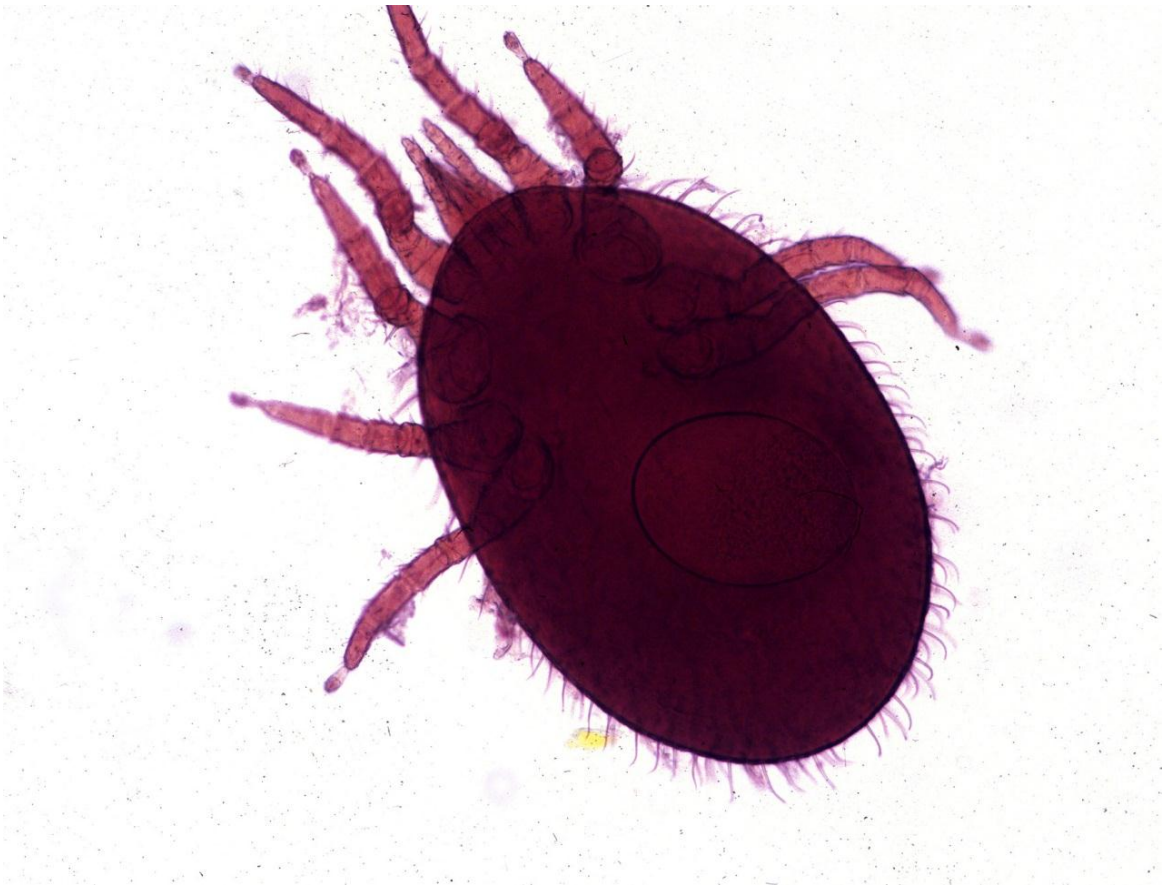

Figure 1-16. Snake mite (*Ophionyssus natricis*). Photomicrograph of a mite removed from a snake. A single egg can be seen within the mite. Courtesy of CRC Press.[1]

CHAPTER 2
THE PRODUCTION OF ANTI-IBDP MONOCLONAL ANTIBODY*

Introduction

In boa constrictors with inclusion body disease (IBD), Wozniak et al.[1] identified a novel antigenically distinct protein within the characteristic intracytoplasmic inclusion bodies. The protein was approximately 68 KDa in molecular weight, and named as inclusion body protein (IBDP). A monoclonal antibody (MAB) was produced against the electro-eluted protein of the excised 68 KDa band obtained from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[4] The MAB was used for immunohistochemical (IHC) staining of the IBD inclusion bodies in frozen liver sections.[4] An antibody recognizing IBDP can be used as a powerful tool for developing molecular or immunobased IBD diagnostic tests. Unfortunately, the hybridoma clone from this study was inactivated after transportation, and the MAB was lost (Wozniak, personal communication).

In the study of Wozniazk et al., the immunogen (IBDP) was not considered to be a highly purified protein since it was obtained from resolved crude liver homogenates on SDS-PAGE.[4] Further, the MAB would only react in sections of frozen material and not react with the IBD inclusion bodies in paraffin embedded tissues.[4] This limited its application for IBDP detection. In order to reproduce an anti-IBDP antibody with broader application, a more purified inclusion body preparation was used as the immunogen in developing a MAB. Immunohistochemical staining of IBD inclusion bodies in paraffin embedded tissues was one of the criteria for hybridoma clone selection. The inclusion

* A portion of this chapter was reprinted from: Chang L, Jacobson ER. Inclusion body disease, a worldwide infectious disease of boid snakes: a review. Journal of Exotic Pet Medicine 2010; 19(3): 216-225, with permission from Elsevier.

body purification procedure was modified from an unpublished project report of a veterinary student's research (Zachary Bissell, 2004), which was adapted from a method for isolation of Mallory bodies.[9]

The entire antibody production procedure was performed in the Hybridoma and Protein Core Laboratories, University of Florida Interdisciplinary Center for Biotechnology Research (ICBR), using the laboratory standard procedures with necessary modifications due to the insolubility of IBDP.

Materials and Methods

IBDP Purification

Tissue homogenization. Unfixed liver and kidney samples obtained from IBD positive or negative boa constrictors were stored in an ultrafreezer at -80°C (IACUC approval #201101156). Approximately, 5 g of liver was thawed, and cut into small portions (approximately 0.3 mm cubes) and homogenized in a Dounce tissue homogenizer with homogenization buffer (HB) containing, 250 mM sucrose, 10 mM EDTA, 10 mM HEPES, pH 7.4. The tissue was processed 1-2 g at a time with 5 mL HB, and the liver homogenate was collected and filtered through two layers of gauze, to remove the larger tissue debris. The unfiltered portion (retentate) was washed with additional 2 mL of HB. One milliliter of the filtered liver homogenate (diffusate) was collected as sample 1, and the remaining homogenate was centrifuged at 1000 x g for 10 minutes. The supernatant was separated from the soft pellet, and subjected to another round of centrifugation using the same conditions as previously described. The supernatant was collected as sample 2, and soft pellets obtained from the first and second centrifugation was pooled together.

Isolation of the inclusion bodies. The pooled soft pellet was resuspended with equal volume of HB, 1 mL of which was collected as sample 3. The remaining suspension was mixed with 1% sarkosyl (Teknova, 2S3380) at a 1:1 ratio, and incubated in 37°C for 30 minutes with frequent vortexing. The suspension was centrifuged at 14,000 x g for 10 minutes in 4°C, and the supernatant was collected as sample 4. The remaining pellet was resuspended with equal volume of 1% sarkosyl, and 500 µL of which was collected as sample 5. The remaining suspension was subjected to one more round of the above procedure, with incubation followed by centrifugation. The supernatant was removed, and collected as sample 6. The pellet was carefully resuspended in 1 mL of HB, and will refer to hereafter as “inclusion body preparation” (IB prep). Ten microliters of IB prep was collected as sample 7, the remaining preparation was stored at 4 °C for future analysis.

Cytospin preparation and hemotoxylin and eosin (H&E) stain. Samples 1 to 7 were diluted 50 fold with water, and 50 µL of each diluted sample was placed in a cytocentrifuge chamber (Biomedical Polymers Inc., BMP-CYTO-S50), and centrifuged for 6 minutes at 800 rpm onto a glass microscopic slide using a cytospin centrifuge (Shandon, Cytospin 2). The slides were air dried, fixed in 10% neutral buffered formalin (NBF; Fisher Scientific, SF100-20) for 10 minutes, washed with distilled water, and stained with H&E stain. The standard cytological H&E staining was performed using an automatic slide stainer (Thermo Shandon, Gemini Varistain), which stained slides as follows: 1) hemotoxylin (Richard-Allan Scientific, 7212) for 2 minutes; 2) incubation with clarifier 2 (Richard-Allan Scientific, 7402) for 15 seconds; 3) followed by incubating with bluing reagent (Richard-Allan Scientific, 7301) for 1 minute, and 4) staining with eosin

(Richard-Allan Scientific, 71311) for 1 minute. In between application of each reagent, the slides were washed with distilled water. Finally, the slides were dehydrated in graded ethanol, dipped in xylene, and coverslipped by permount.

Electrophoresis and gel staining. Equal volume of 2X loading sample buffer (25 mM Tris, 4% SDS, 100mM DTT, and 30% glycerol) was added into aliquots of IB preps or crude liver homogenates, and heated at 95°C for 10 minutes. Then the protein concentrations were estimated by standard protocol of Bradford Protein Assay (Bio-Rad Laboratories, Inc.), using 1:100 dilution of the reduced IB prep. Five micrograms of IB preps and crude liver homogenates were mixed with loading dye, and resolved on a 4%-12% NuPAGE Bis-Tris gel (Novex) with MES buffer (Novex) at 200V constant voltage. The resolved protein was visualized by SimplyBlue (Novex) stain, using the microwave protocol provided by the manufacturer (Novex).

Electro-elution of IBDP. The resolved 68KDa IBDP bands were cut from the gels and packed inside of the glass tubes of the Model 422 Electro-eluter (Bio-Rad, 165-2976). The protein was electro-eluted in Tris-Glycine buffer without SDS, following the protocol provided by the manufacturer (Bio-Rad).

Protein solubilization. Several solubilizing reagents were used alone or in combination in an attempt to solubilize the isolated insoluble inclusion bodies (IB preps). The tested solubilizing reagents included, 8-12 M urea, 6 M guanidine hydrochloride (Gu-HCl), 1% Triton-100, 2% octyl beta-glucoside (OBG), 1% dodecyl maltoside (DDM), 2-4% SDS, 20% lithium dodecyl-sulfate (LDS), 1M DTT, dimethyl sulfoxide (DMSO), bicarbonate buffer and 1% acetic acid. Thirty microliters of IB prep was placed in a 1.5 mL tube, and centrifuged at 12,000 rpm (15,294 x g) for 20 minutes in 4°C using an

Eppendorf 15 Amp Centrifuge Model 5810R (Eppendorf North America, Hauppauge, New York). After centrifugation, the supernatant was removed, and the pellet was resuspended by 30 µL of a solubilizing reagent. After thorough vortexing, the tube was maintained at room temperature (RT) for 30 minutes, followed by observation to see if any inclusion bodies precipitated at the bottom of the tube. Subsequently, the tube with or without precipitation observed was being centrifuged again at 12,000 rpm for 20 minutes. After centrifugation, precipitation of inclusion bodies within the sample that form a pellet was visually confirmed. If the inclusion bodies were solubilized by the reagent, no visible pellet should be seen. If the inclusion bodies were not solubilized by the reagent, a pellet should be seen, and the size of the pellet should be equivalent to the untreated sample. If the portion of the inclusion bodies were solubilized by the reagent, the pellet should appear smaller than the pellet of the untreated sample.

Polyclonal and Monoclonal Anti-IBDP Antibody Production

Mouse immunization. Two female Balb/cByj mice were immunized with approximately 100 µg of isolated inclusion bodies (IB prep of #08-76) diluted in sterile physiologic phosphate buffered saline (PBS) and emulsified in Ribi MPL+TDM adjuvant. The immunogen was administered subcutaneously with a volume of 0.05 mL at both ventral groin sites and 0.1 mL on their dorsal midline on day 1, 21, 44, and 192. The test bleeds were collected 11 to 14 days after the second and third immunizations. Blood samples of 100 µL or less were collected from the tail vein with the mice under a surgical plane of isoflurane anesthesia. The presence of anti-IBDP antibodies in the post-immunized serum were determined by western blots, enyzyme linked immunosorbent assay (ELISA), and IHC staining.

Hybridoma cell production and screening. Six days after the fourth immunization mouse #1 was anesthetized with isoflurane and was exanguinated from the medial canthus of an eye. The mouse was subsequently euthanized using cervical dislocation. The spleen was removed, the ventral margin of the splenic capsule was incised and the B-lymphocyte rich, round cell constituents were flushed from the stroma with sterile serum-free media. Splenic lymphocytes were fused with log phase Sp2/0 mouse myeloma cells with polyethylene glycol (PEG, 1500) at a ratio of 7:1. All resulting hybridomas were suspended in Dulbecco's Modified Eagle Medium (Invitrogen, 11965118) with 1x HAT selective media (Sigma, H0262-10VL) supplemented with 25% sp2/0 conditioned media and 20% fusion tested horse serum (Sigma, H1270). Next they were dispensed into 96 well plates at a concentration of 2 x 10^5 cells per well and grown undisturbed for 5 to 7 days. Two feedings were done by replacing 50% of the cultured media with fresh media before sampling for antibody screening. The cultured media of the growing hybridoma mass cultures were collected and screened for anti-IBDP antibody production by ELISA. The wells that tested positive were transferred to 24 well plates.

Western blot. Aliquots of IB preps were reduced by addition of 4X NuPAGE LDS sample buffer (Invitrogen, NP0008) and 10X NuPAGE reducing agent (Invitrogen, NP0004) with 500 mM DTT, followed by heating over 95°C for 10 minutes. The reduced IBDP was resolved on a 10% or 4%~12% NuPAGE Bis-Tris gel (Invitrogen), with MOPS running buffer (Invitrogen) at 200V constant voltage. The resolved protein was transblotted onto a nitrocellulose membrane using standard protocol of the iBlot dry blotting system (Invitrogen). The membrane was blocked overnight at 4°C with 5% non-

fat dry milk dissolved in PBS, and subsequently washed three times the following day. Each wash was done by incubating the membrane with PBS containing 0.05% Tween 20 (PBST) on an automated rocker for 5 minutes. Each lane of the membrane was separated by a Fast Blot-Developer manifold (Pierce, 88040) and incubated with serum of mouse #1 or mouse #2 in a dilution of 1:100 or 1:500 for 1 hour. For hybridoma mass culture and clone screening, each lane was incubated with undiluted cultured medium collected from each hybridoma clone. The blot was washed three times, and incubated with conjugated rabbit-anti-mouse antibody (Sigma, A1902) in a dilution of 1:1,000 for one hour. The blot was washed three times, followed by application of BCIP/NBT alkaline phosphatase substrate (Sigma, B5655), and developed for 3 to10 minutes until the color of the reacting band reached desired intensity. The blot was then rinsed with water and air dried.

ELISA. Flat bottom 96 well assay plates were coated with IB prep that was diluted in optimal concentrations (10, 20, 30, 40 µg/mL) with bicarbonate buffer. The IB preps isolated from liver and kidney of 2 different IBD positive snakes (#08-76, #08-122) were used as coating antigens on separate plates. The plates were loaded with 50 µL diluted antigen per well, sealed, and incubated overnight at 4°C, followed by a wash procedure with PBST. The wash procedure was performed using a microplate washer (Biotek, ELx405 Select CW). Each well was filled and aspirated four times with 300 µL wash buffer. The plate was blotted on a paper towel to remove the residue buffer. Each coated well was blocked with 250 µL of 1% bovine serum albumin (BSA) in PBS incubated overnight at 4°C, and washed the following day. Fifty microliters of diluted mouse serum or undiluted cultured medium of the hybridoma cells was applied to each

well, and incubated for 1 hour at RT. The plate was washed, incubated with 50 µL per well of a 1:1000 dilution of alkaline phosphate conjugated rabbit anti-mouse IgG antibody (Sigma A, 1902) for 1 hour at RT. After being washed again, 200 µL of para-nitrophenyl phosphate substrate (PnPP; Sigma, N2765) was added into each well and developed for 1 hour at RT. The reaction was stopped with 50 µL of 3M NaOH. The direct optical density (OD) values of each well were recorded using a Spectramax Plus 384 plate reader (Molecular Devices) with the absorbance setting at 405 nm. The sample that had the highest OD reading on four different IB preps were further tested for reactivity to the 68 KDa IBDP by western blot, and by its reactivity to IBD inclusion bodies in tissues using IHC staining.

IHC staining. Sections of paraffin embedded tissue on charged glass microscopic slides were deparaffinized in xylene, followed by rehydrating in graded ethanol, and finally rinsed with water. The slides were either treated or not treated with antigen retrieval (AR) procedure. The antigen in the embedded tissue was retrieved by incubating with trypsin (1:3; ZYMED Laboratory invitrogen immunodetection kit) for 5 minutes at 37°C, quickly rinsed with water, and washed by tris-buffered saline (TBS) twice for 5 minutes. For frozen tissues, sections were cut on a cryostat, mounted on glass slides, and air dried overnight. The frozen tissue sections were fixed in acetone for 5 minutes at -20°C, air dried, and washed with TBS. The slides were blocked with Sniper blocking reagent (Biocare Medical, BS966) for 15 minutes at RT, and washed with TBS. The blocked tissues were covered with diluted mouse serum in antibody dilutent (Invitrogen, 00-3218) or with medium collected from the growing hybridoma clones, and incubated overnight at 4°C. The following day, the slides were washed, the

paraffin embedded tissues were incubated with 3% peroxide in methanol for 10 minutes, and the frozen tissue sections were incubated with Peroxo-block solution (Invitrogen) for 45 seconds. The slides were rinsed with water and washed by TBS before covering the tissue with HRP conjugated goat-anti-mouse antibody (Biocare Medical, MHRP520) for 30 minutes at RT. After being washed, the slides were developed with diaminobenzidine (DAB; Vector, SK-4100) for 1 to 5 minutes until the staining was visualized using a light microscope. The reaction was stopped by rinsing the slides with water. The tissues were counterstained with hematoxylin, dehydrated in graded ethanol, placed in xylene, and cover slipped by Cytoseal XYL (Thermo Scientific).

Monoclonal antibody purification. The final selected hybridoma clone was grown in a CL350 Celline Classic Bioreactor flask (Sigma, Z688037) in medium (BD Biosciences, 220511) containing low IgG serum 10% low IgG fetal bovine serum (Invitrogen/Gibco, 16250-078) until the cell population reached a maximal density. The culture medium was harvested, filtered, and circulated over a protein G column (GE Healthcare Protein G Sepharose 4 Fast Flow). The antibody was eluted, concentrated with Amicon Ultra 15 Centrifugal spin filters (Millipore), and buffer exchanged into PBS. The concentration of the purified monoclonal anti-IBDP antibody was determined and stored at 4°C for future validation.

Results

IBDP Purification

Inclusion body preps and total liver homogenates from 3 IBD positive and 2 IBD negative boa constrictors were obtained. The IBD negative liver did not result in any solid pellet after the incubation with sarkosyl, whereas a tightly bound pellet was obtained from the IBD positive samples (Figure 2-1). The quality of each step in

inclusion body isolation was evaluated by examine collected sample 1 to 7 under the light microscope. The isolated inclusion bodies were most concentrated in sample 7 as anticipated. Among the IB preps, the best quality samples were those with the most inclusion bodies and containing the least amount of extraneous cellular materials. The quality of IB prep was determined by the presence or absence of protein bands other than the 68 KDa band, and the intensity of the resolved 68 KDa protein band on a NuPAGE gel. Under light microscopy, the H&E stained IB prep (sample 7) from snake #08-76 appeared as a suspension of primarily eosinophilic globules of various sizes with minimal extraneous material (Figure 2-2). When resolved on the gel, this IB prep consisted of a major intense band with a molecular weight of slightly less than 68 KDa (Figure 2-3, lane 3). Despite the slight shift in molecular weight possibly caused by the differences in the combination of gel and electrophoresis buffer, and the weight marker references being used. This isolated protein was thought to be consistent with the previously described IBDP.[4] The 68 KDa protein isolated from snake #08-76 was highly concentrated from the liver tissue compared to the resolved crude liver homogenate (Figure 2-3, lane 4). All the attempts to completely solubilize the IB prep using the common reducing and solubilizing agents were unsuccessful. Unfortunately, further protein purification by electro-eluting the 68 KDa bands were not successful due to the insolubility of IBDP. Therefore, the semi-purified IBDP (IB prep) from snake #08-76 was decided to be the best immunogen available for antibody production.

Antibody Production and Monoclonal Antibody Selection

Polyclonal antibody that was reactive to the 68 KDa protein band was detectable in the mouse serum by western blot on day 57 post-immunization (Figure 2-4). Cultured media of hybridoma mass cultures derived from splenic lymphotyes of mouse #1 were

screened for reactivity to the IB preps by ELISA. Four different IB preps were used as coating antigens, including liver and kidney isolates from two IBD positive snakes (#08-76 and #08-122). Each hybridoma mass culture was tested for the reactivity against 2 to 3 different IB preps. Of 303 hybridoma mass cultures screened, only 1 culture (5B3) showed a low positive reactivity (OD reading approximately 5 folds higher than the background or negative control) and cross reacted with 3 different IB preps. Additionally, 32 mass cultures that had significantly higher OD readings compared to the background were also selected. Collected cultured media of the 33 selected mass cultures were further tested for the reactivity to IB preps by western blots. Only antibodies produced by mass culture 5B3 showed reactivity to the 68 KDa protein band (Figure 2-5), and reacted to all four different IB preps.

The mass culture 5B3 was further cloned by limiting dilution and seeded at a single cell per well density. Of 72 single colony wells that were screened for reactivity to IB prep by ELISA, 10 wells that showed low positive reactivity (OD read approximately 5 fold of the negative control or background) were selected, and further tested for their reactivity to all four IB preps. The monoclonal antibodies were isotyped and determined to be IgG subtype by ELISA. Seven out of the 10 selected clones were further tested for reactivity to the 68 KDa band of four IB preps by western blots, and for their IHC reactivity to the inclusion bodies in liver and pancreas of snake #08-76 and #08-122. All the tested clones showed positive reactivity to the 68 KDa and inclusion bodies by western blots and IHC staining respectively. The clone 5B3-3D9 that showed less background in IHC staining was selected (Figure 2-6) and grown to high density for subsequent purification. From a 120 mL of cultured medium that was harvested, and

purified, a total yield of 16.9 mg anti-IBDP monoclonal antibody at a concentration of 10.37 mg/mL was obtained. This antibody was isotyped as IgG1 with kappa light chain by IsoStrip.

Discussion

The most challenging issue encountered during protein purification was the insolubility of semi-purified IBDP (IB prep). The inclusion bodies remained as solid insoluble particles that made it difficult for further purification, sequencing, and other downstream protein analysis.

Insolubility of the IBD Inclusion Bodies

Not solubilized by common solubilizing reagents. Multiple common protein solubilizing reagents were used in an attempt to solubilized the IB prep, including 8-12 M urea, 6 M Gu-HCl, 1% Triton-100, 2% OBG, 1% DDM, 2-4% SDS, 20% LDS, 1 M DTT, DMSO, bicarbonate buffer and 1% acetic acid. All reagents failed to solubilize the inclusion bodies completely. Even when combined with reducing agents in high concentrations, such as 4% SDS, 160 mM DTT, and 12 M Urea, the inclusion bodies still would not completely solubilize. Heating the IB preps with combinations of reducing agents over 95°C for at 5 to 20 minutes was strictly required to reduce the protein, and even with this only a portion of the sample solubilized. The extreme insolubility is a very unique feature of IBDP. Similar degree of protein insolubility had been described in Huntingtin fragment aggregates, a prion-like protein.[11] Heating at 100°C with combination of 1.25% SDS and 1.25% beta-mercaptoethanol had been used for denaturing insoluble prion.[12] The insolubility of IBDP and the nature for aggregate formation may indicate that IBD shares a similar disease mechanism with other protein aggregate forming diseases.

Hydrophobic characteristics. Certain characteristics of the semi-purified inclusion bodies observed during sample processing indicated that the inclusion bodies may have very hydrophobic characteristics. When the IB preps were placed in aqueous solutions (such as water and normal saline), the inclusion bodies form a tight binding pellet after centrifugation, which was difficult to resuspend. The inclusion bodies also bound to the walls of plastic tubes and pipet tips that were subsequently lost during processing. When the IB preps were placed in solution containing urea, Gu-HCl, OBG, sarkosyl and other detergents, the inclusion bodies formed a “less bound” pellet that was easier to resuspend. This also reduced the binding to plastics. In some cases, combinations of Gu-HCl, urea, or OBG with DTT reduced the binding of IBDP. The stickiness of IBDP in aqueous environment may explain the nature of forming cytoplasmic aggregates. And the high hydrophobicity of IBDP made protein analysis more difficult.

Electro-elution of IBDP was unsuccessful. In Wozniak’s study, IBDP (68 KDa band) obtained from liver homogenate was electro eluted from the excised gel, and used as immunogen to produce monoclonal anti-body against IBDP.[4] When semi-purified IBDP (IB prep) were used in this study, the electro eluted IBDP was not soluble. The protein coated onto the membrane inside collecting chambers of the gel eluters (Figure 2-7), and was not possible to dissociate the protein from the membrane. The solution collected from the collecting chamber was tested for protein concentration using Bradford Protein Assay (Bio-Rad) and was found too low to be effectively used for antibody production. Interestingly, this insolubility issue was not reported by Wozniak et al., [4] who first electro eluted the 68 KDa IBDP derived from crude liver homogenates.

Approximately 15 mg of IB prep was resolved into six large SDS-PAGE gels, all eluted protein was not retrievable.

Inaccurate Estimation of Protein Concentration

Bradford Protein Assay is commonly used to estimate protein concentration in solutions by comparing the coloration of protein binding dyes in an unknown sample to a set of standard protein with known concentrations.[10] The insolubility made it difficult to estimate the concentration of IB preps. Heating the IB prep with reducing agent was crucial for the protein to be partially detectable in the Bradford Protein Assay. However, high concentration of detergents in the sample interfered with the coloration of the assay, and resulted in inaccurate estimation of the protein concentration. For example, protein concentration of #08-76 liver IB prep was estimated at 7.6 mg/mL, whereas the HB (without any protein) was estimated to have protein concentration of 6.4 mg/mL. This inaccuracy may resulted in overestimating the amount of protein in the IB prep. For antibody production, protein quantity is very important in the immunization procedure, and for standardization of the assays for antibody screening, such as, ELISA and western blot. Additionally, instead of solely relying on the estimated protein concentration, for each IB prep to be used as an antigen in assays, a standardization run was performed prior to the actual screening test to verify how much volume of IB prep was sufficient to react in assays.

Challenges in Antibody Screening

Ideally when screening for the monoclonal antibody, a purified protein should be used as the antigen for screening assays to avoid selecting an antibody that has non-specific reaction to other undesired protein. Unfortunately, a purified IBDP was not available. Due to the insolubility, further purification methods such as 2-D

electrophoresis, or liquid chromatography could not be performed on semi-purified IBDP (IB prep). Alternatively, IB preps derived from different tissues (liver and kidney) and also from different boa constrictors were used as antigen for antibody screening. When screening the mass cultures, only those that reacted with all IB preps were selected to avoid choosing an antibody that is non-specifically reacting to contaminants from liver or kidney. The specificity of the clones was confirmed by western blot and IHC staining, which ensured the antibody selected reacted to the 68 KDa protein and the inclusion bodies with minimal background staining.

Conclusions

During the ten-month-antibody production process, a monoclonal anti-IBDP antibody producing clone was found in one out of 303 tested viable hybridoma mass cultures using ELISA and western blot. Despite the difficulties working with the insoluble protein, the final selected antibody produced by hybridoma clone 5B3-3C9 appeared to be very specific to the native and reduced IBDP. The MAB was purified, and was isotyped as IgG 1 with kappa light chain. This antibody can be used in various immune-based assays, such as ELISA, western blot, IHC staining on frozen and paraffin embedded tissues. In contrast to the antibody produced by Wozniak et al., [4] which did not react with IBDP in paraffin embedded tissues. The new anti-IBDP antibody has greater advantages, which can be used in retrospective studies and diagnostics on paraffin embedded tissues.

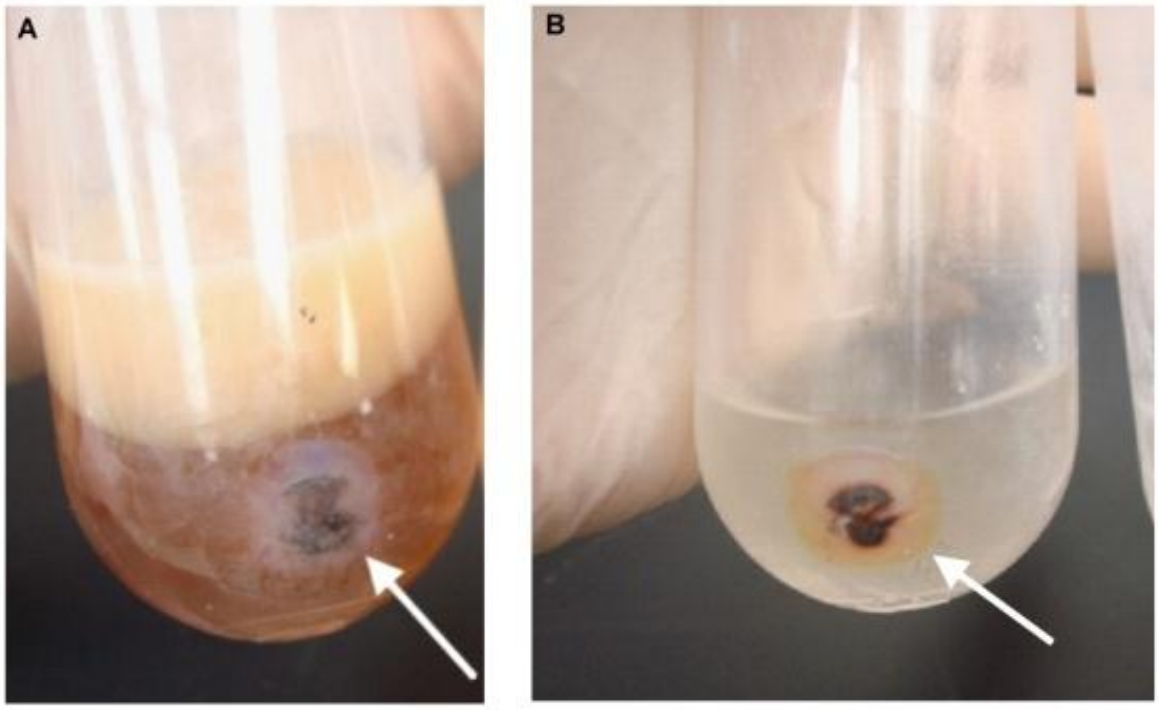

Figure 2-1. The isolated inclusion bodies obtained from 3 g of IBD positive liver. The arrows showing pelleted inclusion bodies in 1% sarkosyl after centrifugation. A. The pellet after first round of incubation with 1% sarkosyl. B. The same pellet after second round of incubation with 1% sarkosyl.

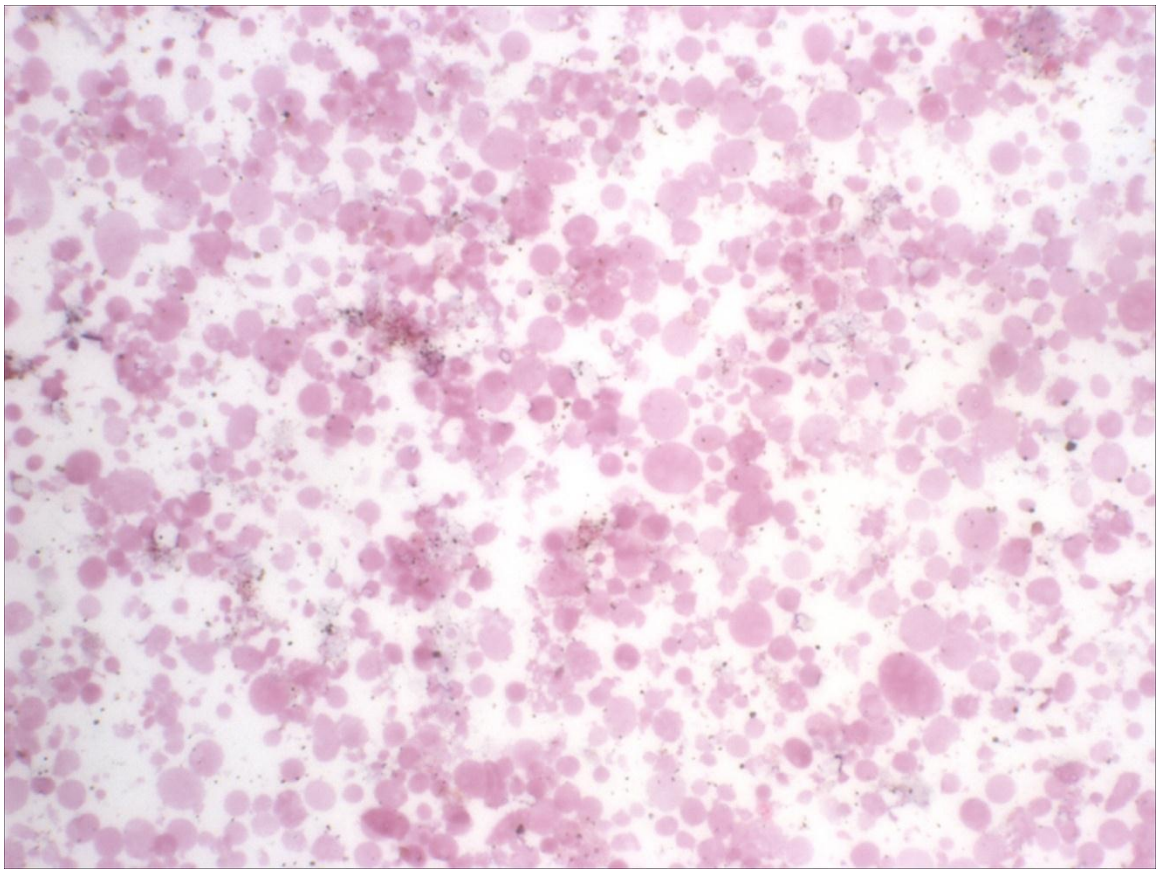

Figure 2-2. The H&E stained IB prep on a microscopic slide. The semi-purified inclusion bodies were used to immunize mice for antibody production.[1]

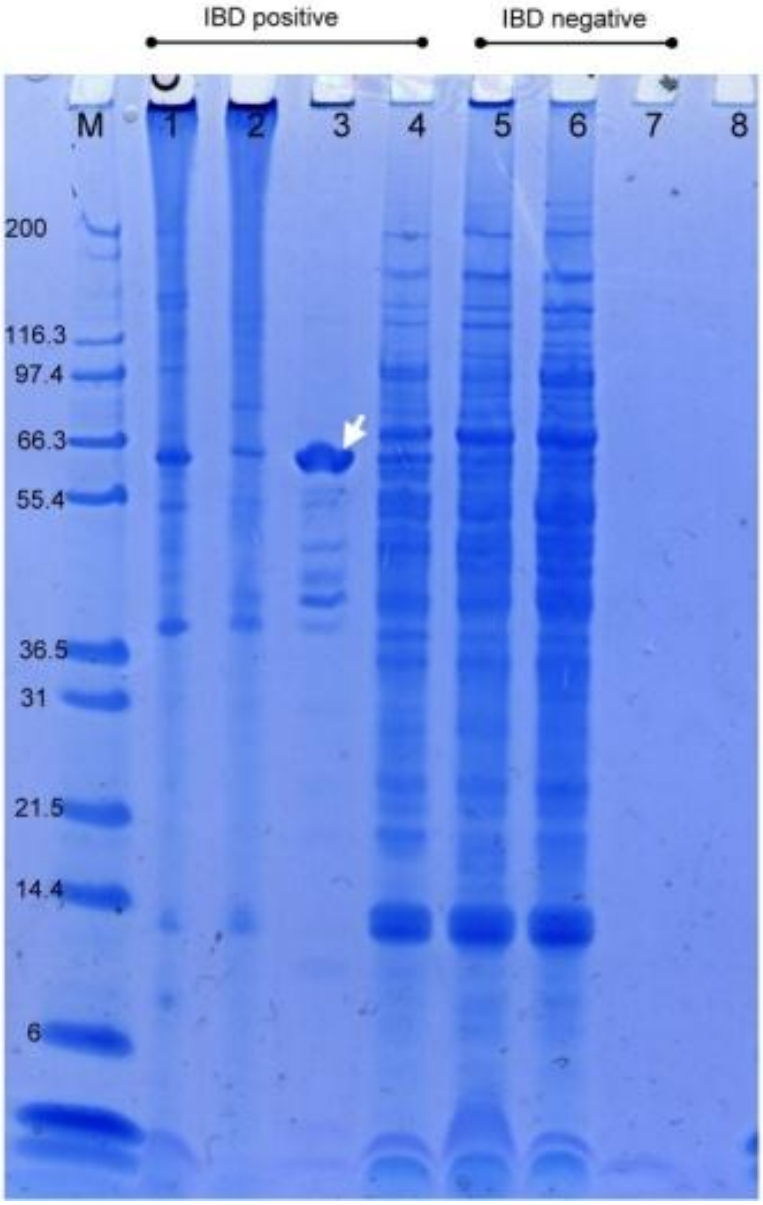

Figure 2-3. The resolved IB preps and liver homogenates on a NuPAGE. Ten microliters of protein were loaded on each lane. Lane 1 to 3 are three different IB preps obtained from three IBD positive boas. Lane 4 is the liver homogenate from the same boa as lane 3. Lane 5 and 6 are liver homogenates from two IBD negative boas. Lane 7 is the IB prep derived from an IBD negative boa, which no pellet were left after incubating with 1% sarkosyl. Lane 8 is HB served as a blank control. The IB prep from #08-76 showed a major intense band approximately at 68 KDa (arrow), showing that the IBDP is concentrated in the IB prep compared to the liver homogenate (Lane 4). M. Molecular weight marker (Invitrogen, Mark 12).

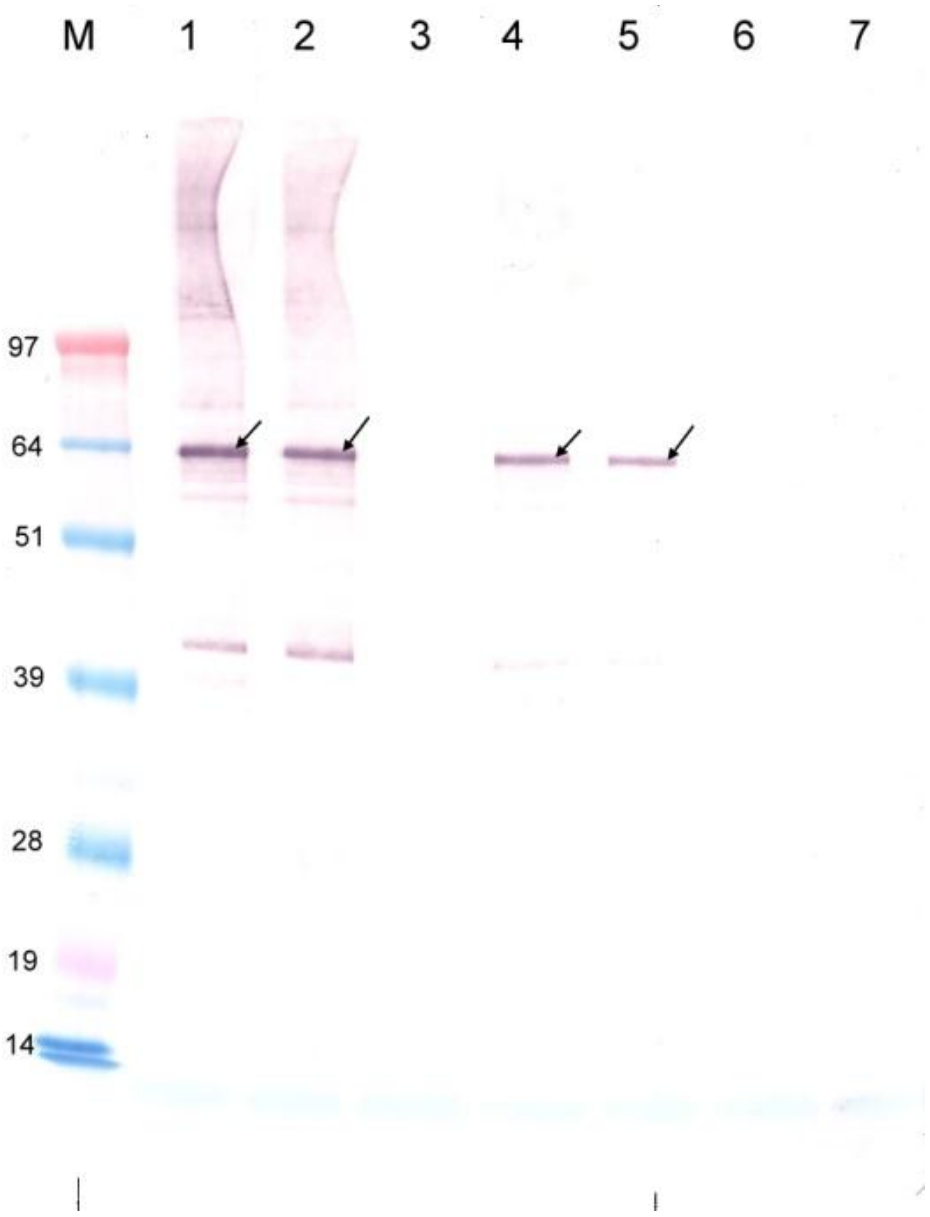

Figure 2-4. Western blot showing the polyclonal antibody reacted with the 68 KDa protein in the IB prep (arrows). Each lane contained 16 µg of IB prep and were detected by post immunized mouse serum collected on day 57 from mouse #1(lane 1 and 4) and mouse #2 (lane 2 and 5). Serum of an un-immunized mouse was used as negative controls (lane 3 and 7). Lane 1 to 3 used mouse serum in 1:100 dilution. Lane 4 to 6 used mouse serum in 1:500 dilution. Lane 7 was a blank control without primary antibody. M. Molecular weight marker (Invitrogen, SeeBlue Plus 2).

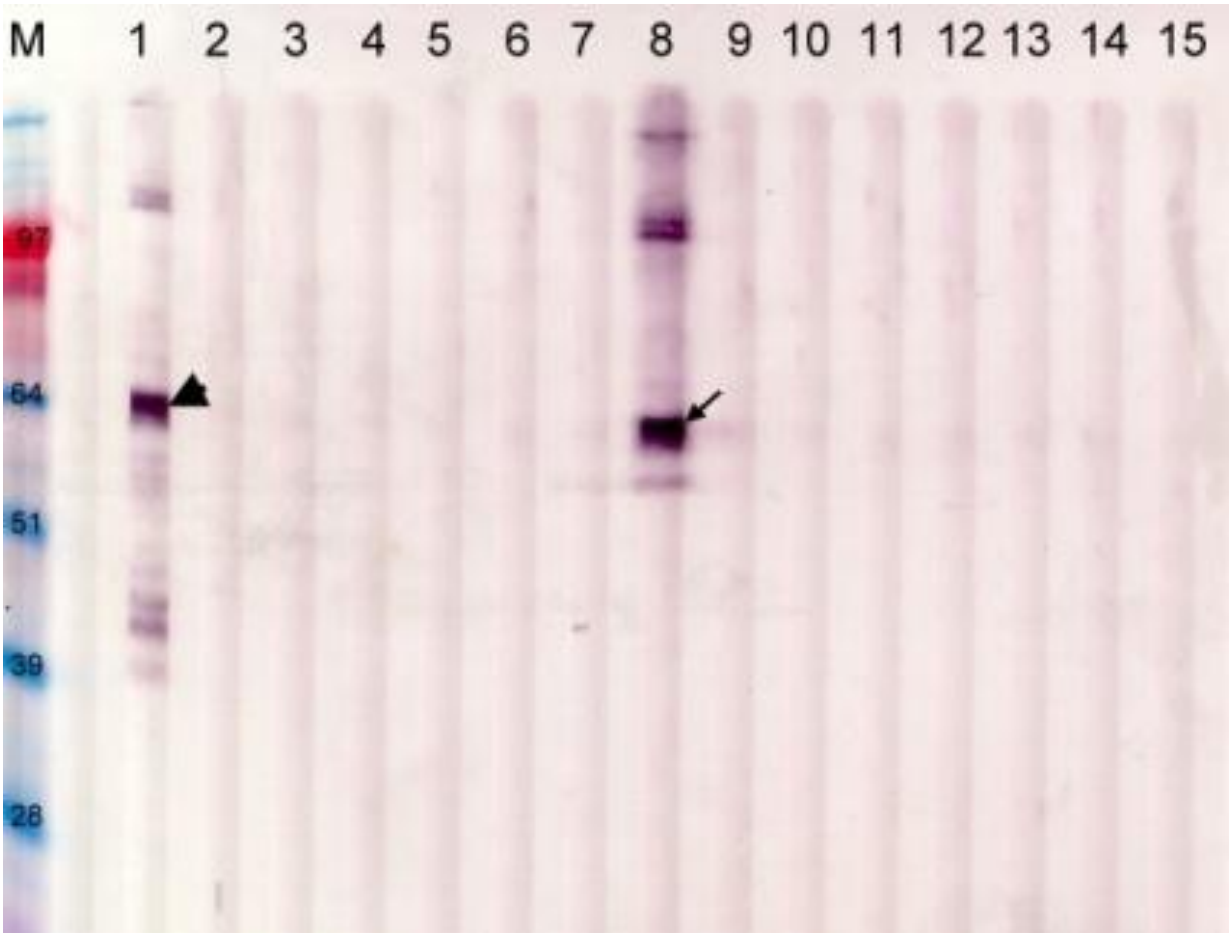

Figure 2-5. Western blot showing presence of antibody that reacted with the 68 KDa protein (arrow) in the cultured medium from hybridoma mass culture 5B3 (lane 8). A total of 140 µg IB prep from liver was blotted onto the membrane, and each lane was detected with a different antibody separated by a Fast Blot-Developer manifold. Lane 1 was detected with mouse #1 serum in 1:1000 dilution as a positive control (arrow head). Lane 2 to 15 were detected with undiluted cultured supernatant collected from different hybridoma mass cultures, only mass culture 5B3 showed reaction to the 68 KDa protein band. M. Molecular weight marker (Invitrogen, SeeBlue Plus 2).

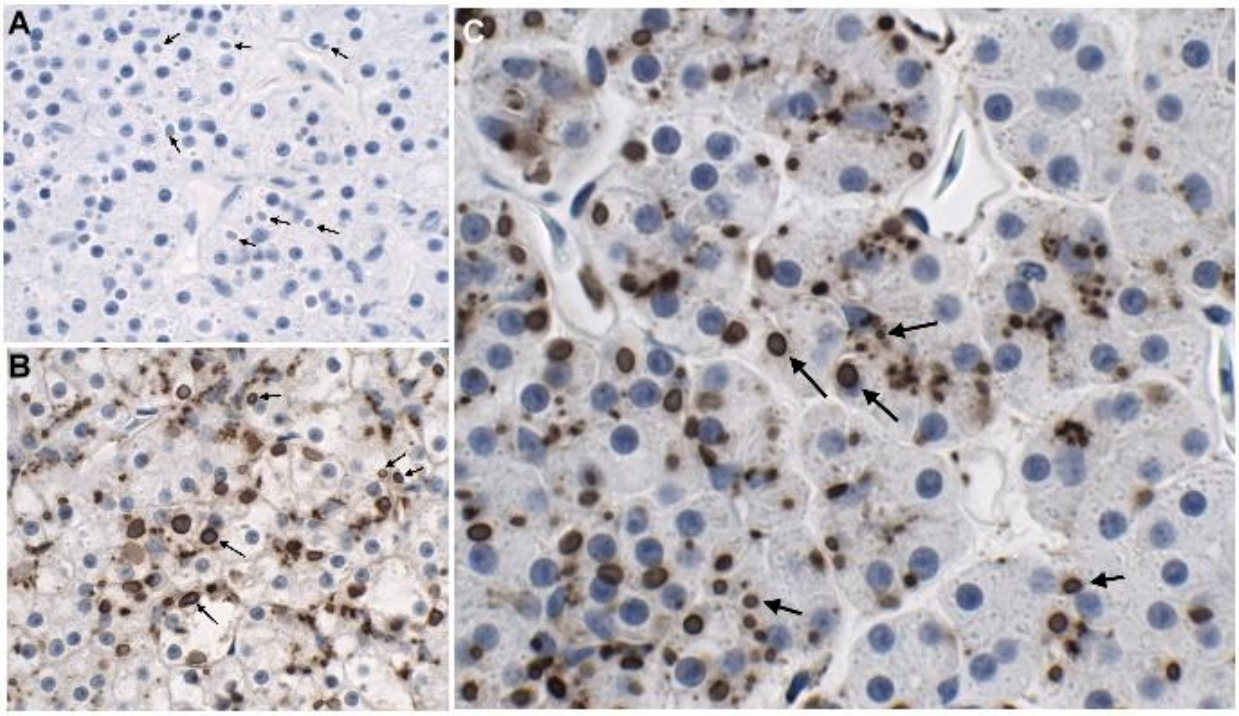

Figure 2-6. IHC staining of paraffin embedded pancreas from an IBD positive boa constrictor using polyclonal and monoclonal antibody. A. A negative control using a non-specific antibody, the inclusion bodies were not stained (arrows). B. A positive control using 1:800 diluted serum from mouse #1, the inclusion bodies were stained (arrows). C. The inclusion bodies are stained with undiluted cultured medium of clone 5B3-3D9, the antibody reacted specifically to the inclusion bodies (arrows) with very little background staining.

Figure 2-7. The collecting chamber of Model 422 Electro-Eluter (Bio-Rad, Instruction Manual M1652976B) (Left), which attached inside of a tank that can be applied with electric current (Right). With electric current, the protein migrated from the gel slices into the collecting chamber, and a membrane cap in the bottom was used to trap the protein inside of the collecting chamber. After being eluted, the insoluble IBDP coated onto the membrane cap (red arrow) instead of staying in the solution within the collecting chamber. Permission to use the photograph and the illustration has been granted by Bio-Rad Laboratories, Inc.

CHAPTER 3
VALIDATION OF AN ANTI-IBD PROTEIN MONOCLONAL ANTIBODY FOR USE IN IMMUNOHISTOCHEMICAL STAINING

Introduction

Inclusion body disease (IBD) had been reported in captive boid snakes worldwide. The specific causative agent(s) and the transmission mechanism remain unclear. Currently, a diagnosis of IBD is made based on identifying characteristic eosinophilic intracytoplasmic inclusion bodies in hematoxylin and eosin (H&E) stained histological slides. In some case, inclusion bodies seen in IBD can be difficult to distinguish from other cellular proteinaceus inclusion bodies or cellular granules that may accumulate in the cytoplasm of affected cells. In some cases, the inclusion bodies may not be abundant in visceral tissue, or early developing smaller inclusion bodies may be overlooked in an H&E stained section. An immunohistochemical (IHC) diagnostic test would be of great value in making a more specific diagnosis.[1,4]

Immunohistochemical staining is a well recommended method with high sensitivity and specificity for diagnosing both infectious and non-infectious diseases. With IHC staining, an antibody reacting to a specific antigen can be used to localize the antigen within affected tissue, which increases the accuracy of a diagnosis.[13] In the study of Wozniak et al., a 68 KDa protein (IBDP) that was found only in the tissue of IBD positive (IBD+) boa constrictors was used to produce a monoclonal antibody, and the antibody reacted to the inclusion bodies in the frozen tissue sections using IHC staining.[4] Unfortunately, the antibody did not react to inclusion bodies in paraffin embedded tissues, and several years later the original hybridoma clone was lost. In my previous chapter, an anti-IBDP monoclonal antibody (MAB) was produced against a preparation of semi-purified inclusion bodies of a boa constrictor that was IBD+. On western blots,

this MAB reacted to the 68 KDa IBDP bands; in IHC staining, the MAB reacted to the inclusion bodies in fresh frozen and paraffin embedded tissues. This antibody can be used as a valuable tool for developing immune-based diagnostic tests for screening cases of IBD.

A problem of IHC staining in veterinary medicine had been discussed by Ramos-Vara et al. that there is a lack of high quality antibody that is specifically made for an antigen but can be used among different species.[13] This problem can be more significant for diagnosing diseases in zoological and exotic animal medicine and pathology where more diverse species are seen. Further, there appeared to be a lack of standardization in IHC staining methodology among different veterinary laboratories. The goal of standardization in IHC staining is to achieve reproducible and consistent results within and among different laboratories.[13] Therefore, MAB needs to be properly validated before being used or offered as a diagnostic test. In this study, the MAB was validated using the format of IHC staining on paraffin embedded tissues following the guidelines suggested by Ramos-Vara et al.[13] The conditions of IHC staining using an anti-IBDP MAB were standardized. The factors that may affect the IHC staining, such as, the fixation time in formalin and the storage time in paraffin were evaluated. The cross-reactivity of MAB in other snake species and other inclusion body-like protein were also evaluated. Finally, the diagnostic performance of the IHC test using anti-IBDP MAB was determined.

Material and Methods

Sample Collection and Management

A repository of paraffin embedded tissue blocks were obtained for the period 1990 to 2007 from the Anatomic Pathology Service and Zoological Medicine Infectious

Disease Testing Laboratory, College of Veterinary Medicine, University of Florida (UF). Additional blocks were provided by a private exotic animal pathology service, Northwest ZooPath, Monroe, WA. A Pathology Report was available for each case, in which the diagnosis of IBD positive or negative (IBD-) was noted by certified pathologists based on histological examination of H&E stained sections. For each case, one block that best represented the IBD pathology was selected for IHC staining, otherwise a block that contained liver, kidney, or pancreas was selected. Twenty blocks were retrieved from the storage facility at a time, sectioned and submitted for one round of IHC staining. When completed, the blocks were returned, and another 20 blocks were retrieved for the next round of IHC staining.

Fresh and formalin fixed tissue samples of year 2008 to 2011 were collected by veterinarians in private practices or UF Veterinary Hospitals, and transferred to my laboratory for IBD diagnosis.

Formalin Fixation and Embedding

Fresh tissues including liver, kidney, and pancreas obtained from an euthanized boa constrictor were dissected into approximately 0.5 mm thick sections, placed in cassettes, fixed in 10% neutral buffered formalin (10% NBF) or 4% paraformaldehyde (4% PF) for 48 hours, and finally embedded into paraffin (IACUC approval #201101156).

In the evaluation of effects of prolonged fixation, freshly obtained liver, kidney, and pancreas of an IBD+ boa constrictor were each cut into 10 sections that were approximately 0.5 mm thick. One piece of each sectioned liver, kidney, and pancreas were placed in a cassette (ten sets of tissues), followed by fixation in ten identical containers filled with 10% NBF. On Day 2 (48 hours after initial fixation), Day 7, Day 8, Day 9, Day 15, Day 23, Day 32, Day 39, Day 50, and Day 58, one cassette was

removed and the tissues were embedded in paraffin. For tissue set of Day 58, only kidney and pancreas were embedded.

Tissues from the repository were embedded in the laboratory of Anatomic Pathology Service in the UF Veterinary Hospital (Lab 1). Using an automated processor (Thermo Electric Corporation, Shandon Excelsior), the fixed tissues were dehydrated in graded ethanol, followed by infiltration of xylene and paraffin. The processed tissues were manually mounted in paraffin blocks. In IHC staining standardization, the fixed tissues were embedded in two different laboratories, Lab 1 and the laboratory of Molecular Pathology Core (Lab 2), College of Medicine, UF. The embedding procedures of the two labs can be found in Table 3-1.

H&E Staining for Paraffin Embedded Tissue

The paraffin embedded tissues were stained using an automatic slide stainer (Gemini Varistain, Thermo Shandon, Illinois, IL), which deparaffinized the slides with xylene, and rehydrated the tissue using graded ethanol. The rehydrated tissues were stained with hemotoxylin (Richard-Allan Scientific, 7212) for two minutes, incubated with clarifier 2 (Richard-Allan Scientific, 7402) for 30 seconds, followed by incubating with bluing reagent (Richard-Allan Scientific, 7301) for one minute, then incubated one minute in 80% ethanol before staining with eosin (Richard-Allan Scientific, 71311) for one minute. In between the application of each reagent, the slides were washed with running water. Finally, the slides were dehydrated in graded ethanol, dipped in xylene, and coverslipped.

IHC Staining for Paraffin Embedded Tissues

Except for the steps described as follow, the general IHC staining procedure remained the same as described in Chapter 2 (IHC staining, Polyclonal and Monoclonal Anti-IBDP Antibody Production).

Antigen Retrieval (AR). The deparaffined slides were either treated with AR reagents or were not treated with AR reagents. The following AR reagents were evaluated, trypsin (Invitrogen, Digest-All2), Trilogy (Cell Marque), Citra (Biogenex), Dako Target Retrieval Solution, pH 6.0 (DAKO), Dako Target Retrieval Solution, pH 9.0 (DAKO). The trypsin AR was done by incubating the slides for five minutes at 37°C. The AR treatments with other reagents were done by incubating the slides for 30 minutes at 95°C. For double AR treatment, the slides were incubated with Trilogy for 30 minutes at 95°C, followed by additional five minutes of incubation with trypsin at 37°C. For the standardized IHC staining protocol, Trilogy was used as the standard AR reagent. For some samples that the standard AR was not strong enough to retrieve the antigen, the double AR procedure was used.

Incubation time for primary antibody. The slides were covered by anti-IBDP MAB in a specific dilution (1:1,000, 1:2,000, 1:5,000, 1:10,000, 1:20,000), and incubated for 1 hour at room temperature (RT), or overnight at 4°C. For the standardized IHC staining protocol, the slides were incubated with 1:10,000 diluted anti-IBDP MAB for 1 hour.

Automated staining machine. For the standardized IHC staining, after AR, blocking, the washes and incubation of primary and secondary antibodies were performed in the automated staining machine (Autostainer Plus, DAKO). The slides

were developed manually using procedures described in Chapter 2. Otherwise, the entire IHC staining procedure was done manually.

Peroxidase chromogenic substrates. The HRP-conjugated secondary antibody was visualized by development with diaminobenzidine (DAB; Vector Laboratory, SK-4100) or VECTOR NovaRED (Vector Laboratory, SK-4800) according to the manufacturer's protocol. For the standardized IHC staining, all slides were stained using NovaRED.

IHC Evaluation

Using light microscopy, the intensity of the IHC stain for each slide was given one of the four scores: 0 (no staining), 1 (faint, barely visible), 2 (moderate), 3 (strong). For each IHC staining run, a positive control slide (IHC score 3) and negative control slides (IHC score 0) were run parallel to the slides to be scored. The negative control of each sample was a duplicated slide that stained with a commercial non-specific mouse IgG instead of MAB (described in Chapter 2: IHC staining, Polyclonal and Monoclonal Anti-IBDP Antibody Production).

For evaluating the Effect of Storage Time in paraffin, Species Cross-reactivity, and Antigen Cross-reactivity, the IHC staining results were interpreted as either positive or negative. A positive IHC stain (IHC+) was defined by a visible staining pattern (IHC score 1 to 3) compared to the negative control (IHC score 0). A negative IHC stain (IHC-) was defined by no visible staining pattern (IHC score 0) compared to the negative control (IHC score 0).

IHC Diagnostic Performance Evaluation

Samples classified as IBD+ or IBD- by H&E examination (current gold standard) were classified as true positive or true negative, respectively. The sensitivity, specificity,

positive and negative predictive values of the IHC test, compared to H&E, were calculated following standard procedures.[14]

To assess the effect of storage time in paraffin, the samples collected during 1990-2000 and 2001-2011 that were classified as IHC+ were compared by using the Fisher Exact X^2 test, following standard procedures.[15]

Results

Standardization of IHC Staining Condition

In order to establish a consistent staining performance, the IHC staining conditions were standardized according to the suggested guidelines by Ramos-Vara et al.[15] The IHC staining conditions for the use of anti-IBDP MAB standardized by this study are summarized in Table 3-3.

Species and tissue type. Tissue of the boa constrictors were assigned as the standard species, due to following reasons: 1. The anti-IBDP MAB was produced using liver tissue of a boa constrictor; 2. Within the sample repository, the majority of the samples were from boa constrictors; 3. IBD in boa constrictors were more frequently diagnosed than other species. In IBD affected snakes, the inclusion bodies were most commonly observed in liver, kidney, and pancreas. Therefore, liver, kidney, and pancreas were selected as the standard material for IHC staining. In a clinical perspective, the liver was preferable, since it is larger tissue compared to the pancreas and kidney, and is an easily accessible tissue for biopsy.

Fixation. In this study, tissues that were fixed in 4% PF were stained with only mild AR treatment or no treatment at all. However, 10% NBF is the most commonly used fixative in routine histopathological evaluation of tissues, and was selected as the standard fixative in this study.

Antigen Retrieval. A variety of AR reagents including, trypsin, Trilogy, Citra, Dako Target Retrieval solution pH 6.0, and Dako Target Retrieval solution pH 9.0 were tested to determine which reagent resulted in the best staining intensity in IHC staining on the selected tissues fixed with 10% NBF. When trypsin was used, the blocks that were made in Lab 2 stained strongly with MAB (IHC score 3), but the blocks that were made in the Lab1 stained faintly (IHC score 1) or no staining (IHC score 0). Using harsher AR treatment such as, Trilogy, Citra, Dako Target Retrieval solution (two different pH, pH 6.0 and pH 9.0), the staining intensity was improved to medium or high (IHC score 2 to 3) (Table 3-2). Trilogy was finally selected as the standard AR reagent, because it generated high staining intensity and less none specific background staining compared to the other reagents tested.

Primary antibody. The standard primary antibody dilution was determined by testing different anti-IBDP MAB dilutions until the staining intensity started to decrease. The staining intensity remained high with very minimal background when a dilution of 1:10,000 was used, but when a dilution of 1:20,000 was used the staining intensity decreased. Thus, the dilution of 1:10,000 was determined as the standard dilution for the anti-IBDP MAB. The incubation of the primary antibody showed no significant differences in the staining intensity when incubated 1 hour at RT or overnight at 4 °C. Thus, the 1 hour incubation protocol was selected as the standard condition, and was compatible with incubation time used in the automated staining machine. There were no significant differences in the staining intensity when IHC stains were performed manually or by machine. Thus, the automated staining procedure was selected as the standard protocol, which enhanced ‘run to run’ and ‘inter-run’ consistency.

Detection system. The chromogenic peroxidase detection system was used throughout all IHC staining, with HRP conjugated goat-anti-mouse antibody detected by DAB or NovaRED. The substrate NovaRED stained the reactive sites with a reddish-purple color that contrasted better in the IHC stain than DAB. The substrate DAB stained reactive sites with a brown color that sometimes could be confused with the brown pigments of the hepatic macrophages in liver tissues. In this study, NovaRED was used as the standardized detection system, which showed better contrast with the blue background hematoxylin counter stain (Figure 3-1).

Validation of anti-IBDP MAB with IHC Staining

The anti-IBDP MAB was validated using the standardized IHC staining conditions. The factors to be evaluated during the validation were determined based on the guidelines suggested by Ramos-Vara et al. [13] The factors included the effects of prolonged formalin fixation and storage time in paraffin, and whether MAB cross-reacted with IBD in other species or other inclusion body forming antigens. The overall IHC test sensitivity and specificity was compared against the result of H&E stain, the current gold standard for IBD diagnosis.

a. Effects of prolonged formalin fixation

Ten blocks (Block 1 to 10) each containing liver, kidney, and pancreas that were fixed for a specific length of time (ranged from standard 48 hours to 58 days), were used in this study. Three slides were made from each block, two stained with anti-IBDP MAB, and one stained with non-specific mouse antibody as negative control.

The inclusion bodies within the embedded tissue remained detectable by MAB up to 58 days following initial fixation (Table 3-4). The staining intensity of pancreas was constantly high (IHC score 3) throughout all tested fixation time periods. The staining

intensity of liver and kidney remained high (IHC score 2-3) up to 32 days of fixation. Some uneven staining (IHC score 2 or 3) were observed in liver and kidney beyond 8 days of fixation. More severe unevenness in staining intensity were observed in the liver (IHC score 1 or 2) and kidney (IHC score 0 or 2 or 3) fixed for 39 days and 50 days. Some areas within the kidney fixed for 50 days were not stained. In all negative control slides, no staining of the inclusion bodies was observed. Overall, prolonged fixation up to 58 days did not affect the IHC staining in pancreas, but in kidney uneven staining pattern were observed in fixation of 9 days, 39 days, and 50 days. In liver, the staining tended to be less intense compared to the staining in kidney and pancreas, and more inconsistence staining pattern was observed throughout the fixation time evaluated in this study (Figure 3-2).

The double AR treatment restored the reactivity of anti-IBDP MAB in liver up to 50 days of fixation in 10% NBF (Block 9), and the uneven staining pattern was not observed. When double AR treatment were used in staining liver tissues of Block 8 and 9 (Table 3-3), the inclusion bodies stained dark red and the intensity was stronger than IHC score 3 (IHC score 3+)(Figure 3-3A). Unfortunately, the double AR treatment was judged to be harsher on tissues, which resulted in loss of cellular structural detail. In kidney and pancreas, double AR treatment reduced the staining of inclusion bodies (Figure 3- 3B and C).

b. Effects of storage time in paraffin

The collection dates for paraffin embedded tissues of 94 (60 IBD+ and 34 IBD-) boa constrictors ranged from 1990 to 2011. Without having specific information, the assumption was that tissues were embedded after a standard fixation time of 48 hours in 10% NBF. One block of each case which included previously evaluated tissues (liver,

kidney, or pancreas) was selected for IHC staining. If IBD inclusion bodies were not described in the standardized tissue, an additional block that contained inclusion body bearing tissue was selected for IHC stain. If the standardized tissues were not available, the block containing tissue with IBD inclusion bodies that were noted in the Pathology Report was selected for IHC staining.

The paraffin embedded tissues were divided into two groups: 1. tissue samples embedded within the time period 1990 -2000 (Group 1: 29 IBD+ and 15 IBD-) and 2. Tissue samples embedded within the time period 2001-2011 (Group 2: 31 IBD+ and 19 IBD-). In Group 1, 50/60 IBD+ cases stained IHC+ and 15/15 IBD- cases stained IHC-, which indicated a sensitivity of 82.8% and a specificity of 100%. In Group 2, 26/31 IBD+ cases stained IHC+ and 18/19 IBD- cases stained IHC-, which indicated a sensitivity of 83.9% and a specificity of 94.7% (Figure 3-4). There were no significant differences (p value = 0.99) of sensitivity and specificity between two year groups analyzed by Fisher Exact test. This suggested that the storage time did not significantly affect the performance of MAB in IHC staining.

c. Species cross-reactivity

The reactivity of an anti-IBDP MAB to IBD inclusion bodies in non-boa constrictor species was evaluated by IHC staining using paraffin embedded tissues in the tissue repository. Inclusion body disease positive tissues of six different non-boa constrictor species were tested with IHC staining, which included annulated tree boas (*Corallus annulatus*) (n=3), ball pythons (*Python regius*)(n=4), carpet pythons (*Morelia spilota*)(n=2), emerald tree boa (*Corallus caninus*)(n=1), palm viper (*Bothriechis marchi*)(n=1), and rainbow boas (*Epicrates cenchria*)(n=4) (Table 3-5). The samples of annulated tree boas [8] and palm viper[16] were obtained from previously reported studies.

The anti-IBDP MAB showed species cross-reactivity to IBD inclusion bodies in ball pythons (1 out of 4) and carpet pythons (2 out of 2).

d. Antigen cross-reactivity

The reactivity of the anti-IBDP MAB to IBD-like inclusion bodies in previously reported corn snakes (*Elaphe guttata*) (n=2)[17] and boa constrictors with adenoviral inclusion bodies (n=1) and pox-virus-like inclusion bodies (n=1) were tested by IHC staining. No antigen cross-reactivity of the anti-IBDP MAB was observed in IHC staining.

e. IHC test diagnostic performance

Sixty samples from boa constrictors that were classified as IBD positive and 34 samples that were classified as negative by H&E stain were tested using IHC stain. The sensitivity of the IHC test was 50/60 or 83% (95% CI = 76%, 91%) (Figure 3-4). The specificity of the IHC test was 33/34 or 97% (94%, 100%). The negative predictive value of the IHC test was 10/43 or 77% (68%, 85%). The positive predictive value of the IHC test was 50/51 or 98% (95%, 100%).

Out of 34 IBD negative cases, one case was suspected to be IBD+ based on clinical signs, but was determined to be IBD- after examination of H&E stained sections. Using IHC stain, the anti-IBDP MAB detected small inclusion bodies in liver and brain of this boa. These small inclusion bodies were not recognized with H&E stain. Thus, the one IBD- (H&E stained negative) case was actually IBD+. When included in with the positive group, the specificity of anti-IBDP MAB in IHC staining was 100%.

In testing boa constrictors, the IHC test is expected to perform with sensitivity above 75.8% and specificity above 93.6% on paraffin embedded tissues stored up to 22 years, and fixation in 10% NBF for up to 58 days.

Discussion

This study was designed to validate an anti-IBDP MAB for use in IHC staining to diagnose IBD in snakes. This validation evaluated the following factors that could potentially affect the staining: 1. Tissue fixation time in 10% NBF; 2. Tissue storage time in paraffin; 3. Antigen variation between species; 4. Reactivity to other antigens. The outcome of studies designed to evaluate these factors are discussed below.

Factors That may Affect IHC Staining

Fixation time in formalin. Prolonged fixation in formalin has been considered a limiting factor for IHC staining, because antigenic epitope(s) can be masked by cross-linking during formalin fixation.[18] For immuno-detection purposes, tissue fixation is typically treated very carefully, and fixation is done strictly within the recommended time period (standard 48 hours), which is thought to be critical for obtaining a positive IHC staining reaction with some antigens. Prolonged formalin fixation is presumed to result in decreased antigen detection.[18] However, the indication of a 'prolonged' time period was not clearly defined.[18] In this study, multiple tissues from a snake with IBD were fixed in NBF for time periods up to 58 days following initial fixation. Interestingly, in this study the kidney and pancreas had moderate to strong reactivity to anti-IBDP MAB up to 58 days of fixation, and the same in the liver up to 32 days of fixation. This finding corresponds to findings in a similar study, in which prolonged fixation affected the quality of the staining intensity, however, under- fixation affected the staining intensity more significantly.[18]

Tissue type. In the standardized staining condition using Trilogy AR treatment, the staining of liver was assessed to be less intense compared to the staining of pancreas and kidney regardless of the fixation time. In liver, the inclusion bodies located

at the margin of the tissue stained more intensively then the inclusion bodies located near the center of the tissue section. In liver fixed beyond 7 days, more unevenness in staining intensity was observed throughout the liver section. The uneven staining was also observed in kidney beyond 9 days of fixation. In fixation up to 50 days, the inclusion bodies were not stained in some areas within kidney. However, in pancreas the staining was not affected at all for fixation up to 58 days (Table 3-4). This suggested that the outcome of IHC staining following extended fixation time is tissue dependent. Possibly differences in pH among different tissues or other biochemical differences affected the efficiency of formalin infiltration. Webster et al. had discussed the possible differences in formalin penetrating rate that may caused the uneven IHC staining.[18] It is likely that the formalin penetration was slower in liver, compared to kidney and pancreas. Whether this is due to physical of biochemical properties of liver compared to the other tissues assessed remains unclear. Compared to the antigen at the margin of tissue, antigen located in the center of a tissue may be under-fixed, resulting in less intensive staining compared to antigen at the margin of tissue. Webster et al., found that many antibodies had variable immune-reactivity among different tissue types.[18] In this study, the validation of the anti-IBDP MAB in IHC staining was evaluated using tissues that are most commonly collected for making a diagnosis in IBD.

Selection of AR treatment. Antigen retrieval is known to be a critical step in IHC staining, which retrieves the cross-linked antigenic epitopes especially when tissues are fixed or remain in fixatives for prolonged periods of time. In prolonged fixed tissue that showed mark decrease in staining intensity, optimization of the AR treatment significantly restored the immune reactivity (Figure 3-3). Prolong fixed liver of Block 9

treated with double AR, showed much improved staining intensity without seeing the uneven staining between the margin and center of the tissue (Figure 3-3C). But when double AR treatment was used, the structural details of the stained tissues were lost due to the harsh treatment. In pancreas and kidney, the staining of inclusion bodies faded when double AR was used (Figure 3-3A and B). Thus, the standard AR using Trilogy should be considered as a routine procedure, but if the staining in liver is not satisfying, then double AR can be used.

Variation of blocks made in different laboratories. During the process of standardization, the same tissue processed and embedded in two different laboratories (Lab1 and Lab2) was found to have different requirements for AR. The tissue processed in Lab2 stained strongly with mild AR (trypsin) or even without AR treatment. But the same tissue processed in Lab1 strictly required harsh AR treatments with incubation at high temperature (95°C) in order for the MAB to stain the inclusion bodies. By comparing the tissue processing procedure between the two labs (Table 3-1), the procedures were not significantly different. It is possible that slight differences during tissue process, such as, temperature, incubation time, and reagents purchased from different suppliers may have affected the intensity of IHC staining. In a case collection of 9 IBD+ and 3 IBD- boa constrictors, 3/9 IBD+ cases the inclusion bodies within liver and kidney could only be stained using double AR treatment (Figure 3-5). This may also be caused by variation in sample processing procedures discussed above among different laboratories, however, the processing procedure of the laboratory was not documented. Fortunately, this study evaluated antigen retrieval methodologies and findings indicated the importance of selecting specific AR reagents. In this study the following AR reagents

were evaluated, trypsin, Trilogy, Citra, Dako Target Retrieval solution pH 6.0, and Dako Target Retrieval solution pH 9.0. Of these, Trilogy worked the best when evaluating tissues embedded in several different laboratories. Embedded tissue from most laboratories that submitted samples for testing stained with moderate to high intensity. When staining was weak, double AR can be used to enhance the staining sensitivity. However, this may increase non-specific background staining in the connective tissues of some samples.

To ensure that insufficient AR was not the cause of those cases that failed to stain with IHC, the blocks that did not stain positive (even though inclusion bodies were seen with H&E staining) in IHC stain (including the IBD- cases) were retested with another run of IHC staining using the double AR treatment. Out of 60 IBD+ boa constrictor cases in the repository, 10 cases did not stain positive following standard AR. However, 3 of these 10 cases stained IHC positive when double AR was used. All IBD- cases remained negative of IHC staining when retested using double AR.

IHC Diagnostic Performance Evaluation

False negative results. The sensitivity of the IHC test was not as high as a typical screening test due to the higher false negative value (10/60 or 16.7%) estimated by this study. When 10 previously diagnosed cases of IBD were re-examined, the characteristic eosinophilic intracytoplasmic inclusion bodies were not found in five of these cases. All five stained negative with IHC. The diagnosis of these cases was classified as questionable. The number of questionable cases resulted in the lower sensitivity of IHC test. Four of the questionable cases were within Group 1, and three of them were reported in 1995. This also suggested that a false positive diagnosis of IBD can likely be made by a pathologist, who may have limited experience diagnosing IBD.

False positive results. The estimated specificity of IHC test was very high which is satisfactory for a screening test. However, the specificity remains under estimated, due to a high calculated false positive value (1/34 or 2.9%). There was one case that was diagnosed negative using H&E staining that clearly stained positive with IHC in liver and brain. The inclusion bodies were so small and sporadic that they were missed with H&E staining. This is an example demonstrated the value of IHC testing for IBD. With the exception of this case, the specificity of IHC test using anti-IBDP MAB would be 100% and false positive would be 0.

Application as a screening test for IBD. The high positive predicted value and low false positive of the IHC test indicated that a case having IHC+ result can be confidently diagnosed as IBD+. However, the low negative predicted value and false negative of the IHC test indicated that for clinical applications when a sample tested by IHC is found to be negative, a diagnosis needs to be evaluated carefully in combination with the findings in H&E stain. In one IBD+ case, the inclusion bodies were only detected in the brain, not in any other tissues including spinal cord. This also addressed the importance of tissue selection for diagnosing IBD. If IBD is ranked high on the differential list of diseases in an ill snake, multiple tissues (including the brain) need to be sampled and tested. Based on the estimated sensitivity, specificity, and positive and negative predictive values, the IHC test has more merit as a confirmatory test than as a screening test for diagnosis of IBD in boa constrictors.

Cross Reactivity Among Non-Boa Constrictors

Of the total number of tissues available for this project, the number of IBD cases in non-boa constrictors was limited. Only 15 cases were found in UF and collaborated laboratories between 1990 to 2011. Within six different non-boa constrictor species

tested, only two carpet pythons and one ball python stained positive with IHC staining. Even when double AR was used, the other 12 cases remained unstained. This may suggest that different species may be infected with different strains of the IBD causative agent, resulting in sufficiently different IBDP (epitopes) from the IBDP in boa constrictors. Therefore, the anti-IBDP MAB produced in this study may not be able to recognize IBDP in all species of snakes that appear to be IBD+. Interestingly, the two species that anti-IBDP MAB cross reacted were more distantly related to boa constrictors than other more close related species tested (Table 1-1) such as, rainbow boas, annulated tree boas and emerald tree boas. Nevertheless, the use of anti-IBDP MAB in diagnosing IBD in boa constrictors is well validated, but when testing in non-boa constrictors the results need to be interpreted carefully.

Conclusions

The anti-IBDP MAB was validated using IHC staining on paraffin embedded tissues of 60 IBD+ and 34 IBD- boa constrictors. The IHC staining condition was standardized with the use of 10% NBF as fixative, Trilogy for AR treatment, 1 hr incubation with 1:10,000 diluted anti-IBDP MAB, and NovaRED as detection substrate. Pancreas, liver, and kidney were used as the standard tissues for IHC testing. In kidney and pancreas the IBD inclusion bodies can be detected up to 58 days of fixation in 10% NBF, and in liver the inclusion bodies can be detected up to 50 days of fixation in 10% NBF. There were no significant differences in the IHC test performances between different storage times in paraffin of Group1 (1990 to 2000) and Group 2 (2001 to 2011). The anti-IBDP MAB had species cross-reactivity with IBDP in carpet pythons and ball pythons, and no antigen cross-reactivity with inclusion bodies of adenovirus, pox virus, and IBD-like inclusion bodies in corn snakes.

In testing boa constrictors using anti-IBDP MAB in IHC test, the sensitivity was 83% (95% CI = 76%, 91%), the specificity of was 97% (94%, 100%), the negative predictive value was 77% (68%, 85%), and the positive predictive value was 98% (95%, 100%). In clinical applications, an IBD+ diagnosis can be made confidently when the sample tested IHC+. However, a diagnosis of IBD- needed to be evaluated carefully in combination with the findings in H&E stain, when the sample tested IHC-.

Table 3-1. The tissue processing procedure of two laboratories.

	Lab1 Processor			Lab2 Processor		
Reagent	Inclubated Time (sec/run)	Number of runs	Temperature	Inclubated Time (sec/run)	Number of runs	Temperature
Formalin	30	2	Ambient	N/A	N/A	N/A
70% EtOH	60	1	30°C	1	1	Ambient
				20	1	Ambient
80% EtOH	60	1	30°C	20	1	Ambient
95% EtOH	60	1	30°C	20	2	Ambient
100% EtOH	60	3	30°C	20	2	Ambient
Xylene	40	3	30°C	20	2	Ambient
Paraffin Wax	40	3	60°C	20	3	60°C

EtOH: ethanol. N/A: not applicable.
In Lab1, the reagents used were Alcohol (Decon Laboratories), Histology Grade Xylene (Fisher), and Paraplast (Fisher). Formalin was made with 37% Formaldehyde (Fisher), Sodium Phosphate Monobasic anhydrous (MP laboratories), and Sodium Phosphate Dibasic, anhydrous (Fisher).
In Lab2, the reagents used were NBF 10% buffered (Fisher), Ethanol (Fisher), Histology Grade Xylene (Fisher), and Paraffin Type9 (Fisher).

Table 3-2. IHC score of tissues processed by two laboratories using different AR treatments.

Tissue embedded	Primary antibody	Sample origin	AR reagents					
			No AR	Trypsin	Trilogy	Dako pH9.0	Dako pH6.0	Citrate
Lab 1	MAB	08-122 Pancreas	2	3	3	2	2	2
Lab 2	MAB	08-122 Pancreas	0	0	3	2	2	2
Lab 2	MAB	08-76 Pancreas	1	1	3	2	3	3
Lab 1	None specific mouse IgG	08-122 Pancreas	0	0	0	0	0	0

MAB: monoclonal anti-IBDP antibody.
Boa 08-76 and 08-122 were two IBD+ boa constrictors, the MAB was produced against the inclusion bodies isolated from boa 08-76.

Table 3-3. Standardized IHC staining conditions for anti-IBDP MAB

	Standardized IHC staining conditions
Species	*Boa constrictor*
Tissue type	Liver, kidney, pancreas
Fixative	10% NBF
Fixation time	48 hours
Antigen retrieval	Triology (95°C 30 minutes)
Primary antibody	Anti-IBDP MAB (1:10,000 dilution)
Incubation time	1 hour RT
Secondary antibody	HRP conjugated goat-anti-mouse antibody (MACH 2 Mouse HRP-Polymer, Biocare Medical)
Incubation time	30 minutes RT
Detection system	Chromogenic peroxidase with NovaRed substrate
Counter staining	Hematoxylin (blue)

Table 3-4. IHC scores of IBD positive tissues fixed in 10% NBF over different time period

Block #		1	2	3	4	5	6	7	8	9	10
Fixation Time (day)		2 d	7 d	8 d	9 d	15 d	23 d	32 d	39 d	50 d	58 d
IHC score											
Slide 1	Liver	2	2	2,3	3	2,3	3	2,3	1,2	1,2	N/A
	Kidney	3	3	3	3	3	3	3	2,3	2,3*	3
	Pancreas	3	3	3	3	3	3	3	3	3	3
Slide 2	Liver	2	3	2,3	2	2,3	3	3	1,2	2	N/A
	Kidney	3	3	3	2,3	3	3	3	2,3	3	3
	Pancreas	3	3	3	3	3	3	3	3	3	3
Slide 3	Liver	0	0	0	0	0	0	0	0	0	N/A
	Kidney	0	0	0	0	0	0	0	0	0	0
	Pancreas	0	0	0	0	0	0	0	0	0	0

The IHC score was not uniformed in some samples, in which two scores were given.
*In some areas the inclusion bodies were not stained by anti-IBDP MAB. N/A: sample not available.

Table 3-5. List of IBD positive non-boa constrictors tested with anti-IBDP MAB

Species	Number of snakes	IHC Staining Results	
		Positives	Negatives
Annulated tree boas[a]	3	0	3
Ball pythons	4	1	3
Carpet pythons	2	2	0
Emerald tree boas	1	0	1
Palm viper[b]	1	0	1
Rainbow boas	4	0	4

[a] Sample of IBD cases reported by Stenglein et al. [8]
[b] Sample of IBD case reported by Raymond et al. [16]

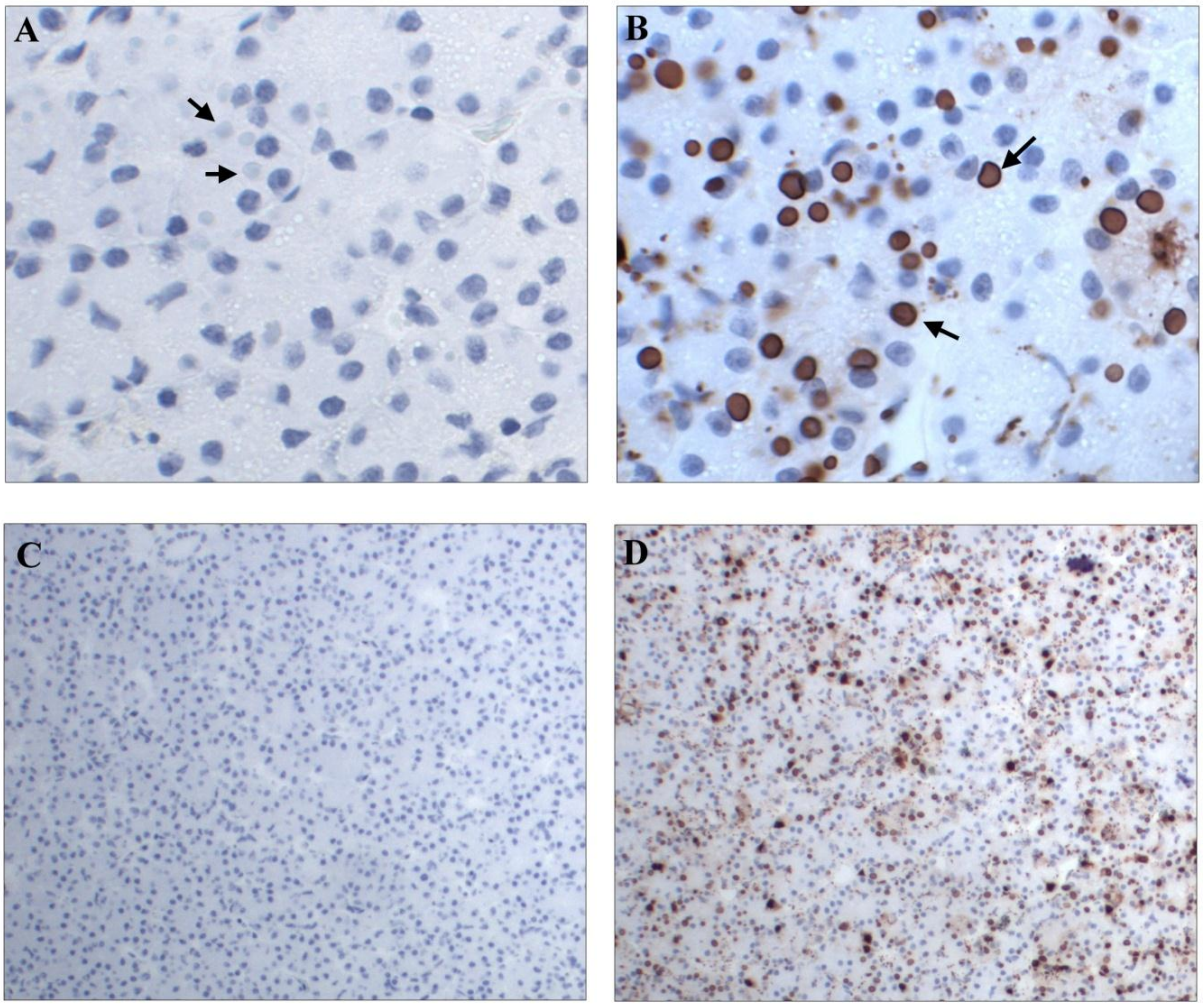

Figure 3-1. Pancreas of an IBD positive boa constrictor stained with standardized IHC condition. The tissue was fixed and stained under standardized IHC staining conditions using NovaRED as substrate. The negative control was stained with non-specific mouse antibody. The cell nucleus stained dark blue with hematoxylin, and the inclusion bodies are indicated by arrows. A. The negative control in high magnification (400x). The inclusion bodies were not stained. B. The inclusion bodies stained dark red by MAB under high magnification (400x). C. The negative control under low magnification (100x). D. The positive stained pancreas by anti-IBDP MAB under low magnification (100x).

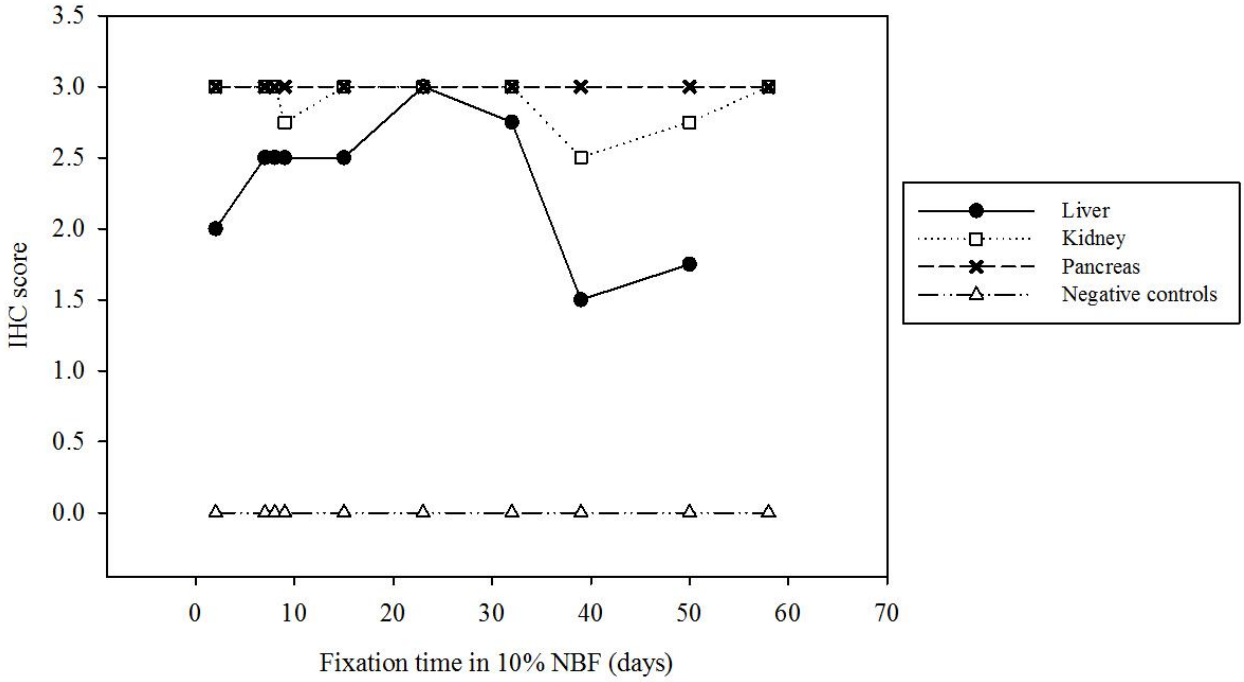

Figure 3-2. Mean IHC score of liver, kidney, and pancreas over fixation time in 10% NBF.

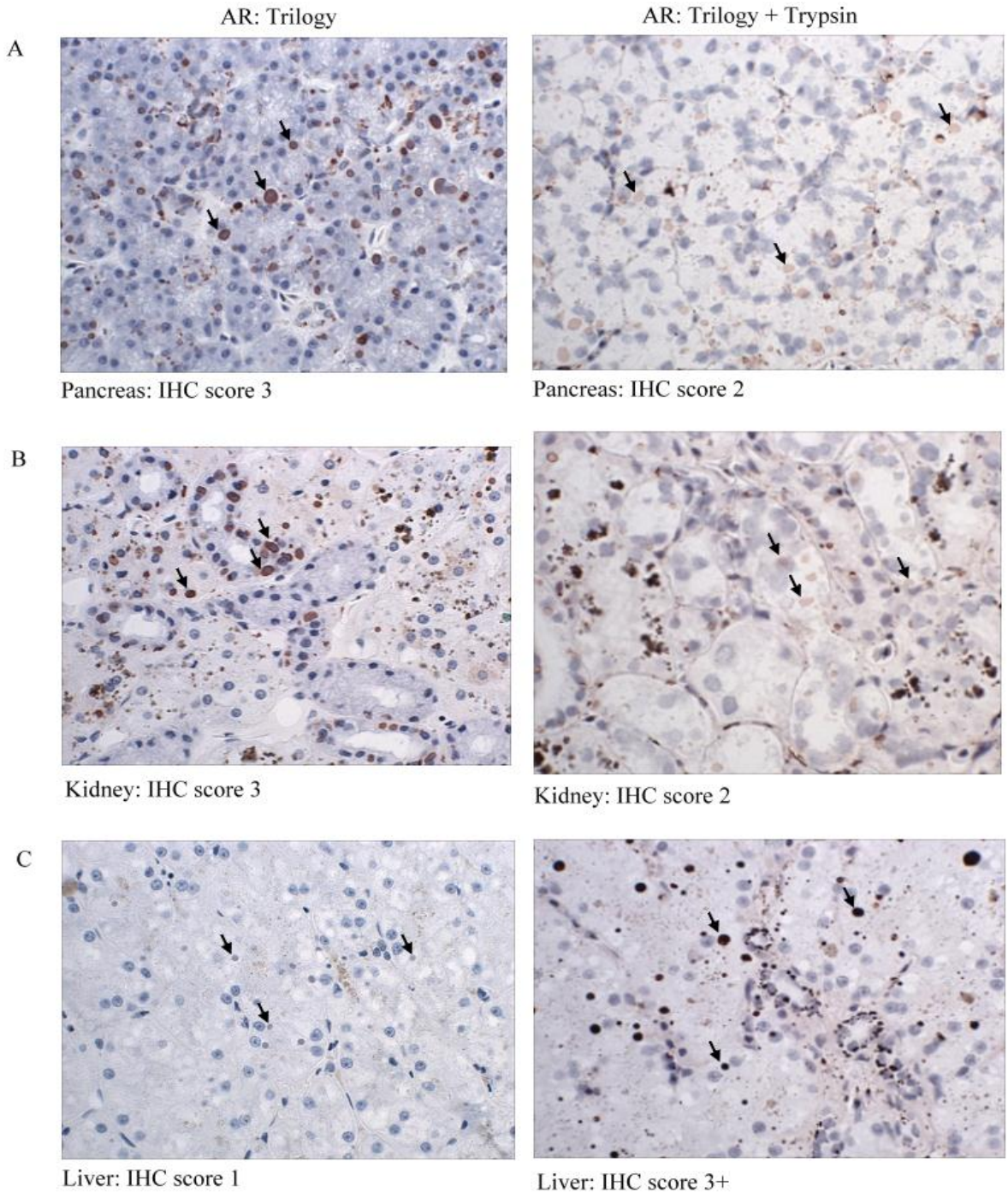

Figure 3-3. Differences in IHC staining between Trilogy and double AR treatment on prolong fixed tissues. Tissue fixed up to 50 days (Block 9) in 10% NBF was stained with standardized Trilogy AR treatment (left) and double AR treatment (right). The inclusion bodies are indicated by arrows. A. In pancreas, the staining of inclusion bodies decreased when double AR was used. B. In kidney, the staining of inclusion bodies decreased when double AR was used.

C. In liver, the staining of inclusion bodies improved when double AR was used.

Overall: Year 1990-2011

	H&E +	H&E -
IHC +	50	1
IHC -	10	33
	60	34

Performance Characteristics		95% CI Lower	95% CI Upper
Sensitivity	83.3%	75.8%	90.9%
Positive predicted value	98.0%	95.2%	100.0%
Negative predicted value	76.7%	68.2%	85.3%
Specificity	97.1%	93.6%	100.0%
False positive	2.9%		
False negative	16.7%		

Year 1990-2000

	H&E +	H&E -
IHC +	24	0
IHC -	5	15
	29	15

Performance Characteristics		95% CI Lower	95% CI Upper
Sensitivity	82.8%	71.6%	93.9%
Positive predicted value	100.0%	100.0%	100.0%
Negative predicted value	75.0%	62.2%	87.8%
Specificity	100.0%	100.0%	100.0%
False positive	0.0%		
False negative	17.2%		

Year 2001-2011

	H&E +	H&E -
IHC +	26	1
IHC -	5	18
	31	19

Performance Characteristics		95% CI Lower	95% CI Upper
Sensitivity	83.9%	73.7%	94.1%
Positive predicted value	96.3%	91.1%	100.0%
Negative predicted value	78.3%	66.8%	89.7%
Specificity	94.7%	88.5%	100.0%
False positive	5.3%		
False negative	16.1%		

Figure 3-4. The performance characteristics and their 95% confident intervals (95% CI) of IHC test on paraffin embedded blocks of boa constrictors date from 1990 to 2011. Group1:1990 to 2000; Group2: 2001 to 2011; Overall: Group1 and Group2.

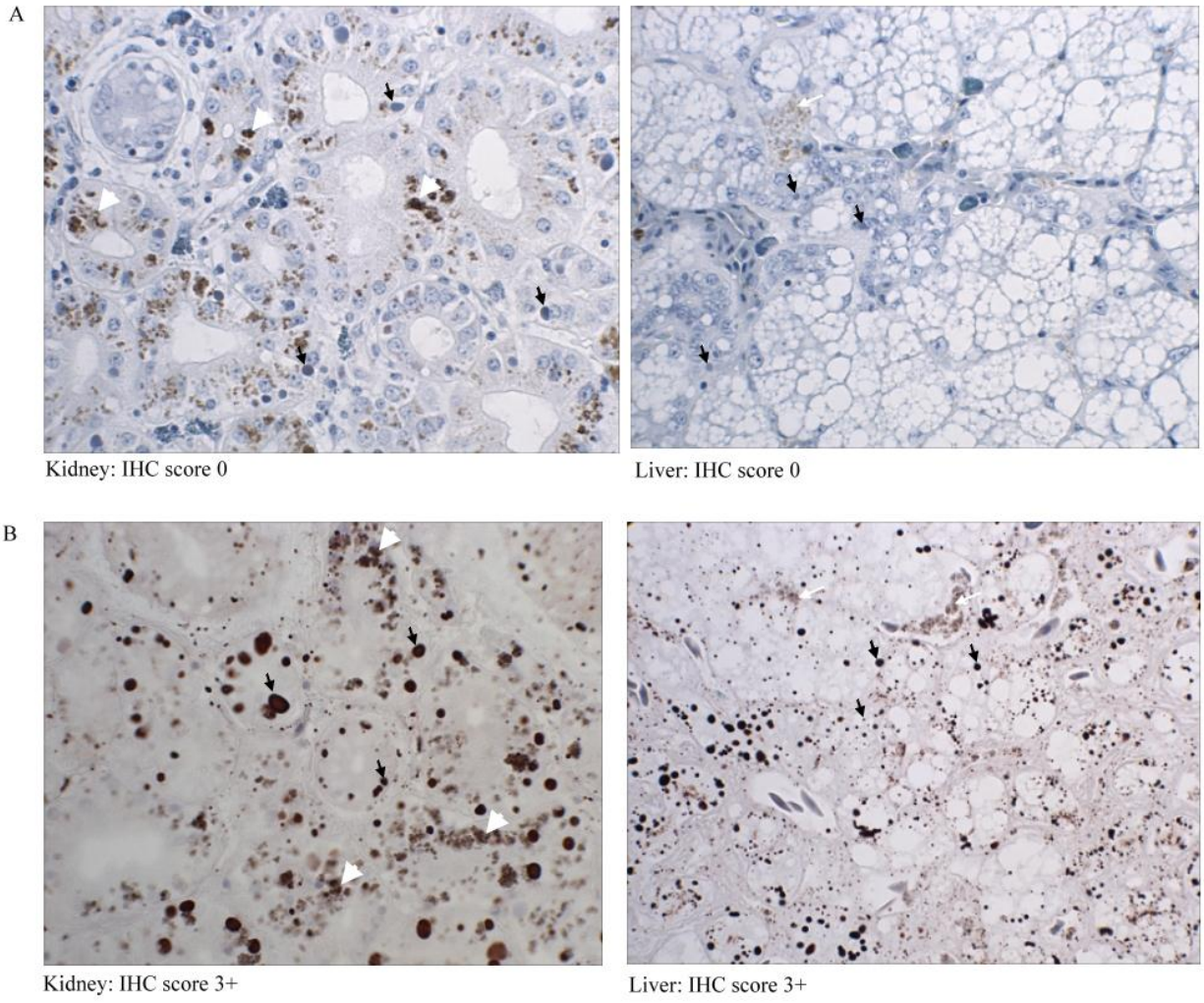

Figure 3-5. IHC staining of embedded tissue that strictly required double AR treatment. The paraffin embedded kidney (left) and liver (right) of an IBD+ boa constrictor were stained with anti-IBDP MAB using standard Trilogy treatment and double AR treatment. In this sample, the inclusion bodies (black arrows) were stained only using double AR treatment. White arrow heads showed the pigmented granules in the kidney, and white arrows showed the pigmented macrophage in the liver. A. The IHC staining of liver and kidney using standard Trilogy treatment. The inclusion bodies were not stained by anti-IBDP MAB. B. The IHC staining of liver and kidney using double AR treatment. The inclusion bodies were stained with high intensity.

CHAPTER 4
IMMUNO-BASED DIAGNOSTIC TESTS FOR SCREENING IBD

Introduction

Inclusion body disease (IBD) is a commonly seen disease in captive boa constrictors, and occasionally in other non-boa species.[1] However, without a screening test the prevalence of IBD is unknown. The disease is characterized by accumulation of an insoluble antigenic unique 68 KDa protein, called IBD protein (IBDP).[4] Up until now, the diagnosis is based on identifying the characteristic intracytoplasmic eosinophilic inclusion bodies in hematoxylin and eosin (H&E) stained tissue obtained from necropsy or biopsy.[1] However, the H&E will also stain inclusion bodies of other protein, and because of this a more specific diagnostic test is needed. A well validated specific and sensitive ante-mortem screening test is still lacking.

In IBD, inclusion bodies had been observed in lymphocytes of the infected snakes, and examination of blood films or buffy coat cytological prepatations had been recommended for diagnosing IBD.[1] But until now, there was a lack of evidence that the inclusion bodies observed in the cytoplasm of peripheral blood cells were IBDP.

A mouse monoclonal antibody (MAB) that is highly specific to IBDP in boa constrictors was produced and validated using immunohistochemistical (IHC) staining. This antibody can be used to develop an immuno-based blood test, which can potentially become a screening test for diagnosing IBD. The goal of this study was to determine whether the circulating antigen (IBDP) within blood samples can be detected by the mouse anti-IBDP MAB and whether there is detectable antibody against IBDP in infected boa constrictors. Being able to diagnose IBD using a blood sample will simplify

and reduce the cost of screening collections of susceptible snakes for this insidious disease.

Materials and Methods

Animal and Sample Collection

The following three different species of captive boid snakes were used in this study: boa constrictor (*boa constrictor*), rainbow boa (*Epicrates cenchria*), and ball python (*Python regius*). The snakes were randomly selected from several snake collections with unknown prevalence of IBD. Approximately 1 mL of heparinzed whole blood samples were collected by veterinarians via cardiocentesis. The samples were transfer to the University of Florida on ice, and stored in 4°C until being processed.

Sample Preparation

Blood film. A blood film of each sample were made on a charged microscopic slide (Thermo Scientific, Shandon Colorfrost Plus Slides), air dried, and fixed in 100% methanol for 15 minutes. After air-dried, the fixed blood film was stained with H&E stain, using the protocol described in Chapter 2 (Cytospin preparation and H&E stain, IBDP Purification).

Plasma separation. The whole blood samples were centrifuged for 10 minutes at 10,000 rpm on a Spectrafuge^{TM} 16M Microcentrifuge (Labnet International). The plasma was separated from the blood cells, collected, and store in -20°C for antibody detection experiments.

Isolating peripheral white blood cells. The pelleted blood cells were resuspended with 500 μL to 1 mL of phosphate buffered saline (PBS) and carefully layered over 300 μL of lymphocyte separtation media (LSM) (Cellgro) in Microtainer^{TM} tubes (BD, REF365971). The tubes were centrifuged for 30 minutes at 10,000 rpm.

After centrifugation, the red blood cells were pelleted in the bottom of the tube, and the peripheral white blood cells (PWBC) were focused on the layer above LSM. All the supernatants in the tube were collected, and the isolated PWBC were pelleted by centrifugation. The PWBC were washed two times with 1 mL of PBS, and pelleted by centrifugation. A portion of the isolated PWBC was resuspended in PBS, and cytospun onto three charged microscopic slides using a Shandon Cytospin2 Centrifuge. The remaining PWBC were stored in -20°C for antigen detection experiments. One of the cytospun slides with isolated PWBC was fixed in 10% neutral buffered formalin (10% NBF) for 15 minutes, rinsed with running water and air dried. The fixed PWBC slides were stained with H&E stain using a protocol described in Chapter 2 (Cytospin preparation and H&E stain, IBDP Purification). The two remaining PWBC slides were stored in a freezer at -20°C until being stained by IHC staining using the mouse anti-IBDP MAB.

Determine IBD Positive or Negative

The H&E stained slides were examined under light microscopes (Figure 4-1 A). If the characteristic cytoplasmic eosinophilic inclusion bodies were identified, the sample was classified as IBD positive (IBD+), if not the sample was classified as IBD negative (IBD-). If the IBD inclusion bodies were identified in either H&E stained blood film or isolated PWBC, the snake was classified as IBD+.

Antigen Detection Using IHC Staining

IHC staining on PWBC. The frozen slides with isolated PWBC were thawed, and air dried overnight in room temperature. The next day, the slides were fixed in 4% paraformaldehyde for 5 minutes, and washed with Tris buffered saline (TBS). Each wash was done by incubating the slides two times in TBS, 5 minutes each time. The

slides were incubated in 0.25% Triton X-100 for 5 minutes to permeabilized the cell wall. The slides were washed, and followed by incubating with Peroxo-Block™ (Invitrogen) ready-to-use solution for 45 seconds. The slides were washed again, and the remaining IHC staining procedures were done manually as described in Chapter 3 (IHC Staining for Paraffin Embedded Tissues). A negative control slide was stained with non-specific mouse IgG (Vector, I-2000) as primary antibody that was performed parallel to the testing slide.

Result interpretation. The IHC positive was defined as positive staining specifically to the inclusion bodies in comparison to the negative control (Figure 4-1). The IHC negative was defined as absence of specific staining to inclusion bodies in comparison to the negative control.

Antigen Detection Using Western Blots

Cell lysing and estimating protein concentration. The PWBC pellet was frozen and thawed three times, followed by addition of 1:1 volume of a 2X lysing buffer (LB) containing 2% sodium dodecyl sulfate (SDS) and 10% beta-mercaptoethanol (2-ME), and solublized by incubation at 95°C for 10 minutes. Two microliters of the solublized cell pellet was removed, diluted 10 fold with water, and the protein concentration was estimated using the Bradford Protein Assay. The Quick Start™ (Bio-Rad) Bradford Protein Assay used in this study was modified to increase the solubility of the IBDP. The bovine serum albumin (BSA) standards used in the Bradford Protein Assay contained the same background of SDS and 2-ME as the diluted PWBC samples. The protein concentration of the original PWBC lysate was estimated by multiplying the dilution factor to the concentration estimated by Bradford Protein Assay.

Western blots. For each sample, 20 μg of PWBC lysate was reduced by incubating with 4X sample buffer (Boston reagent, BP-110R) in 95°C for 10 minutes. The reduced PWBC were resolved on a 10% Tris-Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For each gel, the first well was loaded with molecular weight marker (Bio-Rad, 161-0374), the second well was loaded with semi-purified inclusion body protein preparation (IB prep) as the positive control, and the remaining eight wells each were loaded with a different PWBC isolate. The resolved proteins were transblotted onto a supported nitrocellulose membrane (Bio-Rad, 162-0254), and stained with MemCode™ (Pierce) reversible membrane stain to confirm the protein was properly transblotted. The membrane was destained, and blocked overnight with 5% non-fat dried milk dissolved in wash buffer (0.1% Tween20 in PBS). After washing three times with wash buffer, the blot was cut into half, with the top part containing molecular weight >50 KDa, and the bottom part containing molecular weight < 50 KDa. The top blot was detected using the anti-IBDP MAB in a dilution of 1:3,000 in dilution buffer (1% BSA in wash buffer), the bottom blot was detected by a commercial anti-beta-actin antibody (GenScript, A00702) in a dilution of 1:3,000 as loading controls. The blots are washed three times, and incubated with a HRP-conjugated goat-anti-mouse antibody (Bio-Rad, 170-6516) in a 1:3,000 dilution. Following three washes, the reactive bands were visualized by developing with Opti-4CN™ (Bio-Rad) colorimetric substrate kit. The blots were rinsed with water and air dried.

Result interpretation. The isolated PWBC sample that showed a 68 KDa reactive band detected with the mouse anti-IBDP MAB was interpreted as western blot positive. The sample that did not show a 68 KDa reactive band, but was able to detect beta-actin

was interpreted as western blot negative. The positive band of 42 KDa detected by anti-beta actin antibody demonstrated that equivalent amount of PWBC was loaded in each well (Figure 4-2).

Antibody Detection Using Western Blots

The collected plasmas from boa constrictors were tested for the presence of anti-IBDP antibody. This test assay utilized the previously produced and validated mouse anti-snake IgG monoclonal antibody, hereafter is refer as anti-snake Ab.[19] The application of anti-snake Ab, followed by reaction to goat-anti-mouse antibody was used to determine if IBD+ produced anti-IBDP antibody and could be visualized by Opti-4CN™.

Standardizing anti-snake Ab. The previously produced hybridoma clone HL1785 was thawed and cultured, and the anti-snake Ab was harvested and purified using the protocol described in Chapter 2 (Monoclonal antibody purification, Polyclonal and Monoclonal Anti-IBDP Antibody Production). To confirm the reactivity of the anti-snake Ab to the IgG of boa constrictors, the plasma samples were tested on western blots. Plasma of three boa constrictors were resolved on a SDS-PAGE, transblotted onto a nitrocellulose membrane using the method described. The membrane was cut into portions, with each portion containing three resolved plasma samples. Each portion was detected with a specific dilution of anti-snake Ab, and one portion was not treated with the anti-snake Ab served as the negative control. The membranes were incubated by HRP-conjugated antibody and developed as described in Antigen Detection using Western Blots.

Test strip preparation. Three hundred micrograms of semi-purified IB prep was reduced by incubating with 4X sample buffer in 95°C for 10 minutes. The sample was

resolved on a two-well 10% Tris-Glycing SDS-PAGE, the reference well was loaded with molecular weight marker. The resolved protein was transblotted onto a nitrocellulose membrane, and the transblot was confirmed with reversible membrane stain. After being blocked and washed, the membrane was cut into 5 mm wide strips.

Antibody detection. For each plasma sample to be tested, two (1:500, 1:1,000) or three (1:100, 1:500, 1:1,000) different dilutions were prepared by diluting with 3 mL of diluting buffer. A positive control strip was performed by using anti-IBDP MAB alone in a dilution of 1:3,000. A negative control strip was performed using diluting buffer alone, using the same procedure as the strips receiving snake plasmas. Each strip was incubated with diluted plasma or anti-IBDP MAB inside of a 15 mL nunc^{TM} tube (Thermo Scientific, 362695), and throughout the antibody detection procedures each strip was incubated and washed individually inside the tube to avoid cross contamination. The tubes were laid down in a container and rocked horizontally on a rocker for 1 hour. The diluted plasma or anti-IBDP MAB was ensured to cover the strip at all time during the incubation. The strips were washed three times with 5 mL of wash buffer on the rocker for 5 minutes. Next, each strip was incubated with 3 mL of anti-snake Ab using a dilution of 1:5,000 or 1:30,000 for 1 hour. Then the strips were washed another three times, and incubated with HRP-conjugated anti-mouse IgG in a dilution of 1: 3,000 for 1 hour. After another three washes, the strips were developed by Opti-4CN^{TM}. Until the positive control strip was fully developed, the strips were rinsed with water, and air dried.

Result interpretation. The tested plasma that reacted to the 68 KDa IBDP band aligned with the molecular weight indicated by the positive control was interpreted as

positive. The plasma that did not react to the 68 KDa IBDP band was interpreted as negative.

Standardizing the antibody detection system. Prior to testing the plasma samples of IBD positive and negative samples, the optimal concentrations of each antibody were determined by western blots. Plasma samples of three index IBD positive boa constrictors were tested on transblotted IBDP, using combinations of plasma in three dilutions (1:100, 1:500, 1:1,000) and snake IgG Ab in two dilutions (1:1,000 and 1:500) to ensure the optimal conditions for antibody detection were covered.

Agreement and Association Analysis

Antigen detection. The samples that were tested positive for IBD in H&E stained blood film or isolated PWBC were defined as IBD+ by H&E stain, otherwise were classified as IBD- by H&E stain. The samples testing positive for IBD in IHC stain or western blots were defined as IBD+ by immuno-based tests, otherwise were classified as IBD- by immune-based tests. The agreement between H&E stain and the detection of IBD-antigen by immune-based test was estimated by using kappa statistic with standard protocol provided by Martin et al.[20] Kappa values 0 was considered as no agreement; < 0.4 was considered as poor agreement; 0.4 to 0.75 as fair agreement; > 0.75 as excellent agreement; and 1 as perfect agreement. The 95% confident interval (95% CI) will be calculated by software Win Episcope 2.0.

Antibody detection. The samples that tested positive of IBDP reactive antibody in the plasma were defined as positive in antibody detection. The samples that tested negative of IBDP reactive antibody in the plasma were defined as negative in antibody detection. The agreement between H&E stain and antibody detection was estimated by kappa statistic as described for antigen detection.

Results

Antigen Detection

A total of 78 blood samples were screened for the presence of IBDP, which included 39 boa constrictors, 20 rainbow boa and 19 ball pythons. The test results are listed in Table 4-1 and summarized in Table 4-2.

Antigen detection in boa constrictors. In boa constrictors, inclusion bodies were detected in 13 (of 38) H&E stained blood films, and in 16 (of 39) H&E stained isolated PWBC. When detected with anti-IBDP MAB, 16 (of 39) stained positive in IHC stain, and 15 (of 39) tested positive using western blots (Table 4-2). Using isolated PWBC preparation, inclusion bodies were found in three additional cases that tested negative in direct blood films (Table 4-1). In one case (IB10-53), the inclusion bodies were very sporadic within the blood cells that were only detected in IHC staining (Table 4-1).

Antigen detection in rainbow boas. In rainbow boas, inclusion bodies were detected in one (out of 20) H&E stained blood films, and three (out of 20) H&E stained isolated PWBC. No sample from rainbow boas tested positive in IHC staining and western blots.

Antigen detection in ball pythons. In ball pythons, no inclusion bodies were detected in all 19 blood samples tested. No samples from ball pythons tested positive in IHC staining or Western blots.

Agreement between H&E stain and antigen detection. The diagnosis made by H&E stain and immune-based tests were summarized in Table 4-3. The agreement in rainbow boas and ball pythons were not analyzed, since the reactivity of mouse anti-IBDP MAB in rainbow boas were not validate, and in ball pythons no IBD positive cases

were found. The agreement between H&E stain and immuno-based tests determined by Kappa statistic was 0.894 (95% CI 0.58, 1.00), indicating an excellent agreement.

Antibody Detection

Standardizing the detection system. The reactivity of the purified anti-snake Ab (10 mg/mL) to the IgG of boa constrictors were confirmed by western blots. The reactive concentrations of anti-snake Ab ranged from 3.33 μg/mL (dilution of 1: 3,000) to 0.33 μg/mL (dilution of 1: 30,000) (Figure 4-3). When being used for detecting antibody in the index samples, the anti-snake Ab showed better sensitivity in 2 μg/mL (dilution of 1:5,000) than 0.33 μg/mL (dilution of 1: 30,000) without introducing non-specific background (Figure 4-4). The plasma sample in the dilution of 1:100 showed darker non-specific background, than the dilution of 1:500 and 1:1,000. The remaining plasma samples were tested with dilutions of 1:500 and 1:1,000 using anti-snake Ab with a dilution of 1:5,000.

Antibody detection in boa constrictors. The 39 boa constrictors (16 IBD+ and 23 IBD-) that had been tested for the presence of IBDP, plasma collected from these snakes were tested for the presence of anti-IBDP antibody. By antigen detection in the isolated PWBC using anti-IBDP MAB, 16 of the 39 snakes tested positive of IBDP, and 23 of the 39 snakes tested negative of IBDP. No antibody against IBDP antigen was detected in the plasmas of all 39 boa constrictors.

Agreement between H&E stain and antibody detection. The diagnosis made by H&E stain and the results of antibody detection were summarized in Table 4-4. The agreement between H&E stain and immune-based tests determined by Kappa statistic was 0, indicating no agreements.

Discussion

Modified Bradford Protein Assay

Due to the insolubility of IBDP, IBDP needs to be treated by heating with reducing reagents at 95°C prior to estimating protein concentration. The presence of some reducing reagents may interfere with coloration of the protein-binding dye in Bradford Protein Assay, thus interfering in the estimation of protein concentrations. This issue had been discussed in Chapter 2 (Inaccurate Estimation of Protein Concentration). In this study, using a 2X LB the cell pellets were solubilized by heating in 1% SDS and 5% 2-ME, followed by diluting the sample 10X before binding with the dye for Bradford Protein Assay. Thus, the diluted sample contained a background of 0.1% SDS and 0.5% 2-ME that was found not to cause severe interference in Bradford Protein Assay.[10, 21] The standard BSA protein of known concentrations contained the same background of LB (0.1% SDS and 0.5% 2-ME) as the 10 fold diluted testing samples, and the standard curve made by the BSA standards of known concentrations was used to estimate the protein concentration of the testing samples. The BSA standards with or without LB background were heated or unheated to demonstrate the differences in standard curve (Figure 4-5). Because the heated and unheated LB containing standards formed identical curves, the later standard curve (unheated standard) was used to estimate the protein concentration of isolated PWBC. Using this modified Bradford Protein Assay, protein concentration of the IBDP containing samples could be estimated much more precisely and this improved the accuracy of the assay. This modified assay may benefit other researchers that needed to analyze insoluble proteins.

Assessment of Using Blood Tests for Screening IBD

Antigen detections

H&E stain. Of the 39 boa constrictors examined using H&E stain, three additional cases with inclusion bodies were detected in the isolated PWBC preparation, that were not detected in the blood film. The isolated PWBC preparation increased the sensitivity (16/39 or 41%) of detecting inclusion body bearing cells compared to the sensitivity of blood films (13/38 or 34.2%). In the case of IB10-43 and IB10-44, inclusion bodies having very small and irregular shapes were sporadically seen in the blood films. They were difficult to identify and were missed in the H&E stained blood films. The isolated PWBC preparation concentrated the blood cells and improved the ability to identify cells having IBD inclusion bodies. Therefore, isolated PWBC preparations increase the ability of observing inclusion body bearing cells.

In this study, red blood cells lysis occurred when films were fixed in 10% NBF. The morphology of the blood cells were better preserved when films were fixed in 100% methanol, and the color of cells stained with H&E appeared very similar to the cells fixed in 10% NBF. Fixing with 100% methanol is more versatile, because the fixed slides can also be stained with Wright Geimsa stain.

Possible false positives in H&E stain. In H&E stain, sometimes it is difficult to differentiate IBD inclusion bodies from other intracellular protein. Especially in the case of aged samples, the phagocytosed hemoglobin may appear to be similar to IBD inclusion bodies. In one boa constrictor (IB10-75) and three rainbow boas (IB10-67, IB10-78 and IB10-81) inclusion bodies were observed in H&E stained PWBC, but did not stain with IHC. The inclusion bodies within these cases were found in monocytes, not in lymphocytes where IBD inclusion bodies were more commonly found. Further,

unlike most IBD cases, there were no signs of lymphocytosis in these cases. Thus, these samples are suspected to be false positives caused by prolong storage or handling.

Immuno-based tests. In boa constrictors, except for 2 cases (IB10-53 and IB10-75), the test result of IHC staining and western blot all correlated with the diagnosis in H&E stained PWBC. In the case of IB10-53, the inclusion bodies were extremely sporadic (less than 5 in one preparation) that only IHC staining was able to detect IBDP. Using western blots, IBDP was readily detectable when using only 18 μg of PWBC lysate. All the samples that were negative in western blots (20 μg per load) were repeated with 30 μg per loaded, and the results were consistently negative. In rainbow boas, the PWBC tested positive in H&E staining did not test positive in IHC staining or western blot. This may be due to the following: 1) MAB may not cross-react to IBDP in rainbow boas as previously discussed in Chapter 3 (Cross Reactivity Among Non-Boa Constrictors); 2) the inclusion bodies observed in the samples of rainbow boas may represent some other accumulated protein that is unrelated to boa constrictor IBDP. Although the sensitivity of the immune-based tests were relevant to the H&E stain in isolated PWBC, the immune-based tests were much more specific in detecting IBDP, and provided various options for confirming IBD diagnosis. For blood samples with very small volume (0.2 to 0.5 mL), the IHC staining using cytospin preparation of PWBC is a better choice of diagnosis. Western blots may be a more cost effective method when screening large numbers of samples with larger volumes. Or it can serve as a confirmatory test for samples that test IHC positive.

Possible false positives in IHC stain. Although the inclusion bodies are very easy to identify in the IHC stain, the negative cases are sometimes difficult to define. The granules within heterophils sometimes stained positive in IHC stain, possibly caused by the reaction of the endogenous peroxidase to the substrate. The results needed to be evaluated carefully by comparing against the negative control, which also contained the non-specific background staining.

Possible false negatives in blood tests. Currently, without a proper transmission study, the disease course of IBD is unknown. Based on the inoculation studies done by Wozniak et al. and Schumacher et al., [2, 4] the inclusion bodies were visible in visceral organs approximately 10 week post-infection. But the presence of inclusion bodies in blood cells were not determined. To date, there is no knowledge about whether all IBD infected snake will develop inclusion bodies in the circulating blood cells. It is possible that a snake having IBD may tested negative by the blood tests, if inclusion bodies are not yet in the circulating blood cells. If a snake tested negative by the blood test, it is recommended to re-test the snake 10 weeks later.

Antibody detections

Of 39 plasma samples of boa constrictors tested, which included 16 IBD+ and 23 IBD- samples, none showed reactivity to the 68KDa IBDP. This finding indicated that no snakes had circulating antibody against IBDP, although the snakes are clearly infected with IBD. This agreed with the lack of immune responses at the histological level observed in most IBD snakes. However, when a recently made bulk IB prep was used, all of the tested plasma reacted to a band that was slightly above 50 KDa (Figure 4-6). It is possible the presence of this antibody in boa constrictor is not specific for IBD. It is also possible that additional proteins, other than the 68 KDa IBDP, may be present

within the semi-purified inclusion bodies. While infected boas did not exhibit an immune response against IBDP, they did have antibody response to another protein(s) within the inclusion bodies. The reaction against the 50 KDa protein detected in the IBD- boa constrictors may be due to exposure to another structural protein in the causative agent of IBD. More work is needed to establish the significance of this finding.

Conclusions

The results of antigen detection in boa constrictor demonstrated that the observed inclusion bodies in H&E stain agreed with the detection of IBDP in the immune-based tests. Using antigen detection in whole blood samples can become the first line screening test for diagnosing IBD in boa constrictors. But the results of H&E stain need to be interpreted carefully. The confidence of the diagnosis will increase when the sample is confirmed using the immuno-based test developed in this study.

No antibody against IBDP was detected in the IBD positive boas, and there were no agreement between the H&E stain diagnosis and the detection of antibody against IBDP. The results of antibody detection in boa constrictors showed only moderate agreement. More investigation is needed to determine whether antibody detection can be used for screening IBD.

Table 4-1. Summarized results of antigen detection in blood samples of three snake species

	Boa constrictors n=39		Rainbow boas n=20		Ball pythons n=19	
Test Result	Positive	Negative	Positive	Negative	Positive	Negative
Blood film H&E	13*	25	1	19	0	19
PWBC H&E	16	23	3	17	0	19
PWBC IHC	16	23	0	20	0	19
PWBC WB	15	24	0	20	0	19

*One sample the blood film was not available.

Table 4-2. Results of antigen detection in blood samples of three snake species

Boa constrictors: n=39						Rainbow boas: n=20						Ball python: n=19					
Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB	Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB	Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB
IB10-21	A	Neg	Neg	Neg	Neg	IB10-26	A	Neg	Neg	Neg	Neg	IB10-36	A	Neg	Neg	Neg	Neg
IB10-22	G	Neg	Neg	Neg	Neg	IB10-27	A	Neg	Neg	Neg	Neg	IB10-37	A	Neg	Neg	Neg	Neg
IB10-23	A	Neg	Neg	Neg	Neg	IB10-28	A	Neg	Neg	Neg	Neg	IB10-38	A	Neg	Neg	Neg	Neg
IB10-25	B	Pos	Pos	Pos	Pos	IB10-29	A	Neg	Neg	Neg	Neg	IB10-39	A	Neg	Neg	Neg	Neg
IB10-31	A	Pos	Pos	Pos	Pos	IB10-30	A	Neg	Neg	Neg	Neg	IB10-58	A	Neg	Neg	Neg	Neg
IB10-32	A	Neg	Neg	Neg	Neg	IB10-64	A	Neg	Neg	Neg	Neg	IB10-59	A	Neg	Neg	Neg	Neg
IB10-33	A	Neg	Neg	Neg	Neg	IB10-65	A	Neg	Neg	Neg	Neg	IB10-60	A	Neg	Neg	Neg	Neg
IB10-34	A	Neg	Neg	Neg	Neg	IB10-67	A	Pos	Pos	Neg	Neg	IB10-62	A	Neg	Neg	Neg	Neg
IB10-35	A	Pos	Pos	Pos	Pos	IB10-77	A	Neg	Neg	Neg	Neg	IB10-86	A	Neg	Neg	Neg	Neg
IB10-41	A	Neg	Neg	Neg	Neg	IB10-78	A	Neg	Pos	Neg	Neg	IB10-92	A	Neg	Neg	Neg	Neg
IB10-42	A	Neg	Neg	Neg	Neg	IB10-79	A	Neg	Neg	Neg	Neg	IB10-93	A	Neg	Neg	Neg	Neg
IB10-43	A	Neg	Pos	Pos	Pos	IB10-80	A	Neg	Neg	Neg	Neg	IB10-94	A	Neg	Neg	Neg	Neg
IB10-44	A	Neg	Pos	Pos	Pos	IB10-81	A	Neg	Pos	Neg	Neg	IB10-95	A	Neg	Neg	Neg	Neg
IB10-45	A	Neg	Neg	Neg	Neg	IB10-87	A	Neg	Neg	Neg	Neg	IB10-96	A	Neg	Neg	Neg	Neg
IB10-46	B	Pos	Pos	Pos	Pos	IB10-88	A	Neg	Neg	Neg	Neg	IB11-06	A	Neg	Neg	Neg	Neg
IB10-47	B	Pos	Pos	Pos	Pos	IB10-89	A	Neg	Neg	Neg	Neg	IB11-07	A	Neg	Neg	Neg	Neg
IB10-48	B	Pos	Pos	Pos	Pos	IB10-90	A	Neg	Neg	Neg	Neg	IB11-08	A	Neg	Neg	Neg	Neg
IB10-49	B	Neg	Neg	Neg	Neg	IB10-91	A	Neg	Neg	Neg	Neg	IB11-09	A	Neg	Neg	Neg	Neg
IB10-50	B	Neg	Neg	Neg	Neg	IB11-12	G	Neg	Neg	Neg	Neg	IB11-10	A	Neg	Neg	Neg	Neg
IB10-51	B	Pos	Pos	Pos	Pos	IB11-24	H	Neg	Neg	Neg	Neg						
IB10-52	B	Pos	Pos	Pos	Pos												
IB10-53	B	N/A	Neg	Pos	Neg												
IB10-54	B	Pos	Pos	Pos	Pos												
IB10-68	A	Pos	Pos	Pos	Pos												
IB10-75	A	Neg	Pos	Neg	Neg												
IB10-76	A	Neg	Neg	Neg	Neg												
IB10-84	A	Neg	Neg	Neg	Neg												
IB10-85	A	Neg	Neg	Neg	Neg												
IB11-11	F	Neg	Neg	Neg	Neg												
IB11-14	C	Neg	Neg	Neg	Neg												
IB11-15	C	Pos	Pos	Pos	Pos												
IB11-17	A	Neg	Neg	Neg	Neg												
IB11-18	A	Neg	Neg	Neg	Neg												

Table 4-2. Continued

Boa constrictors: n=39						Rainbow boas: n=20						Ball python: n=19					
Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB	Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB	Test ID	Co	BF HE	WBC HE	WBC IHC	WBC WB
IB11-22	E	Pos	Pos	Pos	Pos												
IB11-25	D	Neg	Neg	Neg	Neg												
IB11-26	D	Pos	Pos	Pos	Pos												
IB11-27	A	Neg	Neg	Neg	Neg												
IB11-28	A	Neg	Neg	Neg	Neg												

Co: snake collection of sample origin; BF: blood film; HE: H&E stain; WBC: peripheral white blood cells; IHC: immunohistochmistry stain; WB: Western blot; Pos: positive; Neg: negative.

Table 4-3. Summarized results of H&E and Immuno-tests

	Boa constrictors n=39		Rainbow boas n=20		Ball pythons n=19	
Test Result	Positive	Negative	Positive	Negative	Positive	Negative
H&E Stain	16	23	3	17	0	19
Immuno-based test	16	23	0	20	0	19

Table 4-4. Agreements between diagnosis by H&E stain and immuno-based antigen detection tests in boa constrictors

	IBD+ (H&E+)	IBD- (H&E-)	Total
Immuno +	15	1	16
Immuno -	1	22	23
Total	16	23	39

Immuno+: immune-based test positive; Immuno-: immune-based test negative.

Table 4-5. Agreements between diagnosis by H&E stain and antibody detection test in boa constrictors

	IBD+ (H&E+)	IBD- (H&E-)	Total
Antibody+	0	0	0
Antibody -	16	23	39
Total	16	23	39

Antibody+: detected positive of antibody; Antibody-: detected negative of antibody.

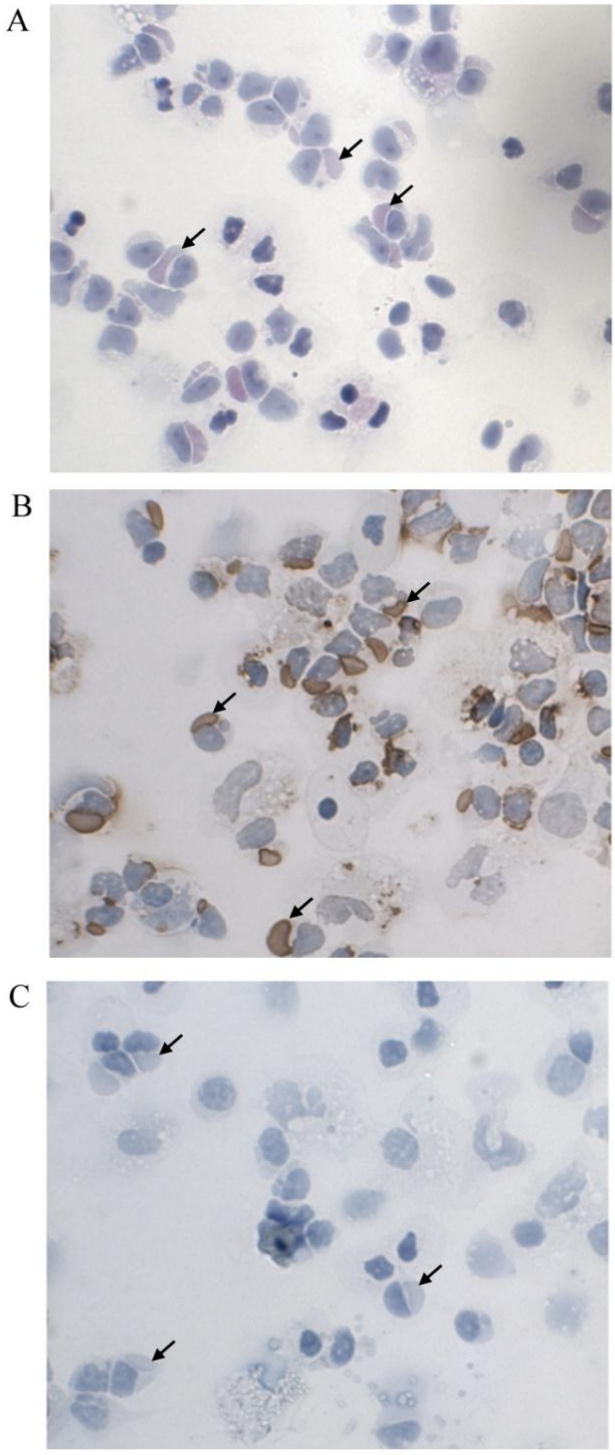

Figure 4-1. Cytospin preparation of PWBC isolated from a boa constrictor. A. The IBD inclusion bodies (arrows) stained eosinophilic in H&E stain. B. Using IHC staining, the IBD inclusion bodies (arrows) stained dark brown with substrate DAB. C. The PWBC stained with nonspecific mouse antibody as negative control for IHC staining. The inclusion bodies were not stained.

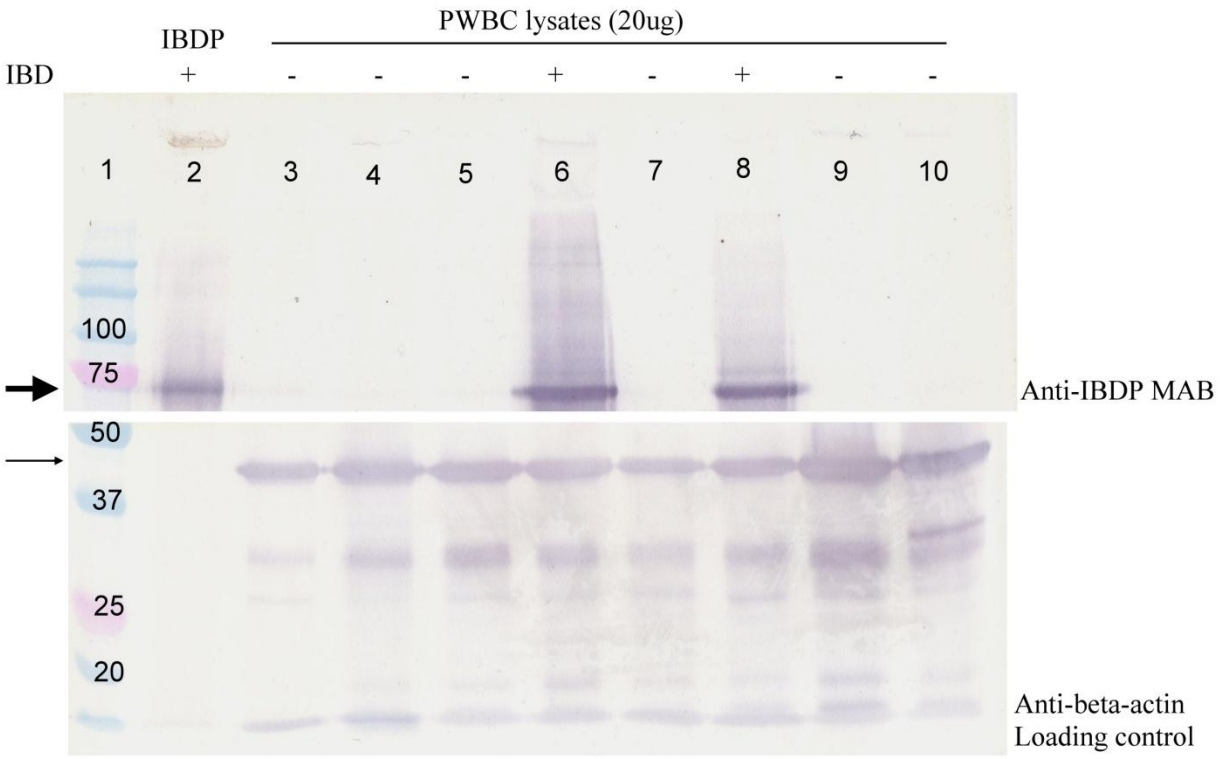

Figure 4-2. Antigen detection by western blot using PWBC isolated from 8 boa constrictors. Lane 1 contained molecular weight marker (Bio-Rad, #161-0347), lane 2 contained 20 μg purified IBDP as positive control. Lane 3 to 10 each contained 20 μg of PWBC lysate from a boa constrictor. The top portion of membrane was detected by anti-IBDP MAB, the thick arrow indicate the molecular weight of IBDP (68 KDa). The bottom portion of membrane was detected by anti-beta-actin antibody as loading control, showing that relevant amount of cell were loaded in each lane. The thin arrow indicated the molecular weight of beta-actin (42 KDa). Lane 6 and 8 were interpreted as western blot positive, whereas others were interpreted as negative.

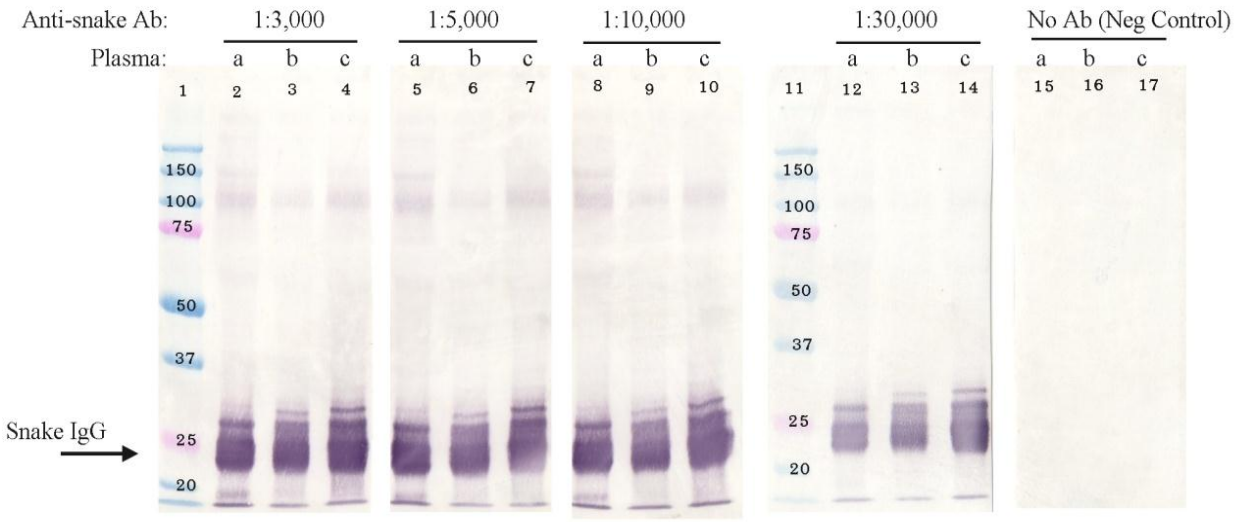

Figure 4-3. Western blots showing the reactivity of purified anti-snake Ab (HL1785) to three plasma samples of boa constrictors. Lane 1 and 11 contained molecular weight marker. Plasma sample of three boa constrictors (a, b and c) were used, lane 2 to 10 and lane 12 to 17 each contained 10 μg of resolved plasma from boa constrictors. The purified anti-snake Ab consistently reacted to the light chain (arrow) of three boa constrictors. The reactive concentrations ranged from 1 μg/mL (dilution of 1:3,000) (lane 2 to 4) to 0.3 μg /mL (dilution of 1:30,000) (lane 12 to14). Lane 15 to 17 were detected with PBS as negative controls.

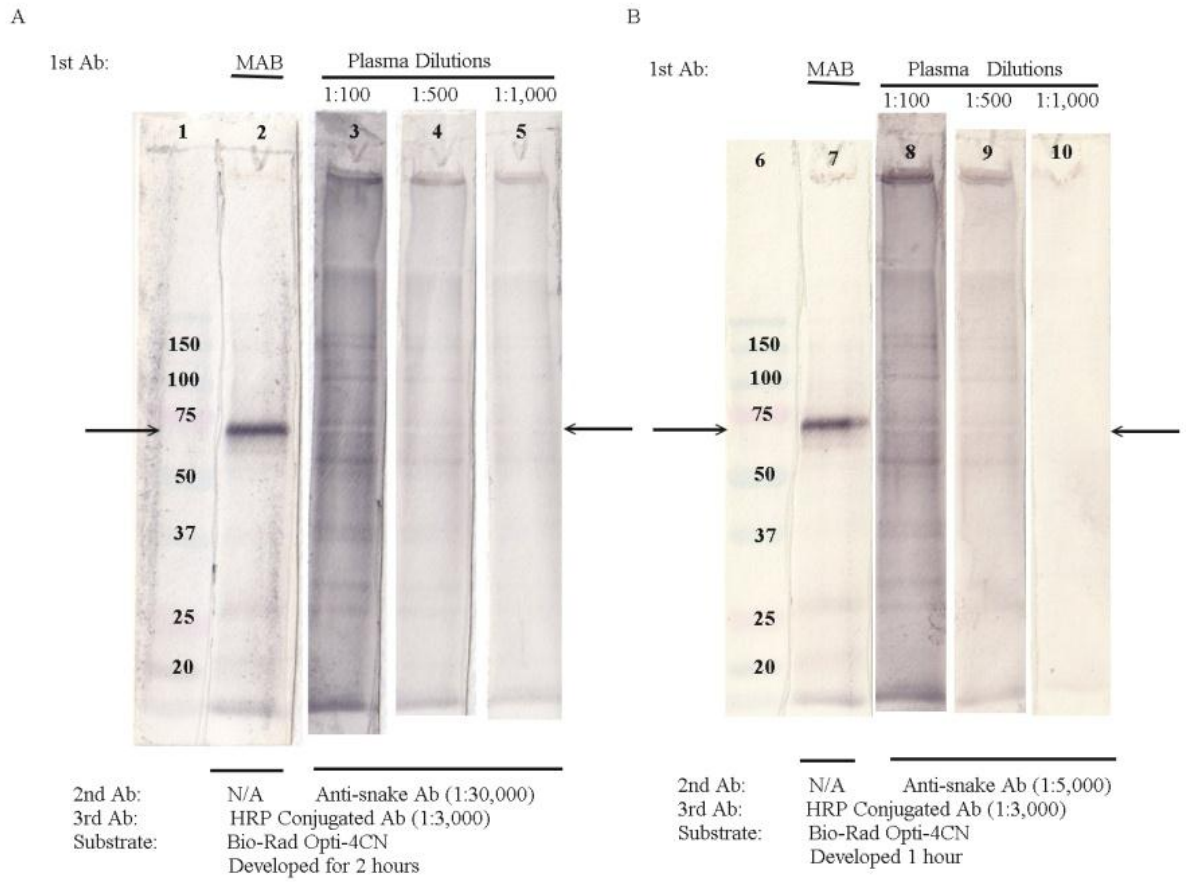

Figure 4-4. Detection of antibody against IBDP within plasma of an IBD positive boa constrictor using western blots. A. Antibody detection using anti-snake Ab with a dilution of 1:30,000 dilution (0.3μg /mL), and developed for 2 hours. B. Antibody detection using anti-snake Ab with a dilution of 1:5,000 (2 μg /mL), and developed for 1 hour. The test strips were purposely over developed to ensure detection of all possible reactivity. The anti-snake Ab with a dilution of 1:5,000 was more sensitive than the dilution of 1:30,000 that reached the maximal development in half of the incubation time. The anti-IBDP MAB was used as positive controls in lane 2 and 7 to label the molecular weight of IBDP (68KDa), indicated by arrows. Higher backgrounds can be seen in the strips detected with 1:100 diluted plasma, but no reactions specific to the 68 KDa IBDP band was detected in the condition listed. The test result of this plasma sample was interpreted as negative of anti-IBDP antibody.

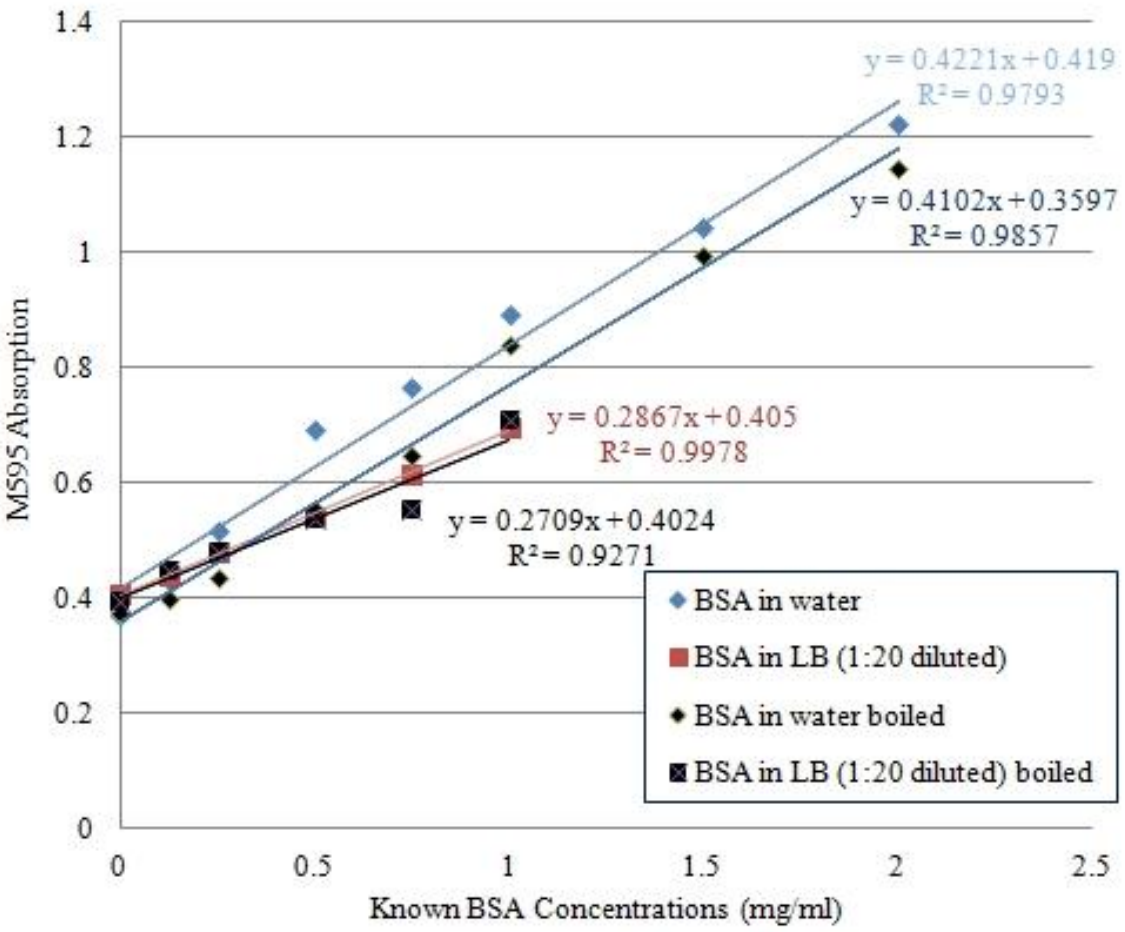

Figure 4-5. The BSA standard curves with or without the background of LB measured by Bradford Protein Assay.

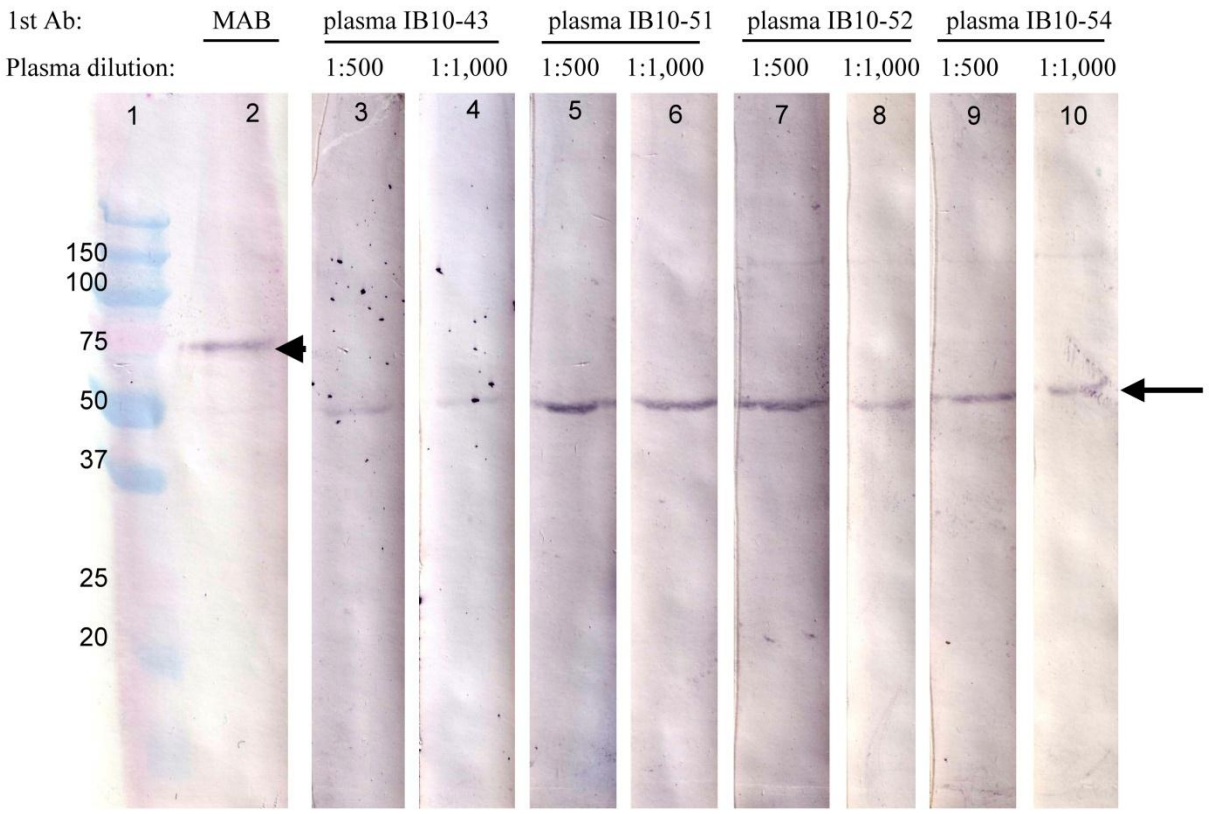

Figure 4-6. Detection of antibody against IBDP within plasma of 4 IBD positive boa constrictors on Western blots. A membrane that was transblotted with resolved IB prep, and cut into strips containing molecular weight marker on lane 1 and 20 μg isolated inclusion bodies on each strip (lane 2-10). Lane 2 was detected with anti-IBDP MAB as positive control reacted to the 68 KDa IBDP (arrow head). The remaining strips were detected with plasma of 4 IBD positive boa constrictors, IB10-43 (lane 3 and 4), IB10-51 (lane 5 and 6), IB10-52 (lane 7 and 8), IB10-54 (lane 9 and 10), with dilutions of 1:500 and 1:1000. The plasma did not show reaction to the 68 KDa IBDP band, but reacted to a protein band approximately 50 KDa (arrow).

CHAPTER 5
SEQUENCING INCLUSION BODY DISEASE PROTEIN

Introduction

Inclusion body disease (IBD) is a commonly seen disease in captive boid snakes.[1] This disease is characterized by the accumulation of an antigenic 68 KDa protein, inclusion body disease protein (IBDP), which formed aggregates as insoluble inclusion bodies in the cytoplasm of affected tissues.[4] While several viruses have been identified in snakes with IBD, [2, 4, 6, 7] a firm connection between these viruses and the formation of IBDP has not been made.

Recently, three strains of arena-like viruses within IBD positive tissues were identified using deep sequencing methods and bioinformatics analysis.[8] Although they have a genome organization of a typical arenavirus, they belonged to a lineage that is very distinct from other known arenaviruses.[8] Additionally, they have a glycoprotein that is more closely related to filoviruses rather the known arenaviruses.[8] Until the morphology of these viruses is verified, they are currently recognized as arena-like viruses. These arena-like viruses were considered the candidate etiological agents for IBD.[8] The genomic sequences of two arena-like viruses were annotated and available in the NCBI GenBank database, which were California Academy of Science Virus (CASV) derived from IBD positive annulated tree boas and Golden Gate Virus (GGV) derived from IBD positive boa constrictors.[8] Stenglein et al. also produced a polyclonal antibody against a synthetic peptide that was derived from the C-terminus of the predicted nucleoprotein (NP) in GGV.[8] The polyclonal antibody reacted to the IBD inclusion bodies by immunohistochemistry and western blots.[8] However, direct amino acid sequencing of IBDP was not performed in this study.

Up until the above recent findings revealing a link between arena-like viruses and IBDP, it was unknown if IBDP represented a host protein that was induced by the causative agent or whether IBDP was a protein that was a component of the causative agent. Sequencing IBDP will determine whether the inclusion bodies were derived from the GGV-NP. Knowing the protein sequence of IBDP will help us better understand the nature of IBDP and its accumulation. A monoclonal anti-IBDP antibody (anti-IBDP MAB) was developed, and validated by immunohistochemical (IHC) staining. Due to the high specificity of this antibody to IBDP, it can be utilized to purify the soluble form of IBDP for protein sequencing. With knowledge of the protein sequence, better diagnostic tests can be developed and used as a tool for screening collections of snakes for IBD.

Material and Methods

Protein Preparation

Semi-purified IBD inclusion bodies. The semi-purified IBD inclusion bodies (IB prep) using methods described in Chapter 2 (IBDP Purification) were reduced using methods described in Chapter 4 (Antigen Detection Using Western Blots), and resolved on a SDS-PAGE or a NuPAGE. Each well was loaded with reduced IB prep with its maximum loading capacity.

Immuno-precipitation (IP). The frozen supernatants of the liver homogenates (boa 0906) made by the procedure described in Chapter 2 (IBDP Purification) were thawed. The protein concentration was determined by the method described in Chapter 4 (Antigen Detection Using Western Blots). For antibody binding, 30 μL of protein A/G coated beads (Santa Cruz Biotechology, sc-2003) was added to each 1.5 mL tube, and incubated with 300 μL of Tris buffered saline (TBS) containing 10 μg of anti-IBDP MAB.

The tubes were placed on ice and agitated overnight. Next day, the beads were pelleted by centrifugation for 1 minute at 1,000 rpm in room temperature on a Spectrafuge™ 16M Microcentrifuge (Labnet International, Edison, NJ). The supernatant of each tube was removed, followed by washing the beads three times. Each wash was done by resuspending the beads in 300 μL RIPA buffer (Thermo Scientific) agitated for 15 minutes, and discarded the supernatant. Except for the negative controls, each tube containing anti-IBDP MAB bound beads were incubated with 500 μL of IBD positive liver supernatant in RIPA buffer with a final protein concentration of 1 μg/mL. For the IP negative control tube, the beads were incubated with RIPA buffer without liver supernatant. For the sham control tube, the beads were incubated with 500 μL of IBD negative supernatant in RIPA buffer with a final protein concentration of 1 μg/mL. The tubes were placed on ice and agitated overnight. The following day, the supernatant of each tube was removed, and the beads were washed four times with RIPA buffer. Finally, the beads in each tube were resuspended in 40 μL of 1X LDS NuPAGE sample buffer (Novex) with addition of 1 μL 1M dithiothreitol (DTT). The tubes were heated at 95°C for 10 minutes, and the supernatants were resolved on a 4-12% NuPAGE gel (Novex). Each well was loaded with reduced IB prep with its maximum loading capacity.

Purified protein bands. The resolved IBDP derived from IB prep or IP was visualized by SimplyBlue™ stain (Novex), using manufacturer's protocol. The IBDP protein bands were cut out and two to three bands were pooled in a tube, and stored at -80°C for later use.

Protein confirmation by western blots. To confirm the quality of protein purification, a portion of the gel containing resolved IBDP was transblotted and detected

by anti-IBDP MAB on western blots using the procedure described in Chapter 4 (Antigen Detection Using Western Blots).

Mass Spectrometry Sample Preparation

Reduction and alkylation. The gel bands containing purified IBDP protein were washed/distained with 50% sequence grade acetonitrile (ACN), then once with 100% ACN, and twice with 50% ACN in 50 mM ammonium bicarbonate (AB) buffer pH 8.4. Each wash was done by agitating the gel with 200 μL of above solutions for 30 minutes. The protein was reduced by incubating the gel bands in 100 μL of 45 mM DTT in AB buffer at 95°C for 10 minutes, followed by incubating the gel bands at 60°C for 50 minutes. The gel bands were chilled, and the DTT was removed. The alkylation was done by incubating with 100 μL of 100 mM iodoacetamide in AB buffer for 1 hour at room temperature in dark. The iodoacetamide was removed, the gels were washed three times with 50% ACN in AB buffer, and dried completely by a CentriVap Vacuum Concentrator (Laconco, Kansas City, MO).

Enzyme digestion. The dried gels were rehydrated either with 10 μL of 12.5 ng/μL trypsin (Promega, V5111), chymotrypsin (Promega, V106A), or Asp-N (Roche, 11-054-589) in digestion buffer (100 mM Tris, 10mM $CaCl_2$, pH 8.0). The tubes were placed on ice for 45 minutes, and more enzyme solution was added as needed until the gels became completely rehydrated. The rehydrated gels were covered with 25 μL digestion buffer. For complete digestion, the trypsin and Asp-N digestion were incubated overnight at 37°C, and the chymotrypsin digestions were incubated overnight at room temperature. For partial digestion, the gels were incubated for 4 hours only. The digestion was stopped by the addition of 5% acetic acid to a final concentration of 0.5%,

and the tubes were store at -80°C until extraction. For each digestion condition, a gel band containing bovine serum albumin (BSA) was used as standard control, and was digested parallel with the IBDP samples using the same protocol.

Peptide Extraction. The supernatants containing digested peptides were collected from the thawed samples in new clean tubes. The gels were extracted twice with 200 μL of 50% ACN in 5% formic acid (FA), vortexed for 15 minutes, and the collected supernatant was combined with the previously collected supernatants. The extracted peptides were lyophilized in a CentriVap Vacuum Concentrator and stored at -80°C.

Tandem Mass Spectrometry

The dried peptide samples were resuspended in a loading buffer containing 3% ACN, 1% acetic acid, and 0.1 % trifluoranoacetic acid. The extracted peptides were then injected onto a capillary trap (LC Packings PepMap) and desalted for 5 min with a flow rate 10 μL/minute of 0.1% v/v acetic acid. Samples were loaded onto an LC Packing® C18 Pep Map HPLC column. The elution gradient of the HPLC column started at 3% solvent A, 97% solvent B and finished at 60% solvent A, 40% solvent B for 60 min for protein identification. Solvent A consisted of 0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H_2O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H_2O. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was carried out on a hybrid quadrupole-TOF mass spectrometer (QSTAR, Applied Biosystems, Framingham, MA). The focusing potential and ion spray voltage was set to 275 V and 2600 V, respectively. The information-dependent acquisition (IDA) mode of operation was employed in which a survey scan from m/z 400–1200 was

acquired followed by collision induced dissociation (CID) of the three most intense ions. Survey and MS/MS spectra for each IDA cycle were accumulated for 1 and 3 seconds, respectively.

Sequence Analysis

Protein identification with Mascot and Scaffold . Tandem mass spectra were extracted by ABI Analyst version 1.1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.0.01). Mascot was set up to search NCBInr database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 0.30 Da. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, were specified in Mascot as variable modifications. Scaffold (version Scaffold-01-06-03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[22] Protein identifications were accepted if they were established at greater than 99.0% probability and contain at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[23]

Protein identification with Protein Pilot v4.2. Using Protein Pilot v4.2 software, The IPI bovine database (60814 total entries) was merged with eight protein sequences that derived from genome of two Arenavirus strains California Academy of Sciences virus (CASV) and Golden Gate virus (GGV) GenBank accession numbers JQ717261 to JQ717264. The eight protein sequences were formatted in the same way as the IPI database and there were assigned IPI-like numbers composed by the date of database creation followed by a later, the name of the protein was input manually, and all other

parameters were defined as 0000. The identification of proteins was performed using ProteinPilot™ Software 4.2. The searching parameters were set as: protein identification, cystein alkylation, iodoacetamide, trypsin (or chymotrypsin, or Asp-N) digestion, and the identification focus for biological modifications.

Results

Protein Preparation

The IB preps made from liver of two IBD+ boa constrictors (0876 and 0906) were reduced, and the resolved 68 KDa IBDP gel bands were collected. Utilizing the anti-IBDP MAB in IP procedure, a soluble form of IBDP within the supernatant of liver homogenate was demonstrated (Figure 5-1). The quality of IP was monitored by the SimplyBlue™ protein stain on the gels and western blots detected with anti-IBDP MAB. The negative control showed adequate binding of anti-IBDP MAB to the protein A/G beads. The IBDP was detected within the IP product from the IBD+ liver, but not detected within the IP product from the IBD- liver. The resolved 68KDa IBDP gel bands from IP preparations were collected. The protein bands were digested with either trypsin, chymotrypsin, or Asp-N, with digestion of a separated BSA control gel using the same protocol that was performed parallel with the IBDP samples.

Sequence Analysis

The tandem mass spectra derived from digested peptides of each sample were searched against the NCBInr database (updated July 2012). The peptides derived from BSA controls matched with the BSA sequence in the database with moderate to high coverage, which indicated a successful enzyme digestion, peptide extraction, and the adequate MS/MS analysis. The peptides derived from IBDP samples did not match with any known protein in the database.

Searching the tandem mass spectra within the in-house protein library built in Protein Pilot v4.2 software, containing the complete Bovine proteins and 8 predicted proteins derived from the genomes of CASV and GGV (each virus has 4 proteins: z protein, L protein, NP, glycoprotein). The peptides derived from BSA controls matched with the BSA protein sequence within the library with high coverage. Each of the digested IBDP samples had produced peptide(s), which in various degrees the tandem mass spectra matched within the predicted protein sequence of GGV-NP (Figure 5-2, 5-3). Besides matching with GGV-NP, the peptides derived from digested IBDP samples did not match with other predicted viral proteins in the library, which included NP of the related annulated tree boa strain (CASV). Compared to the IBDP samples derived from IB preps (Figure 5-2), the IBDP samples derived from IP generated more matching peptides that had coverage above 95% and more overall coverage of amino acids across the predicted GGV-NP sequence (Figure 5-3). The entire length of the predicted GGV-NP is 591 amino acids (a.a.) long (Figure 5-4). When combining all the matching peptides detected, a total of 535 a.a. within the predicted GGV-NP had been seen by MS/MS. Thus, the combined coverage of the matching peptides derived from all sequenced IBDP samples indicated an overall coverage of 90.5% (535/591) to the predicted GGV-NP sequence (Figure 5-4).

Discussion

Challenges in Sequencing IBDP and the Improvements in Methodology

Between the early 2000s and 2010, despite multiple attempts at sequencing IBDP, the protein identity and the origin of IBDP could not be determined. The most challenging aspects of sequencing and identifying a novel protein are the ability to obtain a high quality purified protein sample and the availability of a homolog protein or

reference sequence in the database. Although Edman degradation (N-terminal sequencing) is the ideal method for identifying protein sequences of a novel protein, in which a database or a reference sequence is not required, [24, 25] multiple attempts to direct sequence IBDP by Edman degradation during the past 10 years all turned out unsuccessful. With the preparation of pooling multiple transblotted IBDP protein bands in one sample, no significant amino acid signals was generated after Edman degradation. This suggested that the N-terminal of IBDP may be blocked, and the Edman degradation can not be used for sequencing IBDP. Thus, tandem mass spectrometry became the next best sequencing method available for IBDP sequencing.

Initial studies in the early 2000s used crude materials with less degree of purification for protein sequencing. Starting 2008, a semi-purified inclusion body preparation was used for sequencing. However, due to the insolubility of aggregated inclusion bodies, the efficiency of obtaining enough protein material for MS/MS sequencing was low, only a portion of the reduced IBDP within a sample could be resolved in a gel. Because of this, the amount of purified IBDP within a gel band was not sufficient for sequencing.

Utilizing a newly developed anti-IBDP MAB (Chapter 2) with IP methodology, the process of IBDP isolation was simplified, and the yield of purified IBDP was substantially increased. However, when sufficient amount of IBDP was initially subjected to sequencing (2008 to 2010), no match was found in the existing NCBI databases. At the time it was difficult to determine whether the unsuccessful sequencing was: 1) caused by insufficient sample preparation and processing; 2) the data analysis was insufficient; 3) or simply because of an absence of IBDP homolog proteins in the

database. Until the predicted arena-like virus protein sequences were available in 2012, [8] the previously abandoned MS/MS sequence data were found to match with the GGV-NP. This demonstrated the difficulty in sequencing a protein de novo, and the importance of having related proteins or genomic information in current databases.

The IBDP samples derived from IP preparation generated more matching peptides with higher coverage to the predicted GGV-NP, compared to the samples derived from IB preps (Figure 5-2, 5-3). It is possible that IP preparation improved the quantity and quality (purity) of purified IBDP. Thus, with less contaminants and more concentrated target protein (IBDP) in the sample, resulted in better peptide separation in liquid chromatography, more intensive mass spectra signals, and lead to better protein identifications.

Arenaviruses and Their Protein Divergence

Arenaviruses are enveloped viruses, with a bi-segmented negative-strand RNA genome.[26] According to Sebastien et al. in 2009, the Arenaviridae family comprises 22 viral species, each of them associated with a rodent species.[26] Rodents were thought to be the natural host of arenaviruses, and the infections in rodents were chronic and asymptomatic.[8] Prior to the discovery of the snake arena-like viruses (CASV and GGV) the arenaviruses were though to infect only mammals.[8] Several arenaviruses are known to cause severe disease in human, such as, the lymphocytic choriomeningitis virus (caused lymphocytic choriomeningitis), Lassa virus (caused Lassa fever), and Junin virus (caused Argentine hemorrhagic fever).[8, 26]

Protein sequences among arenaviruses are highly divergent, as well as the snake arena-like viruses.[8, 26] The predicted NP sequence of the snake arena-like viruses only shared approximately 25% amino acid identity between the predicted NP sequences of

known areaviruses.[8] Between CASV and GGV, their predicted NP sequence only shared approximately 55% amino acid identity.[8] Interestingly, the peptides derived from IBDP of boa constrictors (GGV-NP) did not share any amino acid sequence homology with the predicted NP sequence of CASV (the related annulated tree boa strain). This indicated possibly a higher than expected divergence between these two arena-like viruses. Although currently, there had been a lack of agreements in the criteria being used for species demarcation in Arenaviridae, [26] these IBD associated arena-like viruses may eventually form a new clade. Additionally, the diversity among different arena-like viruses may explained the limited cross-species reactivity discussed in Chapter 3 (Cross Reactivity Among Non-Boa Constrictors), in which an antibody produced against NP of one arena-like virus strain could not recognize the NP of other arena-like virus strains.

Confirmation of the Linkage between IBDP and GGV-NP

In addition to a high overall coverage (90.5%) of IBDP derived peptides to the predicted GGV-NP, the repeated detection of peptides at the N-terminus, mid-section, and C-terminus of GGV-NP indicated that the protein identification is highly confident. The peptide 'TKDPTPATI' at the C-terminus of GGV-NP (Figure 5-4) was repeatedly identified in the trypsin digested IBDP samples derived from IP preparations (Figure 5-3). This peptide resembled a portion of the 14 amino acid peptides that were used to produced anti-GGV-NP antibody by Stenglein et al. that recognized IBD inclusion bodies using fluorescence IHC.[8] The sequence result obtained from purified IBDP had confirmed the accuracy of the GGV-NP sequence prediction by genomic sequence analysis. Further, this protein sequence result supported the linkage between GGV infection and the formation of IBD inclusion bodies.

Future Diagnostic Tests Development for IBD

Knowing the protein sequence of IBDP, the marker protein for IBD, this made possible for the development of other higher quality molecular diagnostic tests. For immuno- diagnostic approaches, a better and more consistent immunogen (compared to the semi-purified inclusion bodies) can be obtained through recombinant peptides. The recombinant peptides of IBDP can be produced with consistency to serve as the antigen for serological tests/studies in enyzyme linked immunosorbent assay (ELISA) or western blots. Further, a better and more specific anti-IBDP MAB can be produced using the recombinant peptides of IBDP for immune-diagnostic tests, such as, IHC staining, ELISA and western blots. With the confirmation of the link between IBDP and GGV-NP by this study, a polymerase chain reaction (PCR) screening test can be developed targeting the GGV-NP. Further, a PCR test can be developed for IBD in other non-boa constrictor species by designing primers targeting NP of the particular viral strains.

Conclusions

Purified IBDP derived from 2 IBD+ boa constrictors using two preparation methods, reduced semi-purified inclusion bodies (IB preps) and IP. Using MS/MS sequencing, all of the digested IBDP samples generated peptides that matched within the predicted protein sequence of GGV-NP. The combination of matching peptides derived from all sequenced IBDP samples generated an overall coverage of 90.5% within the predicted GGV-NP sequence. The protein sequence results indicated a highly confident identity of the IBDP to the GGV-NP. This finding further suggested that the GGV is possibly the causative agent of IBD.

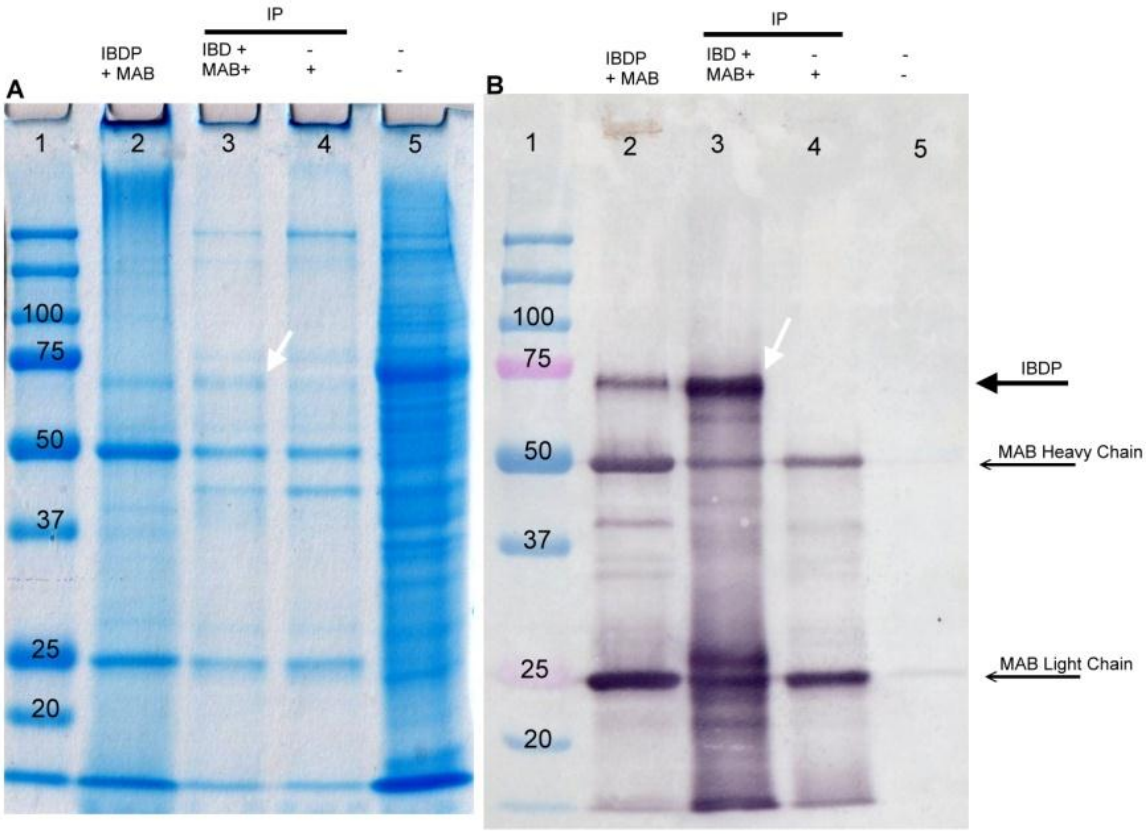

Figure 5-1. The total protein stain and western blot demonstrated a soluble form of IBDP that was immunoprecipitated (white arrows). Lane 1 is the molecular weight marker, lane 2 is the purified IBDP with anti-IBDP MAB to show the molecular weight of IBDP, heavy chain and light chain of anti-IBDP MAB. Lane 3 is the IP product from IBD+ liver homogenate. Lane 4 is the IP product derived from IBD- liver homogenate. Lane 5 is the IBD- liver homogenate. A. The total protein stain of the gel with resolved IBDP derived from IP. The purified IBDP indicated by the white arrow is removed for sequencing. B. The western blot using anti-IBDP MAB. The purified 68 KDa IBDP (large black arrow) purified from the IBD+ liver homogenate (lane 3), but not presence in purification from the IBD- liver homogenate (lane 4). Anti-IBDP MAB did not detect IBDP in the IBD- liver homogenate (lane 5). The heavy and light chains (small black arrows) of the resolved anti-IBDP MAB.were detected by the secondary antibody used for western blot.

Figure 5-2. The coverage of peptides derived from IB preps (IBDP) over the predicted GGV-NP sequence by MS/MS sequencing. Green indicated peptide with coverage equal or above 95%, yellow indicated peptide with coverage equal or above 50% but less than 95%, red indicated peptide with coverage less than 50%, and grey indicated peptide with no coverage.

Figure 5-3. The coverage of peptides derived from immune-precipitated IBDP over the predicted GGV-NP sequence by MS/MS sequencing. Green indicated peptide with coverage equal or above 95%, yellow indicated peptide with coverage equal or above 50% but less than 95%, red indicated peptide with coverage less than 50%, and grey indicated peptide with no coverage.

Golden Gate Virus (GGV) predicted nucleoprotein (NP) full sequence:

MAAFQKAAVNQLALKKKLNKMLAPYQRELNNQIFKDVKALRVGLDINKVNDTLRRLRKET
KGPNDLKNLRDLNETAAGLSGMVATQRTVEIDSSLMSDEELIQCIENIDVIKKKAEYKGGSR
PRISEFESETGMSKSDHEMFNKLFNRFAPKKDPGPAAFSTPKSWVGISPADLANQFGTSP
AITICLIMMRTNSPFKQILDALHDISLLDQGMFVNASVVKAMTSQHPCLDCVEYTVPKNSSG
YNITVKAVVKAANVLSKLPKVEKLVIDDDNRVEIIRTLLTIQRELNIK**IQVNEERGLFEDIFYKIC**
VSPNGPCVVSIRSELTGRGWENTIFRLR**RPPPYAPR**L**YPDLMDLDLDALPPVKGDKKTEEE**
SKIYIFKPAADEIDEYIRSPESASSDSEIPDPRVLYMANACEDLFKGGDSVFMDIEGTAQDPV
EIALFNPDTGKFVHIFRMPKDKDGFKKASKHAHGLLLDDISDHPDLQTDKNIEAFFSKVPLS
ARIFCQGSDIEECLKFFGR**KDLKPTDCK**WKREEFMKYHENILDELSEVFPCKH**SGTVKDKK**
GALTAPHCALLDCLMFSRTASGGKK**TKDPTPATI**

Total protein sequence length= 591 amino acids
Peptides seen by MS/MS = 535 amino acids
Total coverage to GGV-NP = 90.5% (535/591)

Figure 5-4. The overall coverage of peptides derived from purified IBDP over the predicted GGV-NP sequence by MS/MS sequencing. The peptides detected by mass spectrometry were underlined. The C-terminal of GGV-NP was repeatedly detected in many IBDP preparations, which is a portion of the peptide (highlight in yellow) that Stenglein et al. used to produce anti-GGV-NP polyclonal antibody.[8]

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BIOGRAPHICAL SKETCH

Li-Wen Chang also known as Rita Chang was born in Taiwan in 1982. She had strong interests in exotic animals since her childhood, and she presented her first science project about Fresh Water Turtles in the Middle Schools Scientific Exposition. During her high school years, she was actively involved in Taiwan Cetacean Society as a trained volunteer. She volunteered in events for bringing public awareness of the importance in Cetacean conservations. Following her interests, she perused her bachelor degree in Veterinary Medicine at National Chung Hsing University (NCHU), Taiwan. After graduation in 2005, she passed the national certification exam and became a board certified Veterinarian in Taiwan.

Her interests had always been focused in the field of exotic and zoological medicines. She completed externships at Taipei Zoo (mentored by Dr. An-Hsing Lee), and at Ludwig Maximilian University of Munich under the Clinic of Animal Gynaecology, the Equine Clinic, and the Clinic of Small Animal Surgery. In 2006, she completed a one-year residency at the Exotic Animal Clinic of Veterinary Medical Teaching Hospital, NCHU. She also spent one year in Europe-America Biotechnology Co., Ltd as a specialized veterinarian to manage laboratory animals.

In 2006, Dr. Chang received a governmental scholarship awarded by the Ministry of Education in Taiwan to pursue advanced degree in USA. In the spring of 2008, she was fortunate and honored to be admitted to the graduate program at Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida. Being mentored by Dr. Elliott Jacobson, she become deeply involved in the research of diagnostic tests development for Inclusion Body Disease. In 2012 fall, she received her Doctor of Philosophy in Veterinary Medical Science from University of Florida. She will

return to Taiwan and continue to devote herself in the clinical work of exotic animal medicine.