

Diagnosis of Boid Inclusion Body Disease: Challenges and Future Prospects

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Abstract: *Boid inclusion body disease is one of the fatal diseases of captive snakes worldwide. Until recently, cases were diagnosed by the demonstration of eosinophilic intracytoplasmic inclusion bodies under light microscopy. Unfortunately, inclusion bodies are also found in many viral infections. The specific etiologic agent of this disease remained a mystery for over 3 decades. However recently, highly divergent arenaviruses were isolated from some snakes with the disease even though direct causal linkage has not yet been fully established. Research has so far been focused on understanding the formation and nature of the inclusion body protein commonly found in tissues of affected snakes. Isolation of this protein has led to a better understanding of its nature and composition, which has consequently led to the development of monoclonal antibodies for use in immunohistochemical diagnostic techniques. This review describes the diagnostic techniques in use today and those that have potential applications in clinical diagnosis of the Disease. Selected approaches from published literature, as well as those in commercial development have also been discussed.*

Keywords: *Boid Snakes; Arenavirus; Boid inclusion body disease protein; Diagnostic techniques*

I. Introduction

Boid Inclusion body disease (BIBD) is considered as one of the most important diseases of snakes mainly because of its very high morbidity and mortality rates, and the lack of definite information regarding its treatment and prevention. The disease is infectious and highly fatal affecting captive snakes commonly of the families Boidae (Boas and Anaconda) and Pythonidae (Pythons), as well as other species (Schumacher et al., 1994; Orós et al., 1998; Wozniak et al., 2000; Jacobson et al., 2001). The disease was first recognized in the 1970s in captive snakes and was associated with the eradication of entire boid collections (Schumacher et al., 1994). The diagnosis of BIBD is still based on histopathological presence of eosinophilic intracytoplasmic inclusion bodies in tissues and organs of affected animals. However, inclusion bodies are not specific to BIBD alone and are found in many other viral infections. Previously, retroviruses and filoviruses were assumed to be responsible for the disease causation due to consistent isolation in infected snakes (Huder et al., 2002; Schumacher et al., 1994; Jacobson et al., 2001; Pees et al., 2010; Ariel, 2011). However, researchers have recently isolated highly divergent arenavirus from tissue samples of boa constrictors and pythons showing clinical signs of BIBD (Stanglein et al., 2012; Bodewes et al., 2013).

The identification of protein inclusions in tissue sections or blood films has been the gold standard in diagnosis of BIBD for decades. While many snakes with BIBD may have numerous inclusions in multiple tissues (Jacobson, 2007), some snakes may have few, which are confined to certain organs and are easily overlooked. Furthermore, inclusions resembling those seen in BIBD have been seen in snakes with other viral diseases, and distinguishing them from BIBD inclusions can be challenging (Bodewes et al., 2013; Raymond et al., 2001; Jacobson, 2007).

There are few viruses capable of causing clinically significant diseases in reptiles. Ophidian paramyxoviruses and morbilliviruses of the family paramyxoviridae are two important viruses reported in boid snakes causing proliferative pneumonia and eosinophilic intracytoplasmic inclusion bodies. Additionally, secondary infections due to bacterial or fungal pathogens have often been incriminated in the development of BIBD lesions in infected snakes. However, the presence of multisystemic intracytoplasmic epithelial inclusions is distinctively peculiar to BIBD (Frankel et al., 2001; Homer et al., 1995; Kolesnikovas et al., 2006).

Snakes infected with BIBD show variable clinical signs ranging from neurologic signs, increased susceptibility to secondary bacterial infections and neoplasia (Schumacher et al., 1994). Because of the variability of the clinical signs, the list of differential diagnosis is therefore long and may frequently include bacterial infections, parasitic diseases, trauma and exposure to toxins. The disease has no treatment and no vaccine is available. Mortality is 100% once clinical signs appear. Therefore, euthanasia is recommended once evidence of neurologic involvement exists or if there is a risk of transmission to other snakes (Wozniak et al., 2000). Currently, there is no evidence that snakes transmit Boid Inclusion Body Disease Virus (BIBDV) to humans.

Earlier studies have documented that the inclusion bodies in BIBD consists of a unique protein with a molecular weight of approximately 68kDa, this was referred to as Boid Inclusion Body Disease Protein or BIBDP (Wozniak et al., 2000). It is believed that this protein is part of a viral nucleoprotein and understanding its nature and composition may lead to a better understanding of the cause, progression and diagnosis of the disease. This review thus aims to look at the challenges and the future prospects in the diagnosis of BIBD.

Aetiology of BIBD

Retroviruses have been widely incriminated as the causative agents of BIBD for over three decades due to consistent isolation of these viruses from tissue samples of infected snakes. It was later understood that these are endogenous to snakes as they were frequently recovered from genome sequences of uninfected snakes as well. Furthermore, inoculation of samples from infected snakes in some cases failed to reproduce the disease despite the presence of the virus in the samples. Recently, four novel arenavirus genomes were isolated from tissues of snakes diagnosed with BIBD (Bodewes et al., 2013; Stenglein et al., 2012; Hetzel et al., 2013). Therefore, there are currently four highly divergent arenavirus genomes in the sequence database.

Natural Hosts

Snakes belonging to two super families' (Pythonidae and Boidae), which comprise of pythons, boa and anaconda are the natural host of BIBD. However, a similar disease has been reported in colubrid snakes, California king snake (*Lampropeltis getula*) (Raymond et al., 2001), corn snakes (*Elaphe guttata*) (Fleming et al., 2003), and in viperids like the captive palm vipers (*Bitriechis marchi*) (Raymond et al., 2001)

Distribution

Boid species of snakes inhabit Central and South America, Africa, Madagascar, Nepal, India, Burma, southern China, Southeast Asia, and Australia (<http://www.reptile-database.org/>). Although the disease has not been reported in the wild, evidence suggesting its worldwide occurrence in captive snakes exists (Axthelm, 1985; Schumacher et al., 1994; Carlisle et al., 1998; Orós et al., 1998; Collett et al., 1990; Jacobson, 1996).

Epidemiology

Transmission of BIBD involves direct contact with infected snakes. Venereal transmission has been suggested and the snake mite, *Ophionyssus natricis*, has been implicated as a potential vector as they were often present in infected snake collections (Jacobson, 2007). The exact incubation period of the disease varies, with an acute course spanning a few weeks, to a chronic course that lasts for months. It is also believed that some snakes may become carriers as infected boas have been observed for over 12 months without developing clinical signs (Wozniak et al., 2000). The occurrence of latent infections may therefore be a possibility (Schumacher 2006).

Clinical Features

Although clinical signs may be variable, regurgitation which may be on and off is usually among the first signs of the disease in boas followed by anorexia. Dysecdysis (abnormal shedding) may occur. Snakes that develop chronic regurgitation with stomatitis may lose weight, develop pneumonia and have clogged nares. Terminally, snakes develop neurological signs such as head tremor, mydriasis, incoordination, opisthotonus, and a loss of the righting reflex response (Schumacher et al., 1994).

In pythons unlike in boas, clinical signs primarily involving regurgitation are less common features. The neurological signs in pythons develop earlier than in boas and are more pronounced (Schumacher et al., 1994). Although some snakes die within several weeks of first manifesting illness, others may survive for months and commonly develop secondary diseases such as necrotic stomatitis, pneumonia, lymphoproliferative disorders, necrotic dermatitis and round cell tumour. Acutely affected boas also show lower protein and globulin values, and significantly higher aspartate amino transferase values compared to those of chronically affected snakes (Jacobson et al., 2001). Lymphoid depletion with marked leucopenia may occur in advanced stages and Inclusion bodies may be visible within circulating lymphocytes (Schumacher, 2006).

BIBD in palm vipers was found to be similar to BIBD in boas and pythons (Raymond et al., 2001) and is characterized by prolonged anorexia, history of periodic regurgitation and loss of motor function in the posterior half of the body (Raymond et al., 2001; Sheehan and Hrapchak, 1980).

Pathology

BIBD is typically diagnosed at histopathology and stained blood smears through light microscopy (Chang and Jacobson, 2010). Intracytoplasmic inclusions are seen in a wide variety of epithelial and neuronal cells from Haematoxylin-Eosin (H&E) stained tissue sections (Jacobson, 2002; Garner and Raymond, 2004). Inclusions are commonly seen in the liver, kidney, and pancreas, but may not be visible when the inclusions are very few or poorly distributed in the tissue sections (Jacobson, 2007). Thus, inclusions may be missed even by

experienced pathologists in some cases. While the presence of characteristic inclusion bodies is diagnostic for the disease, the absence of inclusions does not necessarily mean the snake is BIBD free. In some cases many tissue sections have to be re-examined to find inclusions. Mild to severe encephalitis with lymphocytic perivascular cuffing may be seen in the brain. Snakes with lymphoproliferative disorders have been identified with lymphoid infiltrates in multiple organs (Jacobson, 2007).

In the blood, inclusions can be seen in lymphocytes and other circulating blood cells in peripheral blood films stained with Wright-Giemsa or Haematoxylin and Eosin (H&E) stained slides.

Lesions such as hepatocyte degeneration, renal tubular necrosis and lymphoid depletion are frequently associated with infected boas (Jacobson, 2007).

Post-mortem Diagnosis

Diagnosis of BIBD at post-mortem is also based on the light microscopic identification of the variably sized eosinophilic intracytoplasmic inclusions in haematoxylin and eosin stained tissue sections (Jacobson, 2007). Inclusions in pythons are mostly found within neurons in the CNS. In boas, the inclusions are commonly observed in neurones and glial cells in the CNS, mucosal epithelial cells, lymphoid cells in oesophageal tonsils, epithelial cells lining the gastrointestinal and respiratory tracts, hepatocytes, pancreatic acinar cells and renal tubular epithelial cells (Vancraeynest et al., 2006).

II. Laboratory Diagnosis

Limited laboratory diagnostic approaches are currently available for the diagnosis of BIBD. These include the following:-

Light Microscopic Examination for Presence of Inclusion Bodies

Tissues and blood smears are stained with Haematoxylin and Eosin (H&E) for the demonstration of the inclusions bodies under light microscope which is currently considered the gold standard for diagnoses of BIBD (Chang and Jacobson, 2010; Banajee et al., 2012). However, inclusion bodies seen in BIBD sometimes can be difficult to distinguish from other cellular proteinaceous materials or cellular granules that may accumulate in the cytoplasm of affected cells (Chang and Jacobson, 2010). Similarly, several viral diseases have been incriminated in producing inclusion bodies in infected cells and may therefore pose a great challenge to the pathologists.

Polymerase Chain Reaction (PCR)

PCR involves the detection of segment(s) of the viral genome. It was the first nucleic acid amplification technique to be described and has since become the most widely used molecular diagnostic technique in clinical use today, with several reviews, chapters and books devoted to its methodology and application (Baumforth et al., 1999; Becker and Darai, 1995).

The recent discovery of four complete arenaviral genomes associated with BIBD has led to the development of specific PCR tests for the detection of these viruses in some Laboratories. The challenge however is that, these are highly divergent RNA viruses, thus making it impossible for the development of a universal Primer for the screening of BIBD- infected snakes worldwide.

Electron Microscopy

Transmission electron microscopy (TEM) can be used to examine the inclusion bodies in infected cells. It was observed that these inclusions vary in shape and size, however, beyond the size and morphology of this protein, the chemical composition of the inclusions remained unknown for many decades, even though it is believed to be associated with the viral nucleoprotein (Wozniak et al., 2000).

Immunohistochemical (IHC) Staining

Immunohistochemical diagnostic technique is being increasingly used, because it is fast, sensitive, specific, less laborious and relatively inexpensive than traditional microbiologic procedures. Immunohistochemistry uses immunologic techniques to detect specific proteins to identify infectious and non-infectious disease agents (Ramos-Vara et al., 2008). It is not yet known whether all BIBD cases in different snake species consist of exactly the same protein or if specie specific variations occur. Polyclonal antibodies were produced against the predicted nucleoprotein of an arena-like virus which recognized the BIBD inclusion bodies in tissue sections of infected snakes (Stenglein et al., 2012; Hetzel et al., 2013).

Researchers have recently isolated inclusion body proteins from a boa constrictor with BIBD and a new anti-BIBDP MAB (Monoclonal antibody) that stains inclusions in paraffin-embedded tissues has been produced. This MAB is undergoing testing and validation studies to ascertain its sensitivity and specificity before applying it toward blood screening (Bodewes et al., 2013). The MAB can be used to develop more

sensitive and specific molecular diagnostic tests such as western blot or ELISA for screening individuals and colonies of snakes at risk.

Furthermore, obtaining the whole genome sequences of the BIBD-associated arenaviruses will help in understanding the relationship between the virus, the protein and the disease pathogenesis.

III. Future Research in the Diagnosis of BIBD

Research into the pathophysiology of infection and inflammation in animals by assessment of serum Acute Phase Protein (APP) concentrations has opened a fascinating aspect of the host non-specific defence mechanism (Johnson, 1997). Acute Phase Protein (APP) is a plasma protein which increases in concentration following infection, inflammation and trauma (Whicher and Westacott, 1992; Eckersall and Conner, 1988; Baumann and Gauldie, 1990). Investigations over the last decade have shown that their quantification in plasma or serum can provide valuable diagnostic information in the detection, prognosis and monitoring of disease processes. APP analysis is becoming a common procedure in clinical and experimental investigations of infectious diseases in companion and farm animals (Myers, 1995; Schijns and Horzinek, 1997; Beck and Habicht, 1986). Assessment of the concentration of acute phase proteins provides a means to estimate the combined effect of the pro-inflammatory cytokine stimulation of systemic functions. In the future, measurement of these proteins could have further applications in the identification of diseased snake before introduction into a collection and for monitoring the presence of sub-clinical disease.

Nanotechnologies currently extend the limits of molecular diagnostics (Jain, 2003). Although the potential diagnostic applications are unlimited (Azzazy et al., 2006), the most important applications are visible in the areas of infectious microorganism detection and the discovery of disease biomarkers (Maharana et al., 2010). Nanobiotechnology when applied in clinical diagnosis of BIBD will therefore play a significant role in the prevention and spread of the disease among captive snakes.

Molecular imaging technologies are providing researchers with exciting new opportunities in the study of small animal models, with continued improvements in instrumentation, identification of better imaging targets by genome-based approach and design of better imaging probes by innovative chemistry may be achieved. These technologies promise to play increasingly important roles in disease diagnosis and therapy (Blasberg, 2003; Weissleder, 2006; Massoud and Gambhir, 2003; Gambhir, 2002).

The use of proteomics in the diagnosis of tuberculosis and other infectious diseases has opened the door for its application in the detection of several diseases of viral origin in companion animals. Proteomic analysis of sera from patients with severe acute respiratory syndrome (SARS) has identified potential protein markers which were consistently found in higher concentrations in the sera of SARS patients compared to healthy controls, that may as well prove useful and have great potential as a diagnostic tool (Ying et al., 2004; Ren et al., 2004). Replication of this principle in snakes with BIBD will result in detection of novel protein markers that are associated with the disease.

The authors' interest is currently focused on the use of proteomic analysis for the identification and quantification of cellular stress proteins associated with BIBD. It is believed that understanding cellular stress responses associated with the infection may lead to a better understanding of the disease pathogenesis and consequently diagnosis.

IV. Prevention and Control

Screening of blood samples from clinically healthy snakes for evidence of leucocytosis and presence of inclusion bodies should be routinely conducted especially at airports and other points of entry, whereas biopsies and tissue sections can be examined for eosinophilic intracytoplasmic inclusion bodies. Quarantine of new snakes and routine disinfection of premises are recommended measures for the prevention of BIBD. PCR testing is recommended for screening collections, even though it may not be specific due to the divergent viral strains available. Snakes should only be sourced from collections with no history of the disease. Good husbandry practice such as treatment for mite infestations in snake collections is important for the prevention of BIBD infection. Sodium hypochlorite and chlorhexidine are recommended for the disinfection of premises in order to prevent spread and limit transmission in snake collections (Schumacher, 2006).

No vaccine against BIBD is currently available, thus initiation of rigorous preventive and control measures is key in limiting its spread. There is also the need for a sensitive and specific blood-based immunodiagnostic test for rapid and early detection of BIBD in both captive and wild populations of boid snakes.

V. Conclusion

Boid inclusion body disease is a fatal disease of boid snakes and therefore has the most enormous implications to global trade in captive snakes as well as in disrupting the ecological balance. The ease with which captive snakes are shipped around the world without restriction certainly contributes to the movement of

BIBD infected snakes from one region of the globe to another, thus helping its spread. It is yet unclear where the disease originated from as no report of its occurrence in the wild population has been documented. Maintaining healthy and disease-free animals should therefore be the primary responsibility of those who keep and breed snakes as pets or in aquariums and zoos.

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