The Veterinary Journal 218 (2016) 13–18

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Detection and prevalence of boid inclusion body disease in collections of boas and pythons using immunological assays

L. Chang [a], D. Fu [b], M.D. Stenglein [c], J.A. Hernandez [a,d], J.L. DeRisi [e], E.R. Jacobson [a,*]

[a] Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, 2015 SW 16th Avenue, Gainesville, FL 32608, USA
[b] Molecular Pathology Core, Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, 1395 Center Drive, Gainesville, FL 32610, USA
[c] Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 200 West Lake St., Fort Collins, CO 80526, USA
[d] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, 2015 SW 16th Avenue, Gainesville, FL 32608, USA
[e] Department of Biochemistry and Biophysics, University of California San Francisco, 1700 4th Street, San Francisco, CA 94158, USA

ARTICLE INFO

Article history:
Accepted 22 October 2016

Keywords:
Boid inclusion body disease
Immunohistochemistry
Snakes
Reptarenavirus
Nucleoprotein

ABSTRACT

Inclusion body disease (IBD) of boas and pythons is characterized by the intracytoplasmic accumulation of an antigenic 68 kDa viral protein IBDP, more recently known as the nucleoprotein (NP) of the reptarenaviruses. Blood samples of 131 captive boas and pythons (53 boa constrictors, *Boa constrictor*; 35 rainbow boas, *Epicrates cenchria*; 22 ball pythons, *Python regius*; 5 carpet pythons, *Morelia spilota*; 6 Burmese pythons, *Python bivittatus*; 4 Jamaican boas, *Epicrates subflavus*; 5 anacondas, *Eunectes spp.*; and 1 green tree python, *Morelia viridis*) were obtained from 28 collections in the USA. Diagnosis of IBD was initially made by the identification of eosinophilic intracytoplasmic inclusion bodies in hematoxylin and eosin (HE) stained blood films and isolated peripheral white blood cells (PWBC).

The overall prevalence of IBD in study snakes was 25/131 or 19% (95% CI = 12.4%, 25.8%) with boa constrictors being more commonly infected (22/53 or 41.5%; 95% CI = 28.2%, 54.8%) than other species in this study. Of the 22 IBD positive boa constrictors, 87% were clinically healthy, 13% had various signs of chronic illness, and none showed signs of central nervous system disease. Using a validated monoclonal anti-NP antibody, NP was confirmed within the isolated PWBC by immunohistochemical staining and Western blots. The presence of reptarenaviruses within blood samples of 27 boa constrictors and three rainbow boas was also assessed by PCR. Among boa constrictors, very good agreements were shown between the observation of inclusion bodies (by HE stain) and the presence of NP (by immunohistochemistry, kappa = 0.92; and Western blots, kappa = 0.89), or the presence of reptarenaviruses (by PCR; kappa = 0.92).

Introduction

Inclusion body disease (IBD) is commonly seen in captive boa constrictors (*Boa constrictor*), and occasionally in other species of boas and pythons. Clinical signs of IBD are highly variable, and can include anorexia, regurgitation, stomatitis, pneumonia, and lymphoproliferative disorders. Some may show signs of central nervous system disease, such as incoordination, disorientation, head tremors, stargazing and opisthotonus. Others with IBD may lack any clinical signs of illness and appear clinically healthy (Schumacher et al., 1994; Carlisle-Nowak et al., 1998; Wozniak et al., 2000; Jacobson et al., 2001; Vancraeynest et al., 2006; Chang and Jacobson, 2010; Pees et al., 2010; Schilliger et al., 2011; Banajee et al., 2012; Stenglein et al., 2012; Schmidt et al., 2013). The percentage of snakes with sub-clinical IBD is unknown.

Inclusion body disease is characterized by the formation of intracytoplasmic inclusion bodies in a variety of tissues. The inclusion bodies are not membrane bound and consist of an insoluble 68 kDa protein (IBDP more recently known as reptarenaviral nucleoprotein, NP; Wozniak et al., 2000; Stenglein et al., 2012; Bodewes et al., 2013; Chang et al., 2013; Hetzel et al., 2013). Several arenaviruses were discovered in IBD affected snakes, and have recently been taxonomically assigned to a new genus *Reptarenavirus* in the family *Arenaviridae* (Stenglein et al., 2012; Bodewes et al., 2013; Hetzel et al., 2013; Radoshitzky et al., 2015). The link between reptarenavirus infection and IBD has been bolstered by a strong correlation between detection of viral RNA and inclusion bodies in infected snakes and by the demonstration that infected cells *in vivo* and *in vitro* contained inclusion bodies recognized by antibodies produced against recombinant or purified reptarenavirus nucleoprotein (NP; Stenglein et al., 2012; Chang et al., 2013; Hetzel et al., 2013). Several studies

* Corresponding author.
E-mail address: jacobsone@ufl.edu (E.R. Jacobson).

http://dx.doi.org/10.1016/j.tvjl.2016.10.006
1090-0233/

confirmed that the IBDP represented reptarenaviral NP: using MALDI-TOF/TOF, the peptide sequences of the viral NP isolated from the infected cells matched with the translated amino acid sequences of the reptarenavirus NP encoding RNA (Hetzel et al., 2013). Further, using MS/MS analysis, the peptide sequences of IBDP purified from the liver of IBD infected boa constrictors had a 90.5% coverage to the amino acid sequences of the predicted reptarenaviral NP (Chang, 2012).

Using hematoxylin and eosin (HE) stained peripheral white blood cells (PWBC) as our standard of diagnosis, 131 boid snakes from 28 collections were categorized into IBD+ or IBD–, and prevalence of IBD was estimated from the studied snakes. Further, we assessed the agreement between the observation of inclusion bodies by HE staining and the detection of reptarenaviral NP by immunohistochemical staining and Western blots, using a previously validated mouse anti-IBDP monoclonal antibody (hereafter referred as anti-NP mAb). Additionally, RNA isolated from PWBC of 27 boa constrictors and three rainbow boas were tested for reptarenavirus by qRT-PCR, and examined the concordance between the observation of inclusion bodies and the presence of viral RNA.

Materials and methods

Animal and sample collection

From 2010 to 2012, whole blood samples were obtained from the following species of 131 captive boid snakes: boa constrictor ($n = 53$), rainbow boa (*Epicrates cenchria;* $n = 35$), ball python (*Python regius;* $n = 22$), carpet python (*Morelia spilota;* $n = 5$), Burmese python (*Python bivittatus;* $n = 6$), anaconda (*Eunectes spp;* $n = 5$), Jamaican boas (*Epicrates subflavus;* $n = 4$), and green tree python (*Morelia viridis;* $n = 1$). The snakes were randomly selected from 28 collections with unknown prevalence of IBD. Approximately 1 mL of whole blood samples collected from each snake by cardiocentesis were added to collection tubes containing heparin, subsequently transferred to a refrigerator (4 °C) at University of Florida (UF) until being processed for IBD testing within 24 h of arrival in the Zoological Medicine Infectious Disease Testing Laboratory. The history of each snake was collected via email or telephone communications. Blood sample collection from the snakes was permitted under University of Florida Institutional Animal Care and Use Committee (Protocol 201101156; Approval date: 24 March 2009).

Blood sample preparation

A blood film of each sample was made on a charged microscopic slide (Shandon Colorfrost Plus Slides; Thermo Scientific), air dried, and fixed in 100% methanol for 15 min. The fixed blood film was stained with a published HE staining protocol (Chang et al., 2013). The remaining whole blood samples were centrifuged for 10 min at 8500 *g*, and the plasma was removed. The pelleted blood cells were resuspended with 0.5 to 1 mL of phosphate buffered saline (PBS) and carefully layered over 300 µL of lymphocyte separation media (LSM; Cellgro) in Microtainer tubes (REF365971, BD). The tubes were centrifuged for 30 min at 8500 *g*, after which the red blood cells were pelleted in the bottom of the tube with the PWBC focused on the layer above LSM. All the supernatants in the tube were collected, and the isolated PWBC were pelleted then washed two times with 1 mL of PBS. A portion of the isolated PWBC was resuspended in PBS, and applied onto three charged microscopic slides using a centrifuge (Cytospin2 Centrifuge, Shandon). The remaining PWBC were stored in –-20 °C for antigen detection experiments. One of the slides with isolated PWBC was fixed in 10% neutral buffered formalin for 15 min, rinsed with running water and air-dried. The fixed PWBC slides were HE stained with protocol used above. The two remaining PWBC slides were stored in a freezer at –20 °C until IHC staining.

Classification of IBD-positive or IBD-negative

For the purposes of this study, identifying HE stained IBD inclusion bodies under light microscopy was considered the standard for making IBD diagnosis. Snakes were classified as IBD+ if the characteristic eosinophilic staining intracytoplasmic inclusion bodies were identified in either a HE stained blood film or isolated PWBC. Otherwise, snakes were classified as IBD–.

Reptarenaviral NP detection by IHC staining

The frozen slides with isolated PWBC were thawed, and air-dried overnight at room temperature. The next day, the slides were fixed in 4% paraformaldehyde for 5 min, and washed two times (5 min each) with Tris buffered saline (TBS). The slides were incubated in 0.25% Triton X-100 for 5 min in order to permeabilize the cell membrane. Subsequently, the slides were washed, and incubated with a ready-to-use blocking solution (Peroxo-Block, Invitrogen) for 45 s. The slides were washed again, and the remaining IHC staining procedures were performed manually using a validated anti-NP mAb following a published protocol (Chang et al., 2013). A negative control slide of PWBC stained with non-specific mouse IgG (Mouse IgG Control Antibody, Vector Laboratories) as the primary antibody was performed parallel to the testing slide. IHC positive (IHC+) was defined as presence of specific staining of the inclusion bodies in comparison to the negative control. IHC negative (IHC–) was defined as absence of specific staining of inclusion bodies in comparison to the negative control.

Reptarenaviral NP detection by Western blots

The PWBC pellet was frozen and thawed three times, followed by the addition of 1:1 volume of a 2X lysing buffer (LB) containing 2% sodium dodecyl sulfate (SDS) and 10% beta-mercaptoethanol (2-ME), subsequently solubilized by incubating at 95 °C for 10 min. Two microliters of the solubilized cell pellet was removed, diluted 10 fold with water, and the protein concentration was estimated using a Bradford protein assay (Quick Start Bradford Protein Assay, Bio-Rad) following the manufacturer's procedure. Except for a slight modification, 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 to be tested. A semi-purified IBD inclusion body preparation (NP) was made from livers of IBD affected boa constrictors as previously described (Chang et al., 2013). The protein concentration of the NP preparation was estimated using the procedure described above, and served as a positive control in the Western blot detection assays.

For each sample, 20 µg of PWBC lysate was reduced by incubating with 4X sample buffer (Laemmli SDS-Sample Buffer, Reducing, 4X, Boston BioProducts) at 95 °C for 10 min, and resolved by 10% Tris-Glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For each gel, a molecular weight marker (Precision Plus Protein Dual Color Standards, Bio-Rad) was used, followed by a NP preparation (positive control), and eight isolated PWBC lysates were loaded respectively. The resolved PWBC proteins were transblotted onto a 0.45 µm supported nitrocellulose membrane (Bio-Rad), confirmed by a reversible membrane stain (MemCode, Pierce), and blocked overnight in buffer (5% non-fat dried milk/0.1% Tween20 in PBS). After washing three times with wash buffer, the blot was cut into half, with the top part containing proteins with molecular weight >50 kDa, and the bottom part containing proteins with molecular weights < 50 kDa. Anti-NP mAb in a dilution of 1:3000 in diluting buffer (1% BSA in wash buffer) was applied to the top half of the blot, whereas a commercial anti-beta-actin antibody (THE beta Actin Antibody, GenScript) in a dilution of 1:3000 in diluting buffer was applied to the bottom half of the blot and served as a control. After 1 h of incubation in room temperature, the blots were washed three times, and incubated with a HRP-conjugated goat-anti-mouse antibody (Goat Anti-Mouse IgG H + L-HRP Conjugate, Bio-Rad) in a 1:3000 dilution for 1 h. Following three washes, the reactive bands were visualized by developing with a colorimetric substrate kit (Opti-4CN, Bio-Rad) following the manufacturer's protocol. The blots were rinsed with water and air-dried.

The PWBC sample that showed a 68 kDa reactive band detected with the mouse anti-NP mAb was interpreted as Western blot positive (WB+). The sample that did not show a 68 kDa reactive band, but was able to detect beta-actin was interpreted as Western blot negative (WB–). The positive band of 42 kDa detected by anti-beta actin antibody demonstrated that equivalent amount of PWBC was loaded in each well.

Reptarenavirus detection by PCR

Frozen PWBC of 27 boa constrictors and three rainbow boas were submitted to the DeRisi Lab, University of San Francisco, for PCR analysis to detect the presence of reptarenavirus RNA. The RNA extraction and qRT-PCR was performed as previously described (Stenglein et al., 2015). Briefly, RNA was extracted from PWBC using the Trizol reagent (Life Tech), further purified using an RNA clean and concentrator-5 column (Zymo Research), and treated with DNase I (NEB). The RNA was reverse transcribed into cDNA using Superscript III RT (Life Tech) and random hexamer priming. Diluted cDNA was used as template in qPCR reactions using primers MDS-400 (5′-TTCATTTCTTCATGRACTTTRTCAATC) and -402 (5′-GGSATAACAAAYTCACTTCAAATATC), which amplify a region of the reptarenavirus glycoprotein gene. Agarose gel electrophoresis and Sanger sequencing of amplicons confirmed positive PCR results.

Data analysis

Snakes were classified as IBD+ or IBD– based on findings from HE stained blood films or PWBC. Further, snakes were classified as NP+ if viral protein was detected by either IHC or Western blots, but were classified as NP– if viral protein was not detected by either method. Snakes in which reptarenavirus RNA was detected by qRT-PCR were classified as PCR+, whereas those in which RNA was not detected were classified as PCR–. The Kappa agreement between observation of inclusion bodies (by HE) and the detection of NP (by IHC or WB), or the detection of reptarenavirus RNA (by PCR) were calculated by a commercial software (MedCalc Statistical Software version 13.2.2, MedCalc Software bvba, 2014[1]). Kappa values 0, <0.4, 0.4–

[1] See: http://www.medcalc.org (accessed 1 November 2016)

0.75, >0.75, and 1 were considered as no agreement, poor agreement, good agreement, very good agreement, and perfect agreement, respectively.

Prevalence of IBD was calculated by dividing the number of IBD+ snakes by the total number of snakes tested. Using the Fisher's exact chi-square test, the proportions of boa constrictors, ball pythons, and rainbow boas classified as IBD+ were compared. Differences at $P < 0.05$ were considered statistically significant.

Results

Detection of reptarenaviral NP

A total of 131 blood samples from boas and pythons were screened for the presence of reptarenaviral NP. Test results for all snakes in this study are listed in Supplementary Table S1 and results from selected species are summarized in Table 1.

Based on examination of the HE stained blood films, the characteristic intracytoplasmic eosinophilic inclusion bodies were identified in 19 of 52 boa constrictors and one of 35 rainbow boas; none were found in other species (Table 1). By expanding the analysis to include HE stained PWBC (Fig. 1A), three additional boa constrictors and two additional rainbow boas were found to be IBD+. Altogether, based on screening with HE stain, 22 IBD+ boa constrictors and three IBD+ rainbow boas were identified (Table 1).

In this study, the PWBC slides of all snakes were stained with IHC using a validated anti-NP mAb (Table 1; Fig. 1; Chang et al., 2013). Of the 22 boa constrictors that were IBD+, 21 of them were IHC+ and one was IHC–. Of the 31 boa constrictors that were IBD–, one was IHC+, and 30 were IHC–. All PWBC slides of the other species in this study were IHC–.

Using Western blots, NP was readily detectable in 18 µg of PWBC lysate obtained from a IBD+ boa constrictor, whereas samples that were WB– when 20 µg of PWBC lysate was loaded, remained WB– when repeated with 30 µg of PWBC lysate (Fig. 2). In this study, the PWBC lysates of 38 (16 IBD+ and 22 IBD–) boa constrictors, 20 (all IBD–) ball pythons, and 20 (3 IBD+ and 17 IBD–) rainbow boas were tested for the presence of NP using Western blots. Of the 16 IBD+ boa constrictors, only one tested WB–. All 22 IBD– boa constrictors were negative for NP on Western blots. The PWBC lysates from ball pythons and rainbow boas tested WB– regardless of being IBD+ or IBD– (Table 1; Table S1).

Among the boa constrictors, the Kappa agreement between the diagnosis of IBD by HE stain and the detection of NP by IHC stain was very good (Kappa statistic = 0.92; 95% CI = 0.81, 1.0; Table 2). The Kappa agreement between the diagnosis of IBD by HE stain and the detection of NP by Western blots was also very good (Kappa statistic = 0.89; 95% CI = 0.74, 1.0; Table 3). Because the reactivity of mouse anti-NP mAb had not been validated for rainbow boas, and no IBD+ cases were found in ball pythons, thus Kappa statistics were not analyzed for these species.

Detection of reptarenavirus RNA

After the discovery of reptarenaviruses in 2012, 27 of the 53 boa constrictors that were tested between 2010 and 2012 with sufficient amounts of banked frozen PWBC pellets were submitted for reptarenavirus testing by qRT-PCR. Among the 12 IBD+ (out of 22 IBD+) and 15 IBD– (out of 31 IBD–) boa constrictors, all IBD+ boa constrictors determined by HE stain were PCR+, and except for one IBD– boa tested PCR+, 14 (of 15) IBD– boa constrictors were PCR– (Table 1 and Table S1). Of the 35 rainbow boas tested in this study, the three tested IBD+ by HE stain were PCR–. The Kappa agreements between the observation of inclusion bodies in the blood cells and the detection of reptarenavirus RNA was very good (Kappa statistic = 0.92; 95% CI = 0.78, 1.00; Table 4).

Occurrence of IBD and clinical signs

Within the 131 captive snake populations studied, the prevalence of IBD was 19% (25/131; 95% CI = 12.4%, 25.8%), which was higher in boa constrictors (22/53 or 42%), compared to ball pythons (0/22) or rainbow boas (3/31 or 10%; $P < 0.05$, Table 1). Of the 22 IBD+ boa constrictors, 82% (18/22) were clinically healthy, 72% (13/18) of those had a history of having contact with IBD+ boa constrictors, and at least one had previously been paired with another boa for breeding. Only 18.2% (4/22) of the IBD+ boa constrictors exhibited signs of chronic illness (such as anorexia, chronic weight loss, respiratory disease, regurgitation), with one that became ill after breeding. None of the IBD+ boa constrictors exhibited signs of CNS disease. However, of the 31 IBD– boa constrictors, 42% (13/31) were showing various clinical signs of illness (such as anorexia, heavy mite infestation, and respiratory disease), including nine (9/31 or 29%) with signs of CNS disease. Four of these snakes were necropsied and no IBD inclusion bodies or NP were identified in any tissues, and two of these snakes' PWBC were found to be PCR–.

Table 1
Summarized test results of comparisons between the observation of inclusion bodies, the detection of reptarenavirus nucleoprotein (NP), and the presence of reptarenavirus RNA in boa constrictors, ball pythons, and rainbow boas.

Test[a,b]	Sample type	Samples (*n*)	Tested Positive			Tested Negative		
			Number (%)	Number checked by PCR	PCR results[c] (+, –)	Number (%)	Number checked by PCR	PCR results (+, –)
Boa constrictors								
HE stain	Blood film	52	19 (37)	9	9, 0	33 (63)	17	3, 14
HE stain	PWBC	53	22 (42)	12	12, 0	31 (58)	15	1, 14
IHC stain	PWBC	53	22 (42)	12	12, 0	31 (58)	15	1, 14
Western blot	PWBC	38	15 (39)	11	11, 0	23 (61)	15	2, 13
Ball pythons								
HE stain	Blood film	22	0	0		22 (100)	0	
HE stain	PWBC	22	0	0		22 (100)	0	
IHC stain	PWBC	22	0	0		22 (100)	0	
Western blot	PWBC	20	0	0		20 (100)	0	
Rainbow boas								
HE stain	Blood film	35	1 (3)	1	0, 1	34 (97)	2	0, 2
HE stain	PWBC	35	3 (10)	3	0, 3	32 (91)	0	
IHC stain	PWBC	35	0	0		35 (100)	3	0, 3
Western blot	PWBC	20	0	0		20 (100)	3	0, 3

HE, Hematoxylin and eosin; IHC, Immunohistochemistry; PWBC, Peripheral white blood cells.
[a] HE stain represented the observation of inclusion bodies.
[b] IHC stain and Western blots represented the detection of reptarenaviral NP.
[c] PCR represents the detection of reptarenavirus RNA.

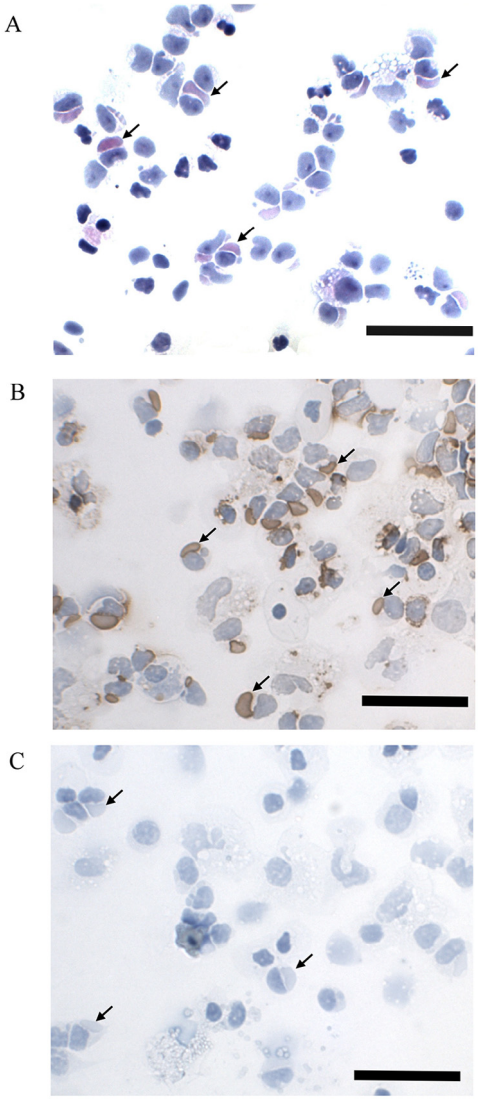

Fig. 1. Cytospin preparation of peripheral white blood cells (PWBC) isolated from a boa constrictor. A. The inclusion body disease (IBD) inclusions (arrows) stained eosinophilic in hematoxylin and eosin stain. B. Using immunohistochemistry (IHC) staining, the IBD inclusion bodies (arrows) stained dark brown with substrate DAB (3, 3 - diaminobenzidine). C. The PWBC stained with nonspecific mouse antibody as negative control for IHC staining. The inclusion bodies were not stained. Bar = 20 μm.

Discussion

To the authors' knowledge, this is the first study that surveyed the presence of reptarenavirus NP in circulating blood cells using IHC. A validated anti-NP specific mAb (Chang et al., 2013) was used to evaluate the staining of intracytoplasmic inclusion bodies in purified PWBC preparations. This study confirmed that inclusion bodies observed in the circulating white blood cells were stainable using an anti-NP mAb (Fig. 1). Using Western blots, NP was demonstrated in the isolated PWBC of IBD+ boa constrictors (Fig. 2), but not in IBD– boa constrictors. In previous studies, IBD inclusion bodies within blood cells were examined by non-specific staining, such as Wright Giemsa stain and Diff-Quik® stain (Pees et al., 2010; Schilliger et al., 2011; Banajee et al., 2012; Keilwerth et al., 2012). However, non-specific stains can label intracytoplasmic inclusion bodies unrelated to IBD, resulting in false positives (Chang and Jacobson, 2010; Chang et al., 2013). In one boa constrictor (IB1075) and three rainbow boas (IB1067, IB1078 and IB1081) the inclusion bodies found in HE stained PWBC were not stained using IHC, indicating either false positive diagnosis or lack of cross-reactivity between the viral NP and the anti-NP mAb (Table S1). These PWBC samples were tested for the presence of reptarenavirus RNA by qRT-PCR, and viral RNA was detected in the boa constrictor (IB1075), but not detected in the rainbow boas. This suggested that the NP produced by the reptarenavirus in snake IB1075 may be a variant that does not cross react with the anti-NP mAb, and the inclusion bodies found in the rainbow boas may be a protein unrelated to IBD or a divergent reptarenavirus that is not detected by our primers.

We found a very good agreement between the observation of inclusion bodies by HE stain and the immuno-based detection (IHC stain and Western blots) of NP (Tables 1, 2 and 3). Only two exceptions were seen: in boa IB1053 inclusion bodies were only found in the IHC stained PWBC, while in boa IB1075 inclusion bodies were only found in the HE stained PWBC (Table S1). However, reptarenavirus RNA was detected in both boas by qRT-PCR, suggesting that they were indeed infected. Therefore, the ability of detecting viral inclusion bodies or NP varies among different methods. In the case of IB1053, although the anti-NP mAb clearly labeled the IBD inclusion bodies, the inclusion bodies were extremely sporadic (<5 in one slide) and were missed in the HE stained preparation, with the amount of NP insufficient to be detected by Western blot. There are cases where IHC staining might have an advantage over Western blots in detecting low concentration of NP, with PCR serving as a more sensitive confirmation tool.

We found generally good agreement between qRT-PCR detection of viral RNA and the other testing methods among the 27 boa constrictors tested by PCR. Samples that tested positive by at least one non-PCR method (HE stained blood film, HE stained PWBC, IHC stain, and Western blots) uniformly tested PCR+ (Table 1 and

Table 3
The agreement between the observations of inclusion body disease (IBD) inclusion bodies by hematoxylin and eosin (HE) stain and the detection of nucleoprotein by Western blots in boa constrictors.

	HE+ (IBD+)	HE– (IBD–)	Total
Western blot+	15	1	16
Western blot–	1	21	22
Total	16	22	38

Observed agreement = (15 + 21)/38 = 36/38 = 0.94.
Kappa statistic = 0.89 (95% confidence interval, 0.74, 1.0).

Table 2
The agreement between[a,b] the observations of inclusion body disease (IBD) inclusion bodies by hematoxylin and eosin (HE) stain and the detection of IBDP by immunohistochemistry (IHC) in boa constrictors.

	HE+ (IBD+)	HE– (IBD–)	Total
IHC+	21	1	22
IHC–	1	30	31
Total	22	31	53

[a] Observed agreement = (21 + 30)/53 = 51/53 = 0.96.
[b] Kappa statistic = 0.92 (95% confidence interval, 0.81, 1.0).

Table 4
The agreement between[a,b] the observations of inclusion body disease (IBD) inclusion bodies by hematoxylin and eosin (HE) stain and the detection of reptarenavirus RNA in boa constrictors.

	IBD + (HE +)	IBD - (HE -)	Total
PCR+	12	1	13
PCR–	0	14	14
Total	12	15	27

[a] Observed agreement = (12 + 14)/26 = 26/27 = 0.96.
[b] Kappa statistic = 0.92 (95% confidence interval, 0.78, 1.00).

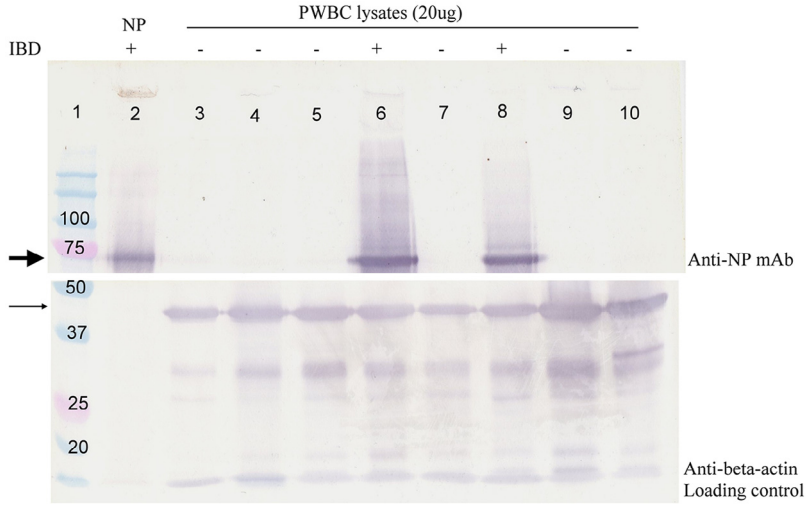

Fig. 2. Antigen detection by Western blot using peripheral white blood cells (PWBC) isolated from eight boa constrictors. Lane 1 contained molecular weight marker (Bio-Rad, #161–0347), lane 2 contained 20 μg purified nucleoprotein (NP) as positive control. Lanes 3 to 10 each contained 20 μg of PWBC lysate from a boa constrictor. The top portion of membrane was detected by anti-NP mAb, the thick arrow indicates the molecular weight of NP (68 kDa). The bottom portion of the membrane was detected using anti-beta-actin antibody as a loading control, showing that relevant amounts of cell were loaded in each lane. The thin arrow indicated the molecular weight of beta-actin (42 kDa). Lanes 6 and 8 were interpreted as Western blot positive, whereas others were interpreted as negative.

Table S1). Some samples that tested negative using a single method were found to be PCR+, suggesting that a single negative test does not rule out the possibility of being viral infected. Multiple methods of blood testing will increase the level of confidence for making antemortem diagnosis of IBD in boa constrictors.

Based on the inoculation studies done by Schumacher et al. (1994) and Wozniak et al. (2000), inclusion bodies were first observed in visceral organs approximately 10 weeks post-inoculation. In those studies, however, peripheral blood cells were not examined to determine the presence or absence of IBD inclusion bodies. Therefore, we do not know the reliability of the HE or IHC stained blood films in making a diagnosis for IBD in early stage of diseases. Further studies will be needed to determine the temporal sequence of appearance of inclusion bodies in various tissues.

In the current study, the prevalence of IBD in boa constrictors was high (41.5%), and of these, 81.8% of the IBD+ boas appeared to be clinically healthy. This may indicate that majority of boa constrictors with IBD are subclinical, and as a result, the true prevalence of IBD may be far greater than realized. In this study, one subclinical boa constrictor (IB1047) that tested IBD+ by the blood tests (HE, IHC and Western blots), remained positive a year later, despite remaining clinically healthy. In the study of Wozniak et al. (2000), no clinical signs were observed in boa constrictors up to a year following inoculation with IBD+ liver homogenates, even though characteristic inclusion bodies were evident in these snakes. Interestingly, none of the IBD+ snakes diagnosed in this study exhibited signs of CNS disease. Further, the snakes that were presented with signs of CNS disease in this study were found to be IBD– (five were confirmed by necropsy and two tested PCR–). Similarly, inclusion bodies have been found in the brain of boa constrictors that did not show any apparent signs of CNS disease. Further studies are needed to determine whether CNS diseases in boa constrictors were associated with another pathogen or additional factors may be involved in the development of CNS signs. It is possible that ill snakes are more likely to be presented to veterinarians for examination, and therefore, IBD (as a primary or concurrent disease) is more likely to be diagnosed and reported. If this holds true, a knowledge bias of the clinical presentation of IBD affected snakes in the literature may be expected.

Conclusions

Ante-mortem testing for IBD was performed using blood films and purified PWBC cytological preparations obtained from 131 boas and pythons. The blood cells were stained by HE or IHC with a validated anti-NP mAb. The IHC staining demonstrated the eosinophilic intracytoplasmic inclusion bodies within the PWBC consisted of a stainable NP. Western blots were used for confirmation of the NP. PCR was used for detection of reptarenaviruses RNA in a subset of samples. In boa constrictors, there was very good agreement between the presence of inclusion bodies and NP or reptarenavirus RNA. In this study, the prevalence of IBD in captive boas and pythons was 19%, with a prevalence of 42% among boa constrictors. Of the IBD+ boa constrictors, 82% were clinically healthy. None of the IBD+ snakes in this study exhibited signs of CNS disease.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

The Morris Animal Foundation[2] funded this project under title ‘Diagnosing Inclusion Body Disease (IBD), A Worldwide Infectious Disease of Boid Snakes’ (Grant ID D10ZO-045). The grant supported the cost for the assays used in this study. The funding foundation had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Portions of this article and figures were from the PhD dissertation of Li-Wen Chang (Chang, 2012. Development of molecular diagnostic tests for inclusion body disease in boid snakes. Dissertation, Doctor of Veterinary Medical Science, University of Florida). This study was previously presented at the 2012 Annual Association of Reptilian and Amphibian Veterinarians Conference as ‘Presumptive routes of

[2] See: Morris Animal Foundation: www.MorrisAnimalFoundation.org (accessed 1 November 2016)

transmission and prevalence of Inclusion Body Disease (IBD) – a clinical survey using a validated antibody against IBD protein (IBDP)'. We are grateful for kind support and technical guidance from Rick Alleman, Francesco C. Origgi, James Coleman, Cruz Fan, Marjorie Chow, April Childress, Diane Duke, and Linda Green.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2016.10.006.

References

Banajee, K.H., Chang, L., Jacobson, E.R., Rich, G.A., Royal, A.B., 2012. What is your diagnosis? Blood film from a boa constrictor. Veterinary Clinical Pathology 41, 158–159.

Bodewes, R., Kik, M.J., Raj, V.S., Schapendonk, C.M., Haagmans, B.L., Smits, S.L., Osterhaus, A.D., 2013. Detection of novel divergent arenaviruses in boid snakes with inclusion body disease in The Netherlands. Journal of General Virology 94, 1206–1210.

Carlisle-Nowak, M.S., Sullivan, N., Carrigan, M., Knight, C., Ryan, C., Jacobson, E.R., 1998. Inclusion body disease in two captive Australian pythons (*Morelia spilota variegata* and *Morelia spilota spilota*). Australian Veterinary Journal 76, 98–100.

Chang, L. 2012. Development of molecular diagnostic tests for inclusion body disease in boid snakes. Dissertation, Doctor of Veterinary Medical Science, University of Florida.

Chang, L., Jacobson, E.R., 2010. Inclusion body disease, a worldwide infectious disease of boid snakes: a review. Journal of Exotic Pet Medicine 19, 216–225.

Chang, L.W., Fu, A., Wozniak, E., Chow, M., Duke, D.G., Green, L., Kelley, K., Hernandez, J.A., Jacobson, E.R., 2013. Immunohistochemical detection of a unique protein within cells of snakes having inclusion body disease, a world-wide disease seen in members of the families *Boidae* and *Pythonidae*. PLoS ONE 8, e82916.

Hetzel, U., Sironen, T., Laurinmaki, P., Liljeroos, L., Patjas, A., Henttonen, H., Vaheri, A., Artelt, A., Kipar, A., Butcher, S.J., et al., 2013. Isolation, identification and characterization of novel Arenaviruses, the etiological agent of boid inclusion body disease. Journal of Virology 87, 10918–10935.

Jacobson, E.R., Oros, J., Tucker, S.J., Pollock, D.P., Kelley, K.L., Munn, R.J., Lock, B.A., Mergia, A., Yamamoto, J.K., 2001. Partial characterization of retroviruses from boid snakes with inclusion body disease. American Journal of Veterinary Research 62, 217–224.

Keilwerth, M., Bühler, I., Hoffmann, R., Soliman, H., El-Matbouli, M., 2012. Inclusion body disease (IBD of Boids) – a haematological, histological and electron microscopical study. Berliner und Münchener tierärztliche Wochenschrift Journal 125, 411–417.

Pees, M., Schmidt, V., Marschang, R.E., Heckers, K.O., Krautwald-Junghanns, M.E., 2010. Prevalence of viral infections in captive collections of boid snakes in Germany. Veterinary Record 166, 422–425.

Radoshitzky, S.R., Bào, Y., Buchmeier, M.J., Charrel, R.N., Clawson, A.N., Clegg, C.S., DeRisi, J.L., Emonet, S., Gonzalez, J.P., Kuhn, J.H., et al., 2015. Past, present, and future of arenavirus taxonomy. Archives of Virology 160, 1851–1874.

Schilliger, L., Selleri, P., Frye, F.L., 2011. Lymphoblastic lymphoma and leukemic blood profile in a red-tail boa (*Boa constrictor constrictor*) with concurrent inclusion body disease. Journal of Veterinary Diagnostic Investigation 23, 159–162.

Schmidt, V., Marschang, R.E., Abbas, M.D., Ball, I., Szabo, I., Helmuth, R., Plenz, B., Spergser, J., Pees, M. 2013. Detection of pathogens in Boidae and Pythonidae with and without respiratory disease. Veterinary Record 172, 236.

Schumacher, J., Jacobson, E.R., Homer, B.L., Gaskin, J.M., 1994. Inclusion body disease in boid snakes. Journal of Zoo and Wildlife Medicine 25, 511–524.

Stenglein, M.D., Sanders, C., Kistler, A.L., Ruby, J.G., Franco, J.Y., Reavill, D.R., Dunker, F., Derisi, J.L., 2012. Identification, characterization, and in vitro culture of highly divergent arenaviruses from boa constrictors and annulated tree boas: candidate etiological agents for snake inclusion body disease. mBio 3, e00180–12.

Stenglein, M.D., Jacobson, E.R., Chang, L., Sanders, C., Hawkins, M.G., Guzman, D., Drazenovich, T., Dunker, F., Kamaka, E.K., Fisher, D., et al., 2015. Widespread recombination, reassortment, and transmission of unbalanced compound viral genotypes in natural arenavirus infections. PLoS Pathogen 11, e1004900.

Vancraeynest, D., Pasmans, F., Martel, A., Chiers, K., Meulemans, G., Mast, J., Zwart, P., Ducatelle, R. 2006. Inclusion body disease in snakes: a review and description of three cases in boa constrictors in Belgium. Veterinary Record 158, 757–760.

Wozniak, E., McBride, J., DeNardo, D., Tarara, R., Wong, V., Osburn, B. 2000. Isolation and characterization of an antigenically distinct 68-kd protein from nonviral intracytoplasmic inclusion bodies in Boa constrictors chronically infected with the inclusion body disease virus (IBDV: Retroviridae). Veterinary Pathology 37, 449–459.