Prevalence of viral infections in captive collections of boid snakes in Germany

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Data on viral infections in apparently healthy snake collections in Germany were obtained with respect to husbandry conditions and health status. Samples from 100 boid snakes (from 14 collections) were examined microbiologically and for the presence of paramyxoviruses (PMVs) using RT-PCR. Blood was tested for the presence of antibodies against PMV, adenovirus and reovirus and for inclusion bodies indicative of inclusion body disease. Nine snakes tested positive for PMV, and inclusion bodies were detected in six snakes. Antibodies against PMV were found in one snake, and two snakes had antibodies against an adenovirus. A significant correlation was found between the origin of the snake and the presence of PMV, and between the presence of remarkable microbiological findings and husbandry conditions.

Materials and methods

Animals and preliminary examinations

The study included 100 boid snakes of nine different species (Table 1) from 14 private and zoo collections across Germany, with an average of seven animals per sample site. Criteria for inclusion were: no clinical signs of disease observed by the owner; no previous findings of viral disease within the collection; and agreement by the owners to complete a detailed questionnaire. Owners were asked 35 questions regarding sex, age, origin (bred in Germany or imported), date of entry into the collection, previous diseases in the collection, and husbandry and feeding conditions. This information and the conditions observed in the collections were assessed to classify husbandry quality into three categories: excellent (all clinical, feeding and technical parameters within the required ranges), average (minor deficits) or poor (major deficits).

A thorough clinical examination of each snake included measurement of body length, body mass, assessment of body and overall condition, and inspection of the oral cavity, including the tracheal opening. On the basis of the clinical findings recorded, the snake was assessed to be clinically remarkable or unremarkable.

A tracheal wash was collected after disinfection of the tracheal opening (Octenisept Wunddesinfektion; Schülke & Mayr) using a sterile flexible tube of appropriate size (Schumacher 1997, Heard and others 2004). Samples were cultured for bacterial and fungal growth according to standard protocols and evaluated as remarkable (presence of moderate or heavy bacterial growth within the tracheal wash sample) or unremarkable (Hilf and others 1990, Rosenthal and Mader 2006, Pees and others 2007).

Examinations for viral infections

Swabs taken from the choana and cloaca, as well as fluid from the tracheal wash sample were examined for the presence of PMV by nested RT-PCR targeting a portion of the L gene (Ahne and others 1999). RNA was prepared from the samples using a QIAamp viral RNA Mini Kit (Qiagen). The first round of the one-tube RT-PCR reaction was carried out in 25 µl reaction mixture containing 2.5 µl of prepared RNA, 1 µM each of primers 5F and 6R, 1 x Taq buffer, 2.5 mM MgCl2, 0.2 mM each of dNTPs, 70 U reverse transcriptase, 6 U ribonuclease inhibitor and 1.25 U Taq polymerase (Qiagen). In a second round, 1 µl of the first-round reaction mixture, 1 µM each of primers 7F and 8R, 1 x Taq buffer with KCl, 2.5 mM MgCl2, 0.2 mM each of dNTPs and 1.25 U Taq polymerase (Qiagen) were included in a 25 µl reaction mixture. Blood was collected from the caudal (ventral) tail vein into a microcontainer tube containing lithium heparin. If the blood sample was of insufficient volume, then, with the agreement of the owner, blood was taken from the heart using a 23 G syringe (Hernandez-Divers 2006). Blood samples were centrifuged immediately after collection, and plasma was separated and refrigerated at ~18°C until serological testing. The remaining part of the blood sample, representing the concentrated blood cells, was sucked into a microcapillary tube and centrifuged in a haematocrit centrifuge at 16.060 g for five minutes to separate the buffy
coated phase. This buffy coat was transferred to a microscope slide using the coverslip method recommended by Strik and others (2007) and stained using Diff-Quik (Dade-Behring). The slide was assessed for the presence of inclusion bodies in the white blood cell count typical of IBD (Strik and others 2007). This technique is a modification of that recommended in the literature (Schumacher 2006) and was used to obtain as high a white blood cell count as possible in the sample.

Plasma was tested for antibodies against PMV, an adenovirus and a reovirus. Plasma was heated to 56°C for 30 minutes before testing for the removal of non-specific inhibitors. Antibodies against PMV were detected using the haemagglutination inhibition (HI) test; antibodies against the adenovirus and reovirus were detected using the virus neutralisation test, as described previously (Gravendyck and others 1998). For both tests, serial twofold dilutions of the plasma up to a dilution of 1:512 were tested. Titres were expressed as the reciprocal of the highest log2 dilution that inhibited haemagglutination or led to complete neutralisation of the virus. For the HI test, four haemagglutinating units of a PMV isolate from a corn snake (Elaphe guttata) (Blahak 1994) were used. This isolate has been shown, as with a number of other snake PMVs, to cross-react serologically with avian PMV type 7 (Blahak 1994), and cluster molecularly with Fer-de-lance virus, the best characterised reptilian paramyxovirus (PMV) (R. E. Marschang, unpublished observations). Plasma from a rattlesnake (Crotalus horridus) in a PMV-infected snake collection was used as a positive control. For the serum neutralisation test, 100 mean tissue infectious dose (TCID50) of an adenovirus isolated from a Boa constrictor (Marschang and others 2005) and a reovirus isolated from a B. constrictor (Wellehan and others 2009) were used. No positive plasma controls were available for the serum neutralisation test against the adenovirus and reovirus. There is no standard cut-off for serological tests in snakes. Titres of 16 and higher were considered positive in the HI test and serum neutralisation test, as described elsewhere (Gravendyck and others 1998). Lower titres (of 8 or less) can be difficult to read, and undiluted snake plasma is often toxic.

### Statistics

Statistical analyses for correlations between clinical and laboratory findings, origin, and husbandry conditions were performed at a 0.05 significance level using commercial statistical software (SigmaStat 11.0 for Windows).

### Results

#### Animals and preliminary examinations

Of the 100 snakes examined, 17 had been imported and 83 had been bred in Germany. Husbandry conditions were assessed to be excellent for 49 snakes, average for 36 and poor for 15. Major problems were inappropriate climatic conditions and feeding practices, and poor hygiene.

In 58 snakes, both clinical and microbiological examinations were unremarkable. For these animals, husbandry was assessed to be excellent in 60 per cent of cases and poor in 7 per cent of cases.

Clinical examination revealed the following remarkable results in 20 snakes from 11 collections. In nine snakes, mucus secretion was increased in the oral cavity. Three snakes showed moderate fibrinopurulent stomatitis, in three the tracheal opening was re- ddened and slightly swollen, and in three fluid from the tracheal wash contained flaky components. Seven snakes showed reduced body condition. No significant correlation was found between the clinical findings and other results of the present study.

The results of the microbiological examination were assessed to be remarkable in 29 snakes from nine collections. These results included the detection of Acinetobacter baumannii (one), Aspergillus fumigatus (four), Aeromonas hydrophila (two), B. zoohelcum (two), Candida species (four), Citrobacter freundii (four), Enterobacter cloacae (four), Enterococcus faecalis (one), Klebsiella pneumoniae (one), Pseudomonas aeruginosa (six), Pseudomonas putida (one), Salmonella species (five) and Streptococcus maltophilia (one). A significant correlation was found between husbandry conditions and the presence of these microorganisms in the tracheal wash sample, with increasing numbers of remarkable findings under poor husbandry conditions (r = 0.267, P = 0.008). No significant correlation was found between the microbiological findings and either clinical findings or the origin of the animals.

#### Examination for viral infections

PMV was detected by RT-PCR in nine animals (five Python regius, three Python molurus and one B. constrictor). Six of these animals had been imported and three had been bred in Germany. A significant correlation was found between the presence of PMV and the origin of the snake (r = 0.415, P < 0.001). Positive animals were from four collections. Within the affected collections, between 10 and 50 per cent of snakes tested were positive for PMV. No significant correlation was found between PMV detected using RT-PCR and the presence of antibodies against PMV. There was also no significant correlation between the presence of PMV and either clinical or bacteriological findings. However, three snakes (33 per cent) that tested positive for PMV were remarkable on clinical examination, showing increased mucus secretion and, in one case, a swollen tracheal opening. Four of the PMV-positive snakes (42 per cent) had remarkable microbiological findings, and only two were classified as unremarkable in the preliminary examinations.

Inclusion bodies diagnostic for IBD were found during the cytological examination in six snakes (three B. constrictor, two P. molurus, one Python reticulatus) from two collections (Fig 1). They were identified as

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**TABLE 1: Number of boid snake species examined from 14 snake collections in Germany, and number of snakes with remarkable clinical and microbiological findings, positive tests for paramyxovirus (PMV) and inclusion body disease (IBD), and positive antibody titre**

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of snakes (collections)</th>
<th>Number of snakes with remarkable/positive findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical exam</td>
<td>Microbiology</td>
</tr>
<tr>
<td>Boa constrictor</td>
<td>32 (6)</td>
<td>5</td>
</tr>
<tr>
<td>Ball python (Python regius)</td>
<td>28 (6)</td>
<td>6</td>
</tr>
<tr>
<td>Burmese python (Python molurus)</td>
<td>16 (5)</td>
<td>5</td>
</tr>
<tr>
<td>Green tree python (Morelia viridis)</td>
<td>11 (3)</td>
<td>2</td>
</tr>
<tr>
<td>Red-tailed python (Python reticulatus)</td>
<td>4 (8)</td>
<td>2</td>
</tr>
<tr>
<td>Garden tree boa (Corallus hortulanus)</td>
<td>4 (1)</td>
<td>2</td>
</tr>
<tr>
<td>Cuban boa (Epicrates angulifer)</td>
<td>2 (1)</td>
<td>2</td>
</tr>
<tr>
<td>Carpet python (Morelia spilota)</td>
<td>2 (1)</td>
<td>2</td>
</tr>
<tr>
<td>Blood python (Python curtus)</td>
<td>1 (1)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

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**Figure 1:** Buffy coat smear from a Boa constrictor. Inclusion bodies (arrows) can be identified in several lymphocytes and are diagnostic for inclusion body disease. Diff-Quik. Bar = 20 μm

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The prevalence of PMV was found to be 9 per cent; nine of the 100 snakes examined in the study, 17 were imported. It is reasonable to assume that those who agreed to take part in the study had a higher-than-average interest in reptiles, with imported snakes being significantly more often affected than those bred in Germany. The increasing danger represented by this viral disease for snake collections.

For this study, a pooled sample from choana, cloaca and tracheal wash fluid was used. The high incidence may be a result of this combination. The tracheal wash in particular can be recommended for the diagnosis of PMV, as this virus was isolated most commonly from the lung in affected snakes (Blahak 1995). Using only one localisation for the examination would probably decrease the chance of virus detection considerably. No significant correlation was found between PMV and husbandry conditions; however, the presence of PMV did correlate with the origin of the snake, with imported snakes being significantly more often affected than those bred in Germany. This result does not necessarily mean that snakes from other countries are generally more susceptible to PMV infections; it is conceivable that owners of imported snakes normally bought them at reptile shows, which may be a risk factor. Animals sold at shows are often of dubious origin, and may have changed owners as well as had contact with other snakes several times. Although there was no significant correlation between the presence of PMV and serological or clinical findings, microbiological results were remarkable in four of the nine PMV-positive snakes. Bacterial secondary infections are often seen following PMV infections and can complicate the clinical signs (Murray 2006). A follow-up call six to eight months after the initial examination revealed that four of the PMV-positive snakes had died, all showing signs of pneumonia. In one case, a postmortem examination was conducted and PMV was confirmed. These data confirm the sensitivity of the method and the importance of PMV detection in apparently healthy collections.

In six of the snakes examined, inclusion bodies were found in the white blood cell count. This is a higher prevalence than expected from checking only peripheral blood cells for inclusion bodies. A wide blood cell count concentration of nearly 100 per cent was achieved using the technique described here. This procedure can therefore result in increased sensitivity compared with a conventional assessment of buffy coat smears. Results are only described as diagnostic when inclusion bodies are identified (Jacobson 2002, Strik and others 2007). Moreover, whereas in Boa species inclusion bodies have been described as being present in all visceral organs, in Python species they may be limited to the tissue of the central nervous system and may generally be lacking in leukocytes (Schumacher 2006). Other methods, such as liver or tonsil biopsies, are more invasive and thus less suitable for screening; therefore, the use of concentrated buffy coat examinations can be recommended as a screening procedure in boids. Further studies are required to evaluate the full potential of this technique. As only two collections were affected, conclusions regarding husbandry conditions and the origin of the snakes are not possible. In three of the six cases, both clinical and microbiological examinations were unremarkable. A follow-up call six months later was answered for five of the six animals in which inclusion bodies had been found. One of the snakes was found to have died during this period showing signs of severe pneumonia, but no postmortem examination was performed. It would therefore be speculative to draw a conclusion regarding the stage of the disease from the presence of inclusion bodies in the peripheral blood cells. Snakes that test positive should be separated, and additional biopsies (for example, liver tissue) should be taken to confirm the diagnosis.

Severe snakes with specific antibodies against PMV and a snake adenovirus were found in the present study. These serological findings did not correlate with clinical signs or with PMV detection by RTPCR. The detection of antibodies against a virus shows that the animal has been infected with that virus in the past, but it is not an indication of acute infection. Virus shedding, on the other hand, is associated with acute infection or recrudescence. For PMV, asymptomatic seropositive snakes and diseased snakes without detectable antibody titres have been described (Gaskin and others 1989, Jacobson and others 1999). Seroconversion in reptiles is dependent on environmental factors and can take eight to 10 weeks. One reason for negative serological results may be that the strain of PMV was not detected with the assay used for the examination (Allender and others...
In this study, results varied between 0 and 100 per cent positive titres depending on the test used, and it is not recommended that these assays be used as the sole technique for the diagnosis of PMV in snakes. The same may be true for antibodies against the reovirus used in this study. Previous studies have shown that reoviruses isolated from reptiles can differ from one another both serologically and molecularly (Blahak and others 1995, Wellen and others 2009). The failure to detect antibodies may therefore be due to the reovirus used in this study. This isolate was obtained from a B. constrictor in Germany and was therefore considered a good candidate for detecting anti-reovirus antibodies in boid snakes in Germany. The clinical importance of antibodies against adenovirus and reovirus is still not known. It has been proposed that reptile adenoviruses evolved with their hosts (Benkó and others 2002), and antibodies against adenoviruses have been found to be widespread in snakes (Marschang and others 2003). Adenovirus has been isolated from different snake species with and without concurrent diseases (Perkins and others 2001). The detection of antibodies against a virus should be interpreted with caution. In some cases, infected animals are known to become carriers, meaning that serologically positive animals may pose a threat to naïve animals. For PMV and snake adenoviruses, no carrier state has been proven, but additional work is required. On the other hand, serologically positive animals may be protected from disease if the virus is present in a collection, so seropositivity can theoretically be an advantage.

In conclusion, the results of the present study show that there is a considerable risk of hidden infectious diseases in apparently healthy boid collections. This is true not only for PMV and IBD, but also for the presence of bacteria in a combination and quantity assessed as remarkable in reptiles. There is a risk for infection of a collection after the introduction of newly acquired snakes and also for infection of snakes introduced into apparently healthy collections.

Screening and entry examinations are strongly recommended and should include swabs from the choana and cloaca, a tracheal wash sample and a blood sample. The following procedures are recommended for basic testing: RTPCR for PMV (pooled sample from choana, cloaca and trachea), bacteriology and mycology (tracheal wash) and auffy coat smear for the presence of inclusion bodies (for IBD). In cases of suspected IBD, a liver or tonsil biopsy should then be performed to increase the sensitivity of the test. Serology can be used to determine whether the animal has been exposed to specific diseases in the past, and screening for antibodies against PMV is recommended at the beginning and end of quarantine. A detailed history is important to put the results into the context of husbandry deficits and to establish appropriate measures for the rehabilitation of a collection.

Acknowledgements
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References