Virological Screening of Bearded Dragons (*Pogona vitticeps*) and the First Detection of Paramyxoviruses in This Species

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Abstract: In May 2011, 22 cloacal swabs and plasma samples were collected from 22 bearded dragons (*Pogona vitticeps*) from a rescue center in Munich, Germany. All of the lizards swabbed were healthy throughout the observation period and did not show any clinical signs during the course of the study. The swabs were tested for the presence of ferlaviruses (*Paramyxoviridae*) and adenoviruses (AdVs) using reverse transcriptase polymerase chain reaction (RT-PCR) and PCR methods. Virus isolation attempts were carried out on two iguana heart cells. Plasma was tested for antibodies against several ferlavirus isolates using a hemagglutination inhibition (HI) test. A second round of testing was carried out on 27 animals four months later. Of all swabs tested in the first round, 11 (50%) were positive for ferlaviruses using an RT-PCR. Sequences of the PCR products of all detected ferlaviruses were 100% identical to corresponding sequences of isolate Pyth-GER01 that was originally isolated from a python. One swab (4.5%) was positive for an AdV, and the PCR product was 100% identical to Agamid AdV-1 (Acc. No. DQ077706). No virus was isolated from the cloacal swabs. The HI test revealed low titers of ≤8 against Pyth-Ger01 for all samples tested. Four months later, no ferlaviruses were detected in any of the samples tested by RT-PCR. However, four animals (14.8%) were found positive for an AdV. Sequence analyses of a portion of the DNA-dependent DNA polymersase gene revealed three new AdVs that were 99% similar to Agamid AdV-1 (Acc. No AY576678). Antibody titers of 16–32 against at least one ferlavirus were detected in six of the animals sampled four months later. This is the first report documenting the occurence of ferlavirus infection in bearded dragons.

Key Words: Adenovirus, bearded dragons, ferlavirus, paramyxovirus, *Pogona vitticeps*, RT-PCR/PCR.

Introduction

Paramyxoviruses (PMV) are important pathogens in snakes (Fölsch and Leloup, 1976; Jacobson *et al.*, 1992; Essbauer and Ahne, 2001; Marschang *et al.*, 2009; Abbas *et al*., 2011). However, there is little information available on PMV infections in lizards (Marschang *et al.*, 2002, 2009; Gravendyck *et al*., 1998). The first report of a PMV-like virus in a lizard was in a false tegu (*Callopistes maculatus*) (Ahne and Neubert, 1991). No clinical signs associated with the infection were reported. An epidemic of proliferative pneumonia caused by PMV infection in a group of caiman lizards was documented by Jacobson *et al.* in 2001. A PMV was also isolated from a clinically healthy wild-caught Mexican lizard (Marschang *et al.*, 2002).

The family *Paramyxoviridae* contains pleomorphic enveloped single-stranded RNA viruses. Most reptilian PMV described thus far are grouped together in the genus *Ferlavirus* (ICTV, 2012), which is named after the first PMV isolate obtained from a group of Fer-de-Lance vipers during an outbreak in a Swiss serpentarium in 1972 (Clark *et al.*, 1979). Partial sequence analyses of L, HN, and U genes of a number of ferlaviruses from squamates (snakes and lizards) has shown that these can be divided into three genogroups: A, B, and C (Abbas *et al.*, 2011), whereas a single ferlavirus isolate from a Hermann's tortoise (*Testudo hermanni*) (THer-GER99) clusters within the genus *Ferlavirus* but separately from all squamate ferlaviruses based on partial genome sequences (Marschang *et al.*, 2009).

Adenoviruses (AdVs) have been documented in four species of bearded dragons: *Pogona vitticeps*, *Pogona henry-lawsoni*, *Pogona barbatus*, and *Pogona minor* (Julian and Durham, 1982; Jacobson *et al*., 1996; Kim *et al.*, 2002; Wellehan *et al.*, 2004; Jacobson, 2007; Hyndman and Shilton, 2012), and AdV infections are frequently detected in captive bearded dragons. Clinical signs in infected lizards often include acute depression, anorexia, diarrhea, ophisthotonus, and death. Some animals infected with AdVs may develop systemic infection associated with gastroenteritis, hepatitis, nephritis, pneumonia, and encephalitis, whereas others may remain clinically healthy (Jacobson, 2007). Intracellular basophilic inclusion bodies are commonly seen in hepatocytes, bile ducts, and intestinal mucosa (Jacobson, 2007). Ante mortem diagnosis is usually done through polymerase chain reaction (PCR) testing of cloacal swabs, followed by sequencing (Papp *et al*., 2009). Thus far, all AdVs of squamate reptiles have been classified as members of the genus *Atadenovirus*. It has been hypothesized that this

genus may have coevolved with this group of reptiles (Benkö *et al.*, 2005).

The purpose of this study was to screen healthy bearded dragons that were collected at a reptile rescue station for the presence of viral infections. This screening led to the first detection of a ferlavirus in this species of lizard.

Materials and Methods

In May 2011, 22 cloacal swabs and plasma samples were collected from 22 bearded dragons from a rescue center in Munich, Germany. All animals were healthy and did not show any clinical signs during captivity. Therefore, many were given away to new homes. A second round of testing was carried out on 27 animals along with 22 plasma samples four months later (including nine lizards from the first testing round) (Table 1). The bearded dragons had all been taken into the rescue center from private keepers with various types of husbandry (kept in pairs, alone, or in groups of males only). All animals were clinically examined upon arrival at the rescue center and integrated into the existing collection if they were determined to be healthy. Most animals remained in the rescue center for several weeks or months (in some cases even years). In the winter, the animals are kept in terraria inside the station, whereas in the summer, they are kept in outside enclosures protected from rain. Because most of the bearded dragons were male, they were housed individually. None of the contact animals

Table 1. Results of virus positive lizards samples. Only animals that tested positive for at least one of the tests are included in the table. Abbreviations: AdV = adenovirus, PMV = Paramyxovirus, Acc No = accession number, HI = hemagglutination inhibition test. Virus isolates used for HI test are as follows: A = Xeno-USA99; B1 = Pyth-GER01; B2 = Crot-GER03; C = Pan gut-GER09; and T = THer-GER99. Virus genotypes are as follows: Squamatid ferlavirus isolates: Pyth-GER01 and Crot-GER03, both belonging to subgroup B; Xeno-USA99 from subgroup A; and Pan gut-GER09 from subgroup C. Chelonian ferlavirus: THer-GER99. # = PMV, a 566 nt long partial sequence of the RNA dependent RNA polymerase (RDRP) gene (L-gene) was amplified (Ahne *et al*., 1999). For AdV, a 318 nt long partial sequence of the DNA-dependent DNA polymerase gene was amplified (Wellehan *et al*., 2004). π = (RT)-PCR detection was always done from swabs. Ω = Antibody detection was done from plasma.

	Identification number	Case history	(RT-)PCR results#π		HI results and virus isolates usedΩ					Comment
			PMV	AdV	A	B1	B2	C	T	
ROUND ONE	I 1.2	Swabs and plasma collected from 22 animals on 27.05.11	+	–	low antibody titers of ≤8 were detected against Pyth-GER01 for 20 samples of the 22 tested					The other 11 lizards: I 1.1, I 1.7, I 1.8, I 1.9, I 1.10, I 1.12, I 1.14, I 1.16, I 1.17, I 1.19, and I 1.22, were (RT-) PCR negative for the presence of PMV and Adenoviruses.
	I 1.3		+	+						
	I 1.4		+	–						
	I 1.5		+	–						
	I 1.6		+	–						
	I 1.11		+	–						
	I 1.13		+	–						
	I 1.15		+	–						
	I 1.18		+	–						
	I 1.20		+	–						
	I 1.21		+	–						
ROUND TWO	II 1.16	Most of the animals tested in the 1st round were dispersed except nine lizards including (II 1.3, II I.5, II 1.11, II 1, 16, II 1.19, II 1.20, II 1.21). 18 new animals were introduced into the previously established groups. Swabs from 27 animals and 22 plasma were collected four months after the first round of testing.	–	–	8	–	8	16	8	All lizards tested in this round were RT-PCR negative for PMV. Low antibody titers of 4 –8 were detected in the second round of testing from 7 sera including those sera tested in the first round: II 1.5, II 1.11, II 1.19 and II 1.21.
	II 1.20		–	–	8	8	8	4	16	
	II 2		–	–	8	4	4	8	32	
	II 3		–	+	8	8	4	8	32	
	II 4		–	+	4	–	4	4	4	
	II 5		–	+	8	–	4	4	8	
	II 6		–	+	–	–	–	–	–	
	II 7		–	–	8	–	4	16	32	
	II 8		–	–	4	–	4	16	16	

kept in the same room as the bearded dragons had respiratory signs at the time of sampling, and none of the animals developed respiratory signs later.

Plasma from the first round of samples were tested against one squamate ferlavirus isolate (Pyth-GER01) belonging to subgroup B using a hemagglutination inhibition (HI) test as described previously (Thayer and Beard, 1998; Marschang *et al.*, 2002), whereas plasma collected from the second round of samples were tested for antibodies against four squamate ferlavirus isolates (Pyth-GER01 and Crot-GER03, both belonging to subgroup B; Xeno-USA99 from subgroup A; and Pan gut-GER09 from subgroup C) and one chelonian ferlavirus (THer-GER99) (Marschang *et al.*, 2009; Abbas *et al.*, 2011). Because of nonspecific reactions, low titers can be measured using this test, which are considered false positives. For that reason, a cut-off was defined at a titer of 16 (Jacobson *et al.*, 1992; Blahak, 1994; Marschang *et al.*, 2002).

Cloacal swabs were immersed in 3-ml Dulbecco's modified Eagle's medium (DMEM, Biochrom AG, Berlin, Germany) supplemented with antibiotics (Penicillin 400 U/ml, gentamycin sulfate 12.8 U/ml, streptomycin sulfate 760 U/ml, and Amphotericin B 1 µg/ml; Biochrom AG). Samples were then sonicated for the destruction of cells and centrifuged at 3,000xg for 10 min for the removal of bacteria and cell debris. Virus isolation was attempted on iguana heart cells (IgH2, ATCC, CCL-108) as described previously (Abbas *et al.*, 2011). Two hundred µl of supernatant was inoculated onto cell monolayers and incubated at 28°C for two weeks. Cells were observed microscopically twice a week for the development of cytopathic effects (CPE).

RNA was prepared from 300 µl of the sonicated samples using the guanidinium isothiocyanate method described by Boom *et al.* (1990) and resuspended in 75 µl RNase free water. A nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay targeting a portion of the L gene was carried out in 25-µl reaction mixtures, using the primers described by Ahne *et al.* (1999) as described previously (Abbas *et al.*, 2011).

DNA was prepared from 200 µl of sonicated samples using the DNeasy Blood and Tissue Kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's instructions for a final elution volume of 100 µl. For AdV detection, a nested PCR targeting a portion of the DNA-dependent DNA polymerase gene was used as described previously (Wellehan *et al.*, 2004; Papp *et al.*, 2009).

Positive amplicons of all PCRs were gel purified (Invisorb Spin DNA Extraction Kit, Ivitek GmbH, Berlin, Germany) and sequenced by a commercial service (Eurofins MWG Operon, Ebersberg, Germany). Sequences were downloaded from the company's home page, edited, assembled, and compared using the STADEN Package version 2003.0 Pregap4 and Gap4 Programmes (Bonfield *et al.*, 1995). The sequences were compared to data in GenBank using BLASTN and BLASTX options (National Center for Biotechnology Information). Multiple alignments of nucleotide sequences were performed using the ClustalW algorithm of the BioEdit Sequence Alignment Editor programme (Hall, 1999).

Results

Of all animals tested in the first round, 11 (50%) were positive for ferlaviruses using the RT-PCR. The PCR products of all detected PMV were 100% identical to isolate Pyth-GER01 (Acc. No: GQ277612) (Marschang *et al.*, 2009), which was used in the serological tests. One animal (No. I 1.3) (4.5%) that was ferlavirus positive was also positive for an AdV, and the PCR product was 100% identical to the corresponding sequence of Agamid AdV-1 (Acc. No. DQ077706). The HI test revealed low antibody titers of ≤8 against Pyth-GER01 for 20 (90.9%) of the 22 samples tested.

Four months later, no ferlaviruses were detected in any of the samples tested by RT-PCR. However, four animals (14.8%) of the 27 tested were positive for AdVs. The sequences of three of these were unique and found to be 99% identical to Agamid AdV-1 (Acc. No. AY576678). Six (27.2%) of the 22 plasma samples collected in this round had detectable antibody titers of 16–32 against at least one PMV isolate; three animals (13.6%) had antibodies against PanGut GER09 and five animals (22.7%) against the tortoise isolate. Two animals had antibodies against both of these PMV isolates. Nonspecific antibody titers of 1:8 were detected in 11 animals against the rest of the PMV isolates (Pyth-GER01, Crot-GER03, and Xeno-USA99).

No virus was isolated from the cloacal swabs in vitro. Of the antibody-positive animals, two had been tested in the first round, one of which had tested PMV positive by RT-PCR, whereas the other had not.

Discussion

The importance of PMV infections in lizards, and especially in *Pogona*, is still unknown. Thus far, there are limited reports available concerning PMV infection in lizards (Gravendyck *et al.*, 1998; Jacobson *et al.*, 2001; Marschang *et al.*, 2002). Two studies have detected the presence of antibodies against both PMV and reoviruses in free living healthy iguanas and wild-caught Mexican lizards (Gravendyck *et al.*, 1998; Marschang *et al.*, 2002), indicating that ferlaviruses may be endemic in lizards in Central America. This is the first report in which a ferlavirus infection has been documented in captive bearded dragons. It is interesting to note that the animals in this study were considered healthy and did not show any clinical signs during the course of the study. This corresponds to findings in several other reports of ferlavirus infection in lizards (Marschang *et al.*, 2002). To date, only one report is available on a disease outbreak in ferlavirus infected lizards, with pneumonia the predominant finding (Jacobson *et al.*, 2001).

Serological testing of the bearded dragons against the ferlavirus detected in cloacal swabs was nonspecific in the first round of testing and negative in the second round of testing. It is surprising that positive antibody titers were detected against different ferlavirus genotypes four months after infection was documented but not against the genotype detected in this group. Freshly infected animals may give negative results on the HI test. This can be attributed to many factors, including the time required in reptiles for the development of an immune response, which has been shown to take about eight to 10 weeks in infected snakes and depends on several parameters including environmental factors and the animal's condition (Jacobson *et al.*, 1991).

Previous studies have demonstrated serological differences between different strains of ferlaviruses (Blahak, 1995; Richter *et al.*, 1996), and a comparison of HI titers measured in different laboratories using different ferlavirus isolates showed extreme differences in results (Allender *et al.*,

2008). Why a measurable antibody response does not always appear to match the virus genotype detected is, however, unknown and has not been previously documented.

AdVs are often detected in bearded dragons, and their clinical relevance is not always clear. Concurrent infections have often been described, especially with parvoviruses and coccidial protozoa (Jacobson *et al.*, 1996; Kim *et al.*, 2002). Ferlavirus infections in reptiles have also been reported with concurrent infection, particularly infection of a single animal with several ferlavirus strains (Papp *et al.*, 2010), as well as with other viruses including reoviruses (Gravendyck *et al.*, 1998; Marschang *et al.*, 2002; Abbas *et al.*, 2011) and AdVs (Abbas *et al.*, 2011) or with several types of bacteria (Pees *et al.*, 2010).

The current study highlights the importance of diagnostic screening for viruses in clinically healthy animals, especially when dealing with animals from mixed provenances. Although the infected bearded dragons in this study did not develop disease, the detected viruses could pose a danger to other species in mixed collections. In addition, many infectious agents can be involved in factorial disease processes. The ability of ferlaviruses to cause disease in bearded dragons under different circumstances is unknown.

We emphasize using at least two of the ferlavirus subgroups for the detection of antibodies in reptiles through HI assay. Using only one isolate can lead to false negative results because some animals in the current study only had detectable antibodies against one of the isolates. The origin of the infections in these animals remains unknown.

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