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Prevalence of Agamid adenoviruses of the bearded dragons (*Pogona vitticeps*) in Japan

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Abstract

In this study, we surveyed the prevalence and characteristics of agamid adenovirus (*genus Atadenovirus*) infections in bearded dragons (*Pogona vitticeps*) in Japan. Swab samples were collected from the oral cavity and pharynx of 44 healthy bearded dragons and 24 bearded dragons with clinical signs of respiratory disease. PCR confirmed agamid adenovirus in 25 of the 44 healthy lizards (56.8%). Of the 24 bearded dragons with respiratory clinical signs, 14 were agamid adenovirus-positive (58.3%). Sex was determined for 21 of the 24 bearded dragons with respiratory clinical signs (9 males and 12 females). Agamid adenovirus was confirmed in two of the nine males (22.2%) and 10 of the 12 females (83.3%), indicating a higher prevalence of adenovirus in the females. Overall, the prevalence of agamid adenovirus in bearded dragons with respiratory clinical signs was almost the same as that in clinically healthy bearded dragons, suggesting that the virus is widespread in this species. In addition, we detected no apparent seasonality in the occurrence of agamid adenovirus infection. The mean value of globulin was slightly higher in seven of the female lizards with confirmed agamid adenovirus.

Key Words: Agamid adenovirus, bearded dragon, PCR, Pogona vitticeps, respiratory disease

Introduction

Adenoviruses are nonenveloped, double-stranded DNA viruses that form spherical regular icosahedrons, approximately 80–100 nm in diameter. In humans, adenovirus infections cause respiratory organ disease, pharyngoconjunctivitis, epidemic keratoconjunctivitis, hemorrhagic cystitis, and gastroenteritis[11]. In veterinary medicine, adenovirus type 2 infections that cause respiratory disease are well known in dogs[3]. In reptiles, the typical clinical signs of adenovirus infection are nonspecific signs such as poor appetite, lethargy, and emaciation, as well as neurological signs such as head tilt and circling, and gastrointestinal signs. However, in some cases, the infected animals do not exhibit any clinical signs[7]. One case report has described

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Table 1. Adenovirus prevalence in healthy bearded dragons and in those with respiratory clinical signs

	Number of adenovirus-positive bearded dragons/number examined	Adenovirus prevalence
Healthy bearded dragons	25/44	56.8%
Bearded dragons with respiratory clinical signs	14/24	58.3%

infection in the esophagus and trachea of a Jackson's chameleon, whereas in another study, adenovirus-like agents were detected in the organs of a corn snake with pneumonia[6,9]. Infection has also been reported in a boa constrictor with a skin disease[16].

Agamid adenoviruses belonging to the genus *Atadenovirus* are among the adenovirus species that can infect lizards[1], and adenovirus infection in lizards has been reported in North America, Europe, and Australia. The first report of adenovirus infection in lizards was published in 1982, and since that time it has been confirmed that a range of different species can be infected[2,10]. The liver and enterocytes of neonatal bearded dragons (*Pogona vitticeps*) have been reported to be co-infected with an adenovirus-like virus and an even smaller dependovirus-like virus[8]. In addition, group outbreaks of adenovirus infection among hatched bearded dragons were reported and the authors of this report observed neurological signs of head tilt and circling in lizards[12]. However, although histopathological abnormalities were observed in the liver and intestines of lizards showing such clinical signs, no histopathological abnormalities were seen in the brains of lizards showing neurological signs[12]. Histopathological tests of bearded dragons infected with adenovirus have commonly shown basophilic intranuclear inclusion bodies in the liver and small intestine, accompanied by characteristic necrotic lesions of the liver[18]. Inclusion bodies have also been reported in other sites, including bile duct epithelial cells, renal tubules, the large intestine, acinar cells of the pancreas, and squamous epithelial cells of the oral mucosa[15].

Recently, adenoviruses have been successfully isolated from lizards and a cell line from bearded dragon embryos has been established[1]. However, although isolation of adenovirus from bearded dragons has been reported[1], whether adenovirus infection is truly pathogenic in the absence of visible clinical signs is unclear. The purpose of this study was to clarify the infection rate of adenovirus in bearded dragons in Japan and to determine if it is related to respiratory clinical signs. We also aimed to investigate the characteristics of bearded dragons infected with adenovirus.

Materials and Methods

Healthy bearded dragons and bearded dragons with respiratory clinical signs

The study was performed using 44 clinically healthy bearded dragons (*Pogona vitticeps*) with no evidence of physical abnormality, which were obtained from pet shops or owners of bearded dragons. The lizards were healthy and good appetite, consuming crickets and vegetables on an almost daily basis. Swab samples were collected from the oral cavity and pharynx of each lizard using sterile cotton swabs. In addition, 28 samples were collected from the oral cavity and pharynx of a further 24 bearded dragons displaying clinical signs of respiratory diseases, such as copious viscous secretion in the oral cavity or abnormal sounds such as mouth breathing, forced breathing, or actions like blech when breathing. These 24 bearded dragons were taken to veterinary hospitals by their owners and were also bred in Japan or overseas. For four of these lizards, sampling was performed twice at different time points to enable a temporal assessment of infection status.

PCR amplification and next generation sequencing

A 250-μL volume of PBS was added to each of the samples and nucleic acids were extracted using viral DNA and RNA purification kits (High Pure Viral Nucleic Acid Kit; Roche). Nested-PCR was performed in the first PCR analysis using polFouter (5'-TNMGNGGNGGNMGNTGYTAYCC-3') and polRouter (5'-GTDGCRAANSHNCCRTABARN GMRTT-3') as the forward and reverse primers, respectively, and the second PCR analysis was performed using polFinner-tag (5'- TAATACGAC TCACTATAGGGCCGTNTWYGAYATHTGYGG HATGTAYGC -3':) and polRinner-tag (5'- GGGAT ATCACTCAGCATAATATCGCCANCCBCDRTT RTGNARNGTRA -3') as the forward and reverse primer, respectively, and the product of the first PCR as the template[21]. Underline indicates tag sequences designed in this study. After the second PCR, agarose gel electrophoresis was carried out, and a band with a predicted size of 364-370 bp was confirmed. The PCR products were purified from the agarose gels. PCR was then performed direct sequencing using primers of tag sequences of polFinner-tag and polRinner-tag.

The nucleic acids extracted from samples by High Pure Viral Nucleic Acid Kit were pooled and treated with Ribo Minus Eukaryote Kit (Thermo Fisher) for depletion of ribosomal RNA. Then, reverse transcription was done using SuperScript III Reverse Transcriptase (Themo Fisher). Double-strand DNA was generated using GenomiPhi V2 DNA Amplification Kit (GE Healthcare). NGS library was prepared with Nextera XT DNA Library Prep Kit (Illumina) and was sequenced using MiSeq bench-top sequencer (Illumina) with MiSeq Reagent Kit v3 (150 Cycles). The sequence data was deposited in SRA (accession number DRA008935).

Biochemical analysis and blood tests

For the 24 lizards suspected of having respiratory diseases, the sex and the month in which they were first taken to a hospital were determined from medical records. Blood was collected from seven animals determined to have adenovirus infection, and plasma total protein (TP), albumin, and globulin levels were measured. A 1-mL syringe with a 25-G needle was used to collect blood from the caudal vein in the tail, and the blood was immediately transferred to a 0.5-mL tube and processed in heparin lithium. The sample was centrifuged immediately at 1,900 × g for 6 min to obtain plasma. Values for TP, albumin, and globulin were measured using a DRI-CHEM 7000V apparatus (Fujifilm Medical).

Statistical analysis

Statistical analysis of adenovirus prevalence was performed to compare the infection rate between genders using Excel Statistics 2015 (Social Survey Research Information). Obtained values were assessed for gender differences using Fisher's exact test.

Results

For the gene sequences obtained by PCR in this study, a BLASTn search of the National Center for Biotechnology Information database was performed and the sequences showing homology with both agamid adenovirus 1 and agamid atadenovirus well. There were few information of nucleotide sequences regarding difference of these two viruses. We could not find any difference of amino acid sequences among agamid adenovirus 1, agamid atadenovirus well, bearded dragons with healthy and respiratory clinical signs in this study. The nucleotide sequences were deposited in GenBank as accession numbers, LC503890 and LC503891. Although we tried to get whole genome sequences using next generation sequencer, we could not obtain enough reads of adenoviruses (data not shown). Therefore, in this study, we called PCR-positive as agamid adenovirus-positive. Of the 44 healthy bearded dragons, 25 were confirmed as agamid adenovirus-positive (56.8%), whereas 14 of the 24 bearded dragons with respiratory clinical signs were found to be positive for agamid adenovirus (58.3%)

Table 2. The four cases with respiratory clinical signs sampled twice

sample number	sex	Sampling period	1st sampling		2st sampling	
			clinical signs	PCR	clinical signs	PCR
1	male	379days	anorexia mouth breathing respiratory distress breathing like belch	-	respiratory distress oral discharge	-
2	female	421days		-	breathing like belch	-
3	female	20days		-	mouth breathing respiratory distress oral discharge	+
4	female	313days	anorexia breathing like belch oral discharge	+	anorexia mouth breathing oral discharge	+

Table 3. Adenovirus-positive rates in bearded dragons with respiratory clinical signs

Sex	Female	Male	Total
Adenovirus (-)	2	7	9
Adenovirus (+)	10	2	12
Prevalence rate	83.3%	22.2%	57.1%

(Table 1). In one of the four bearded dragons that were sampled twice, one sample was agamid adenovirus-nagative and the other was agamid adenovirus-positive. In another, both samples were positive for agamid adenovirus, whereas in the remaining two lizards, both samples were negative (Table 2).

The month in which lizards were first brought to hospital for investigation of the clinical signs of respiratory disease was recorded for each of the 24 bearded dragons showing respiratory clinical signs. Six animals were brought in spring (March to May), four in summer (June to August), nine in autumn (September to November), and five in winter (December to February). Excluding March, July and December, at least one agamid adenovirus-positive bearded dragon was brought to a hospital each month (Figure 1).

Sex was determined for only 12 of 44 healthy bearded dragons (3 males and 9 females), among which agamid adenovirus were confirmed in two of the three (66.6%) males and in one of the nine (11.1%). Fisher's exact test yielded a P value of 0.1273. Sex was determined for 21 of the 24 bearded dragons with respiratory clinical signs (9 males and 12 females), among which agamid adenoviruses were confirmed in two of the nine (22.2%) males and in 10 of the 12 (83.3%) females (Table 3). Fisher's exact test yielded a P value of 0.0092, indicating that significantly more females showed clinical signs than males by the infection of agamid adenovirus. Of the three bearded dragons of unknown gender, two were positive for agamid adenovirus. The seven bearded dragons that tested positive for agamid adenovirus and were used for blood biochemistry tests were all females. Plasma mean (range) values of TP, albumin, and globulin were 6.2 g/dL (4.7–8.0 g/dL), 2.24 g/dL (1.9–2.6 g/dL), and 3.957 g/dL (2.8–5.7 g/dL), respectively. According to a report by Tamukai and Kanazawa, plasma mean (reference range) values of TP, albumin, and globulin in healthy females were 5.6 (3.6–8.2 g/dL), 2.2 (1.5–3.2 g/dL), and 3.4 (2.1–5.0 g/dL)[19].

Discussion

Adenovirus infections in lizards are generally detected via histopathological tests of tissue from the liver, intestines, and kidneys, and several case reports have been published[4,7,18]. However, agamid adenovirus has also been detected in clinically healthy animals. In a previous study conducted in the UK, 27 bearded dragons classified by sex and age tested positive for agamid adenovirus-1, of which five (18.5%) tested positive for adenovirus[14]. In addition, the positive rate was highest (40%) in

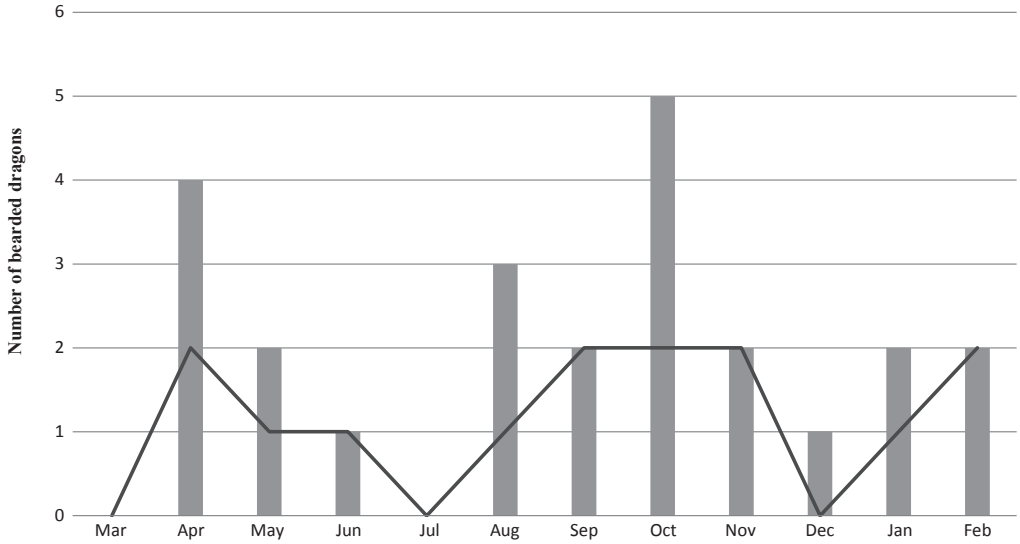

Fig. 1 Months in which respiratory clinical signs were first observed in bearded dragons and the number of cases per month when agamid adenovirus was confirmed in these lizards. Blue bars: number of bearded dragons with respiratory clinical signs; orange line: number of bearded dragons in which adenovirus was confirmed.

lizards aged 13 to 18 months old. In the present study, agamid adenovirus was observed in 25 of 44 (56.8%) healthy bearded dragons, which is clearly a higher percentage than that observed in the aforementioned previous study. In the UK report, the pharyngeal swab and the cloaca swab were combined and used for detection of virus. On the other hand, only the pharyngeal swab was used in this study. Furthermore, while the samples in the UK report were collected from geographically different areas, the samples in this study were collected from a limited area in Japan. These may influence the difference of the positive rate between UK report and this study. The propagation mode of this virus is not yet fully understood, and therefore further studies on the modes of infection are necessary.

We predicted that the prevalence of agamid adenovirus in bearded dragons with clinical signs of respiratory disease would be considerably higher than that in healthy bearded dragons. However, even the clinically healthy bearded dragons showed evidence of a relatively high prevalence of agamid adenovirus. Accordingly, we consider it unlikely that agamid adenovirus is the sole cause of the clinical signs of respiratory disease observed in bearded dragons.

With the exception of March and July, animals with clinical signs of respiratory disease were brought to the hospital all months of the year. Given that the rates of positive agamid adenovirus tests were the same for hospital visits throughout the year, there appears to be no seasonal epidemic of bearded dragon adenovirus.

In this study, we detected a significantly higher prevalence of agamid adenovirus in female bearded dragons with respiratory clinical signs. Reproductive diseases such as egg binding and follicular stasis are very common among reptiles and birds, and cases of mortality have been reported[13]. Indeed, altered immune function during the gestational period has been demonstrated in *Chalcides ocellatus*[17]. In addition, increased progesterone concentration during the oogenesis period and a decrease in the spawning period have been reported in *Chamaeleo calyptratus*[13]. These changes in sex hormone concentrations are probably associated with immunity[5]. Therefore, there may be a correlation between the balance of sex hormones, immune status, and the presence of agamid adenovirus.

We found that the mean albumin value of the seven females positive for agamid adenovirus was well within the reference range[19]. Similarly, all

the observed values TP fell within the reference range[20], although the mean value was slightly higher than the reference mean. The mean globulin value observed in this study was higher than the reference mean[19, 20]. These results may indicate that female bearded dragons with respiratory clinical signs and high TP and globulin values are carriers of agamid adenovirus.

Conclusions

The prevalence of agamid adenovirus in both clinically healthy bearded dragons and those with suspected clinical signs of respiratory disease was almost the same, thereby indicating the probable widespread distribution of these viruses and that agamid adenovirus infection in bearded dragons is unlikely to be the cause the respiratory disease. Furthermore, we detected no seasonality in the prevalence of agamid adenovirus. Prevalence was, however, higher in females than in males with respiratory clinical signs. Given that high values of TP and globulin were observed in female bearded dragons with respiratory clinical signs, they may be carriers of agamid adenovirus.

Acknowledgements

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