

REPTILE ADENOVIRUS PCR AND SEQUENCING AT THE UNIVERSITY OF FLORIDA CVM

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Foreword: This document is a brief review of adenoviruses, testing methods, and the current issues associated the adenoviral infections in bearded dragons and was prepared by Drs. Elliott Jacobson, Jim Wellehan, and Brian Stacy of the Jacobson laboratory. Every attempt has been made to clearly distinguish facts and principles cited in the peer-reviewed literature from ideas based on preliminary data and our experience with infections diseases of reptiles and agamid adenovirus 1. The Jacobson laboratory has been performing molecular testing for agamid adenovirus 1 since 2003. As more laboratories begin to offer molecular testing for this virus, it is anticipated that valuable new information will be generated in the near future. The information herein will be updated as new studies and data become available. Individuals not familiar with the methodology and terminology of this document are advised to consult their veterinarian.

1. BACKGROUND:

1.1. Adenovirus Biology. Members of the family Adenoviridae are large unenveloped DNA viruses. Compared to enveloped viruses, unenveloped viruses generally tend to be more stable in the environment, and adenoviruses are no exception, making environmental cleanup more difficult. Examples of other families of unenveloped viruses seen in veterinary medicine include parvoviruses and caliciviruses. Very meticulous sanitation and biosecurity practices are needed to prevent the spread of an adenovirus in a reptile collection. Large DNA viruses tend to change more slowly than smaller viruses, and many appear to have coevolved along with their hosts. The complexity of large DNA viruses makes them generally less well adapted for different species, and there appears to be a fair degree of host specificity. Adenoviruses jumping to new host species can be expected to be rare. Examples of other families of large DNA viruses seen in veterinary medicine include herpesviruses and poxviruses. There are currently four genera in the [family Adenoviridae](#); all characterized reptile adenoviruses belong to the genus [Atadenovirus](#).

1.2. Human Adenoviruses. All human adenoviruses are in the genus [Mastadenovirus](#). No members of *Atadenovirus* have been found to infect humans to date. In humans, adenoviruses frequently result in non-lethal upper respiratory disease, gastroenteritis, and conjunctivitis. Adenoviruses have been found to be the most

common cause of infectious conjunctivitis in humans (Ishii et al, 1987, Woodland et al, 1992).

1.3. Agamid Adenovirus 1. Agamid adenovirus 1 (Agamid AdV1) is a virus of bearded dragons. The first detection of adenovirus-like particles in bearded dragons was reported from New Zealand in 1982 (Julian and Durham). Adenovirus-like particles have been found histologically associated with enteritis and hepatitis in bearded dragons (Julian and Durham, 1982, Frye et al, 1994, Jacobson et al, 1996, Kim et al, 2002, Wellehan et al, 2004), and the first characterization of the virus to the species level was done in 2004 (Wellehan et al, 2004). To date, this virus has not been successfully cultured, and critical information on subjects including epidemiology, strain pathogenicity, and co-factors of clinical disease is poorly understood and requires further studies.

It is very apparent that there is a high prevalence of Agamid AdV1 in bearded dragons in the United States. The majority of samples we have tested from pet bearded dragons are positive. Additionally, **the presence of virus in cloacal swabs demonstrates that these infections are active and virus is being shed.** The majority of these dragons are not reported to have signs of disease at the time of sample collection. The long-term effects of these infections have not been identified or studied. In regard to this virus in other countries, [sequence information from an Austrian group](#) studying Agamid AdV1 samples from Austria has been submitted to Genbank, although the accompanying publication is not yet available.

The gene we initially chose to obtain sequence information is a very conserved gene (DNA polymerase), meaning that it is slow to change evolutionarily. This is a very useful characteristic when looking for new viruses, as parts of the gene may be expected to change less for designing PCR primers. It is also much more useful when looking at how more distantly related viruses evolved. However, it is not as useful for identifying strain differences within a species. We are initiating studies to look at less conserved genes to detect strain differences, but do not have results yet. **Therefore, no information is available on strain differences in Agamid AdV1.**

1.4. Other Lizard Adenoviruses. Adenoviruses have been found in a number of other lizard species, including savannah monitors (*Varanus exanthematicus*) (Jacobson

and Kollias, 1986), mountain chameleons (*Chamaeleo montium*) (Kinsel et al, 1997), Jackson's chameleons (*Chamaeleo jacksoni*) (Jacobson and Gardiner, 1990), blue tongued skinks (*Tiliqua scincoides*) (Wellehan et al, 2004), leopard geckos (*Eublepharus macularius*) (Wellehan et al, 2004), Tokay geckos (*Gekko gecko*) (Wellehan et al, 2004), fat tailed geckos (*Hemitheconyx caudicinctus*) (Wellehan et al, 2004), and Gila monsters (*Heloderma suspectum*) (Wellehan et al, 2004). The viruses from the savannah monitors and Jackson's chameleons were not characterized beyond the family level, and samples are not available to do so. As stated above, all characterized adenoviruses found to date in reptiles are in the genus *Atadenovirus*. The only characterized lizard adenovirus found in more than one species is Eublepharid adenovirus 1, which was found in both leopard geckos and fat tailed geckos, both members of the family Eublepharidae. Clinical findings suggest that this virus is more pathogenic in fat tailed geckos. Of reported pathology associated with adenoviruses in lizards, enteritis and hepatitis are most commonly found, but tracheitis and esophagitis have also been found. No information on prevalence or strain differences in these viruses is known.

1.5. Snake Adenoviruses. Adenovirus-like particles have been found in a number snake species, including California kingsnakes (*Lampropeltis zonata*) (Wozniak et al, 2000, Raymond et al, 2003), Pueblan milksnakes (*Lampropeltis triangulum*) (Wellehan et al, unpublished), four-lined ratsnakes (*Elaphe quatuorlineata*) (Heldstab and Besetti, 1984), Aesculapian snakes (*Elaphe longissima*) (Heldstab and Besetti, 1984), corn snakes (*Elaphe guttata*) (Juhasz and Ahne, 1992), rosy boas (*Lichanura trivirgata*) (Schumacher et al, 1994), *Boa constrictor* (Heldstab and Besetti, 1984, Jacobson et al, 1985, Perkins et al, 2001), ball pythons (*Python regius*) (Ogawa et al, 1992), palm vipers (*Bothreichis marchi*) (Raymond et al, 2002), and Gaboon vipers (*Bitis gabonica*) (Heldstab and Besetti, 1984). Based on very limited data, adenoviruses appear to be a significant cause of gastrointestinal disease in colubrids, especially kingsnakes. Of reported pathology associated with adenoviruses in snakes, enteritis and hepatitis are most commonly found, but encephalitis and esophagitis have also been observed. No information on prevalence or strain differences in these viruses is known.

2. TESTING METHODS

2.1. Important concepts about testing. Numerous tests have been devised and are utilized for determining the presence of pathogens (viruses, bacteria, fungi, parasites) in tissues or biological samples. **Sensitivity** of a test refers to how good a test is at correctly identifying animals that have the specific pathogen. The sensitivity is the proportion of infected animals that test positive in the population. Specificity, on the other hand, is concerned with how good the test is at correctly identifying an animal who is free of the disease or pathogen. **Specificity** is the proportion of uninfected animals that test negative in the population. No test is 100% sensitive and specific. Errors can occur with every test that has been developed to determine presence or absence of a pathogen. The best tests are those with high sensitivity and specificity. A test result that is positive from a patient that is actually negative is defined as a **false positive result**. A test result that is negative from a patient that is actually positive is defined as a **false negative result**.

2.2. Polymerase chain reaction (PCR) testing. Most DNA, including adenovirus DNA, is composed of two complementary strands oriented in opposite directions. For a PCR test, primers (short single stranded segments of DNA) are designed to match parts of the sequence of each strand of adenovirus DNA in a manner such that they are directed inward toward one another. The DNA is heated so that the two strands separate. The temperature is then decreased to an appropriate temperature for the primers to anneal (bind) with the DNA. If the primers do not match with the DNA sequence, they will not anneal and no product will be formed. The temperature is then increased to an appropriate temperature for thermostable DNA polymerase to extend the primer, making a matching strand for the DNA. The amount of DNA between the primers present is thus doubled. The cycle starts over again as the DNA is reheated to separate the strands. The cycle is repeated many times and the amount of DNA increases geometrically, resulting in a large amount of product from a small amount of template very rapidly.

2.2.1. Nested PCR. A nested PCR is an additional second round of PCR that is run using additional internal primers on the product of the first PCR. This results in an assay that is both more sensitive due to further amplification, and more specific

from the requirement for binding of the additional primers. **The adenovirus PCR run at the University of Florida is a nested PCR.**

2.2.2. Product identification.

2.2.2.1. Product sequencing. **After a PCR is run, the PCR product must then be validated, meaning that it must be determined to be the appropriate product** and not an accidental binding of the primers to an unexpected site on a different DNA template. This is a topic that is not discussed enough and the veterinarian needs to be aware of how this is done by the lab they are using. **The most definitive way to determine the identity of the PCR product is to sequence it. All products of PCRs run in the Jacobson Laboratory at the University of Florida College of Veterinary Medicine are identified by sequencing.** Any lab offering PCR diagnostics should at least offer the option of confirmation of the PCR product by sequencing at a reasonable additional cost. Sequencing costs have decreased dramatically with the advent of newer technologies.

2.2.2.2. DNA Probe. Alternatively, a labeled piece of DNA containing part of the target sequence may be used to probe the PCR product. The probe must be designed to a sequence unique to the pathogen found within the PCR product. When done under the appropriate conditions, binding of the probe to the PCR product indicates that the PCR product is correct. Some protocols, such as TaqMan, have the probe incorporated in the PCR routine. While potentially less specific than sequencing, and not flexible for identification of new virus species, this technique may provide information on the amount of virus present, and is more specific than product confirmation methods other than sequencing.

2.2.2.3. Restriction enzyme digestion. Another method of determining the identity of the PCR product is to use restriction enzyme digestion. A restriction enzyme recognizes a short sequence of DNA, typically 4 to 6 base pairs, and cuts at the site. The sizes of the pieces generated are then typically measured by electrophoresis on a gel. When the DNA product is cut into pieces of the expected size, this suggests that the appropriate product has been generated. This is known as restriction fragment length polymorphism (RFLP). This is less specific than sequencing or probing, but indicates that the PCR product was of the appropriate size and that

restriction enzyme digestion sites were spaced to result in the expected fragment sizes. Due to time, labor, and availability of better methods, this method is now considered somewhat antiquated.

2.2.2.4. Measuring the size of the product. A fourth method of checking the identity of the PCR product is to measure the size of the product, typically by electrophoresis on a gel. This is the least rigorous method of product identification, and has the highest rate of false positive results. The veterinarian needs to use more caution when interpreting these results.

2.2.3. Pitfalls of PCR. PCR is a highly sensitive technique, and the potential for false positives from slight contamination throughout the process from collection to laboratory is high. As such, it is critically important that measures are taken to avoid contamination during sample collection and transport. Appropriate negative controls need to be used each time a PCR is run to look for lab contamination of the PCR reaction, and positive controls need to be used each time to ensure that the reaction is working properly. When possible, it is best to use a positive control that can be differentiated from the expected product as another means of detecting laboratory contamination.

2.3. Virus isolation. Virus isolation can be very difficult, and successful culture conditions have not been established for a number of adenoviruses, including Agamid adenovirus 1. Thus, virus isolation currently is not a method of diagnosing adenoviral infections in bearded dragons. Successful isolation of Agamid adenovirus 1 would greatly facilitate critical research, such as infection and transmission studies.

2.4. Electron microscopy.. Electron microscopy is another option that can look for the presence of adenovirus-like particles in feces or tissues. However, the species of adenovirus cannot be differentiated by electron microscopy. While further testing is needed to compare the sensitivity of PCR and electron microscopy, studies with many other viruses have consistently found electron microscopy to be much less sensitive than PCR for detection (Biel et al, 2004, Castriciano et al, 2007, Cubitt et al, 1999, Hoet et al, 2003, Jain et al, 2001, Pratelli et al, 2000, Tang et al, 2005). Typical detection limits of electron microscopy are several orders of magnitude higher than PCR detection limits. **It can therefore be expected that electron microscopy will result in a**

significantly higher rate of false negative results.

2.5. DNA in-situ hybridization. DNA in-situ hybridization is another method of confirming adenovirus infection (Perkins et al, 2001). However, currently available methods do not identify virus species, and it requires histologically prepared tissues, so the invasiveness of sample collection makes it less suitable as an antemortem screening test.

3. Testing in the Laboratory of Elliott Jacobson, University of Florida. Adenovirus nested polymerase chain reaction (PCR) and sequencing is performed in the Jacobson Laboratory at the University of Florida College of Veterinary Medicine. The protocol for the procedure is found in [Wellehan et al, 2004](#). This PCR directly tests for the presence of nucleic acid from an adenovirus (a member of the family Adenoviridae), and sequencing of the PCR product identifies the species of Adenovirus that was amplified by PCR. **Like other tests that look directly for a pathogen (such as electron microscopy), this requires that the veterinarian submit an appropriate sample that contains the actual pathogen. Choice of sample for these tests is highly important.** For example, in a patient with a viral infection that is not viremic (does not have virus in the blood) at the time of sample collection, test results on blood will be negative. This does not mean that there is not virus elsewhere in the patient.

The presence of an infectious agent does not necessarily indicate disease. While adenoviruses, including Agamid adenovirus 1 (the most common bearded dragon adenovirus), are capable of causing disease, animals may be subclinically infected. In a subclinical infection the host harbors the pathogen but the host does not show any signs of illness. Other host and environmental factors play significant roles in potential disease manifestation. A veterinarian needs to correlate this information with other appropriate diagnostic information to diagnose disease. Agamid adenovirus 1 infection is very common in bearded dragon populations in the United States, and the rate of disease is significantly lower. Improved husbandry can be expected to significantly lower the rate of disease in infected animals.

The Jacobson laboratory requires that bearded dragon owners submit samples and obtain results through their veterinarian. This precaution is in place to

avoid misinterpretation of test information and to insure that medical advice and management decisions are guided by veterinarians directly involved with individual cases and collections. Submission forms can be obtained from our laboratory scientist, Ms. April Childress, at: ChildressA@mail.vetmed.ufl.edu

4. Summary comments about agamid adenovirus.

4.1. What we know.

- 4.1.1.. Adenovirus is associated with disease in bearded dragons.
- 4.1.2.. Prevalence of agamid adenovirus is very high in captive bearded dragons.
- 4.1.3. Many infected animals are asymptomatic (exhibit no signs of disease).

4.2. What we suspect.

- 4.2.1. Disease caused by adenovirus is affected by other cofactors, such as immune status, husbandry, and other sources of stress.
- 4.2.2. Agamid adenovirus is transmitted by viral particles in the feces, which contaminate the environment.
- 4.2.3. Of the currently available diagnostic tests, PCR on feces or cloacal wash is the most sensitive screening method.
- 4.2.4 Viremia is not common, making blood a poor sample choice for diagnosis.
- 4.2.5. Eradication of agamid adenovirus from the bearded dragon population will be very difficult due to high prevalence and the stability of the virus in environment.

4.3. What we don't know.

- 4.3.1. Specifically when or by what means agamid adenovirus entered the U.S. bearded dragon population.
- 4.3.2. Whether or not there are any collections/groups completely “free” of agamid adenovirus, i.e. groups that do not include infected animals.
- 4.3.3. What are the long term/life long effects or duration of adenoviral

infection in asymptomatic animals.

4.3.4. Whether there is periodicity to adenovirus shedding.

4.3.5. Whether there are different strains of agamid adenovirus 1 present in the US.

4.3.6. Whether or not agamid adenovirus is transmitted from females on or in their eggs to their young.

4.4. What needs to be done.

4.4.1. Additional screening to better characterize prevalence of agamid adenovirus in the captive bearded dragon population.

4.4.2. Long term monitoring of asymptomatic infected dragons.

4.4.3. Long term monitoring of adenovirus shedding.

4.4.4. Investigation of potential virus strain variability.

4.4.5. Studies of transmission, especially vertical transmission (parent to offspring).

4.4.6 Work toward vaccine development

4.4.7 Studies of the effects and pharmacology of drugs found to be effective against mammal adenoviruses, such as cidofovir, on reptile adenoviruses.

5. Frequently Asked Questions

5.1. What is an adenovirus?

Viruses are very small, very simple life forms that survive and reproduce by infecting cells of other organisms, which are referred to as “hosts.” Adenoviruses are one group of viruses and they are found in a variety of different animals, including reptiles, birds and mammals. **Adenoviruses typically are specific for their hosts.** For example, a reptile virus may infect other reptiles, but is unlikely to infect a human or other mammal.

5.2. Does adenovirus cause disease in bearded dragons?

Yes, illness and death have been documented in bearded dragons with confirmed adenovirus infection and organ injury consistent with viral infection. Technically, “causation” is proven by experimentally infecting animals with a virus and producing

disease; however, there are enough well-documented cases of adenoviral disease in bearded dragons that veterinarians and virus experts agree that adenovirus can cause disease. Now it gets a little complicated. Some types of viral infection are called “asymptomatic” or “subclinical.” These terms are used when virus is present, but the infected animal does not have any detectable signs of disease. Many factors may determine if a host develops signs of disease. These include the host’s immune response, infection by other pathogens, and other influences on general health, such as husbandry conditions. In addition, it is not known whether or not there are different types or strains of Agamid adenovirus 1. There may be strains of adenovirus that are more likely to cause disease and others that are less pathogenic. **However, at this time it is unknown whether or not there are multiple strains of bearded dragon adenovirus. The currently available PCR testing will identify the species of adenovirus, but does not distinguish different strains.**

5.3. How do bearded dragons become infected by adenovirus?

Adenoviruses are very stable in the environment. Dragons likely become infected by exposure to the feces of other dragons that are shedding virus. It is unknown whether infected females pass the virus to their offspring during egg laying (vertical transmission).

5.4. How is adenoviral infection diagnosed in bearded dragons?

The two most commonly used available tests for diagnosing adenovirus in bearded dragons are **polymerase chain reaction (PCR) and sequencing, and electron microscopy**. These tests are most commonly performed on tissues (usually liver from deceased animals) or feces/cloacal swabs.

When tissues of dead animals or tissue samples taken surgically are examined by light microscopy, inclusions in cell nuclei may be seen by light microscopy. However, these inclusions may be very difficult to find or absent in some infected animals, and other processes than adenoviral infection may cause inclusions in cell nuclei, so light microscopy is neither sensitive nor specific, and either a positive or negative diagnosis of infection by light microscopy needs to be confirmed by other means. Light microscopy is very useful for differentiating whether or not an animal that is known by other means to be infected with Agamid AdV1 has disease associated with the infection.

5.5. What does it mean if my dragon tests “positive” for adenovirus by PCR at the University of Florida?

PCR detects adenoviral DNA, thus **a positive result indicates the presence of adenovirus in the sample submitted.** All positive results at the University of Florida are confirmed by genetic sequencing, which is included in the test fee, and all tests are performed with the appropriate positive and negative controls. The sequence of the region of viral DNA detected by this PCR is unique to each adenovirus species and the sequence of each positive result is analyzed by University of Florida personnel. The sequence data is what is used to determine the virus species amplified. If adenoviruses other than Agamid adenovirus 1 are present, they are easily distinguished. **There is no risk of other adenoviruses of humans or other species being reported as a positive result for Agamid adenovirus 1.**

5.6. What does it mean if my dragon tests “negative” for adenovirus by PCR at the University of Florida?

A negative PCR result indicates that there is no detectable virus present in the sample at the time of testing. Some viruses are shed intermittently, meaning that sometimes virus is shed by the host and sometimes it is not. **It is not known if Agamid adenovirus 1 is shed intermittently.** It is possible that an animal will test negative at one time point and positive at another, or vice versa. There are no studies of adenovirus shedding over long periods of time in bearded dragons, and it is therefore not known how many times an animal needs to be tested to be “confirmed” as negative. **All that can be confidently stated is that multiple negative test results from different time points means more than a single negative test result.**

5.7. What is the difference in testing by electron microscopy of feces and PCR of feces/cloacal swabs? Why was my dragon negative by electron microscopy and positive by PCR?

While further testing is needed to compare the sensitivity of PCR and electron microscopy, studies with many other viruses have consistently found electron microscopy to be much less sensitive than PCR for detection (Biel et al, 2004, Castriciano et al, 2007, Cubitt et al, 1999, Hoet et al, 2003, Jain et al, 2001, Pratelli et al,

2000, Tang et al, 2005). **It can therefore be expected that electron microscopy will result in a significantly higher rate of false negative results.**

5.8. How common is adenoviral infection in bearded dragons?

The Jacobson laboratory has been using PCR to detect adenoviral infections in bearded dragons since 2003. Bearded dragons that are positive for Agamid adenovirus 1 have been detected in all groups studied thus far. **Very few negative animals have been identified in our sample population.** These results support that **Agamid adenovirus 1 infection is very common in bearded dragon populations in the United States.** **The rate of disease, i.e. animals that are sick or dying from adenoviral infection, is significantly lower.** **Many animals that test positive (apparently) do not have any clinical signs of disease.** Many factors may play a role in whether or not animals become ill from adenoviral infection. Possibilities include, husbandry conditions, other disease problems and differences in strains of Agamid adenovirus 1; however, none of these issues have been studied.

5.9. Are there different strains of Agamid adenovirus 1?

This is an excellent question and a needed area of further research. It is unknown whether or not there are different strains of Agamid adenovirus 1. **The current methods used to detect Agamid adenovirus 1 will not identify different strains of the virus.** Differences in the pathogenicity (ability to cause disease) in strains may explain why some groups of animals become ill and others do not.

5.10. Can adenoviral infection be managed in the bearded dragon community?

The ability to detect adenoviral infection in bearded dragons has far outpaced our understanding of this disease. The implications of having positive animals in a collection are unknown at this time. Investigation of key issues, such as the potential for different strains of Agamid adenovirus 1, hopefully will shed light on the many unanswered questions regarding management of infected animals. There is far too little definitive information known about adenoviral infections to offer specific recommendations. General good management practices are indicated, such as quarantine of new animals entering collections, isolation of sick animals, and providing optimal husbandry conditions. For those individuals that are regularly testing animals for

Agamid adenovirus 1, negative animals should be housed separately. **To date, we have seen no definitive evidence of collections that are completely free of Agamid adenovirus 1.**

5.11. Is Agamid adenovirus 1 infection something I should just ignore?

The high prevalence of infection of bearded dragon adenovirus, lower rate of disease, and environmental stability of this virus certainly make this a frustrating problem. Much like other viral diseases of intermediate pathogenicity, such as hepatitis C virus in humans (HCV), equine infectious anemia virus (EIAV) feline immunodeficiency virus (FIV), porcine reproductive and respiratory syndrome virus (PRRSV), and bovine viral diarrhea virus (BVDV), this is something that many infected animals can live with for a long time. However, like these other diseases, it also has a significant negative impact on the health of both individual animals and populations. A good deal of effort and expense will be needed to establish negative colonies, and adenovirus would easily spread amongst animals if only one infected animal were brought into the colony. It is certainly tempting to give up and ignore this problem. At this point, our best recommendation is testing and honesty, with the goal of establishing adenovirus-free colonies. If the decision is made to accept that your bearded dragon colony is infected, the ethical thing to do is to inform anyone accepting a lizard from your colony of the infection.

15.12. I am considering purchasing a bearded dragon. What should I ask the seller?

As a reptile owner or someone considering purchasing a bearded dragon, there are key issues regarding adenovirus that you should be familiar with that are outlined in this document. This is a poorly studied topic and there are many gaps in our understanding that have been filled with rumor and misinformation. Some breeding colonies include infected animals and do not report any major episodes of illness or deaths associated with adenoviral disease, while others have had major health problems in their animals. Some of the possible explanations for these observations are given in this text. To date, we are not aware of any source of bearded dragons that has been proven to be free of agamid adenovirus. This statement does not imply that adenovirus is not an important health problem in these animals or that adenovirus-free

colonies will not be available in the future. Given an opportunity to acquire an infected vs an uninfected animal, the choice is clear. However, be aware that there likely are major differences in the sensitivity of different methods to detect adenovirus (see 2. TESTING METHODS). The primary considerations for purchasing any reptile should be taken into account when purchasing a bearded dragon. Talk with the seller and decide for yourself whether they are knowledgeable regarding proper husbandry and proper care. Only purchase robust, actively feeding animals and consult a veterinarian for a general health examination or any possible medical problems.

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