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Prevalence of neutralising antibodies against adenoviruses in lizards and snakes

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ABSTRACT

Adenoviruses (AdVs) are relatively common in lizards and snakes, and several genetically distinct AdVs have been isolated in cell culture. The aims of this study were to examine serological relationships among lizard and snake AdVs and to determine the frequency of AdV infections in these species. Isolates from a boa constrictor (*Boa constrictor*), a corn snake (*Pantherophis gutattus*) and a central bearded dragon (*Pogona vitticeps*), and two isolates from helodermatid lizards (*Heloderma horridum* and *H. suspectum*) were used in neutralisation tests for the detection of antibodies in plasma from 263 lizards from seven families (including 12 species) and from 141 snakes from four families (including 28 species) from the USA and Europe. Most lizard and snake samples had antibodies against a range of AdV isolates, indicating that AdV infection is common among these squamates. Neutralisation tests with polyclonal antibodies raised in rabbits demonstrated serological cross-reactivity between both helodermatid lizard isolates. However, squamate plasma showed different reactions to each of these lizard isolates in neutralisation tests.

Introduction

Adenoviruses (AdVs) are a group of non-enveloped, icosahedral, double-stranded DNA viruses with a diameter of 80–100 nm. The family *Adenoviridae* consists of five accepted genera: (1) *Mastadenovirus*, which contains mammalian AdVs; (2) *Aviadenovirus*, containing AdVs of birds; (3) *Siadenovirus*, with chelonian, amphibian and avian AdVs; (4) *Ichtadenovirus*, with a single AdV isolated from a white sturgeon (*Acipenser tranmontanus*); and (5) *Atadenovirus*, containing all squamate AdVs described so far, as well as AdVs of birds and several mammals (Harrach et al., 2012). Recently, a sixth genus, *Testadenovirus*, has been proposed for AdVs in testudinoid hosts (Doszpoly et al., 2013). Atadenoviruses appear to have co-evolved with reptiles and, subsequently, have made several host switches (Harrach, 2000).

AdVs are regularly detected in various species of lizards (Frye et al., 1994; Wellehan et al., 2004; Papp et al., 2009; Hyndman and Shilton, 2011; Ball et al., 2012, 2014a); in particular, central bearded dragons (*Pogona vitticeps*) (Kim et al., 2002; Wellehan et al., 2004; Moormann et al., 2009). They have also been described in a range of different species of snakes (Heldstab and Bestetti, 1984; Farkas et al., 2002; Marschang et al., 2003; Garner et al., 2008; Papp et al., 2009; Abbas et al., 2011).

The pathogenicity of AdVs for their reptile hosts is not always clear and, for the most part, evidence is circumstantial. Although there are reports of detection of AdVs in clinically healthy squamates (Kubiak, 2013), pathological changes in infected animals include enlarged, mottled or diffusely pale livers, dilatation of the duodenum and hyperaemia of the intestinal mucosa (Jacobson and Kollias, 1986; Hyndman and Shilton, 2011; Marschang, 2011). However, the pathogenicity of these viruses has been demonstrated only in snakes in an experimental transmission study (Jacobson et al., 1985).

Lizard AdVs seem to be relatively species specific and mostly have been found in single hosts. However, there are a number of exceptions, e.g. eublepharid AdV-1 has been described in two different gecko genera, a leopard gecko (*Eublepharis macularius*) and a fat-tail gecko (*Hemitheconyx caudicinctus*) (Wellehan et al., 2004). An AdV with 99% identity to helodermatid AdV-2, which was originally detected in a beaded lizard (*Heloderma horridum*) (Papp et al., 2009) was detected in a western bearded dragon (*Pogona minor*

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minor) in Australia (Hyndman and Shilton, 2011). A closely related virus, helodermatid AdV-1, has been found only in Gila monsters (*Heloderma suspectum*) (Wellehan et al., 2004; Papp et al., 2009). An AdV identical to helodermatid AdV-2 has also been identified in a captive central bearded dragon in the USA (Wellehan et al., 2012).

There appears to be a relative lack of species specificity among snake AdVs. Snake AdV-1 has been found in both colubrid and boid snakes (Farkas et al., 2002; Marschang et al., 2003), while snake AdV-2 has been described in viperid and colubrid snakes (Garner et al., 2008; Papp et al., 2009) and snake AdV-3 has been detected in different species of colubrid snakes (Garner et al., 2008). Thus, AdVs are not necessarily host-specific and do not always follow a co-speciation model under which host and virus phylogenies are concordant (Ascher et al., 2013).

Among 12 collections of snakes in four different countries, neutralising antibodies against a snake AdV-1 isolated from a boa constrictor (*Boa constrictor*) were found in 15/113 (13.3%) plasma samples tested from Costa Rica and Germany, including a sample from a wild boa constrictor from Costa Rica (Marschang et al., 2003). Antibodies against snake AdV-1 were also found in plasma samples from 4/86 (4.7%) boid snakes in Germany (Pees et al., 2010).

Until 2009, AdVs had been isolated only from snakes and these viruses were closely related (Jacobson et al., 1985; Juhász and Ahne, 1992; Farkas et al., 2002, 2008; Marschang et al., 2003). In 2009, helodermatid AdVs-1 and -2 were isolated in cell culture from different species of helodermatid lizards (*H. horridum* and *H. suspectum*) (Papp et al., 2009). Subsequently, an agamid AdV-1 was isolated in cell culture from a central bearded dragon (Ball et al., 2014b). There have been no previous studies to determine the presence of antibodies against AdVs in lizards or the serological cross-reactivity of lizard AdVs.

The aims of this study were to raise polyclonal antibodies against two closely related lizard AdVs (helodermatid AdV-1 and -2), to establish neutralisation tests (NTs) with a panel of AdV isolates from lizards and snakes, and to determine the prevalence and cross-reactivities of neutralising antibodies against squamate AdVs in plasma from lizards and snakes.

Materials and methods

Samples

Plasma samples were collected from lizards and snakes in the USA and Europe from 2006 to 2013, generally using heparin as an anticoagulant (Table 1). Plasma was collected from 263 lizards, including 12 different species from five different families. Ten of 263 (3.8%) lizards were wild-caught helodermatid lizards, while 101/263 (38.4%) were captive-bred; the origins of the remaining lizards were unknown. Snake plasma was collected from 141 snakes from 28 different species from four families. One-hundred-and-four of 141 (75.2%) snakes were wild-caught and 2/141 (1.4%) were captive-bred, while the origins of the remaining snakes were unknown (Table 1). The plasma samples were tested for the presence of antibodies against different AdVs using NTs.

Viruses

NTs were developed using three lizard AdVs (helodermatid AdVs-1 and -2, and agamid AdV-1) and two snake AdVs (snake AdVs-1 and -2) isolated in our laboratory (Table 2).

Polyclonal antibodies from rabbits

Helodermatid AdV-1 and -2 were purified by equilibrium centrifugation in CsCl gradients and desalted on a 10 DC column (Bio-Rad) (Pérez-Berná et al., 2009). Purified viruses were diluted 1:10 in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffered saline (HBS) solution composed of 20 mM HEPES (Biochrom), 0.15 M NaCl pH 7.8 (Merck) and 10% glycerol (Carl Roth), and stored at –80 °C.

Virus suspensions of helodermatid AdV-1 with a 50% infectious dose (ID_{50}) titre of $10^{5.5}$ per mL and helodermatid AdV-2 with an ID_{50} titre of $10^{6.5}$ per mL were used to inoculate rabbits. Two rabbits were used for each virus. The initial immunisation was performed via intradermal injection with 500 µL purified virus suspension combined with 500 µL adjuvant (saponin; Sigma-Aldrich Chemie). The immunisation was repeated on days 14 and 28. Blood was collected from the saphenous vein of each rabbit before each immunisation. The final hyperimmune serum was collected once every 7 days for 3 weeks after the final immunisation. The inoculation of rabbits at the Veterinary Medical Faculty of the University of Leipzig was carried out following notification of the appropriate committee (Landesdirektion Leipzig, Referat 24) as an 'Anzeige von Eingriffen und Behandlungen zur Herstellung, Gewinnung, Aufbewahrung oder Vermehrung von Stoffen, Produkten oder Organismen' according to Section 10a of the German Animal Protection Law (date of notification 30 November 2009).

Neutralisation tests

All plasma samples were clarified by low speed centrifugation (800 *g*) for 10 min and incubated at 56 °C for 30 min for heat inactivation of complement and other nonspecific inhibitors. Plasma samples from rabbits were first tested for the presence of antibodies against helodermatid AdV-1 and -2 by NT (Beard, 1989). These plasma samples were used as positive controls for all NTs to determine cross-reactivity and since they contained high titres of antibodies against lizard AdVs.

Lizard and snake plasma were pre-diluted 1:10 with phosphate-buffered saline (PBS) composed of 1.463 g/L $Na_2HPO_4.2H_2O$ (Karl Roth), 8 g/L NaCl (Th. Geyer), 0.2 g/L KCl (Karl Roth) and 0.245 g/L KH_2PO_4 (Merck). Pre-diluted plasma (25 µL) were serially diluted from 1:20 to 1:2560, in 96-well tissue culture plates (BD Falcon). Virus suspension (25 µL) containing 100 50% tissue culture infectious doses ($TCID_{50}$) of virus was added to the plasma. The tissue culture plates were incubated for 2 h at 28 °C in an atmosphere of 5% CO_2. Freshly subcultured cells from the same cell line on which each virus was originally isolated were then added to each well. The plates were examined for cytopathic effects after 10–14 days. A titre ≥1:20 (the lowest detectable titre) was considered to be significant, since previous studies have used a cut-off value ≥1:16 for similar tests (Pees et al., 2010).

Post-immunisation polyclonal antibodies from rabbits were tested for the presence of antibodies against the original viruses using NTs. VN antibody titres of 2560 (end point of the dilution series) were detected against both helodermatid AdVs in plasma from rabbits inoculated with each virus.

Statistical analysis

General linear models and general linear mixed models (GLMM) were performed using the procedures PROC GENMOD and PROC GLIMMIX of SAS software Version 9.4 (SAS Institute). The GLMM allows the calculation of odds ratios and relative risks for significant effects. Separate analyses were performed for lizards and snakes. Sampled animals were assumed to be independent. The probability of finding antibodies was compared depending on families, species, sex and habitat for each virus. It was assumed that the data would follow a binomial distribution and, therefore, the logit link was used. Main effects were fitted for families, species within families, sex and habitat, and tested using an F-test. In a second analysis, we tested whether the probabilities of finding antibodies against individual viruses were independent. A log linear model was therefore fitted and included fixed main effects for four viruses and all possible interactions between them. A likelihood ratio test was performed for testing main effects and interactions.

Results

The results of the NTs against different squamate AdVs are presented in Tables 3, 4 and 5. The highest seroprevalences were detected in lizards (89/263, 33.8%) and snakes (62/138, 44.9%) against agamid AdV-1. Antibodies against two or more AdVs were detected in 32/263 (12.2%) lizards and 14/141 (9.9%) snakes.

In plasma samples from lizards, the median (±standard deviation, SD) titre was 1:40 ± 1:186. In plasma samples from snakes, the median (±SD) titre was 1:40 ± 1:192. On statistical analysis no significant correlations between families, species, habitat or sex were found ($P < 0.05$). In lizards, the presence of antibodies against agamid AdV-1 was associated with a higher probability of the presence of antibodies against helodermatid AdV-1 ($P = 0.0144$). In lizards, there were no significant interactions between the presence of antibodies against helodermatid AdV-1 and helodermatid AdV-2 ($P = 0.058$). In snakes, the presence of antibodies against agamid AdV-1 was associated with a higher probability of the presence of antibodies against snake AdV-2 ($P = 0.0148$) and the presence of antibodies against helodermatid AdV-2 was associated with a higher probability of the presence of antibodies against snake AdV-1 ($P = 0.0011$).