Szent István University
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Detection and characterisation of adeno-, irido- and paramyxoviruses in reptiles

Brief Summary of Doctoral Thesis

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2012

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Introduction

Reptile virology dates back approx. 50 years, and began by surveying zoonotic arboviruses (flaviviruses, togaviruses) in potential reptile reservoir hosts. Discoveries of reptile specific viruses started about a decade later. The most important pathogens among the reptile viruses turned out to be diverse for the different reptile taxa. In chelonians (turtles and tortoises) rana- (*Iridoviridae*) and herpesviruses, in crocodilians poxviruses, in ophidians (snakes) paramyxo- and reoviruses, and in lacertilians (lizards) reo- and adenoviruses were the predominantly diagnosed illness-related agents.

Among reptiles, adenoviruses (AdVs) have most often been identified in lizards, but have also been detected in various species of snakes, chelonians, and a few crocodiles. AdV infection appears to have a world-wide distribution in captive populations and wild collected snakes have also been tested positive for antibodies against AdV. In squamates, gastroenteritis and hepatitis are most commonly associated with AdV infection, and central nervous symptoms also occur occasionally. The associated virus has only been isolated and/or genetically characterised in a very few cases. Isolates were avaible only from snakes at the beginning of our studies. As a single reptilian AdV type, one of these isolates has been completely sequenced in Budapest by 2005.

Iridoviruses (family *Iridoviridae*) are important pathogens of lower vertebrates (poikilotherm vertebrates) and various invertebrates. Of the five accepted genera, members of two genera (*Iridovirus* and *Chloriridovirus*) had earlier been described only in invertebrates and are shortly named as invertebrate iridoviruses (IIV). At the end of the 1990's, two research groups in Germany isolated and characterised a new IIV from crickets. In both cases, the animals derived from commercial breeders that produced crickets as food for reptiles. Later, these IIVs were described in lizards by two German research groups. A host-switch of this virus from prey insects to the predator lizards was hypothesized. The virus was characterised phenotypically and genetically, and considered a variant of Chilo iridescent virus (CIV or IIV-6), type species of the *Iridovirus* genus.

The first reptilian paramyxovirus (rPMV) was described in a serpentarium in Switzerland in 1972 with severe respiratory distress, CNS signs and 30% mortality. The isolated virus (Fer de Lance Virus - FDLV) is considered the type species of all rPMVs and its genome has an extra transcriptional unit between the N and P genes coding for a short protein of unknown fuction (U gene). Further squamatid (snake and lizard) rPMV have been detected and characterised since, a number of them by partial gene sequences. Phylogenetic analysis and the unique genome-organisation both justified the establishment of a new genus *Ferlavirus* for these rPMV within the *Paramyxovirinae* subfamily (family *Paramyxoviridae*, order *Mononegavirales*). Yet, subgrouping within this genus remained controversial, prevalence of the PMVs in captive populations was not surveyed properly and very little information with no genetic sequences was available from non-squamate ferlaviruses.

The objectives in our studies were to further characterise these important and common reptile viruses, to describe new types and to extend our understanding of their prevalence, host specificity and phylogenetic relationships.

Materials and methods

Samples

Routine diagnostic samples from a total of 72 lizards, snakes and tortoises were surveyed for the presence of adenoviruses using a consensus nested PCR and the cell culture isolation methods. In the case of the Gila monsters (*Heloderma suspectum*) and Mexican beaded lizards (*Heloderma horidum*) at a Danish zoo, follow-up tests with four consecutive sampling times were performed.

Eight different iridovirus isolates were used in this study (Table 1). Further diagnostic samples (45 lizards, 27 crickets) arriving at the laboratory were also tested for the presence of IIV by virus isolation as well as by conventional PCR (nPCR) and real-time PCR (qPCR) methods.

Paramyxoviruses from six snakes, three lizards and one tortoise were included in a genetic comparison study based on three genes. Except for the two most recent field samples from a German corn snake and a Hungarian masked water snake case (PanGut-GER09 & HoBuc-HUN09), the viruses were isolated in cell culture. A total of 203 clinical samples (organs, swabs and tracheal washes) originating from 102 snakes, representing 21 boid, viperid, pythonid and colubrid species, were screened for the presence of PMV by RT-PCR and virus isolation.

Table 1. Origin of IIV isolates

	Host	Id. Nr.	Owner	IIV positive organs*	Case history
Squamata, **Lacertilia**	**high-casqued chameleon** (*Chamaeleo hoehnelii*)	**Ir.iso.1**	A	kidney, liver, spleen, *lung,* intestine	emaciation, kerato-conjunctivitis
	bearded dragon (*Pogona vitticeps*)	**Ir.iso.2**	B	*lung,* heart, tongue	unknown
	bearded dragon (*Pogona vitticeps*)	**Ir.iso.3**	B	*lung,* brain, tongue, stomach, intestine	unknown
	spiny tailed lizard (*Uromastyx sp.*)	**Ir.iso.4**	B	*skin*	hyperkeratosis
	four-horned chameleon (*Ch. quadricornis*)	**Ir.iso.5**	C	*liver*	emaciation, several animals died suddenly
	green iguana (*Iguana iguana*)	**Ir.iso.6**	D	*skin*	hyperkeratosis
Arthropoda	**house cricket** (*Acheta domesticus*)	**Ir.iso.7**	E	*whole body*	prey insects, massive die offs
	emperor scorpion (*Pandinus imperator*)	**Ir.iso.8**	F	*abdominal organs*	loss of UV coloration

*Isolates used in the sequence comparison are printed in italics, isolates compared in a cricket bioassay are underlined.

Cell culture-based methods

Isolation of viruses from samples was attempted on the iguana heart cell line (IgH-2, ATCC: CCL-108) and/or on Russell's viper heart cells (VH-2, ATCC: CCL-140) and/or Terrapene heart cells (TH-1, ATCC: CCL-50) depending on the origin of the sample. For the purpose of the animal infection studies or the antibody production or the partial genome sequencing experiments, in which higher virus yield or pure concentrated DNA was required, two helodermatid lizard AdVs and selected IIV isolates (Ir.iso.1 to 8) were further propagated and purified.

Animal infection studies

A series of transmission experiments were carried out at two different constant temperatures (20ºC, 30ºC) with crickets (*Gryllus bimaculatus*) to compare the virulence of three different IIV isolates originating from different hosts (Table 1).

Two groups (2 x 5 animals) of young (5 months) bearded dragons were infected using the lizard IIV isolate Ir.iso.3 (Table 1). At the beginning of the study group A & B members were infected via stomach catheters, and lizards in group B with an additional intra-coelomic injection. Both groups were force-fed with infected dead crickets for 45 days and only on vegetable diet in the last two weeks. Negative control lizards received the same treatment with the mock-solutions and were fed with IIV free crickets. Animals were kept at temperature of 19-26ºC and relative humidity of 28-38%. After 60 days, surviving animals were euthanized, gross pathology was performed and 12 different organs were collected for further tests to detect IIVs.

Microscopic techniques

Following *post mortem* examination, organs from the bearded dragons as well as longitudinally dissected body halves of crickets were fixed in paraformaldehyde, routinely processed for histology, and stained with haematoxilin-eosin. Digoxigenin (DIG) labeled probe targeting the major capsid protein (MCP) gene of IIV was prepared, and used for *in situ* hybridisation (ISH) studies. Polyclonal antibodies to bearded dragon IIV isolate (Ir.iso.3) were prepared in rabbits and used for immunohistochemical (IHC) studies on the histological slides.

In the case of the AdV isolates, cell culture supernatant was negative-stained with 2% potassium phosphotungstate and examined using a JEM-1011 transmission electron microscope for the presence of viral particles.

Molecular biological techniques

Nucleic acids were extracted from sample homogenates and virus suspension. Random cloning of the HindIII and PstI digested DNA from the lizard AdV isolates was performed using Phagemid pBluescript® II KS(+/-). For the RT-PCR targeting RNA of PMV, the first round single tube reverse-transcription-polymerase chain reaction was carried out (Fermentas, St Leon-Rot, Germany). All other PCRs targeting viral DNA or

copy DNA were performed as follows. Thermal protocols with an initial denaturation at 95°C for 5 min followed by 35-45 cycles at 95°C for 30 s, 45-60°C for 30-60 s, and 72°C for 1-4 min and a final extension at 72°C for 7 min were run. In the amplification cycles, the annealing temperatures for specific primers were set to T_m -4ºC, but not lower than the standard for the degenerated primers at 45ºC. Elongation times were set according to the expected product size, calculating 1 minute for 1 kb. For the AdV studies, six primers were previously described in the literature, whereas thirty-eight others were newly designed and synthesised. For IIV studies, fourteen primers targeting six different genes were taken from the literature. Further fifty-seven primers were newly designed. For PMV studies, ten primers targeting three genes were taken from the literature. Further twelve targeting HN- and U-genes were newly designed.

If direct sequencing of PCR products was unsuccessful or gave dubious results, then purified PCR amplicons were blunt-end cloned into pJET 1.2 vector (Fermentas) following the manufacturers recommendations. Gel purified PCR amplicons (Invisorb Spin DNA Extraction Kit; Invitek GmbH, Berlin, Germany) and kit purified plasmids (Plasmid Mini Kit; Qiagen) were sequenced using a BigDye Terminator Cycle Sequencing Kit v.1.1 applying the PCR primers, pJET-F & -R primers and T3 & T7 primers for the PCR amplicons, cloned amplicons and random cloned genome fragments, respectively.

Raw were sequences edited, assembled and compared using the STADEN Package version 2003.0. The sequences were identified by homology search programs run against GenBank. Multiple alignments of sequences were performed with ClustalW algorithm of the BioEdit Sequence Alignment Editor programme. The alignments were used for phylogenetic calculations in the PHYLIP program Package version 3.6. (Felsenstein, 1989) applying various methods (distance based & maximum likelihood) to obtain an optimal tree. Bayesian trees were constructed using the MrBayes on the TOPALi v2.5 platform. SimPlot and BootScan analyses of the sequenced IIV and PMV genes with their homologues retrieved from GenBank were performed using the SimPlot for Windows v.3.5.1 programme. Structural analysis of the predicted IIV exonuclease and the DNA polymerase proteins was done using the GENtle program v. 1.9.4. and SWISS-MODEL automated protein structure homology-modeling server (http://swissmodel.expasy.org).

Sequence of the major capsid protein (MCP) genes of IIV isolates (Table 1) were determined. This gene was found conserved enough for being target of diagnostic PCRs. The MCP gene sequences were applied in primer and probe design using the Primer Express Version 3.0 programme. Reactions were carried out in a StepOne™ Real-Time PCR System, using MicroAmp™ 48-well reaction plates and the StepOne™ Software version 2.0. Purified PCR products of the house cricket isolate (Ir.iso.7) and the clone of pJET plasmid inserted 1st round MCP products of a spiny tailed lizard (*Uromastyx* sp.) IIV isolate (Ir.iso.4) were used as references in different dilutions for the quantification.

Results

Adenovirus studies

We found AdVs in 25 animals (32%) representing 4 lizard and a snake species, and successfully isolated AdVs from individuals of two helodermatid species. Two different types of AdV could be detected and isolated from the two species: Gila monster and Mexican beaded lizards. Hereafter, these isolates will be referred to as lizard AdV type 1 (LAdV-1) and LAdV-2, respectively. Furthermore, five different variants of the same agamid AdV, a varanid AdV and snake AdV type 2 were detected by PCR and sequencing. The G+C contents of the DNA-polymerase gene fragments ranged between 42.5-58.3%. In phylogenetic analysis, each newly detected squamatid AdV sequence clustered to the genus *Atadenovirus.*

From the genome of LAdV-1 and LAdV-2, the sequence of three and two larger fragments could be determined. The added up size of the LAdV-1 sequence was 13.4 kb with G+C content of 43.3%, whereas that of LAdV-2 was 17.4 kb and G+C content of 43.8%. The partial genome of the two LAdVs showed high degree of similarity (81-99% and 77-99% for nucleotide and deduced protein sequence). Lengths of completely determined genes, as well as lengths of intergenic regions (IGR) were found to be identical in most cases. LAdVs seemed to have SnAdV-1 as the closest relative with the major difference being that both LAdV types possess a second fiber gene downstream of the surprisingly short, SnAdV-1 homologue first fiber gene.

Iridovirus studies

In the newly established qPCR, the sensitivity and efficiency, as well as intra- and inter-assay reproducibility values of the reaction were very good. A comparison of the sensitivity of the nested PCR and the real-time PCR with DNA preparations of serial dilutions of two lizard IIV isolates (Table 1, Ir.iso.3 & 5) showed that both assays were capable to detect the same low number of copies. Cell culture based virus titration was 10^2-10^3 less sensitive than the PCR methods.

We characterised IIVs isolated over the course of six years from crickets and insectivorous pets fed with crickets (Table 1) based on complete (8 gene) and partial gene (7) sequences. Identity values compared to corresponding genes of Chilo iridescent virus (CIV), type species of genus *Iridovirus*, are shown in Table 2. The isolates included in this study were all identical to cricket iridoviruses in the GenBank, based on the available partial MCP gene data. The studied isolates were found to be identical by using four different endonucleases and by comparing sequences of several genes. However, a very low inter-isolate variance (up to 0.4%) was detected in genes coding for DNA synthesis and degrading enzymes (DNA polymerase, exonuclease).

Table 2. Identity values (%) between Cr-LizIV isolates and CIV

Region:	Exonuc.	Polym.	ATPase	Viral Antibiotic Peptide and flanking genes					Ligase	Thym. synt.	Major Capsid Protein and flanking genes				I.E. prot.
ORF (CIV)	012L	037L	075L	155L	157L	159L	160L	161L	205L	225R	**orf011** (WIV)	274L	281R	282R	393L
DNA	91.2	97.0	97.8	97.4	95.4	**54.5***	**73.6**	99.3	95.8	95.7	**43.8***	94.5	84.9	98.1	97.8
PROTEIN	91.6	96.7	100	99.2	98.0	**46.0***	**39.6**	100	97.2	92.4	**NA**	95.5	78.8	97.6	96.3

*shows recombination spots

The results of the cricket bioassay did not show remarkable difference between the pathogenecity of the three different IIV isolates (Table 1). In the infected groups, apparents signs of infection were increased activity, swollen abdomen and moulting abnormalities. The so called “patently” infected crickets with very high virus loads and most often showing blue iridescence proved to have high virus copy numbers: 10^7 to 10^{10} according to qPCR, while the so-called covertly infected animals had considerably lower (10^2-10^4) copy numbers in their fat bodies. In selected patently infected crickets, the viral DNA could be detected by ISH as well.

None of the bearded dragons (except for A5) showed any signs of clinical disease during the course of the study. They continued eating normally and gaining weight during the entire 60-day period. Animal A5 started to loose weight (with 15% loss) during the second week and showed no appetite. It died emaciated on day 16 post infection (dpi) and was dissected. Gross pathology did not reveal remarkable alterations, except for a yellowish friable liver with glycogen depositing and lipidosis in the histopathology. The fourteen surviving animals were dissected at the end of the study. No changes were noted on gross pathology. A1 and A4 showed similar liver changes to those seen in A5.

No virus was detected in any of the negative control animals. In cases of the infected animals, however, virus isolation and PCRs were positive from several organs. No specific macroscopic or histological changes could be detected in any of these organs, and the other detection methods (ISH, IHC, EM) failed to detect the traces of replication of IIV in the lizard tissues unequivocally.

Paramyxovirus studies

In the case of the PMV isolate from the Hermann’s tortoise (THer-GER99), only the L gene PCR has given a specific amplicon. From every other (squamatid) isolate, all three targeted genes could be amplified and sequenced. Two lizard isolates (Xeno-USA99; Var-GER95) had identical L, HN and U gene sequences. The third lizard isolate (Igu-GER00) had an HN gene sequence identical to that of two snake isolates from this study (Pyth-GER01, Crot-GER03) and of three other isolates from previous studies. The phylogenetic analysis showed that all of our isolates were related to snake PMVs available in GenBank and formed together the lately established genus *Ferlavirus*. Within the genus, the Hermann’s tortoise isolate branched off as an ancient

sister to a monophyletic squamatid PMV cluster. This squamatid PMV cluster could be further subdivided into monophyletic groups A, B and C.

Diagnostic RT-PCR screening of snakes for ferlaviruses was positive for a total of 50 samples (24.6% of the 203 tested organs/swabs) from 28 (27.5%) different animals. PMV isolation was attempted with all samples, but was successful in three animals only (10.7% of PMV positive animals). Nested RT-PCR for the HN-gene was also carried out with 47 of the L gene-PCR positive diagnostic samples, but gave products of specific size in 16 (34%) cases only. Predominantly, CNS signs and pneumonia were the known in the anamnesis of these cases. Thirty-eight amplicons were sequenced, originating from 25 different individuals representing 11 different snake species. PMV was detected in swabs from twelve live animals (26.1%) out of 46 tested.

From the 38 selected amplicons, thirteen different partial L gene sequences ("sequence variants") were obtained, of which six clustered to "group A", and the rest "group B". Eight sequences were identical to earlier characterised isolates. In some cases, identical sequences were obtained from different animals in the same collection and from different organs of the same snake. However, in several cases, amplicons from different tissues of the same animal (or amplicons from different animals in the same collection) indicated that snakes can be infected with more than one PMV during an outbreak. The multiple infection was proven also by cloning PCR-amplicons in two cases, where the direct sequencing results resembled a mixed sequence pattern. In the case of the leopard tortoise (*Geochelone pardalis*) which died with severe respiratory distress, 12 different organs were tested. Squamatid ferlaviruses of at least two different types were amplified from the liver, heart, small intestine and cloacal samples, resembling 4 different sequence variants, and being rather divergent from the Hermann´s tortoise isolate.

Discussion

Adenovirus studies

We successfully detected AdV in altogether 25 squamatid reptiles. Five different AdV types were detected; supposedly all of them are being specific for the host. Two of these were novel ones, from a Mexican beaded lizard and an emerald monitor. Furthermore we found four novel agamid AdV variants. Each of the viruses had a more or less balanced G+C content (42.5-58.3%) in the sequenced region, which might indicate a long coevolutionary history with their hosts, and supports the hypothesis on the squamatid origin of the genus *Atadenovirus*.

Prior to the start of our studies, all reptilian AdV isolates originated from snakes. Previous attempts to isolate the agamid AdV, resulted in mixed cultures of AdV and reoviruses, and these letter ones "overgrew" the AdV upon passages. From swabs of two Gila monsters (LAdV-1) and from organs of a Mexican beaded lizard (LAdV-2),

here we report the first pure lizard AdV isolates ever. We made these isolates available to cooperating partners for further studies, including three dimensional structural imaging. We started the determination of genome sequences in Stuttgart in cooperation with collegues at the VMRI, HAS in Budapest.

The G+C content of LAdV-1 & LAdV-2 was lower in each of the examined genes than in the corresponding genes of SnAdV-1. For the concatenated fragments, LAdV-1 had a 43.3% and LAdV-2 had 43.8% G+C content, compared to 50.2% of SnAdV-1 genome. We also discovered the first cases when more than one fiber genes were present in an atadenovirus. This phenomenon has already been reported in three other genera: *Mastadenovirus*, *Aviadenovirus* and *Ichtadenovirus.* Analysis of the deduced protein sequences predicted that both fiber-1 and fiber-2 of LAdVs can be functional. In their tail regions, all three conserved motifs could be localised. The relatively lower G+C content and the presence of two different fiber genes in LAdVs, raise the possibility that these are not genuine helodermatid viruses, but come from another host species and are now in an adaptation process. This hypothesis needs further investigations.

Iridovirus studies

As IIVs are a significant problem for insect breeders and are considered as potential threat to pet lizards, it was important to elaborate sensitive and specific tools for the detection of these viruses in clinical samples from a wide range of animals. The qPCR method developed by us, is specific, and as sensitive as the previously described nested nPCR detecting the MCP gene, but requires less handling steps and provides more informative data faster.

The studied IIV isolates were found to be identical to one another by RFLP and by comparing six complete and six partial gene sequences. A very low inter-isolate variance (up to 0.4%), however, was detected in two other genes. These IIVs are therefore considered a single virus type (cricket iridovirus; GbIV). Similarity plot analysis showed recombination spots in two genes (Table 2) of GbIV compared to CIV. Alteration was found in GbIV in the secondary structure of one of the active centres (DEDDY domain) of the DNA polymerase gene compared to CIV. Thus, based on the considerable genomic differences described in here, we suggest that GbIV should be classified as a separate type of group II invertebrate iridoviruses. The limited number of point mutations and the observed insertions/deletions, recombination spots in the analysed genes are compatible with the hypothesis that the cricket-lizard iridovirus in question evolved relatively recently from CIV.

In the cricket bioassays, our aim was to compare the infectious characteristics of three different IIV isolates originating from three distinct hosts (Table 1). The experiments were performed at two different regulated temperatures (20 & 30ºC). Due to technical reasons, not all the planned experiments could be perfectly accomplished in the planned two repetitions. Nevertheless the correlations and differences revealed in this study are interesting. The data indicate that there might be differences in the

pathogenecity of the different isolates (lower for the lizard isolate), yet the repeated infection studies with the same isolate under the same conditions also varied significantly, so the observed differences between isolates could be due to interassay variability rather than true differences between the isolates.

Koch's postulates could not be sufficiently fulfilled in the bearded dragon infection trial. None of the bearded dragons showed any signs of clinical disease during the course of the study. Virus was detectable by isolation and PCRs from the oral and cloacal swab of the infected animals from the 1st week on (after feeding infected crickets), while the negative control lizards remained negative in these tests. The early death of lizard "A5", the smallest animal included in the study, could not be connected to infection by iridovirus. The yellowish friable liver, as sole pathological finding, resulted from glycogen depositing and lipidosis, might indicate hepato-toxic effect of the viral proteins, as it had been recorder in mice injected with CIV. The successful re-isolation of virus from every tissue and the positive nPCR results suggested viremia which was only partly supported by the repeated qPCR results with the later established, re-evaluated controls. In the ISH, IHC tests, the viral DNA and proteins could not be detected. In the gastrointestinal tract, the large intestine (colon) showed the highest virus load by both virus isolation and qPCR, which is logical if we consider possible remnants of the infected food-crickets, despite the 2 weeks of vegetable diet at the end of the study. Relatively high virus loads were detected from the proximal portions of the digestive tract of some survivors. Other organs (lung, heart, liver, skin) also had higher virus loads (by qPCR), but in a 3 to 6 fold lower range compared to the GI system. No macroscopic or histological changes, indicative of virus replication, could be detected in any of these organs, and the other detection methods (ISH, EM) were also unable to detect IIVs in the tissues. The elaborated IHC test seemed to give aspecific signals, thus its results were omitted during the analysis.

Paramyxovirus studies

Up to now, occurrence of PMVs has been described in the snake (sub)families Boidae, Colubrinae, Elapidae and Viperidae. Our study included the first field isolate characterised from a homalopsid snake (subfamily of Colubridae). This study was also the first to partiallly characterise PMV isolates from varanid, xenosaurid and iguanid lizards, and from a chelonian host. All sequence information obtained in the study indicated that PMVs of snakes and lizards were closely related, while the tortoise isolate (THer-GER99) differed from the others, although part of a monophyletic cluster with the squamatid isolates. This cluster could be identified as the genus *Ferlavirus* within the family *Paramyxovirinae*.

Based on our findings, the term "ophidian PMV" is inappropriate for the denomination of these viruses as similar types appear in lizards and tortoises as well. The name "ferlavirus" is preferred to be used as a collective name for the candidate members of this genus. Our proposed new grouping within the genus considered the seniority rule in nomenclature, and the high bootstrap monophyletic clusters of the L,

HN and U gene trees. The single tortoise isolate, being apparently the most ancient branching of ferlaviruses, is regarded as the first representative of a supposed chelonid group separate of all other squamatid ferlaviruses, that form three groups: A, B and C. These groups might serve as basis for establishing the species demarcation criteria within this new genus.

Based on the above observations with the PMV isolates, we set up a more comprehensive survey with snake samples arriving to our laboratory in Stuttgart. Virus isolation and nested RT-PCR targeting the L gene were used as a primary selective tool, and an additional RT-PCR to detect the HN gene was performed with the positive samples. This was the first study to characterise PMVs directly from clinical samples of snakes. We found evidence for concurrent infection with several strains at the same time within populations and even within animals. Using the sequence data, suspected transmissions could be traced.

Considering the best tissue for the PMV diagnostics (to give hints for practitioners for sampling), our data support that the lung, intestine and kidney “triad” is the best choice from dead specimens. These were simultaneously examined 11 and 19 times among the PMV positive and negative animals, respectively. There was not a single case in which these tissues were all negative and another organ was positive in the L-gene RT-PCR. From live snakes, swabs (and trachea washes) were positive in 12 of the 46 examined cases (26.1%). This prevalence in swabs, similar to that detected in tissue samples, demonstrates sensitivity of this nested RT-PCR method.

In a separarate study, a surprising finding was the presence of multiple squamatid ferlaviruses in a leopard tortoise with severe pneumonia. This case supported our finding multiple PMVs in snakes and also contributed to the hypothesis that a switch from a squamatid to a chelonian host can happen. Although the clinical impact of the PMV on the leopard tortoise is not clear based on our results, this finding underlines the apparent lack of strict species specificity of the squamatid ferlaviruses that appear to be able to infect a wide range of reptilian hosts.

New scientific results

1. Describing two novel squamatid adenoviruses, altogether five reptilian AdV types, that all proved to be members of genus *Atadenovirus*; supporting the theory of the coevolution and squamate origin of atadenoviruses.

2. Isolating the first pure lizard AdVs in culture, and determining their partial genome sequence; revealing the existence of two fiber genes in atadenoviruses for the very first time.

3. Establishing new methods (qPCR, ISH) for the detection of invertebrate iridoviruses (IIVs) in lizards and prey insects, and applying these methods in transmission trials.

4. Comparison of partial genom segments (14 kb) from 15 genes of IIV isolates from lizards, a scorpion and a cricket; finding mutations, insertions/deletions, and two spots with evidence for recombination compared to the type species CIV; proving a need for taxonomic revision and showing widespread presence of stable variants circulating in Germany.

5. With a transmission study, demonstrating that even the administration of high loads of IIV does not cause the clinical signs in bearded dragons, previously attributed to these viruses.

6. Molecular characterisation of novel paramyxovirus isolates from snakes, lizards, and (the first time) a tortoise; supporting their classification as members of the new genus *Ferlavirus*; the tortoise ferlavirus being the most ancient branch, while the other squamatid isolates separating into a new and two redefined groups. The genus characteristic U gene was undetectable in the tortoise isolate.

7. Describing fifteen ferlavirus sequence variants with surprisingly high (27.5%) prevalence, and (the first time) revealing concurrent infection with more than one PMV types in snake specimens and/or populations and in a leopard tortoise.

Publications

Related articles

Weinmann, N., Papp, T., Alves de Matos, A.P., Teifke, J.P., Marschang, R.E.: **Experimental infection of crickets (*Gryllus bimaculatus*) with an invertebrate iridovirus isolated from a high-casqued chameleon (*Chamaeleo hoehnelii*),** *J. Vet. Diag. Invest.* 19, 674-679. 2007.

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1. Acknowledgements

First and most, I would like to express my gratitude to my supervisor in Stuttgart, *PD Dr. Rachel Marschang*. She has not only given me interesting topics and provided a great deal of professional and financial help, but also supported my private life difficulties in a foreign country. The friendly atmosphere that she has established in her laboratory with excellent leader's qualities transmitted her affection for the reptile virology to all of us. Her husband, *Dietmar Marschang* should also be thanked for a lot of technical help in constructing the animal housing facilities and for the friendly conversations.

With equally strong gratitude I would like to thank *Prof. Dr. Balázs Harrach*, my supervisor in Budapest. He and his wife, *Prof. Dr. Mária Benkő* were the ones helping me at the first steps in virology during my diploma work, then encouraging me for my leave to Germany and finally warmly welcoming me on my return. They were regarded as role models for both their professional expertise and their humanity.

In Stuttgart, I would like to thank also my consultant Prof. Dr. Arthur Pfitzner (Institute for Genetics) for the openness, the advises and for his contribution. In the virology lab of our Institute (Environmental- and Animal Hygiene), the excellent and vivacious assistance and the "German language course" by *Christa Schäfer* and *Silvia Speck* are warmly remembered. Other colleagues of the lab (*Nina Apfelbacher, Renate Haumacher, Lorenza F. Poschetto, Reinhild Rösler, Anke Stöhr, Petra Stumpf, Martin Buhl*, *Anthony Ike,* and *Jawad Nazir,* among others) are also thanked for their help, the offered cooperations, and mostly for being there. *Inna Ball [Romanova]*, *Maha D. Abbas*, *Nadine Weinmann* and *Dirk Spann* deserve special thanks for contributing minor parts to the work presented here, as cited at the appropriate spots. Their help is greatly acknowledged and will be rewarded. Two further institutes of the Hohenheim University: Institute of Anatomy and Physiology and Institute of Animal Husbandry and Breeding provided me facilities for tissue embedding and cutting. All vets sending us diagnostic samples are also acknowledged. Members of the *Stumpf family* are also thanked for their support.

I have performed histology, ISH and IHC tests in the FLI, Insel Riems, Germany with the kind help of *Gabrielle Czerwinski* and the supervision of *Prof. Dr. Jens Teifke*. The pleasing month spent there will long be remembered. EM examinations were performed by *Valerij Akimkin* at CVUA, Stuttgart and *Prof. Dr. A.P. Alves de Matos,* Curry Cabral Hospital, Lisbon, Portugal.

I also want to thank my colleagues at the Institute for Veterinary Medical Research in Budapest for the good homely atmosphere. Four of them*: Dr. Szilvia L. Farkas, Judit Pénzes*, *Dr. Andor Doszpoly* and *Dr. Győző Kaján* contributed to the projects presented in this dissertation and are cited accordingly. Repeated EM and IHC was kindly offered by *Renáta Pop* in Pathology Department of the Faculty of Veterinary Science, Budapest in courtesy of *Dr. János Gál.*

Lot of friends should be thanked from Stuttgart and here in Hungary, but the place is limited... Let me name just one now: Thanks, *Sándor*.

Our beloved high school biology teacher *István Sipos*, "Sipos Tanár Úr" at Sárospatak cannot be forgotten. His devotion to his subjects as well as to many other disciplines poured the enthusiasm into his pupils and first showed us the joy and beauty of science.

At last, but not at least I would like to acknowledge my *family*, especially my *parents* for their endless sacrifices, their support and love along these long years, that enabled me to achieve my goals.

Financial supports were granted by the German and Hungarian Scientific Research Funds, DFG MA 2534/2-5 and OTKA-K72484 as well as by the Morris Animal foundation: MAF- D09ZO-023.