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From the Clinical Virology[1] and Clinic for Diagnostic Imaging[3] (Clinical Department for Diagnostic Imaging, Infectious Diseases and Clinical Pathology) and the Clinic for Avian, Reptile and Fish Medicine[2] (Department for Farm Animals and Herd Management), University of Veterinary Medicine Vienna

First report of an iridovirus (Genus *Ranavirus*) infection in a Leopard tortoise (*Geochelone pardalis pardalis*)[1]

V. BENETKA[1], E. GRABENSTEINER[2], M. GUMPENBERGER[3], C. NEUBAUER[2], B. HIRSCHMÜLLER[1] and K. MÖSTL[1]

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Summary

An adult Leopard tortoise (*Geochelone pardalis pardalis*) with severe clinical disease characterised by nasal discharge, severe necrotising stomatitis, dehydration, apathy and anorexia was presented at the Clinic for Avian, Reptile and Fish Medicine of the University of Veterinary Medicine in Vienna. Shortly before the onset of clinical symptoms the animal had been imported from Ethiopia. Specific PCRs for iridovirus and Chelonid herpesvirus (ChHV) gave positive results with pharyngeal swabs and necrotic layers of the oral cavity. The positive result could be confirmed by sequencing for iridovirus specific nucleic acids, the band achieved for ChHV was too weak for sequencing. Additionally various bacterial infections (*Staphylococcus* spp., *Citrobacter freundii, E. coli*, *Salmonella* spp., coagulase negative *Staphylococcus* spp.) and mycosis (*Candida* spp.) were found, probably all secondary due to the virus induced epithelial damage. Sequence analysis of the detected iridovirus revealed nucleotide identities of 98 to >99 % with various amphibian and reptilian isolates of the FV-3 group. After one month of intensive treatment the animal was released and recovered fully after 4 months. Our results show that an iridovirus infection has to be considered as possible coagent or differential diagnosis to ChHV. This is the first report of iridovirus infection in the species *Geochelone pardalis pardalis*.

Schlüsselwörter: Schildkröte, Reptil, Pantherschildkröte, *Iridoviridae*, *Ranavirus*, Herpesvirus, *Geochelone pardalis pardalis*, CT, PCR.

Zusammenfassung

Erstbeschreibung einer Iridovirusinfektion (Genus *Ranavirus*) bei einer Pantherschildkröte (*Geochelone pardalis pardalis*)

Eine erwachsene Pantherschildkröte (*Geochelone pardalis pardalis*) wurde mit hochgradigen klinischen Symptomen bestehend aus Nasenausfluss, hochgradiger nekrotisierender Stomatitis, Dehydrierung, Apathie und Anorexie an der Klinik für Geflügel, Ziervögel, Reptilien und Fische der Veterinärmedizinischen Universität Wien vorgestellt. Kurz vor Auftreten der Symptome war das Tier aus Äthiopien importiert worden. Spezifische PCRs auf Iridovirus und Chelonid Herpesvirus (ChHV) ergaben an Pharynxtupfern und nekrotischem Material aus der Maulhöhle jeweils ein positives Ergebnis, welches für Iridovirus-spezifische Nukleinsäuren durch Sequenzierung bestätigt werden konnte, während die für ChHV erhaltene Bande für die Sequenzierung zu schwach war. Zusätzlich konnten verschiedene bakterielle Infektionen (*Staphylococcus* spp., *Citrobacter freundii, E. coli*, *Salmonella* spp., Koagulase-negativer *Staphylococcus* spp.) sowie eine Mykose (*Candida* spp.) nachgewiesen werden, welche wahrscheinlich als Sekundärinfektionen zu betrachten sind. Die nachgewiesenen Iridovirussequenzen wiesen Homologien von 98 bis zu >99 % mit verschiedenen Amphibien- und Reptilien-Isolaten aus der FV-3-Gruppe auf. Nach einer intensiven Therapie über 1 Monat wurde das Tier entlassen und erholte sich vollständig nach 4 Monaten. Die Ergebnisse zeigen, dass Iridovirusinfektionen als mögliche Kofaktoren oder Differentialdiagnosen zu ChHV-Infektionen in Betracht gezogen werden müssen. Dies ist die erste Beschreibung einer Iridovirusinfektion bei der Spezies *Geochelone pardalis pardalis*.

Abbreviations: ATV = Ambystoma tigrinum virus; BIV = Bohle iridovirus; CFV = Catfish virus; ChHV = Chelonid herpesvirus; CT = computed tomography; DEPC = Diethylpyrocarbonate; EHNV = Epizootic haematopoietic necrosis virus; FV = *Frog virus*; GV = Gutapo virus; LT = Lucké triturus virus; MCP = major capsid protein; PCR = polymerase chain reaction; SFV = Sheatfish virus; TEV = Tadpole edema virus; TV = turtle / tortoise virus; WV = Wamena virus

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Introduction

Members of the family *Iridoviridae* are icosahedral virions containing a double stranded linear DNA genome of 140-303 kb in length. The viral genome codes for up to 30 different proteins depending on the genus (FAUQUET et al., 2005; ICTVdB Management, 2006) of which the major capsid protein (MCP) is the most abundant in the viral particle (40-45 %). The MCP gene is highly conserved but distinct enough to identify different iridovirus genera (MAO et al., 1997; TIDONA et al., 1998). The virions can be enveloped by the host cell membrane, which is not necessary for infectivity.

Among the 5 genera of the family, 2 comprise insect viruses (Genera *Iridovirus* and *Chloriridovirus*), the other 3, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* infect poikilothermic vertebrates. Several fish, reptile and amphibian iridovirus isolates have been assigned to the genus *Ranavirus* with the type species *Frog Virus 3* (FV-3). Exemplary members of the genus are the reptile Box turtle virus 3 (TV3) and Tortoise virus 5 (TV5; MAO et al., 1997), the amphibian Bohle iridovirus (BIV), Lucké triturus virus 1 (LT-1; ESSANI and GRANOFF, 1989), Ambystoma tigrinum virus (ATV, salamander; JANCOVICH et al., 2003) and Tadpole edema virus (TEV, salamander; DOCHERTY et al., 2003), Epizootic haematopoietic necrosis virus of fish (EHNV), Catfish virus (CFV; POZET et al., 1992) and Sheatfish virus (SFV; AHNE et al., 1989). Numerous further closely related isolates have been described: Gutapo virus (GV) (amphibian), Terrapene carolina ranavirus (turtle), Testudo horsfieldii ranavirus (tortoise), Testudo hermanni ranavirus (tortoise), Wamena Virus (WV) (green python) (HELDSTAB and BESTETTI, 1982; MAO et al., 1997; MARSCHANG et al., 1999; ESSBAUER and AHNE, 2001; HYATT et al., 2002; DE VOE et al., 2004) and a gecko (*Uroplatus fimbriatus*) iridovirus (MARSCHANG et al., 2005).

In the MCP gene, iridovirus strains and isolates of fish, amphibians and reptiles are very closely related. Nucleotide identities among these viruses in parts of the MCP gene vary between 97 and 100 % (HYATT et al., 2000; MARSH et al., 2002).

A variety of clinical symptoms have been associated with iridovirus infections in chelonids. HELDSTAB and BESTETTI (1982) first described iridovirus-like particles in a Mediterranean land tortoise (*Testudo hermanni*) affected with hepatitis, splenitis and enterocolitis. WESTHOUSE et al. (1996) described respiratory disease in a free living gopher tortoise (*Gopherus polyphemus*). Upon necropsy the animal showed necrotising ulcerative pharyngitis, oesophagitis, tracheitis and pneumonia. Transmission electron microscopy revealed iridovirus-like virions. MARSCHANG et al. (1999) isolated and characterised by restriction enzyme and sequence analysis (MCP) an iridovirus isolated from an outbreak of fatal disease in a group of 7 Hermann´s tortoises. Pathological examination revealed in all animals similar alterations: hyperaemic oral, lingual, pharyngeal, oesophageal, intestinal and cloacal mucosa with disseminated ulcerations covered by thick yellow plaques of necrotic material. Sequence analysis showed that the virus isolated was very closely related (97 % homology) to FV-3. In the same year, CHEN et al. (1999) described a virus showing the typical appearance of the family *Iridoviridae* by electron microscopy isolated fromsoft-shelled turtles (*Trionyx sinensis*) with ”red neck disease”. The most recent report (DE VOE et al., 2004) described fatal course in 5 of 7 diseased captive eastern box turtles (*Terrapene carolina carolina*) with respiratory symptoms, oral ulceration and cutaneous and oral abscessation. The virus isolated from 2 fatal cases was sequenced (MCP) and assigned to the genus *Ranavirus*. Currently, JOHNSON et al. (2006) performed transmission experiments with an iridovirus of the genus *Ranavirus* in box turtles and red-eared sliders (*Trachemy scripta elegans*). Pathomorphological investigation of the experimentally infected turtles showed changes consistent with those seen in naturally infected cases including multicentric necrotising vasculitis with fibrin thrombi, splenitis and glomerulonephritis, and sinusoidal thrombi in the liver with multifocal necrosis.

Clinical symptoms similar to iridovirus infection can be caused by the widely distributed Chelonid herpesvirus (ChHV). In Mediterranean land tortoises herpesvirus infection is related to an upper respiratory tract disease referred to as ”mouth rot” characterised by stomatitis and rhinitis. In mild cases the animals show clear nasal discharge, palpebral edema and conjunctivitis. Severely affected tortoises suffer from diphtheroid-necrotising stomatitis, glossitis, mucopurulent rhinitis and hepatitis and may also show signs of enteritis (HARPER et al., 1982; JACOBSON et al., 1986; COOPER et al., 1988; BRAUNE et al., 1989; LANGE et al., 1989; MUELLER et al., 1990; PETTAN-BREWER et al., 1996; MARSCHANG et al., 1997; DRURY et al., 1998, 1999; KUEBBER-HEISS et al., 1999; UNE et al., 1999; TEIFKE et al., 2000; ORIGGI et al., 2004).

We describe a case of a double infection with iridovirus and Chelonid herpesvirus in a *Geochelone pardalis pardalis* imported from Ethiopia to Austria.

Materials and methods

Clinical history

An adult female Leopard tortoise (*Geochelone pardalis pardalis*) of unknown age, weighing 62 kg, was presented by its owner at the Clinic for Avian, Reptile and Fish Medicine of the University of Veterinary Medicine, Vienna, Austria, because of apathy, anorexia and respiratory signs. The animal had been imported from Ethiopia about 3 weeks before and kept separately from 3 other Leopard tortoises in the same household. The owner reported the onset of clinical symptoms shortly after arrival. Clinically a high grade serous rhinitis and ptyalism as well as respiratory sounds were observed (fig. 1). Furthermore the animal was dehydrated. A severe stomatitis of the whole oral cavity was diagnosed (GRABENSTEINER et al., 2006). Pharyngeal swabs and necrotic material in the oral cavity were collected for virological and bacteriological examination. In order to determine possible affection of the lung, computed tomography was performed. The animal was treated symptomatically (antibiotics and liquid substitution) as described by GRABENSTEINER et al. (2006) over a period of one month with only moderate improvement of the clinical status. Another 4 months later the animal had, according to its owner, fully recovered.

Fig. 1: *Geochelone pardalis pardalis* presented at the Clinic for Avian, Reptile and Fish Medicine

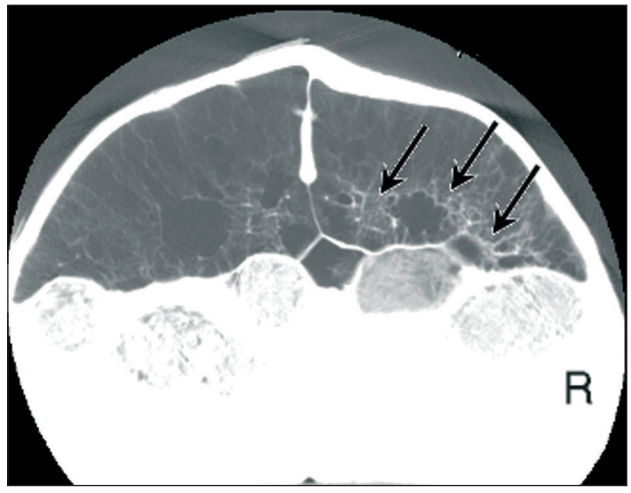

Fig. 2: Transversal CT image of the caudal part of the lung of the *Geochelone pardalis pardalis*; a larger area of hyperdense reticular interstitial lung tissue (arrows) is visible in the caudoventral part of the right lung.

Bacteriological investigation

Oral, nasal and cloacal swabs were analysed for the presence of bacteria and fungi at the Clinic for Avian, Reptile and Fish Medicine. The swabs were directly streaked out on Columbia agar supplemented with 5 % sheep blood (BioMérieux, Vienna, Austria), Chromocult® Coliform Agar with Coliform Selective-Supplement (Merck, Vienna, Austria), SM2 agar (BioMérieux, Vienna, Austria) and Sabouraud Gentamicin Chloramphenicol agar (BioMérieux, Vienna, Austria). Except the Sabouraud Gentamicin Chlorampenicol agar, all agar plates were incubated at 37 °C aerobically for 24 h. The Sabouraud Gentamicin Chloramphenicol agar plates were incubated at 28 °C aerobically for 48 h.

Parasitological investigation

Faecal samples were examined parasitologically using the flotation technique as described by BOCH and SUPPERER (1992). Briefly, 5 g of faeces were mixed thoroughly with 10 volumes of zinc sulfate solution. The suspension was then filtered (mesh size 500-800 µm) collecting the flow through in a centrifuge tube. Afterwards the solution was centrifuged for 3 minutes at 300 x g allowing worm eggs, oocysts and protozoal cysts to float to the top. 3 to 5 drops were subsequently removed from the top to a microscope slide using a wire loop and examined under the light microscope (100x and 400x magnification).

Virological investigation

Extraction of viral DNA

Swabs were stored in DEPC treated water at -80 °C. Necrotic material was incubated in 180 µl Buffer ATL (Qiagen, Hilden, Deutschland) and 20 µl proteinase K (Promega, Mannheim, Deutschland) at 37 °C until complete lysis. A volume of 140 µl of the supernatant served as sample for DNA extraction using a commercially available kit (Qiagen, QIAamp®Viral RNA Kit). Extracts (60 µl) were stored at -20 °C.

ChHV and FV-3-specific PCRs

For the detection of ChHV specific DNA a nested PCR was employed. This PCR had shown the highest sensitivity when compared to other ChHV specific PCRs by VAN DEVANTER et al. (1996), MURAKAMI et al. (2001) and ORIGGI et al. (2004). The sequences of the primers used in the first assay were the following: Forward 5'-CCGTAAAGCCATTCGACGTA-3, Reverse 5'-TCGTTCCATCCGGTGATAGA-3' and for the second round of amplification: Forward 5'-CAGCTTGCCATCAAAGTAAC-3', Reverse 5'-AAATACCGAATCCGTGTCTC-3' amplifying 258 bp of the DNA polymerase gene. The assays were carried out in a volume of 25 µl (22.5 µl master mix and 2.5 µl of DNA extract). The PCR mixture contained 10 mM Tris-HCl (pH 9 at 25 °C), 50 mM KCl, 0.1 % Triton X-100, 200 µM of 2'-desoxynucleotide 5'-triphosphates each, 0.5 µl DMSO, 1.5 mM $MgCl_2$, 0.63 U Taq polymerase und 0.4 µM of the primers each.

For iridovirus specific PCR primers published by MAO et al. (1997) and modified by MARSCHANG et al. (1999) were used to amplify a segment of the MCP gene (500 bp) following the protocol by the latter. Primer sequences were the following: sense primer: 5'-GACTTGGCCACTTATGAC-3', antisense primer: 5'-GTCTCTGGAGAAGAAGAAT-3'.

To avoid contaminations in particular during ChHV nested PCR the template for the second round was added to the PCR mixture in an air flow unit of a separate laboratory used only for this purpose. For all PCR procedures, the following measures were taken to minimize the risk of contaminations: sample preparation, nucleic acid extraction and preparation of PCR mixtures were all carried out in separate laboratories, the latter in an air flow unit. All components of these procedures including pipets, surfaces and centrifuges were treated with disinfectants and/or UV-light before use. Negative controls of RNAse free water were carried along with the diagnostic samples through all procedures. For iridovirus specific PCR no positive control was available, for ChHV PCR the use of a positive control was dismissed due to the high contamination risk during nested PCR.

Extraction of amplified DNA, sequencing PCR and sequence analysis

Amplified DNA was extracted from the PCR product using a commercially available kit (NucleoSpin®Extract, Machery-Nagel, Düren, Germany) following the instructions in the users manual. DNA extracts served as template for sequencing PCR.

The latter was carried out in a volume of 20 µl with a ready to use sequencing PCR mixture (DNA Sequencing Kit, Applied Biosystems, Rotkreuz, Switzerland) and a primer concentration of 0.2 µM each.

The forward and reverse sequences were determined. Sequencing PCR products were analysed with the ABI Prism 310 Genetic Analyser (Applied Biosystems). The obtained sequences were aligned with each other and sequences available in the GenBank database using the program Align Plus 4 for Windows 95 (Scientific & Educational Software, Cary, NC, USA).

Computed Tomography (CT) examination

The patient was fixed with belts in ventral recumbency on a first aid bed without sedation. Subsequently axial CT examinations (CT HiSpeed 3rd generation, single slice, General Electric, Milwaukee, US) with 3 mm and 7 mm thick consecutive slices were performed. The images were evaluated in bone, lung and soft tissue windows.

Results

Bacteriological investigation

Staphylococcus aureus and *Citrobacter freundii* were isolated from the nose and the oral cavity, respectively. *E. coli*, *Salmonella* spp., coagulase negative *Staphylococcus* spp. and *Candida albicans* were isolated from the cloaca. The bacteria found were sensitive to Marbofloxacin, which was used for treatment at a dosage of 10 mg/kg s.c. per day for 3 weeks. For the treatment of the infection with *Candida albicans* Nystatin would have been the drug of choice which has to be medicated orally. Since it was not possible at all to open the mouth of the tortoise without sedation, the mycotic infection was left untreated.

Parasitological investigation

A low number of eggs of the nematode *Oxyuris* spp. was detected in faecal samples.

Virological investigation
ChHV and FV-3-specific PCRs

ChHV specific PCR revealed only in the second nested round very low amounts of amplified DNA with only a faint band of the estimated length visible after ethidium bromide staining whereas FV-3 specific PCR resulted in a high yield of amplified DNA.

Sequence analysis

The sequence obtained after amplification with FV-3 specific primers was submitted to a blast search in the GenBank database. The search of 513 bp of the obtained sequence revealed the highest nucleotide identity of 99.6 % with an amphibian (*Rana grylio*) iridovirus isolate (iridovirus RGV-9806, accession number AF192509) from China and of 99.4 % with FV-3 (complete genome, accession number AY548484). Similar high identities (≥98 %) were found with various amphibian and reptile isolates (U82553, Terrapene Carolina ranavirus; AY187046, Bohle iridovirus; AF389451, Rana tigrana iridovirus; AF114154, Testudo hermanni ranavirus; U82554, Testudo horsfieldii ranavirus), the virus clustering clearly in the FV-3 group.

The very faint band obtained after ChHV-PCR was not suitable for sequence analysis.

CT examination

A larger area of hyperdense reticular interstitial lung tissue was found in the caudoventral part of the right lung (fig. 2). A second almost triangular area within the cranial left lung between the stomach and carapace appeared much denser but smaller. No fluid accumulation was visible in the entire lung. The CT diagnosis was multifocal interstitial non exudative pneumonia with the possible differential diagnosis of remnants of an old pneumonia.

Discussion

We report an iridovirus infection in an adult Leopard tortoise *(Geochelone pardalis pardalis)* imported from Ethiopia. Additionally a very faint band was detected for ChHV specific nucleic acid. Whereas the specificity of the iridovirus PCR result could be confirmed by sequencing, this was not possible for the ChHV PCR result, as the band was too weak. However, as both PCRs were performed without using positive controls and severe measures avoiding contaminations were performed, a contamination seems very unlikely. The estimated position of the ChHV band supports the assumption of a double infection.

The tortoise displayed severe clinical symptoms characterised by necrotising stomatitis and severe seromucous rhinitis. These symptoms are in accordance as described for iridovirus infections. ChHV infection causes a very similar clinical image called “mouth rot” characterised by diphtheroid-necrotising stomatitis, glossitis and mucopurulent rhinitis (HARPER et al., 1982; JACOBSON et al., 1986; COOPER et al., 1988; BRAUNE et al., 1989; LANGE et al., 1989; MUELLER et al., 1990; PETTAN-BREWER et al., 1996; MARSCHANG et al., 1997; DRURY et al., 1998, 1999; KUEBBER-HEISS et al., 1999; UNE et al., 1999; TEIFKE et al., 2000; ORIGGI et al., 2004). It is therefore impossible to determine the causative involvement of the 2 agents or which infection triggered the other. It is likely that the double infection worsened the course of the disease.

The ChHV specific PCR used had been compared to various PCRs available in literature (VAN DEVANTER et al., 1996; MURAKAMI et al., 2001; ORIGGI et al., 2004) and had shown a ten up to 10^4 times higher sensitivity (unpublished results).

Sequence analysis of the highly conserved MCP revealed the highest nucleotide identities of the Austrian iridovirus with an amphibian isolate from China (99.6 %) and with FV-3 (99.4 %). In this part of the genome it clearly clusters with FV-3 and related amphibian and reptile isolates.

Several additional infectious agents, bacteria and fungi, respectively, were detectable in the upper oral and respiratory tract. Since the *Geochelone pardalis pardalis* had been pretreated with antibiotics by a local veterinarian before examination and sampling at the clinic, these results may not reflect the actual situation of the bacteria originally present in the animal. These infections were probably secondary,

due to viral induced epithelial damage.

Signs of focal interstitial pneumonia were found in CT, but were assumed to be an unlikely cause of the severe nasal discharge.

The seroprevalence of ChHV was estimated with approximately 10 % in captive tortoises in France (MATHES, 2003). There are no epidemiological data available for iridoviruses. Iridovirus infections are described world wide as probable agents of amphibian population declines and mass deaths (CUNNINGHAM et al., 1996; DOCHERTY et al., 2003; JANCOVICH et al., 2003) and in systemic fish diseases with high morbidity and mortality (HEDRICK et al., 1992; HETRICK and HEDRICK, 1993; MAO et al., 1997). In turtles and tortoises only sporadic outbreaks and single cases are reported from different countries: Switzerland (HELDSTAB and BESTETTI, 1982; MARSCHANG et al., 1999), USA (WESTHOUSE et al., 1996; DE VOE et al., 2004) and China (CHEN et al., 1999). The species affected were *Testudo hermanni*, *Gopherus polyphemus*, *Terrapene carolina carolina*, *Trionyx sinensis* and *Gopherus polyphemus*. Recently, JOHNSON et al. (2006) performed infection experiments with box tortoises and red-eared sliders reproducing clinical signs and histological changes consistent with those seen in naturally infected tortoises and turtles. However, to our knowledge, this is the first report in the species *Geochelone pardalis pardalis*.

The source of infection in the present case could not be identified. As iridovirus infections have been reported from Switzerland, it is possible that iridoviruses of the genus *Ranavirus* circulate in reptile, amphibian and fish populations in Austria. It seems nevertheless more likely that the animal had acquired the infection in its original environment or from contacts during transport and due to the stress of capture, transport and settlement in a new environment developed clinical disease.

Surprisingly, the tortoise had, although severely diseased, overcome clinical disease after approximately 4 months.

As a conclusion, this case demonstrates that iridovirus infection must be considered as a possible differential diagnosis to Chelonid herpesviruses in particular in animals imported from eventually endemically infected areas.

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Authors' address:
Dr. Viviane Benetka, Dr. Elvira Grabensteiner, Dr. Michaela Gumpenberger, Dr. Claudia Neubauer, Mag. Birgit Hirschmüller, Univ. Prof. Dr. Karin Möstl, Veterinärplatz 1, A-1210 Wien.
e-Mail: viviane.benetka@vu-wien.ac.at

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