A novel alphaherpesvirus associated with fatal diseases in banded Penguins

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Abstract

A novel avian alphaherpesvirus, preliminarily designated sphenicid alphaherpesvirus 1 (SpAHV-1), has been independently isolated from juvenile Humboldt and African penguins (Spheniscus humboldti and Spheniscus demersus) kept in German zoos suffering from diphtheroid oropharyngitis/laryngotracheitis and necrotizing enteritis (collectively designated as penguin-diphtheria-like disease). High-throughput sequencing was used to determine the complete genome sequences of the first two SpAHV-1 isolates. SpAHV-1 comprises a class D genome with a length of about 164 kbp, a G+C content of 45.6 mol% and encodes 86 predicted ORFs. Taxonomic association of SpAHV-1 to the genus Mardivirus was supported by gene content clustering and phylogenetic analysis of herpesvirus core genes. The presented results imply that SpAHV-1 could be the primary causative agent of penguin-diphtheria-like fatal diseases in banded penguins. These results may serve as a basis for the development of diagnostic tools in order to investigate similar cases of penguin diphtheria in wild and captive penguins.

Penguin diphtheria, a disease of wild and captive juvenile penguins, is characterized by diphtheroid stomatitis [1–3] and necrotizing gastroenteritis [4] and causes high mortality. Although it was frequently discussed that the disease may be of primarily viral cause, this was never proven [5]. Here we describe a novel alphaherpesvirus that appears to be associated with cases of penguin diphtheria in banded penguins. The virus was isolated independently from captive African and Humboldt penguins (Spheniscus demersus and Spheniscus humboldti) suffering from diphtheroid-necrotizing oropharyngitis/laryngotracheitis and enteritis, respectively.

In June 2012, three 5- to 6-week-old female Humboldt penguin chicks (cases A1–A3 in Table S1, available in the online Supplementary Material) from the Tierpark Cottbus (Cottbus, Germany) died during hand rearing. The birds showed lack of weight gain, and shortly before death, the zoo technician noted diarrhoea. At necropsy, the three animals were emaciated, anaemic and dehydrated. The main finding in all cases was an acute necrotizing and haemorrhagic jejunitis. Histopathologically, the jejunal epithelium showed multiple coalescing foci of necrosis and haemorrhages. Significant lesions were absent from the other inner organs except for a mild myotic airsacculitis (Aspergillus fumigatus) in two of the chicks.

In December 2013, a 4-week-old African penguin chick from the Berlin Zoological Garden (Berlin, Germany) was found dead in the breeding burrow (case B1). The zookeepers did not note preceding clinical symptoms. At necropsy, the chick was in a good body condition but dehydrated. The main finding was a severe diffuse diphtheroid and necrotizing oropharyngitis and laryngotracheitis. Histopathologically, the stratified squamous epithelium of the oral cavity, the epithelium of the submucous glands and the respiratory epithelium of trachea showed multiple to coalescing foci of necrosis.

In all cases, infections with virulent bacteria, endoparasites, avian paramyxovirus type 1, avian influenza viruses

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Abbreviations: EAHV-3, equine alphaherpesvirus 3; SpAHV-1, sphenicid alphaherpesvirus 1.

The GenBank/EMBL/DDBJ/PIR accession numbers for the complete genome sequences of SpAHV-1 isolates GER/2012/S.humboldti/A1A2-01003 and GER/2013/S.demersus/B1-01004 are LT608136 and LT608135, respectively.

One supplementary figure and four supplementary tables are available with the online Supplementary Material.
and chlamydia were excluded by standard diagnostic methods (data not shown). The remaining epithelial cells bordering the necroses were enlarged with swollen nuclei containing amphophilic inclusion bodies (Fig. S1) indicative of an alphaherpesvirus infection. Tissues from the intestinal tract of two of the Humboldt penguins and from the oropharynx of the African penguin reacted positive in a panherpes PCR [6].

Virus isolation in primary chick embryo fibroblasts was successful for organ material from two Humboldt penguins (isolate GER/2012/S.humboldti/A1A2-01003, hereinafter A1A2-01003) and the African penguin (isolate GER/2013/S. demersus/B1-01004, hereinafter B1-01004). Chick embryo fibroblast cultures showed typical herpessviral cytopathic effect and were PCR positive. Virus was passaged in quail muscle cells (QM9) and electron microscopy of these revealed typical electron-dense herpesvirus particles (Fig. 1).

DNA was isolated from viral particles obtained by ultracentrifugation of infected QM9 cell cultures. Sequencing libraries were prepared and sequenced using Ion Torrent PGM as described by Höper et al. [7]. The obtained datasets for A1A2-01003 and B1-01004 were subjected to metagenomic analysis using Riem [8] and approximately 0.3 and 0.1% of the reads, respectively, were classified as herpesviral sequences. For determination of highly reliable complete genomes, the Ion Torrent datasets were supplemented with Illumina data, generated as described [9] from identical input DNA. An iterative de novo assembly/mapping procedure using the 454 Sequencing Systems Software suite (version 3.0; Roche) was used to build contigs representing the complete viral genomes of A1A2-01003 and B1-01004. For determination of the lengths of repetitive regions, these were PCR amplified (for primer sequences, see Table S2) and the products sequenced using Sanger sequencing. The overall positional identity of the A1A2-01003 and B1-01004 genomes in a nucleotide alignment was 95.1%. Annotated full genome sequences of SpAHV-1 were uploaded to the European Nucleotide Archive and published under the accession numbers LT608136 and LT608135 for A1A2-01003 and B1-01004, respectively.

To date, a relation of the virus isolates described here and previously published cases of (putative) alphaherpesvirus infections in penguins cannot clearly be established because only either pathology or viral sequences are available. In two reported cases of herpesviral infections in African penguins [10, 11] putatively caused by sphenicid herpesvirus 1 [12], only partly similar pathology was observed, but no viral sequences are available. Alterations in the African penguin (case B1) are consistent with the aforementioned cases of herpesviral infections in penguins, where lesions in the respiratory tract were predominant. In contrast, oropharyngitis and enteritis as described here for cases B1 and A1–A3, respectively, have to our knowledge not so far been reported for herpesviral infections in banded penguins. For two other putative penguin herpesviruses derived from samples from Magellanic penguins (Spheniscus magellanicus), no pathological information has been published. From these, only partial sequences of the DNA polymerase encoding gene are available in the INSDC databases (KJ720217, 439 nt; KR338839, 259 nt). In an alignment of the overlapping regions (263 nt), the identity of A1A2-01003 with these was 51.2 and 87.7%, respectively, whereas the identity between the A1A2-01003 and B1-01004 sequences was 98.0%. Based on the above information, we propose to designate the penguin-associated herpesvirus described here as sphenicid alphaherpesvirus 1 (SpAHV-1), in accordance with the most recent nomenclature [13].

The SpAHV-1 genome with an overall length of approximately 164 kbp and a G+C content of 45.6 mol% comprises the elements TR1-U1-IR1-R1-U5-TR5 (Fig. 2a), characteristic for a class D genome [14]. Slightly different genome lengths of both isolates are attributed to repetitive regions. Furthermore, the genome contains three identical putative origins of replication (Or1, Or1S1, Or1S2; Fig. 2b) comprising a central 41 bp A+T-rich stretch flanked up- and downstream by the sequence motif YGYTCGCACT that may serve as binding site for the origin binding protein [15]. Their sequences form a nearly perfect 61 bp palindrome. In addition, pac-1 and pac-2 sites associated with cleavage and packaging of viral genomes [16] were identified. The pac-1 site consists of an A+T-rich stretch flanked by homopolymeric stretches of C [17] and is located 33 nt upstream of the right terminus of the SpAHV-1 genomes. The pac-2 site was identified as an A-heptamer that is conserved in both SpAHV-1 genomes close to the left terminus.

**Fig. 1.** Electron microscopy of a SpAHV-1-infected syncytial cell. (a) Syncytial cell containing intranuclear capsids of uniform shape (black arrows) and a cytoplasmic virion (white arrow) 110 and 134 nm in size, respectively. Bar, 500 nm. (b) Virion budding from the nuclear membrane (NucM), immediately before entering the perinuclear space (pNuc); the cytoplasm (Cyt) is also visible. Bar, 100 nm. (c) The virion structure is typical for alphaherpesviruses: the capsid (Cap) is surrounded by the amorphous tegument (Teg), and the virion is encapsulated by a lipid-bilayer envelope (Env). Bar, 100 nm.
Using EMBOSS getorf [18], a total number of 86 ORFs was predicted, 71 of which are located within UL, nine in US and three being duplicated in IRs/TRs (Fig. 2b). In order to check for sequence similarity with other herpesviruses, the translated ORFs of A1A2-01003 were compared to aa sequences from 27 alphaherpesviruses (Table S3) using BLASTP [19]. Of the 83 unique ORFs, the highest BLASTP identities were found with members of the genera Mardivirus (45/83) and Varicellovirus (35/83). Forty-four ORFs represent herpesvirus core genes [20], which are of common ancestry in alpha-, beta- and gammaherpesviruses. An additional 26 ORFs were exclusively conserved in alphaherpesviruses. The aa identity between both SpAHV-1 isolates ranged between 89.4 % (envelope glycoprotein J) and 99.3 % (envelope glycoprotein M). A complete list of predicted ORFs along with standardized protein names, adopted from Davison [20], can be found in Table S4. The sequential arrangement of the predicted genes strictly corresponds to the prototypic human alphaherpesvirus 1 genome. No partial inversion or rearrangements of UL, as reported for iltoviruses [21], have been recognized. A polyadenylation signal sequence AATAAA was predicted in 55 3'-UTRs of

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**Fig. 2.** Genomic arrangement and organization of SpAHV-1. (a) Schematic illustration of the genomic arrangement observed in SpAHV-1 (not drawn to scale). SpAHV-1 comprises a class D genome, consisting of two unique regions flanked by inverted tandem repeats. Positions of the repeats are indicated for both SpAHV-1 isolates. (b) Green arrows represent the structural elements UL and US, respectively. Grey arrows depict IR and TR. The 86 predicted ORFs are depicted as arrows labelled with gene names; three of them exist as duplicates in IRs/TRs. ORFs highlighted in blue (n=13) encode proteins with highest similarity to proteins so far solely reported for mardi-, varicello- or iltoviruses; ORFs highlighted in purple (n=3) encode proteins without sequence similarity to other alphaherpesvirus proteins. The three predicted independent origins of replication, located in UL and IRs/TRs, are indicated as black stem loops.
potential mRNAs. Eleven predicted ORFs shared sequence identity with proteins that have solely been reported for either varicello-, mardi- or illoviruses and seem to characterize SpAHV-1 as a unique evolutionary intermediate. Their genomic position, ancestry and potential function are discussed in the following paragraph.

(i) Downstream of UL56, we identified an ORF encoding a 186 aa protein and predicted a hydrophobic transmembrane domain near the C terminus using TMHMM [22] (www.cbs.dtu.dk/services/TMHMM). The predicted protein showed 27.0 % aa identity with the type 2 membrane protein V1 of equine alphaherpesvirus 3 (EAHV-3). (ii) A second predicted protein (132 aa), located between UL27 and UL26, was 48.9 % identical to V32 of EAHV-3. Proteins V1 and V32 are of unknown function and have so far only been observed in varicelloviruses. To our knowledge, V1- and V32-like proteins have not been reported for avian alphaherpesviruses before. (iii) In SpAHV-1, US1 and US10 are duplicated within the tandem repeats IRg/TRg. This combined duplication has so far only been reported in varicelloviruses. Contrarily, in mardi- and illoviruses, only duplication of either US1 or US10 alone has been observed. (iv) Furthermore, we predicted an ORF encoding a potential homologue of the myristylated tegument protein CIRC, a protein thought to be involved in immunomodulation [23]. It has only been reported for some varicelloviruses and mardiviruses. (v) Two ORFs with 25.8 % pairwise protein sequence identity, provisionally designated LORF2I and LORF2II based on sequence similarities with LORF2 of anatid alphaherpesvirus 1 (AnAHV-1), are located at the right terminus of U5. We suggest that both ORFs represent paralogues which have emerged from an ancient duplication event. A similar constellation has been observed for gallid alphaherpesvirus 1 [24]. (vi) Furthermore, four putative proteins with highest identities to avian alphaherpesviral proteins of unknown function (LORF5, LORF4, the lipase and SORF2) were predicted.

Three predicted ORFs shared no significant sequence similarity with other herpesviral proteins and were therefore preliminarily designated hypothetical proteins 1–3 (HP1–3). HP1 encodes a putative 149 aa protein with a C-terminal hydrophobic domain. Transmembrane prediction suggested a membrane anchor-like structure very similar to that of the UL56 protein. HP2 is located between UL22 and OrIL and encodes a putative 116 aa protein. Ilovirus encode a cluster of five ORFs (A–E) of unknown function flanked by UL22 and OrIL; however, all without detectable similarity to HP2. Hence, the potential function of HP2 and its relation to ORFs A–E remains unclear. A third hypothetical protein HP3 (405 aa) is located at the right terminus of U5, between LORF2II and ICP0. Some mardiviruses encode predicted ORFs at the respective location, but none of them shared significant sequence similarity with HP3. Submission of the hypothetical protein sequences to InterPro [25] (www.ebi.ac.uk/interpro) and Phyre2 [26] (www.sbg.bio.ic.ac.uk/~phyre2) web portals yielded no relevant results. All hypothetical proteins show high conservation at both the nucleotide and the protein level between the SpAHV-1 isolates and maintain their own regulatory sequences. We therefore assume that these predicted ORFs encode hitherto unknown proteins that have evolved independently, were lost in other herpesviruses, or have been captured from host genomes. However, further experiments have to prove their existence.

The detected unique pattern of predicted proteins of SpAHV-1 prompted us to perform a gene content phylogeny as described by Montague and Hutchison [27] based on Tatusov et al. [28]. They showed that gene content clustering can be used to generate robust phylogenetic trees for herpesviruses. Therefore, a subset of 60 conserved proteins from Davison [20] was used and the respective SpAHV-1 proteins were added to the matrix. This binary matrix was used for distance analysis and subsequent hierarchical clustering utilizing the functions heatmap.2 [29] and dist.binary [30] in R (version 3.1.1, www.R-project.org). The resulting tree (Fig. 3a) displays the relationship of the 21 analysed alphaherpesvirus genomes, placing SpAHV-1 in close relation to mardiviruses. The closest relative of SpAHV-1 is AnAHV-1. In order to elucidate the phylogenetic and taxonomic position of SpAHV-1 in relation to other alphaherpesviruses with higher resolution, we compared the protein sequences of 52 core genes from 27 members of the alphaherpesviruses with those of SpAHV-1. A maximum-likelihood tree was inferred from an alignment concatenated from individually aligned protein sequences (in total 44 592 aa; Fig. 3b) using IQ-TREE [31] with automatic model selection (LG+F+I+G4) and statistical support through 10 000 ultra-fast bootstrap replicates [32]. The topology of the resulting tree reflected the taxonomic classification of the viruses into the five alphaherpesvirus genera. Like in the gene content tree, all members of the genus Mardivirus and SpAHV-1 form a monophyletic group. SpAHV-1 is the most distant member within this group and its closest relative is again AnAHV-1.

The high sequence identity between the herpesvirus isolates from Humboldt and African penguins both at the protein and nucleotide level is in accordance with the assumption that the recent banded penguins descended from a relatively young common ancestor some 6.1 (4.3–8.4) million years ago [33]. Following the concept of viral co-evolution [34], SpAHV-1 should have undergone cospeciation with its natural host towards the modern form present in Humboldt and African penguins. Based on the divergence times for the genus Spheniscus and uncorrected p-distances, as calculated by MEGA6 [35], the substitution rate for complete coding nucleotide and protein sequences of A1A2-01003 and B1-01004 was estimated to be 4.9 × 10⁻⁹ nt (3.5–6.9 × 10⁻⁹) and 4.2 × 10⁻⁹ aa (3.0–6.0 × 10⁻⁹) substitutions (site year)⁻¹. This approximately fits published mutational rates of mammalian and avian herpesviruses [2.7 × 10⁻⁹ nt, 4.4 × 10⁻⁹ aa [36], 3.5 × 10⁻⁸ nt [37], 3.3 × 10⁻⁹ aa [38] substitutions (site year)⁻¹]. A cospeciation of SpAHV-1 alongside the genus...
Fig. 3. Phylogenetic relation of SpAHV-1 to other alphaherpesviruses. The presented phylogeny is identical with the established phylogenetic grouping of alphaherpesviruses and puts the novel SpAHV-1 (highlighted in red) in close relation to mardiviruses. Virus species are highlighted according to their genus classification and biological hosts are presented as black silhouettes. (a) Gene content analysis of orthologous genes from 21 alphaherpesviruses and SpAHV-1. The presence of a specific gene in a specific genome is denoted as ‘1’ and its absence as ‘0’ in the resulting binary matrix. Furthermore, a phylogenetic tree was calculated from pairwise binary distances. AnAHV, anatid alphaherpesvirus; BoAHV, bovine alphaherpesvirus; CeAHV, cercopithecine alphaherpesvirus; EAHV, equid alphaherpesvirus; GaAHV, gallid alphaherpesvirus; HAHV, human alphaherpesvirus; MeAHV, meleagrid alphaherpesvirus; PsAHV, psittacid alphaherpesvirus; SuAHV, suid alphaherpesvirus. (b) Phylogenetic tree based on a 44,592 aa alignment from 52 core genes of 27 alphaherpesviruses and the novel SpAHV-1 isolates. Bootstrap support values are indicated in italics for each branch.
Spheniscus could therefore be plausible and supports the hypothesis that banded penguins are natural rather than accidental hosts of SpAHV-1. Nevertheless, a possible accidental transmission from free-range birds cannot be excluded since all examined penguins were kept in open-air compounds. However, this assumption seems to be implausible because SpAHV-1 has been isolated independently from penguins in two different German zoos. Another source of transmission between the examined penguins could be the exchange of animals between the zoos. Representatives of both zoos stated that penguins had never been exchanged, and other exchanged birds never had contact with the affected penguin colonies. Further screening of wild Humboldt and African penguins needs to be done in order to elucidate the predicted connection between banded penguins and SpAHV-1 in their natural habitats. If the genus Spheniscus represents the natural host for SpAHV-1, a similar virus could possibly also be isolated from Magellanic and Galapagos penguins (Spheniscus mendiculus). It is also supposable that a very similar virus could be causative in currently unresolved cases of diphtheritic stomatitis in yellow-eyed penguins (Megadyptes antipodes) [5] since the diphtheroid inflammation of the affected mucosal surfaces is identical in all these cases. However, whether SpAHV-1 is the primary agent of penguin diphtheria or whether other primary pathogens are involved remains a subject for further investigations.

In conclusion, the obtained sequences of SpAHV-1 may help to improve the knowledge about herpesviral diversity and evolution. Furthermore, they can serve as a basis for the development of diagnostic tools like real-time PCR, in order to check for virus presence in the natural habitats of the penguins, both in current and, retrospectively, in unresolved cases of penguin diphtheria. This is especially interesting since many penguin species are rated as vulnerable and endangered by the International Union for the Conservation of Nature and are the subject of special breeding programmes in zoos worldwide.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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