Novel divergent nidovirus in a python with pneumonia

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The order Nidovirales contains large, enveloped viruses with a non-segmented positive-stranded RNA genome. The order Nidovirales is currently divided into four different families, the Arteriviridae, Coronaviridae, Mesoniviridae and Roniviridae. Although the genome size of different nidoviruses varies between 12.7 and 31.7 kb, sequence identity in replicase proteins and similarities in overall genome organization, gene order and replication strategy indicate that nidoviruses share the same ancestor (Cavanagh, 1997; Cowley et al., 2000; Lauber et al., 2012; Snijder et al., 1993).

Nidoviruses have been detected in humans and various animal species, including fish, insects, birds and terrestrial as well as marine mammals, but they have not been identified in reptiles yet (de Groot et al., 2012). In the present study, we describe a case of an 8-year-old captive Indian python (Python molurus) from a zoological garden in northern Germany that was submitted for pathological examination after sudden death. Necropsy was performed according to standard procedures and samples of various tissues were collected and fixed in 10 % (v/v) non-buffered formalin. In addition, tissue samples were stored at −20 °C. Pathomorphological examination revealed evidence of a severe, acute, diffuse, necrotizing pneumonia with associated mild, heterophilic inflammation and hypersecretion (Fig. 1a). Moreover, multifocal pancreatic necroses were found and focal thrombosis of a coronary venous blood vessel was detected, while the connective tissue within the body cavity displayed diffuse oedema. Observed macroscopic

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and microscopic changes indicated a viral infection of the lungs, presumably with a secondary bacterial infection and septicaemia that resulted in increased vascular permeability with subsequent oedemas and increased intravascular coagulability with thrombi formation. Aerobic microbiological investigation of lung samples resulted in the detection of Salmonella and Bordetella spp., while random PCR in combination with 454 sequencing was used to identify potential viral causative agents as described previously (Bodewes et al., 2013; van den Brand et al., 2012). Analysis of the obtained reads using BLASTX revealed sequences that were most closely related to various viruses belonging to the subfamily Torovirinae, while no other viral sequences, except for python endogenous retroviruses, were detected. Additional analysis of the obtained sequence reads revealed the consensus sequence of the partial genome of a previously unknown nidovirus, tentatively called python nidovirus (PNV; GenBank accession number KJ935003). Using specific primers, the obtained sequence was confirmed partially using methods described previously (van Leeuwen et al., 2010) and the 3’ end of the PNV genome was obtained using 3’ RACE PCR. Primer sequences are available on request. The consensus sequence of the partial genome of PNV consisted of 33 559 nt and a 3’ poly(A) tail of at least 12 nt, which is the largest RNA virus genome currently known. The partial genome contained eight predicted major ORFs with a genome organization similar to nidoviruses (Fig. 2a; Table S1, available in the online Supplementary Material), while the 3’ UTR was 904 nt.

In the overlapping region of ORF1a and ORF1b, no sequences were detected that were identical to the slippery sites that facilitate ribosomal frameshifting of nidoviruses (Nga et al., 2011; Zirkel et al., 2011). However, the putative slippery sequence AAAAAAC was identified 87 nt downstream of the start codon of ORF1b and 15 nt upstream of the stop codon of ORF1a (Fig. 2b). Although the AAAAAAC sequence was not identified as a potential slippery sequence for nidoviruses previously, it was identified as such for the families Astroviridae and Retroviridae (Brierley, 1995). In addition, a putative stem–loop RNA structure was identified downstream of this putative slippery sequence ($\Delta G = -17.8$ kcal mol$^{-1}$; Fig. 2c) as performed by Mfold and RNAfold webservers (rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Zuker, 2003). Experimental evidence for the role of the AAAAAAC sequence as a slippery sequence in the PNV is necessary to prove its function.

Analysis of sequences of polyprotein 1ab (pp1ab) based on alignments with other nidoviruses and predictions using HMMER3, HHpred (toolkit.tuebingen.mpg.de/hhpred) and Phyre2 revealed similarity to other nidoviruses (Finn et al., 2011; Kelley & Sternberg, 2009; Söding et al., 2005). In PNV, amino acid residues 2290 to 2495 were most closely related to the catalytic domain of serine/threonine protein kinase ($E$ value = $8.2 \times 10^{-48}$). In addition, a domain (residues 2126–2236) immediately 5’ from the predicted kinase may show some similarity to the ADP-ribose-$1’$-phosphatase domain present in viruses from the family Coronaviridae (Finn et al., 2011). Additional analyses are necessary to elucidate the function of both domains.

The region adjacent to these domains comprises three regions containing disordered sequences (residues 570 to 1105, 1144 to 1184 and 1672 to 1755) that did not show any similarity with other nidoviruses. Three hydrophobic regions with multiple putative transmembrane domains (TM1, amino acid residues 4092–4302; TM2, amino acid residues 4739–4843; TM3, amino acid residues 5219–5411) were identified using Phobius (Käll et al., 2004) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM), these regions were located at relative positions in the genome similar to the corresponding regions in coronaviruses and toroviruses (Fig. 2a).

Between TM2 and TM3, a putative main protease (Mpro) domain was present with several conserved regions similar to nidovirus Mpro domains, including the conserved GX(S/C)G region of chymotrypsin-like proteases (Fig.

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**Fig. 1.** Pathomorphological findings and in situ hybridization (ISH) in the lungs. (a) The pulmonary epithelium of the trabeculae (E) contains numerous necrotic cells and respiratory lumina (L) of the bronchi and faveolae are filled with necrotic cells (arrows) and cellular debris. Sections were stained with haematoxylin and eosin. Magnification $\times 400$. (b) Detection of PNV RNA by means of ISH with evidence of positive viable cells (arrowheads) within the pulmonary epithelium (E) as well as of positive necrotic cells (arrows) within the lumen (L). Magnification $\times 400$. 

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- **Table S1**
- **Fig. 2a**
- **Fig. 2b**
S2). PNV appears to be no more closely related to torovirus Mpro domains than those from other nidoviruses, but it apparently uses serine instead of cysteine as its principal nucleophile, like the families Arteriviridae and Torovirinae and in contrast to the families Mesoniviridae, Coronavirinae and Roniviridae. The other counterparts of a prototypical His–Ser–Asp catalytic triad seem to be conserved as well. Nidovirus Mpro show a preference for Gln or Glu at the position immediately preceding the scissile bond (referred to as the P1 residue and the following residues are referred to as P1’, P2’), which is facilitated by a conserved His and Thr residue located in the S1 subsite; these side chains form hydrogen bonds with the carboxamide/carboxylate moiety of the P1 Gln/Glu residue (Matthews et al., 1994; Mosimann et al., 1997; Snijder et al., 1996). These His and Thr residues are conserved in the Mpro of PNV, suggesting a substrate preference of Gln/Glu at the P1 position similar to other nidoviruses. However, in vitro experiments are necessary to
confirm this substrate preference and to make predictions on cleavage sites present in pp1ab.

In pp1b, various regions with high similarity to other protein domains of nidoviruses were identified in the following order: RNA-dependent RNA polymerase (RdRP), zinc-binding domain (Z), helicase domain (Hel), 3' to 5' exoribonuclease domain (ExoN), nidoviral uridylate-specific endoribonuclease (NendoU), ribose-2'-O-methyltransferase domain (OMT), similar to what was shown for members of the Torovirinae (Fig. 2a) (Gorbalenya et al., 2006). Experimental evidence, including identification of all cleavage sites, is necessary to confirm the function and exact location of these domains in this virus.

In the 3' proximal region of the PNV genome, six ORFs of more than 100 amino acids were identified (ORFs 2–7) (Table S1, Fig. 2a). Analysis of the amino acid sequences of the ORFs 2, 3, 6 and 7 revealed a putative transmembrane domain at the C-terminus in all these ORFs, at least six potential N-linked glycosylation sites and a signal peptide at the N-terminus (only ORFs 2, 3 and 6), suggesting that they are integral membrane proteins. Based on size, location in the genome and BLASTX results, ORF2 encodes

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**Fig. 3.** Phylogenetic analysis of PNV. (a) Bayesian phylogenetic reconstruction of the complete polyprotein 1ab of the family Coronaviridae including the novel python virus. Numbers at nodes indicate statistical support of grouping from posterior probabilities (only values above 0.7 are shown for clarity of presentation). GenBank accession numbers: SARS-related human coronavirus, AAP33696; Rousettus bat coronavirus HKU9, YP_001039970; Middle East respiratory syndrome coronavirus, AFS88944; mouse hepatitis virus, NP_068668; transmissible gastroenteritis virus, CAB91143.1; HCoV 229E, NP_073549; infectious bronchitis virus, NP_066134; beluga whale coronavirus, ABW87819; munia coronavirus, ACJ12061; thrush coronavirus, YP_002308496; fathead minnow nidovirus, ADN95978; white bream virus, YP_803213; porcine torovirus, YP_008798230; bovine torovirus, YP_337905. (b, c) Analysis of pairwise identities of RdRP and HEL domain of PNV and WBV with members of the genera Bafinivirus, Torovirus and representative members of the subfamily Coronavirinae. Bars indicate mean ± SD.
the spike protein (S), but no putative furin cleavage site could be detected, although a potential cathepsin L cleavage site is present (KLFE ↓ET; residues 570–575). ORF4 (215 aa) contained three putative transmembrane domains at residues 29–50, 62–81 and 93–120, which is similar to the M protein of coronaviruses and toroviruses (de Haan & Rottier, 2005; Schütze et al., 2006). No transmembrane domains were detected in ORF5, although analysis of the amino acid sequence suggested that it was most closely related to the nucleocapsid protein (N) of porcine reproductive and respiratory syndrome virus (PRRSV). Notably, the order of the main structural proteins is 5′–S–M–N–3′, similar to that in coronaviruses and toroviruses.

Analysis of ORF7 using Phyre2 (Kelley & Sternberg, 2009) revealed that amino acid residues 55–138 were most closely related to the sialidase domain of haemagglutinin–neuraminidase (HN) proteins of paramyxoviruses with a confidence of 87.9 %. However, no similarity was detected between the haemagglutinin domain of the HN of paramyxoviruses, the hemagglutinin–esterase (HE) of nidoviruses, or any other protein and ORF7 of PNV. Alignment of ORF7 with HN within members of the family Paramyxoviridae using MAFFT software (Katoh & Standley, 2013) suggested that five out of seven conserved active site residues of neuraminidases and sialidases were present (Table S2, Fig. S3) (Yuan et al., 2005). In addition, maximum-likelihood phylogenies reconstructed with PhyML (Guindon et al., 2010) using the Whelan and Goldman (WAG) amino acid substitution model and 1000 bootstrap replicates suggested that this ORF may belong to the group of HN proteins of paramyxoviruses (Fig. 2d). However, experimental data are necessary to confirm that this ORF indeed encodes a HN and to discover possible biological implications.

Phylogenetic analysis of pp1ab was performed using Mafft software to align the complete pp1ab amino acid sequence of various members of the family of Coronaviridae (Katoh & Standley, 2013). Bayesian inference of phylogeny was performed for the pp1ab using the MrBayes program (Ronquist & Huelsenbeck, 2003), a WAG amino acid substitution model and 2 000 000 generations sampled every 100 steps resulted in 20 000 trees, of which 25 % were discarded as burn-in. Cavally virus (family Mesoniviridae) was used as an outgroup. Results of this analysis indicated that PNV belongs to the subfamily Torovirinae, with high statistical support (Fig. 3a). In addition, pairwise identity analysis of the putative RdRP (pp1b residues 217–889) and Hel (pp1b residues 1128–1414) domains performed in the MEGA6 software (Tamura et al., 2013) indicated that pairwise identities were 40 % or lower for both domains with members of the subfamily Torovirinae and 26 % or lower with members of the subfamily Coronavirinae (Fig. 3b). Interestingly, pairwise identities of the RdRP and Hel domains of white bream virus were similar compared to PNV for viruses of the genus Torovirus and the subfamily Coronavirinae, while the pairwise identities were respectively 77 and 66 % for the fathead minnow nidovirus that belongs to the same genus as white bream virus (Fig. 3c, FHMMV and WBV, respectively). Since pairwise identities of conserved domains were less than 46 % (de Groot et al., 2012), these findings suggest that PNV belongs to a new genus within the subfamily Torovirinae.

The presence of viral RNA in the lungs of an Indian python with pneumonia was evaluated by in situ hybridization using a synthetic gene construct (Eurofins MWG Operon) encoding a 95 bp spanning region of PNV RdRp gene as described previously (Gaedeke et al., 1997; Grötters et al., 2005; Hahn et al., 2013). Using this technique, a finely granular cytoplasmic signal, specific for PNV RNA was detected in viable as well as degenerated pulmonary epithelial cells and in single epithelial cells of the trachea and pharynx, suggesting that the virus infected respiratory epithelium only. No viral RNA was detected in other tissues of the python by in situ hybridization. In addition to tissues of the Indian python, formalin-fixed and paraffin-embedded tissues from ten boid snakes dating from 2009 to 2013 from the archive of the Department of Pathology, University of Veterinary Medicine, Hannover, were investigated using in situ hybridization, but PNV RNA was not detected in the organs examined.

In conclusion, a previously unknown nidovirus was identified in lungs of an Indian python with pneumonia. Phylogenetic analysis of PNV suggests that this virus belongs to a novel genus within the subfamily Torovirinae, while various genetic characteristics were observed that are distinct from other members of the order Nidovirales, including a putative frameshift signal identical to that of astroviruses and retroviruses and an accessory gene that was most closely related to the HN of paramyxoviruses. Additional analyses, including the elucidation of the transcription mechanism of this virus and the function of various domains and proteins, are of great interest and will provide more information about the genetic characteristics of this virus and might help to understand the phylogeny of nidoviruses in general.

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References

