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Short Communication

Concurrent ranavirus and *Batrachochytrium dendrobatidis* infection in captive frogs (*Phyllobates* and *Dendrobates* species), The Netherlands, 2012: A first report

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ABSTRACT

A ranavirus infection with concurrent *Batrachochytrium dendrobatidis* infection and mortality in captive *Phyllobates* and *Dendrobates* species is reported. Greyish skin with hepato- and reno-megaly were evident. Microscopically, *Batrachochytrium dendrobatidis* was present in the stratum corneum of the hyperkeratotic skin. Intracytoplasmic inclusion bodies were present in erythrocytes and multiple organs. All samples examined tested positive using PCR for the major capsid protein (MCP) gene of ranavirus and the ITS-1–5.8S region of *B. dendrobatidis*. The sequence obtained showed a 99% identity with the deposited sequence of the MCP gene of the common midwife toad virus (CMTV). This is the first report of mortality in captivity in poison dart frogs caused by a ranavirus, CMTV or like virus, and *Batrachochytrium dendrobatidis* infection.

Ranavirus and *Batrachochytrium dendrobatidis* infections have been associated with amphibian mass mortalities and population decline worldwide. They are considered as emerging infections and have been listed as notifiable by the World Organization for Animal Health (Schloegel et al., 2010). In The Netherlands, ranavirus infection has recently been discovered in wild water frogs (*Pelophylax* spp.) and common newts (*Lissotriton vulgaris*) (Kik et al., 2011). Ranavirus infection in captivity in Belgium and The Netherlands has only been described in imported red tailed knobby newts (*Tylototriton kweichowensis*) (Pasmans et al., 2008). We are aware of *B. dendrobatidis* infections described in captive poison dart frogs in Germany (Mutschmann et al., 2000) and a captive Central American bolitoglossine salamander (*Bolitoglossa dofleini*) (Pasmans et al., 2004).

We report here the first ranavirus with concurrent *B. dendrobatidis* infection and mortality in captive *Phyllobates* and *Dendrobates* species in Europe. The die-off began with two *P. bicolor* (black-legged poison) frogs, followed by five *P. vittatus* (Golfo Dulce poison) frogs and five *D. auratus* (green and black poison dart) frogs – 12 animals in total. Young as well as adult animals were involved. Clinical signs in the frogs, as reported by the owner, were dry, greyish skin, animals spending more than 90% of their time in the water, anorexia and then death. Young metamorphosed tadpoles that just emerged onto land lay dead without prior signs other than anorexia, and still others did not grow well. Some lay dead in the water.

Necropsy was performed on 10 of the frogs. The weight of the animals varied from 1 to 6 g. The skin of eight adult frogs showed greyish foci and two juvenile frogs showed slight hyperaemia of the skin of the hind legs. The livers of all frogs were enlarged, pale brown and friable. The spleens were enlarged and hyperaemic, measuring 3–4 mm in 8/10 frogs. The other two adult frogs had a spleen measuring 1 mm. The kidneys were enlarged and beige. The gastro-intestinal tract was empty. Tissue samples from the skin, liver, spleen, kidney and gastro-intestinal tract were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with haematoxylin and eosin (H&E).

Histological examination of the skin showed epithelial hyperplasia and focal hyperkeratosis with multifocal intracellular sporangia (spore-containing bodies) of *B. dendrobatidis* in the stratum corneum (Fig. 1). The livers showed granulomatous lesions and microvacuolation of hepatocytes. Necrosis was detected in the enlarged spleens of eight frogs, and lympho-depletion in the two small spleens. The kidneys showed interstitial and glomerular heterophilic granulocytes with proteinaceous material in the tubular lumina. The stomachs showed no abnormalities. Mild intra-epithelial infiltrates of heterophilic granulocytes were present in the intestines.

Multifocal basophilic intracytoplasmic inclusion bodies, suggestive of viral infection, were evident in the erythrocytes, hepatocytes, villus enterocytes and renal tubular epithelium (Fig. 2). However, in contrast to other cases (Kik et al., 2011), but not based on morphometric analysis, lower numbers of the basophilic cytoplasmic inclusion bodies were seen in hepatocytes, villus enterocytes of the small intestine or kidney cells.

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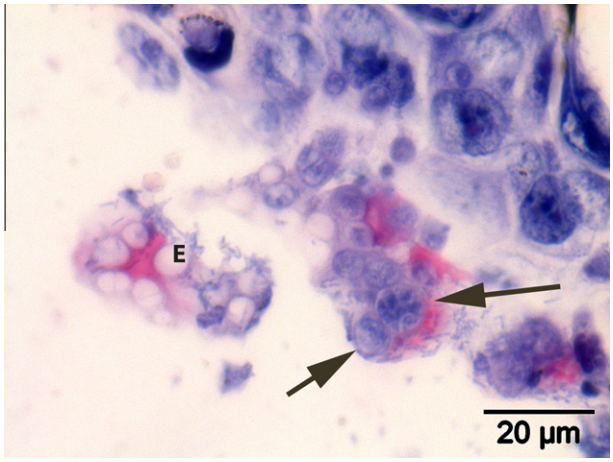

Fig. 1. Skin of a *Phyllobates bicolor* with sporangia (spore-containing bodies) consistent with *Batrachochytrium dendrobatidis* (arrows). Empty sporangia, having discharged all zoospores (E).

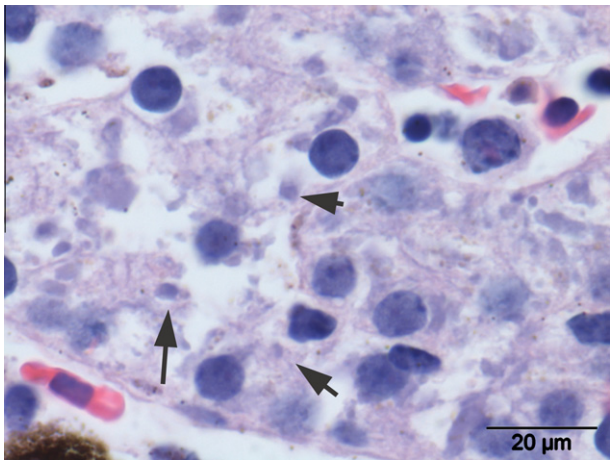

Fig. 2. Liver of a *Phyllobates bicolor* showing multifocal intracytoplasmic inclusion bodies in the hepatocytes as indicated by arrows (H&E).

Total DNA was isolated from formalin fixed, paraffin embedded tissue using the DNeasy Tissue Kit (Qiagen) according to the manufacture's protocol. The final eluate was used in a PCR for either the major capsid protein (MCP) gene of ranavirus or for the ITS-1–5.8S region of *B. dendrobatidis*. The PCR for the MCP of ranavirus was performed using the forward primer 5′-GACTTGGCCACTTATGAC-3′ and the reverse primer 5′-GTCTCTGGAGAAGAAGAA-3′ (Mao et al., 1997). Primers TTS1–3Chytr (CCTTGATATAATACAGTGTGC-CATATGTC) and 5.8S Chytr (AGCCAAGAGATCCGTTGTCAAA) (Boyle et al., 2004) were used in the PCR for the ITS-1–5.8S region of *B. dendrobatidis*. PCR reaction mixes were subjected to gel electrophoresis and generated products of the expected size (MCP, 533 bp; ITS-1–5.8S region, 146 bp) were purified from gel using the Agarose Gel DNA Extraction Kit (Roche). Purified PCR products were sequenced using the Sanger method (BaseClear). Sequences obtained from independent PCRs and from the various samples were analyzed using the DNASTAR Lasergene Core Suite 9.1 program (DNASTAR) and after removing the primer sequences, were subjected to a homology search in the NCBI database using BLAST.[1] The sequence of the MCP-PCR product showed a 99% identity (99% coverage) with the deposited (partial) sequence of the MCP gene of the common midwife toad ranavirus (accession numbers JQ231222.1 and FM213466.1). The sequence of the ITS1–5.8S region PCR product showed a 98% identity (100% coverage) with the deposited partial sequence of the ITS1–5.8SrRNAgene-ITS2 of *B. dendrobatidis* strain CW34 clone I, as well as with ITS1–5.8SrRNAgene-ITS2 sequences of other clones of the same or other *B. dendrobatidis* strains.

We conclude that the frogs probably died from a ranavirus infection, most likely the common midwife toad virus (CMTV), or a CMTV-like virus, combined with *B. dendrobatidis* infection. The pathological changes in the livers, spleens and kidneys of the frogs that had intracytoplasmic inclusions are consistent with ranavirus infection. In addition, these frogs also had pathological changes in the skin and intralesional *B. dendrobatidis*. However, it is difficult to determine which of the present pathogens contributed more to the deaths of the frogs.

To date, CMTV is known to infect tadpoles of the common midwife toad and juvenile alpine newts, adult water frogs and common newts (Balseiro et al., 2009, 2010; Kik et al., 2011). This is the first report of a combined ranavirus and *B. dendrobatidis* in captive frogs in The Netherlands. More importantly, it is also the first time CMTV or a CMTV-like virus has been reported in these host species in captivity. The findings highlight the importance of monitoring ranaviral and *B. dendrobatidis* infections in captive as well as wild amphibians.

Especially with the worldwide trade in amphibians, the risk of introducing these infections into wild populations with (un)intentionally released animals is high (Fisher and Garner, 2007). Outbreaks of ranavirus infection have been linked with trafficking of tiger salamanders (*Ambystoma tigrinum*) in the USA. Chytridiomycosis in the wild in the UK was associated with introduction of infected North American bullfrogs (*Rana catesbeiana*) (Schloegel et al., 2010). Kik et al. (2011) were not able to find a source of infection in the wild amphibians that they diagnosed. A spill-over of CMTV or CMTV-like ranavirus and *B. dendrobatidis* present in a captive population of frogs is a serious threat to wild populations of amphibia.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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[1] See: http://www.ncbi.nlm.nih.gov/blast.

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