Epidemic ranaviral disease in imported captive frogs (Dendrobates and Phyllobates spp.), Japan, 2012: a first report

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Introduction: Ranavirus infection is associated with mass die-off and population decline in amphibians worldwide, and is listed by the World Organization for Animal Health (OIE) as a notifiable disease under the Aquatic Animal Health Code. Work is ongoing to control the spread of ranavirus, because this agent is considered an emerging pathogen of amphibians. In Japan, ranaviral diseases have been detected at each episode of die-off of wild bullfrog juveniles since 2008. However, ranaviral disease has not been found in captive anurans.

Case presentation: Epidemic and lethal ranaviral disease in captive Dendrobates and Phyllobates species imported from the Netherlands is reported. Poison dart frogs of three genera were imported from the Netherlands in March 2012. Disease and death were noted approximately 10 days after importation. Fifty-three adults of five species died in about 1 month, including frogs that had been kept previously. Microscopic examination revealed necrosis and intracytoplasmic basophilic inclusion bodies in the parenchyma of multiple organs. Electron microscopy showed cytoplasmic ranavirus-like particles within haematopoietic cells of the kidney. These particles were icosahedral, with a diameter of approximately 144 nm. Thirty-three of 41 dead frogs showed positive PCR results for the major capsid protein gene of Ranavirus. The sequence obtained from five frogs was identical and did not match the registered sequence of any ranavirus.

Conclusion: This is the first report, to our knowledge, of ranaviral disease in imported anurans of the family Dendrobatidae and genera Phyllobates and Dendrobates in Japan.

Keywords: frog; infectious diseases; ranavirus.

Introduction
Ranavirus infection is associated with mass die-off and population decline in amphibians worldwide, and is listed by the World Organization for Animal Health (OIE) as a notifiable disease under the Aquatic Animal Health Code. Work is ongoing to control the spread of ranavirus, because this agent is considered an emerging pathogen (OIE, 2012; Schloegel et al., 2010). In Japan, ranaviral diseases have been detected at each episode of die-off of wild bullfrog juveniles (Une et al., 2009) since 2008. Although ranaviral disease has not been found in captive anurans, we discovered epidemic and lethal ranaviral disease in anurans imported from the Netherlands. In the Netherlands, ranaviral diseases have been discovered in free-range frogs (Pelophylax spp.), common newts (Lissotriton vulgaris) (Kik et al., 2011), and imported red-tailed knobby newts (Tylototriton kweichowensis) (Pasmans et al., 2008). Further, ranaviral disease has recently been confirmed in breeding poison dart frogs (Kik et al., 2012). We describe here the first occurrence to our knowledge of ranaviral disease and mortality in captive Dendrobates and Phyllobates species in Japan.

Case report
Thirty poison dart frogs of three species (Dendrobates auratus, Dendrobates azureus, Dendrobates tinctorius) were imported from the Netherlands in mid-March 2012. Onset of disease and death occurred approximately 10 days after importation. Subsequently, four species of frog (D. auratus, Dendrobates leucomelas, D. tinctorius, Phyllobates terribilis) that were bred from a previous stock of frogs also
began to die. Before this, there was indirect contact between the imported frogs and frogs that had been kept previously in the same container at the importer’s facility. Symptoms were nonspecific – sloughing failure, difficulty moving, lethargy and sudden death. Surviving frogs were treated in medicated baths containing an antymycotic drug (itraconazole) for chytrid fungus; however, death of frogs continued. In total, 53 adults of 5 species died in about 1 month (Table 1).

Examinations were performed on 52 (41 dead and 11 surviving) of the frogs. There were no skin lesions except adhesion of sloughed skin. Total DNA was extracted from fresh kidneys by using the DNeasy Tissue kit (Qiagen) according to the manufacturer’s protocol. We used three primer sets [1, RanaM68F (5’-GCACCCACCTCTACTCTTATG-3′) and BIV MCP 154 (5’-CCATCGAGCCGTTCATGAT-3′)], 230 bp; 2, FV3MCP4F (5’-GACTTGGCACCTATGTAC-3′) and FV3MCP5R (5’-GTCTCTGGAGAAGAA-3′), 530 bp (Mao et al., 1997); and 3, RanaJP556F (5’-GTTCTTCCCCCTCACCTCTCTTT-3′) and RanaJP772R (5’-GGTCTGAGGCGGCTGCAC-3′), 217 bp] and amplified a *Ranavirus*-specific gene encoding major capsid protein (MCP) from the 52 frog specimens. If two or more of these primer sets were positive, that sample was determined to be positive. As a consequence, 33 of the 41 dead frogs and 3 of the 11 surviving frogs tested positive. The nucleotide sequences obtained were aligned using the Clustal W method of the program MEGA v. 5.2. The phylogenetic tree was constructed using the neighbour-joining algorithm with the Tamura three-parameter model. To determine the robustness of the tree, a bootstrap test of 2000 replicates was applied. The corresponding *European catfish virus* (FJ358608, FJ358609) and short-finned eel ranavirus (FJ358612) were used as an outgroup. The MCP gene sequence (1392 bp) was separately determined from five frogs. Sequence alignment showed no variation in the sequence from these frogs. The comparison of the sequence obtained from the poison dart frogs with previously published data in GenBank/ DDBJ/EMBL databases using the BLAST system showed that the sequence obtained in this study had a 99 % identity (1386/1392 bp) with the MCP gene sequence of *Frog virus 3* (FJ459783). The neighbour-joining tree based on the MCP gene sequences confirmed that the ranavirus obtained in this study clustered with *Frog virus 3* with a 64 % bootstrap value (Fig. 1). Nested PCR examination for *Batrachochytrium dendrobatidis* was performed using samples obtained by swabbing the skin of frogs with cotton swabs and using a primer for the BdITS gene region, as described by Goka et al. (2009).

Of 33 frogs positive for ranavirus on PCR (Table 1), the livers were blackish and friable in 22 and splenomegaly was observed in 17. Tissue samples (skin, liver, spleen, kidney and digestive tract) from 20 dead frogs were fixed in 10 % phosphate-buffered formalin, embedded in paraffin, sectioned at 3–4 μm, and stained with haematoxylin and eosin. Histological lesions common to all cases included: necrosis and degeneration in varying degrees in the glomeruli and tubules of the kidney and in the hepatocytes; basophilic or amphophilic intracytoplasmic inclusion bodies present in interstitial haematopoietic cells, hepatocytes, renal tubular epithelial cells and some glomerular cells; and apoptosis in the haematopoietic cells of the liver and kidney (Fig. 2). No lesions associated with chytridiomycosis or chytrid fungus were found in the skin. In addition, no bacterial infection was seen. For ultrastructural examination, a portion of formalin-fixed kidney tissue was refixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide, and embedded in epoxy resin. Ultrathin sections (90–150 nm) were stained with uranyl acetate and lead citrate. Electron microscopy showed cytoplasmic ranavirus-like particles within haematopoietic cells of the kidney. These particles were icosahedral, with a diameter of ± 144 nm.

### Discussion

Considering the setting of the epidemic together with characteristic microscopic lesions and molecular investigations, we decided that a ranaviral disease caused the outbreak in the imported poison dart frogs of this report. As far as we know, this is the first documented case of significant morbidity and mortality associated with a ranavirus in captive anurans in Japan. There are

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**Table 1. Profile of the frogs**

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Total number</th>
<th>Dead</th>
<th>Surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>PCR+/dead*</td>
<td>n</td>
</tr>
<tr>
<td>D. auratus</td>
<td>26</td>
<td>16/15</td>
<td>10</td>
</tr>
<tr>
<td>D. azureus</td>
<td>12</td>
<td>7/5</td>
<td>5</td>
</tr>
<tr>
<td>D. tinctorius</td>
<td>21</td>
<td>14/14</td>
<td>7</td>
</tr>
<tr>
<td>D. leucomelas</td>
<td>10</td>
<td>9/3</td>
<td>1</td>
</tr>
<tr>
<td>P. terribilis</td>
<td>10</td>
<td>7/4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>79</td>
<td>53/31</td>
<td>26</td>
</tr>
</tbody>
</table>

*Number examined by the PCR method. ND, Not done.
two reports of concurrent infection with ranavirus and *B. dendrobatidis* in the poison dart frog (Kik et al., 2012; Miller et al., 2008). In these reports, it was difficult to determine which of the present pathogens contributed more to the deaths of the frogs, because either agent has the pathogenic potential to kill amphibians. This is the first report that shows clearly that ranavirus itself has lethal pathogenicity and the potential to induce an outbreak of epidemic proportions in the poison dart frog.

Fig. 1. Phylogenetic tree based on MCP sequences of *Ranavirus*. The comparison of the obtained sequence from the poison dart frogs with previously published data in GenBank/DBJ/EMBL databases using the BLAST system showed that the sequence obtained in this study had a 99 % identity (1386/1392 bp) with the MCP gene sequence of *Frog virus 3* (FJ459783). The neighbour-joining tree based on the MCP gene sequences confirmed the ranavirus obtained in this study clustered with *Frog virus 3* with a 64 % bootstrap value.

Fig. 2. Kidney. (a) Glomerular necrosis and degeneration of renal tubular epithelium. Apoptosis of interstitial haematopoietic cells is abundant. Inset shows an intracytoplasmic inclusion body (arrowhead) in a renal tubular epithelial cell. H&E stain. ID no.12065X (*D. auratus* that was imported and died in March 2012). (b) Ranavirus-like particles in the cytoplasm of haematopoietic cells. Bar, 3 μm; electron microscopy. (c) The particles are icosahedral, with a diameter of 144 nm. Bar, 0.5 μm; electron microscopy. ID no.12067X (*D. auratus* that was imported and died in March 2012).
Cunningham et al. (1996) identified two main disease syndromes: one characterized by skin ulceration (ulcerative syndrome) and one characterized by systemic haemorrhages (haemorrhagic syndrome). Among affected common frogs (*Rana temporaria*) in Britain, individuals were found with lesions common to both of these syndromes. Ulceration of the skin is a common lesion in infected amphibians (Cunningham et al., 1996; Une et al., 2009). The ulcer is an important lesion, and can lead to secondary infection such as a bacterial infection in aquatic animals. However, we examined 52 frogs and did not find a distinct skin ulcer. Type and severity of lesion will vary depending upon the species and condition of the host, and the strain of the virus. In addition, lesion formation is related to the length of the clinical course, which is affected by the sensitivity of the species of frog to the specific virus. A possible explanation for the absence of skin ulcers is that the frog species in this report had high susceptibility to the ranavirus, and the clinical course was short. Therefore, diagnosis should be based on synthetic methods, which are not influenced by the presence or absence of a specific lesion such as the skin ulcer. In ranaviral disease of the poison dart frog in this case, degeneration and necrosis of parenchymal organs with intracytoplasmic inclusion bodies were found to be characteristic, which concurs with common findings in previously reported ranaviral disease (Miller et al., 2011; Une et al., 2009).

Course of onset and detection of the same sequence of the MCP portion in all *Ranavirus* sequences suggest horizontal transmission at the facility in Japan; a ranavirus outbreak as in this case has never previously been confirmed in Japan. In The Netherlands, the common midwife toad virus (CMTV), or CMTV-like virus, and *Frog virus 3* have been identified in both captive frogs and free-range frogs (Kik et al., 2011, 2012; Pasmans et al., 2008), and the virus in this report is closely related to *Frog virus 3*. The importer went to the Netherlands and brought the frogs directly back to Japan himself. Further, the importer had not experienced previous outbreaks, and this epidemic started after the introduction of these frogs. Therefore, the frogs imported from the Netherlands might have represented the original route of entry into the facility. A spill-over of ranavirus present in a captive population of frogs is a serious threat to wild amphibian populations. Considering the worldwide trade in amphibians, the risk of introducing this infection into wild populations with intentionally released animals is high (Picco & Collins, 2008). In conclusion, this is, we believe, the first report of ranavirus in imported animals of the Dendrobatidae in Japan.

Acknowledgements

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References


