Rustrela Virus Infection – A New Emerging Neuropathogen of Red-necked Wallabies (Macropus rufogriseus)

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Abstract

The rustrela virus (RusV) was recently described as a novel pathogen in a circumscribed area of northern Germany close to the Baltic Sea. Up to now, the virus has been detected in cases of fatal non-suppurative meningoencephalitis in zoo animals of different species and a single wild carnivore as well as in apparently healthy yellow-necked field mice (*Apodemus flavicollis*). Data regarding the background of this previously undiscovered pathogen, including clinical presentation of the disease, host range, and distribution of the virus, are still limited. Here, three euthanized red-necked wallabies (*Macropus rufogriseus*) from zoos of different areas in northeastern Germany were submitted for necropsy after presenting with apathy and therapeutically unresponsive neurological symptoms. A moderate to severe, non-suppurative meningoencephalitis was diagnosed in all three cases. RusV was consistently detected via RT-qPCR and RNA *in situ* hybridization in the brains of all wallabies. Other, commonly known neuropathogens could not be detected. Overall, red-necked wallabies appear to be highly susceptible to RusV as novel neuropathogen, which is broader distributed in northeastern Germany.

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Keywords : rustrela virus; rubivirus; encephalitis; Macropodidae; red-necked wallaby

Summary

The rustrela virus (RusV) was recently described as a novel pathogen in a circumscribed area of northern Germany close to the Baltic Sea. Up to now, the virus has been detected in cases of fatal non-suppurative meningoencephalitis in zoo animals of different species and a single wild carnivore as well as in apparently healthy yellow-necked field mice (*Apodemus flavicollis*). Data regarding the background of this previously undiscovered pathogen, including clinical presentation of the disease, host range, and distribution of the virus, are still limited. Here, three euthanized red-necked wallabies (*Macropus rufogriseus*) from zoos of different areas in northeastern Germany were submitted for necropsy after presenting with apathy and therapeutically unresponsive neurological symptoms. A moderate to severe, non-suppurative meningoencephalitis was diagnosed in all three cases. RusV was consistently detected via RT-qPCR and RNA *in situ*hybridization in the brains of all wallabies. Other, commonly known neuropathogens could not be detected.

Overall, red-necked wallabies appear to be highly susceptible to RusV as novel neuropathogen, which is broader distributed in northeastern Germany.

Introduction

Recently, the rustrela virus (RusV; species *Rubivirus strelense*) has been described as a new fatal pathogen of different zoo and wild animal species (Bennett et al., 2020a, 2020b; Pfaff et al., 2022). Before the discovery of RusV and its relative ruhugu virus (RuhV;*Rubivirus ruteetense*) (Bennett et al., 2020b), rubella virus (RuV; *Rubivirus rubellae*), the cause of "German measles" in humans (Lambert et al., 2015), was the sole member of the genus*Rubivirus* , family *Matonaviridae* (Rubing Chen, 2018). The highly contagious RuV occurs worldwide, however, is restricted to humans (Lambert et al., 2015).

So far, there are only individual reports of RusV infections associated with fatal non-suppurative meningoencephalitis in a donkey (*Equus asinus*), a capybara (*Hydrochoeris hydrochaeris*), a red-necked wallaby (*Macropus rufogriseus*), and a South American coati (*Nasua nasua*) all originating from the same zoo in northern Germany as well as a wild Eurasian otter (*Lutra lutra*) from the proximity of this zoo. The virus was also detected in brain tissues of wild, yellow-necked field mice (*Apodemus flavicollis*), which had been collected in the vicinity of the zoo and did not exhibit detectable encephalitis (Bennett et al., 2020a, 2020b; Pfaff et al., 2022).

The knowledge on this new pathogen and the disease associated with the infection is limited so far. The full host range and the geographic distribution of the virus are unknown and the clinical and pathological data are limited. However, this information is of high importance for a suitable risk management and prevention strategies.

Here, we describe the clinical symptoms and pathology of three new cases of affected red-necked wallabies (*Macropus rufogriseus)* from different locations in northeastern Germany.

Material and Methods

Three red-necked wallabies were clinically examined after noticed to show neurological symptoms (Table 1). Therapeutic attempts failed and the animals were euthanized. A complete necropsy and histopathology were performed. Samples from different tissues, including lungs, liver, kidney, heart, stomach, small and large intestine, as well as brain, were routinely fixed in 4% formalin, paraffin embedded (FFPE) and cut into 3 µm sections followed by hematoxylin and eosin staining (H&E) for light microscopic evaluation. Consecutive sections were standardly stained with Luxol fast blue/cresyl violet (LFBKV) to evaluate myelin sheaths and Nissl substance, with von Kossa to demonstrate dystrophic mineralization and with Prussian blue reaction to detect hemosiderin as previously described (Bennett et al., 2020b). Immunohistochemistry for CD3 as a T cell marker, ionized calcium-binding adaptor molecule 1 (iba-1) as a marker for microglia and macrophages, and glial fibrillary acidic protein (GFAP) for astrocyte detection was performed as previously described (Bennett et al., 2020b) using 3-amino-9-ethylcarbazole as a substrate (AEC, Dako Carpinteria, CA, USA)

for chromogen labeling. Mayer's hematoxylin was used for counterstaining. In addition to formalin fixation, tissue samples of the brain, lung, spleen, liver and small intestine were stored at -80°C for subsequent molecular analysis.

Total RNA extraction and RT-qPCR was performed as previously described (Pfaff et al., 2022). For all three animals, partial E1 protein-encoding sequences of 715 nucleotides (nt) length were determined via conventional RT-PCR with subsequent Sanger sequencing of the amplicons by Microsynth Seqlab (Balgach, Switzerland). A Juke-Cantor Neighbor-Joining tree was calculated from the three new sequences together with all RusV sequences available in public databases using Geneious Prime 2021.0.1 (Biomatters Ltd., Auckland, New Zealand). In addition, the RNAScope 2-5 HD Reagent Kit-Red (Advanced Cell Diagnostics, USA) was employed according to manufacturer's instructions for RNA *in situ* hybridization (ISH) using a custom-designed probe targeting the RusV non-structural protein (p200, NSP) open reading frame. A probe against the dihydrodipicolinate reductase (*DapB*) gene was used as a technical negative control. Brain tissue from a red-necked wallaby diagnosed with lumpy jaw disease without signs of meningoencephalitis served as a negative control for detection of RusV in ISH.

To exclude other known neuropathogens, brain samples were tested via PCR for rabies virus (Fischer et al., 2014), mammalian bornaviruses (Schlottau et al., 2018), West Nile virus (Eiden et al., 2010), Usutu virus (Jöst et al., 2011), tick-borne encephalitis virus (Klaus et al., 2010), herpesviruses (Ehlers et al., 1999) and *Toxoplasma gondii* (Talabani et al., 2009).

Results and Discussion

The three red-necked wallabies, aged 1.5 to seven years, showed sudden signs of a therapy-resistant neurological disease and an underlying non-suppurative meningoencephalitis was initially diagnosed histopathologically in all cases (Fig. 1a). Subsequently, the brains were analyzed for putative causative agents. Only RusV was consistently detected in the brain tissue from all animals via RT-qPCR (Table 1), whereas no other neuropathogens analyzed were detectable.

The animals originated from three different zoos in northeastern Germany (Fig. 2). Case 1 occurred in the zoo in Stralsund in which RusV had been originally discovered in encephalitic zoo animals, including a further red-necked wallaby that had died in 2018 (Bennett et al., 2020a, 2020b). Cases 2 and 3 originated from zoos in Perleberg and Paaren, respectively, in which RusV had not been detected before. Notably, a previous case of fatal neurological disease in a red-necked wallaby had been reported also for each of these two zoos but the cause had been unknown as no diagnostic material from these animals was available.

The three investigated wallabies of this study showed a sudden onset of disease with a wide spectrum of neurological signs affecting the locomotor system shortly before euthanasia (Table 1). In cases 2 and 3, the animals were euthanized within 24 h after the reported onset of disease (Table 1). The affected animal of case 1 exhibited hind leg paralysis and ataxia five months prior following a fox attack. It fully recovered until re-occurrence of more severe clinical signs a few hours before euthanasia (Table 1). The nutritional condition of the animals varied from very good (case 2) to cachectic (case 1; Table 1), which is consistent with previous cases (Bennett et al., 2020b; Pfaff et al., 2022). The reduced nutritional condition could indicate a prolonged course of disease, although obvious neurological signs had been observed only shortly before euthanasia. This observation needs to be assessed when data regarding the incubation period of RusV and further factors affecting the infection such as immune status are available.

The necropsies revealed only non-specific gross findings such as acute congestion of the liver, lungs and kidneys and acute, alveolar edema. However, a non-suppurative meningoencephalitis of varying degrees with perivascular lymphocytic cuffing and few microglial nodules was histopathologically detectable in the cerebrum, cerebellum and brain stem in all cases (Table 1 and Fig. 1a). RusV was consistently identified via RNA ISH in these brain localizations predominately in neuronal cell bodies and their processes (Fig 1b), as reported previously for other RusV-infected hosts (Bennett et al., 2020b; Pfaff et al., 2022). Although neurons seem to be the main target cell of the virus, no evidence of significant neuronal degeneration or necrosis was contemporaneously found. In addition, neither dystrophic mineralization was detectable by

von Kossa stain, nor demyelination or loss of Nissl substance by LFBKV stain in any wallaby analyzed. Immunohistochemistry revealed that the perivascular cuffs consisted of numerous CD3-positive T cells and fewer iba-1 labelled microglia/macrophages (Fig. 1c-d). The microglial nodules largely comprised of iba-1 positive cells, with few interspersed T-cells (Fig. 1c-d). Remarkably, there was often no direct local connection between inflammatory reaction or microglial nodules and RusV RNA detection via ISH. Plump, perivascular astroglia were evident in only one localization of case 2 by GFAP labelling (Fig. 1e-f) indicating that an activation of astroglia seems not to be a prominent feature of this infection. Minimal iron deposits were only detected in a focal area in two out of three animals (cases 1 and 3) by Prussian Blue reaction. The pathogenesis of these minimal intravital hemorrhages remains unclear. A possible traumatic impact as a consequence of the neurological disorder could be speculated. Based on the focal, minimal occurrence we hypothesize that RusV-infection does not systematically affect blood vessels. These results are in line with the previously published reports of RusV infection (Bennett et al., 2020b; Pfaff et al., 2022).

In contrast to the striking inflammation of the brain and the detection of the virus in this organ, no other lesions were consistently traceable in the three animals and no RusV-specific RNA was found in other tissues tested by ISH, including heart, stomach, small and large intestine. The restriction of lesions and the viral distribution to the brain is typical for spill-over hosts of neuropathogens such as the Borna disease virus 1 (Schulze et al., 2020). These animals act as dead-end hosts and do not contribute to the spread of the virus. It seems likely that red-necked wallabies may play a similarly restricted role in the epidemiology of RusV. However, further investigations, including a comprehensive, systemic investigation of the route of entry, incubation period, potential further factors that may contribute to the disease, such as stress, viral load, time point of infection, preexisting conditions, such as trauma or parasite infestation, the viral tissue distribution and the analysis of secretions and excretions for the virus, are recommended to clearly address the pathogenesis of this new disease and the epidemiological significance of red-neck wallabies as hosts.

RusV seems to infect a wide range of mammalian hosts (Bennett et al., 2020a, 2020b; Pfaff et al., 2022) and our study raises the number of reported cases of RusV infections in red-necked wallabies to four (Bennett et al., 2020a, 2020b). This species, which is popular in zoological gardens as well as in private holdings, seems to be particularly susceptible to RusV infection, raising the question for putative preventive strategies. In addition to the previously published cases of encephalitic zoo animals and one wild Eurasian otter (Bennett et al., 2020a, 2020b; Pfaff et al., 2022), RusV was also detected in apparently healthy yellow-necked field mice (Bennett et al., 2020b). Certain small mammal species are known reservoir for infectious viruses, such as the Borna disease virus 1 (Hilbe et al., 2006; Puorger et al., 2010), and seem to play a role in the transmission of infections. Whether and how the RusV is transmitted from yellow-necked field mice to other animals is not clarified yet. At this point, pest control seems to be a putative preventive method and is recommended for zoological gardens and other holdings of red-necked wallabies in Northern Germany.

In addition to the first publications of this new disease (Bennett et al., 2020b; Pfaff et al., 2022), our data show that the virus seems to be broader distributed in northern Germany (Fig. 2), indicating a wider epizootic range. Partial RusV E1-encoding sequences of 715 bp lengths were generated for all three animals and revealed a minimal nucleotide identity of 92.7% among the available RusV sequences (Fig. 3). The sequence originating from case 1 from Stralsund was most closely related to the previously determined sequences from this location ([?]98.9% nt identity), whereas the sequences from the two other wallabies were more distantly related to the sequences from Stralsund and among each other (92.9 to 93.7% nt identity), which is in line with their origin from separate locations (Fig. 2 and 3). Novel RusV genomes from this study are available under DDBJ/ENA/GenBank accession numbers: (in preparation).

Taken together, RusV infections should be included on the list of differential diagnoses for neurological disorders and non-suppurative meningoencephalitis in zoo and wild animals, especially red-necked wallabies, at least in Germany. The complete geographic distribution and the entire host range of the virus need to be further investigated.

Ethics statement The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure 1. Histopathology of the brain associated with RusV infection in red-necked wallabies: **(a)** Non-suppurative meningoencephalitis with marked perivascular cuffing, H&E stain.**(b)** RNA ISH, detection of RusV specific RNA in neuronal bodies and processes, fast red as chromogenic labeling, Mayer's hematoxylin counter stain. **(c)** Immunohistochemistry for iba-1 as a marker for microglia and macrophages revealing few microglial nodules (asterisk). **(d)** Immunohistochemistry for CD3 as a T cell marker on a consecutive slide to (e), indicating perivascular lymphocytic cuffing consisting of numerous T cells (arrow) and microglial nodules containing few interspersed T cells (arrow head). **(e)** and**(f)** Immunohistochemistry for GFAP as a marker for astrocyte detection, indicating **(e)** plump appearance as sign of activation of astrocytes in one region and in comparison, **(f)**normal appearance of astrocytes in other regions. (c)- (f) AEC for chromogen labeling, Mayer's hematoxylin counter stain. Scale bar 50 µm (a)-(b) and (e)-(f), 100µm (c) and (d).

Figure 2. Current geographic distribution of RusV infections in Germany in red-necked wallabies. Further cases of RusV infections occurred in other zoo and wildlife species in the region of case 1 of this study.

Figure 3. Phylogenetic analysis of partial RusV E1-encoding sequences (715 nucleotides, representing genome positions 8,208 to 8,922 of the RusV reference genome MN552442.2) was performed using the Neighbor-Joining algorithm and Jukes-Cantor distance model in Geneious 11.1.5. RusV sequences from red-necked wallabies determined during this study are depicted in bold. Values at branches represent support in 1,000 bootstrap replicates. Only bootstrap values [?]70 at major branches are shown. GER: Germany; BB = Brandenburg; MV: Mecklenburg-Western Pomerania.

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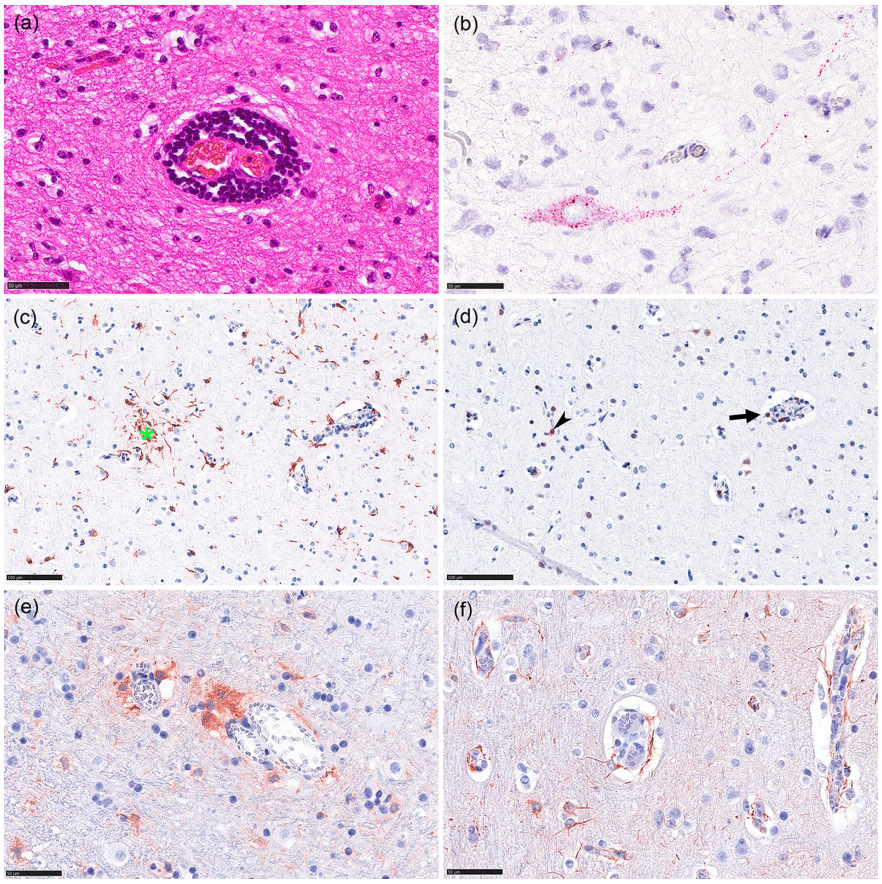

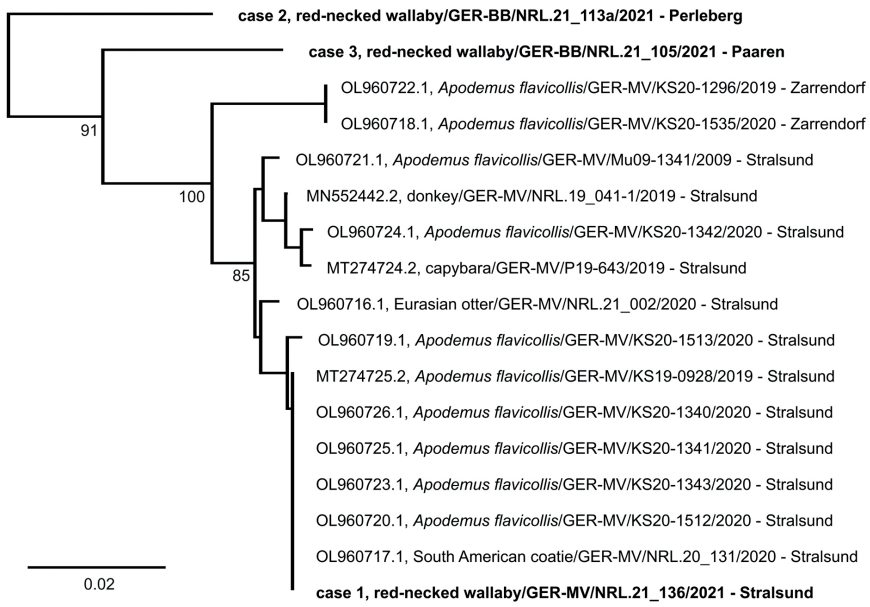

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