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ORIGINAL ARTICLE

A novel alphaherpesvirus and concurrent respiratory cryptococcosis in a captive koala (*Phascolarctos cinereus*)

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A novel alphaherpesvirus was detected in a captive adult, lactating, female koala (*Phascolarctos cinereus*) admitted to James Cook University Veterinary Emergency Teaching & Clinical Hospital in March 2019, showing signs of anorexia and severe respiratory disease. Postmortem examination revealed gross pathology indicative of pneumonia. Histopathology demonstrated a chronic interstitial pneumonia, multifocal necrotising adrenalitis and hepatitis. Intranuclear inclusion bodies were detected by light microscopy in the respiratory epithelium of the bronchi, bronchioles, alveoli, and hepatocytes, biliary epithelium and adrenal gland associated with foci of necrosis. *Cryptococcus gattii* was isolated from fresh lung on necropsy, positively identified by PCR, and detected histologically by light microscopy, only in the lung tissue. A universal viral family-level PCR indicated that the virus was a member of the Herpesviruses. Sequence analysis in comparison to other known and published herpesviruses, indicated the virus was a novel alphaherpesvirus, with 97% nucleotide identity to macropodid alphaherpesvirus 1. We provisionally name the novel virus phascolarctid alphaherpesvirus 3 (PhaHV-3). Further research is needed to determine the distribution of this novel alphaherpesvirus in koala populations and establish associations with disease in this host species.

The koala (*Phascolarctos cinereus*) is a native, Australian marsupial with global recognition, and listed as vulnerable to extinction under the *International Union for Conservation of Nature* (IUCN) Red List of threatened species.[1] Koalas have been identified as one of ten species most vulnerable to climate change.[1] They are arboreal marsupials, inhabiting native *Eucalyptus* trees in forests and woodlands, and specialist folivores, with a diet consisting largely of specific *Eucalyptus* leaves, supplemented with *Eucalyptus* stems, bark and flowers and occasional leaves of other Myrtaceous species.[2]

In Australia, many wild koala populations exist in coastal bushland areas and in offshore Islands including Magnetic Island, Townsville.

Those in northern Queensland are under threat from disease; ecosystem destruction from environmental degradation, rapid urban and coastal development, habitat displacement by invasive pest plant and weed species; severe extreme weather events, such as tropical cyclones, tropical monsoon floods, bushfires, droughts and extreme heat.[3] Commonly reported causes of koala mortality or morbidity in Australia include vehicular trauma; domestic animal, feral cat and native animal attacks or predation and disease.[4–15]

Koalas in the Townsville region of northern Queensland appear to be susceptible to trauma, and infectious diseases including melioidosis, cryptococcosis, chlamidiosis and respiratory disease of unknown cause (unpublished data, James Cook University, Veterinary Pathology Diagnostic Services). Pathology studies done on koalas necropsied in south-east Queensland and in South Australia have similarly shown that trauma and infectious diseases make up a large percentage of the diagnoses, with neoplasia, viral infection, mange and nephrosis less frequently diagnosed.[15, 16]

The *Herpesviridae* family is composed of enveloped, double-stranded, DNA viruses that infect mammals, birds, reptiles and fish. The viral family is further subdivided into three subfamilies, the *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. The Alphaherpesviruses are a large subfamily containing 5 genera with numerous species and naturally infect a large range of marsupials.[17]

Herpesviruses have been documented since 1976 in Australian marsupials causing mortalities in various captive and wild species, including; grey dorcopsis wallabies (*Dorcopsis luctuosa*), parma wallabies (*Macropus parma*), tammar wallabies (*Macropus eugenii*), eastern grey kangaroos (*Macropus giganteus*), western grey kangaroos (*Macropus fuliginosis*), red kangaroos (*Macropus rufus*), common wombats (*Vombatus ursinus*), long-nosed potoroos (*Potoris tridactylus*), Quokkas (*Setonix brachyurus*), woylies (*Bettongia penicillata*), rufous bettongs (*Aepyprymnus rufescens*), greater bilbies (*Macrotis lagotis*) and Lumholtz's tree kangaroos (*Dendrolagus lumholtz*).[18–31] The alphaherpesviruses identified causing mortalities in marsupials include Macropodid alphaherpesvirus 1 (MaHV-1), Macropodid alphaherpesvirus 2 (MaHV-2), and Macropodid alphaherpesvirus 4 (MaHV-4).[27, 31–34] To date, no known alphaherpesviruses have been described causing disease in koalas, but two gammaherpesviruses, Phascolarctid gammaherpesvirus 1 and 2 (PhaHV-1 and PhaHV-2), have been well described from koalas, but do not appear to be associated with clinical disease.[35–37]

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Cryptococcosis is a disease that has been well described in koalas but has not been previously associated with alphaherpesvirus infection.[7] This paper aimed to describe the clinical, histopathological and molecular biology findings associated with a novel alphaherpesvirus that was detected in, and isolated from a sick, captive, koala showing severe respiratory signs and concurrently infected with the yeast *Cryptococcus gattii*. To our knowledge, this is the first alphaherpesvirus detection and associated disease in a koala. Further research is needed to determine the role of this virus in causing disease in captive and wild koala populations.

Materials and methods

Clinical history

A 9-year-old captive female koala weighing 4.8 kg, was admitted to the Veterinary Emergency and Clinical Teaching Hospital, James Cook University, Townsville, Australia on March 27th, 2019. The koala presented with clinical signs of lethargy, weakness, anorexia and severe dyspnoea. The koala was bred in captivity and reared at a local wildlife park. It was housed in an enclosure with six other koalas. Other resident marsupials at the wildlife park included red-legged pademelons (*Thylogale stigmatica*), red kangaroos (*Macropus rufus*), eastern grey kangaroos (*Macropus giganteus*), agile wallabies (*Macropus agilis*) and wombats (*Vombatus ursinus*). The original breeding colony of koalas came from a mainland population from the Mackay region some years ago. Treatment was immediately initiated and consisted of oxygen support via an oxygen cage at 100% oxygenation. Intravenous catheterization was placed and Hartmann's with 5% dextrose was administered intravenously (IV), at 3 mls/kg/hr. Furosemide was administered 1 mg/kg IV, Trimethoprim-sulphamethoxazole 15/mg/kg subcutaneously and butorphanol intramuscularly (IM) at 0.2 mg/kg. Thoracic radiographs showed a nodular diffuse, bronchial interstitial pattern with mild pleural effusion. Despite treatment the koala developed increasing respiratory distress and humane euthanasia was opted due to significance of disease and poor prognosis. The koala was administered butorphanol for analgesia at 0.2 mg/kg IM and euthanased with IV pentobarbitone.

Pathology

A necropsy was performed within 48 hours. Organs and tissues examined and sampled included the brain, eyes, heart, lungs, trachea, liver, spleen, kidney, adrenal gland, thyroid gland, tracheobronchial and medullary lymph nodes, tongue, oesophagus, stomach, pancreas, small and large intestine, caecum, ovary, uterus, cervix, bladder, skeletal muscle and bone marrow. All tissues were fixed in 10% buffered neutral formalin for 48 hours, then processed routinely for histology. Paraffin embedded tissues were sectioned at 5 μm and stained with Hematoxylin and Eosin for light microscopy. Special stains performed on lung tissue included Brown-Hopp's Gram, Periodic acid-Schiff (PAS), Giemsa, and Grocott-Gomori's methenamine silver stain (GMS).

Bacteriology and mycology

A deep nasal swab was taken for fungal culture. Fresh lung and heart were sampled for aerobic and anaerobic bacterial and fungal culture. Cloacal, oropharyngeal and conjunctival swabs were taken for real-time PCR (qPCR) testing for *Chlamydia* and for fungal culture. Fresh lung was stored at -80°C for virology.

Lung and heart tissues were processed aseptically in a Biosafety Class II cabinet (ESCO), and were dilution streaked onto a variety of selective and enrichment agars using standard microbiological procedures.[38] Tissues were incubated for up to 7 days at 37°C both aerobically and aerobically. The nasal, cloacal, oropharyngeal and conjunctival swabs were grown under aerobic conditions for the presence of fungi. Representative colonies were identified using morphological and biochemical identification protocols for the bacteria and morphological and genotyping protocols for fungi.[38]

DNA was extracted from the nasal, cloacal, oropharyngeal and conjunctival swabs for *Chlamydia* identification using the QuickGene DNA tissue kit according to the manufacturer's instructions. A realtime polymerase chain reaction (qPCR) was then carried out on 2 μL replicates of the extracted DNA using the following reagents: 10 μL SensifastSYBRHiRox (Bioline, Australia); 0.6 μL (6 pmol) forward primer Chuni1-23S-rRNA (5′-GGGCTAGACACGTAACCTA-3′), 0.6 μL (6 pmol) reverse primer Chuni2-23S-rRNA (5′-CCATGCTTCAACCTGGTCATAA-3′) (Macrogen, South Korea) and 6.8 μL ultrapure water.[39] A MIC magnetic induction cycler (Biomolecular Systems) amplified target DNA for 35 cycles at the following temperatures: denaturation at 95°C for 5 seconds, annealing of the primers at 60°C for 10 seconds and extension of the target DNA for 10 seconds. Once the variable heating cycles had completed the melting point of the PCR product was determined and a single melting point of 84°C which matched the known positive control was considered positive. The same protocol using different primers was used to identify whether cultured *Cryptococcus* species was *Cryptococcus neoformans* (Forward primer CnIGS1F 5′-GCTTGGAT TGCACACACACTCTCATC-3′; Reverse primer CnIGS1R 5′-CTTACTTGCTGCAAGGCGTCTTACC-3′) or *Cryptococcus gattii* (Forward primer CgIGS1F 5′-GTCATTTCAGCTGGCATCGAT-3′; Reverse primer CgIGS1R 5′-TTGCCGCATAACGCTCTTAGCCA-3′) (Macrogen, South Korea). If the test DNA and positive *C. gattii* control had a melting point of 85.4°C, with no amplification with the *C. neoformans* primers, the yeast was identified as *C. gattii*.

Virology

DNA was extracted from the koala lung biopsy (sample L9-329) using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A previously published pan-herpesvirus PCR assay, targeting the DNA polymerase gene (UL30) was used to screen the sample for herpesvirus DNA.[40] PCR products of the expected size (~300 bp) were sent to the Australian Genome Research Facility (AGRF) for Sanger capillary sequencing.

Contiguous nucleotide sequences were assembled and edited, followed by multiple sequence alignment using Geneious Prime version 2020.2.4 (Biomatters Ltd). Phylogenetic analyses were conducted on herpesvirus sequences using MEGA X version 10.1.7 including selected alphaherpesvirus sequences sourced from the GenBank Database.[41]
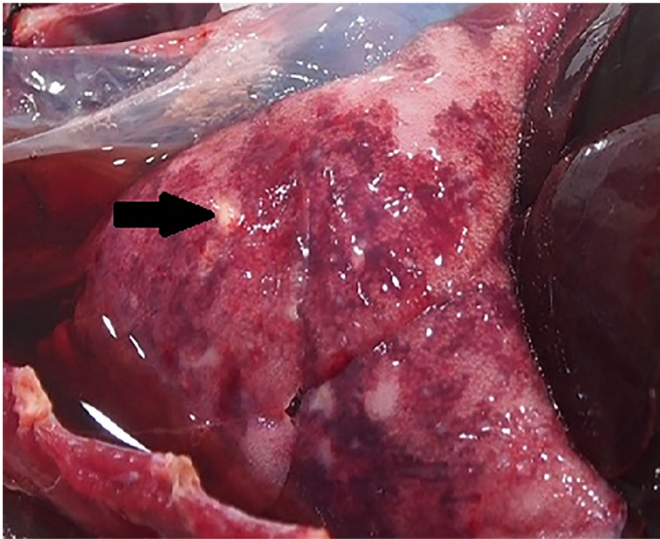
FIGURE 1. Lungs from a koala (*Phascolarctos cinereus*), are mottled, red to pink, with a confluent, pale to cream coloured granuloma (arrow)

FIGURE 2. Photomicrograph of lung from a koala (*Phascolarctos cinereus*) showing pulmonary interstitial fibrosis and hyaline membrane formation. Inset shows higher power magnification of the respiratory epithelium with nuclear hypertrophy, eosinophilic, intranuclear inclusion bodies, and margination of nuclear chromatin (arrowheads), and necrosis. 1,000X, H&E stain

Results

Gross pathology

On external examination, the koala was in fair body condition and had mild tartar build up on the teeth. The mandibular gingivae had several small 2 mm diameter, oval ulcers with hyperaemic borders, and the maxillary gingivae, lips and ventral surface of the tongue had patchy erythema and mild petechiation. There was mild faecal staining on the perineum. Internally the distal third of the trachea contained a moderate amount of stable, red-tinged foam. A moderate amount of serosanguinous fluid was observed in the thoracic cavity. The lungs were diffusely emphysematous, and mottled pink to red in colour (Figure 1). Multifocally and randomly distributed throughout all lung lobes were 1–3 mm, round to oval, cream to white, confluent, subpleural granulomas, that contained a creamy-white caseous exudate on cut surface and multifocal areas of haemorrhage. All other organs and tissues appeared normal.

Histopathology

The main histopathological changes were observed in the lungs, liver and adrenal glands. In the lungs, there was a severe chronic bronchopneumonia with multifocal granulomas and interstitial, peribronchial and peribronchiolar fibrosis, with variably associated, partial to complete effacement of the bronchioles. There was a marked necrotising alveolitis, with Type II pneumocyte hyperplasia and hyaline membrane formation, with an accompanying increase in the thickness of the alveolar walls (Figure 2). Multifocally, the epithelium lining the bronchi, bronchioles and alveoli had enlarged, vesicular nuclei, with marginated nuclear chromatin and eosinophilic, intranuclear inclusion bodies, often associated with necrosis of adjacent respiratory epithelial cells (Figure 2).

Within the granulomas were numerous, narrow-based budding yeasts, measuring 5–10 µm in diameter with a thick mucinous capsule, consistent with *Cryptococcus* sp. These organisms were admixed
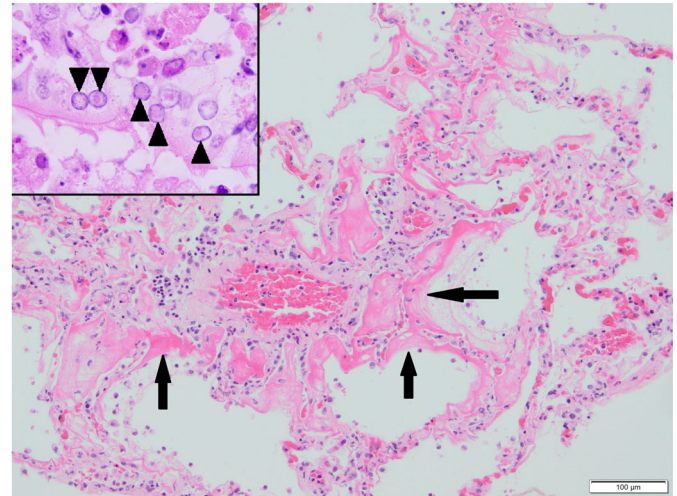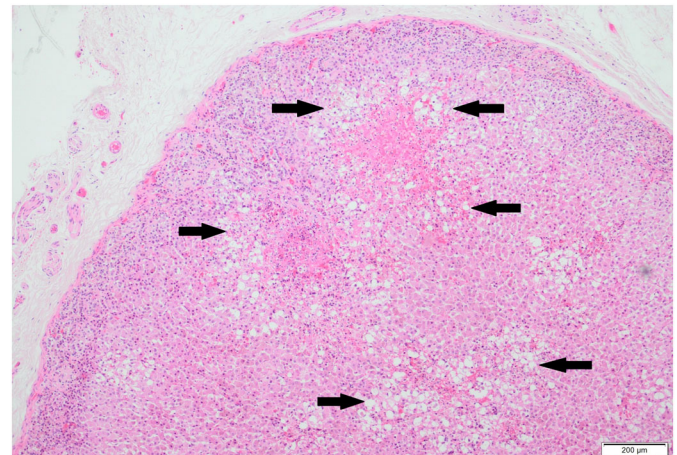

FIGURE 3. Photomicrograph of adrenal gland from a koala (*Phascolarctos cinereus*), with adrenocortical vacuolar degeneration and necrosis. H&E stain

with inflammatory cells and necrotic cellular debris. At the periphery were variable numbers of macrophages and multinucleated giant cells, bordered by a thick layer of fibrous connective tissue.

The adrenal gland had multifocal to coalescing areas of necrosis, haemorrhage and vacuolation. Nuclear changes included nuclear hypertrophy, margination and peripheral beading of chromatin and the presence of eosinophilic intra-nuclear inclusion bodies in the secretory cells of the cortex and medulla (Figure 3). Foci of necrosis were randomly distributed throughout the hepatic parenchyma and the hepatocytes and biliary cells immediately adjacent to these sites
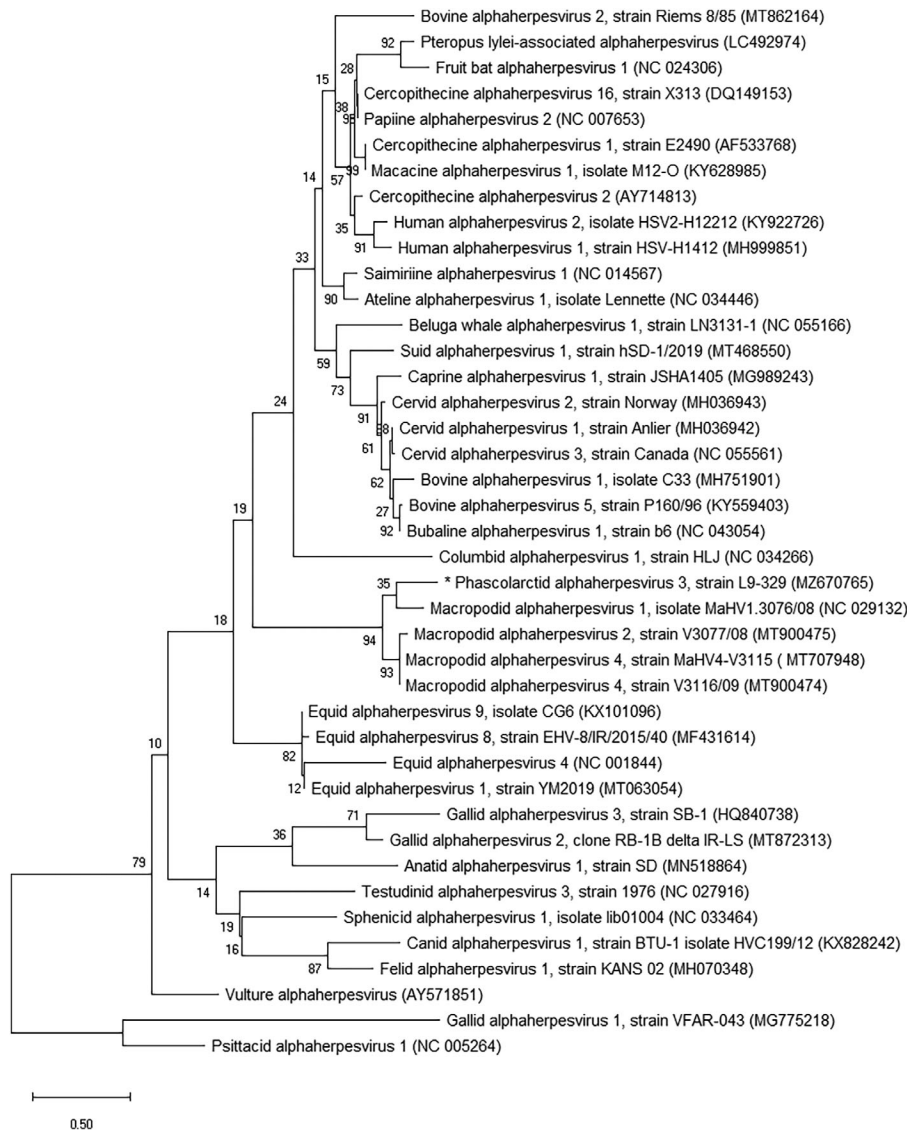
FIGURE 4. The evolutionary relationship of Phascolarctid alphaherpesvirus 3 compared to representative alphaherpesviruses based on partial DNA polymerase (pol; UL30) gene sequences. The tree was built using the maximum likelihood method based on the Tamura 3-parameter model to compute the evolutionary distances. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA X (version 10.1.7)

occasionally contained eosinophilic to basophilic, intranuclear inclusion bodies.

The spleen and medullary lymph nodes had marked lymphoid depletion with necrosis and haemorrhage, and mild histiocytic hyperplasia of the red pulp.

Bacteriology and mycology

No aerobic or anaerobic bacteria were cultured from the heart or lungs and PCR was negative for *Chlamydia* species. A *Cryptococcus* sp. was cultured from the nasal swab and lung tissue, identified as *Cryptococcus gatti* by PCR.

Virology

Screening of the koala lung sample (L9-329) using the universal herpesvirus PCR assay[40] resulted in an amplicon of approximately 300 bp. Subsequent phylogenetic sequence analysis, showed that the

herpesvirus clustered with other alphaherpesviruses that have been detected in Australian marsupials, including macropodid alphaherpesvirus 1 (MaHV-1), macropodid alphaherpesvirus 4 (MaHV-4), and macropodid alphaherpesvirus 2 (MaHV-2) with 77.2%, 77.2% and 74.4% nucleotide identity in the UL30 region, respectively (Figure 4).

Discussion

A captive koala (*Phascolarctos cinereus*) that presented with severe dyspnoea, and died from chronic bronchointerstitial pneumonia, was found to be concurrently infected with a novel alphaherpesvirus and *Cryptococcus gattii*. The histopathology lesions were consistent with a herpesvirus infection, with multiple, eosinophilic to basophilic, intranuclear, inclusion bodies detected in several organs. To the authors' knowledge, this is the first alphaherpesvirus reported in a koala.

Histopathology revealed the presence of viral inclusion bodies that were associated with necrosis of the respiratory epithelium and interstitial tissue of the lungs, hepatocytes, biliary epithelium and cortico-medullary secretory cells of the adrenal gland. Similar lesions associated with the presence of viral inclusion bodies have been detected, variously, in the same organs of other marsupials infected with alphaherpesviruses.[18, 20–22, 25, 31, 42–44] The lesions observed in this koala were very similar to those described from a colony of 9 greater bilbies (*Macrotis lagotis*), that died suddenly from acute, severe respiratory disease, with infection of an alphaherpesvirus closely related to MaHV-1.[29] As described in the bilbies, the koala had eosinophilic inclusion bodies in the respiratory epithelium of the bronchi, bronchioles, alveoli, and adrenocortical cells and hepatocytes associated with cellular necrosis and hyaline membrane formation in pulmonary alveoli.[29] Other histological changes noted in this koala included peribronchial, peribronchiolar and interstitial fibrosis, indicating a chronic respiratory disease process.

Domesticated mammals infected with herpesviruses have shown similar pathology to that of the koala and bilbies.[45] Hyaline membrane formation in the lungs can result from a legion of other possible aetiologies in animals including larval parasite migration, acid-induced injury from aspiration of sterile vomitus, thermal injury from inhalation of smoke or steam during bush fires, chronic exposure to inhaled toxic gases such as nitrogen dioxide, sulphur dioxide,100% oxygen, ammonia, ozone, ventilator-induced lung injury, inhalation of fibreglass or silo dust, ingestion of toxic plants such as *Brassica* sp., or Crofton weed, septicaemia, endotoxaemia, adverse drug reactions or acute hypersensitivity pneumonitis.[45] In this case, the koala had no known exposure to any chronic airway irritants, poisons, toxic gases or other noxious substances and had not ingested any toxic plants. All seven koalas in the same enclosure were fed on the same diet, of freshly picked eucalyptus leaves. The other six koalas co-inhabiting the enclosure did not develop clinical signs of respiratory disease. Other known causes of respiratory disease in koalas include *Chlamydia pecorum*, *Bordetella brontiseptica*, and *Burkholderia pseudomallei* however no bacteria were isolated from the lung of this koala, the histopathology did not indicate a bacterial infection, and PCR was negative for *Chlamydia* species.[6]

A single *Cryptococcus gattii* colony was isolated from fresh lung tissue. Histologically, these organisms were confined to the lungs with no histopathological evidence of haematogenous dissemination to other organs or tissues. Koalas can carry *Cryptococcus* sp. in their nasal cavities or turbinates subclinically and can progress to develop upper or lower respiratory tract symptoms with pneumonia in 73% of respiratory tract cases.[7, 46] It is possible that the alphaherpesvirus was a latent herpesvirus infection reactivated in this koala during a period of physiological stress, from the increased demands of lactation, adverse weather events, and the concurrent infection with *Cryptococcus gattii* leading to the severe respiratory disease. The koala was negative for *Chlamydia* species and no other bacteria, fungi or yeasts were isolated from other organs or tissues.

Herpesviruses previously isolated from koalas include two gammaherpesviruses; Phascolarctid gammaherpesvirus 1 (PhaHV-1) and Phascolarctid gammaherpesvirus 2 (PhaHV-2).[35–37] A recent molecular and serological study of wild koala populations in South Australia showed both PhaHV-1 and PhaHV-2 were highly prevalent, with over 66% of koalas actively shedding the virus, but the koalas studied showed no clinical signs of disease.[47] To our knowledge, this is the first alphaherpesvirus reported in a koala, associated with severe respiratory disease.

Three members of the *Alphaherpesvirinae*; (MaHV-1), (MaHV-2) and (MaHV-4) have been previously identified causing respiratory disease and sudden deaths in various species of Australian marsupials. In 1974, a group of captive Parma wallabies (*Macropus parma*) developed severe respiratory disease, hepatitis and sudden death, from infection with MaHV-1.[18] A subsequent experimental challenge trial was done in healthy Parma wallabies, where after inoculation of the virus into wallabies, the disease developed, and the virus was re-isolated from clinically infected wallabies. The virus was then characterised and formerly named "Parma Wallaby herpesvirus."[19, 21] MaHV-1 has also been isolated from a quokka (*Setornix brachyurus*) that had chronic conjunctivitis for over 1 month but then died suddenly.[22] An alphaherpesvirus similar to MaHV-1 has been described causing mild upper respiratory disease and sudden death with 100% mortality in a captive breeding colony of greater bilbies (*Macrotis lagotis*).[29] Experimental challenge studies in eastern grey kangaroos (*Macropus giganteus*) infected with MaHV-1, demonstrated that MaHV-1 was latent and was reactivated following immunosuppression induced by corticosteroid treatment, the authors proposing dissemination of the herpesvirus via the respiratory route.[48]

MaHV-2 has caused two separate disease outbreaks with acute respiratory disease, hepatitis and sudden deaths in two geographically isolated groups of grey Dorcopsis wallabies (*Dorcopsis muelleri luctuosa*).[22] MaHV-2 was also implicated in the death of an orphaned western grey kangaroo (*Macropus fuliginosus*) from the same study.[22] MaHV-4 has been isolated from the nasal swab of an eastern grey kangaroo (*Macropus giganteus*) that displayed chronic rhinitis and conjunctivitis.[27] A captive wombat from a Hungary zoo, died despite 3 days of intensive therapy. Postmortem examination showed the wombat had oedematous lungs, a necrotising non-suppurative myocarditis, glomerulonephritis and focal hepatic necrosis, associated with intranuclear inclusion bodies in the necrotic hepatocytes. Transmission electron microscopy studies done on hepatocytes with inclusion bodies revealed herpesvirus-like virions, and was positive by PCR for herpesvirus. It is unknown if the virus was sequenced.[44]

Several early serological and molecular epidemiological studies have shown that herpesviruses are widespread among Australian native marsupial populations, with a higher prevalence of infection in captive zoo populations compared to wild populations.[19-24,28] Herpesviruses in mammalian species are known for their ability to cause life-long infection and to lie "latent" in their infected mammalian host, becoming "reactivated" following a physiological stressful event, after infection with another disease agent, or with immuno-suppression. In Australia, nearly all herpesvirus disease outbreaks or isolated deaths in marsupials have been recorded in captive zoo populations, where animals were held in higher densities than would naturally occur in the wild or were in contact with another species. Many marsupial species have succumbed to herpesvirus infection and death, following stressful events including recent capture from the wild, translocation from another country or facility, reintroduction into a new facility, or after inter-mixing and contact with a new cohort of marsupials of the same

or with a different marsupial species, or following induced immuno-suppression after corticosteroid treatment.[19–25, 28, 29, 42, 47–49]

In this case, the alphaherpesvirus infection occurred in a single, captive, older, lactating koala, held in a high-density enclosure at a wildlife park, following a 1 in 500-year, heavy rainfall and flooding event in Townsville. It is not known if the koala was a naive host, contracting the novel koala alphaherpesvirus from a co-inhabitant of the wildlife park or wild marsupial species or whether it had a latent infection that was reactivated after a period of physiological stress. The latter scenario would be more likely, given that the koala was lactating with a pouch young, was an older animal of 9 years, and was concurrently infected with cryptococcosis. The six other koalas co-habiting the same enclosure were exposed to the same environmental conditions yet were clinically normal and did not succumb to infection.

The discovery of a novel alphaherpesvirus associated with severe respiratory disease in an adult female lactating koala, is of concern, given that the koala is listed as vulnerable to extinction under the IUCN Red List of threatened species, and is already threatened throughout Australia from infectious diseases such as chlamydiosis and cryptococcosis, habitat destruction from bushfires and deforestation, and extreme weather events such as monsoon flooding and drought.[1, 4, 7, 9–12, 14, 15] Further research is needed to determine the threat that this novel alphaherpesvirus poses to vulnerable populations of koalas and possible interactions with other pathogens such as *Cryptococcus gattii*.

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