Co-localization of hedgehog arterivirus 1 (HhAV-1) and histologic lesions in the European hedgehog (*Erinaceus europaeus*) with neurological disease

Fabian Z. X. Lean[1,7]*, Mark F. Stidworthy[2], Akbar Dastjerdi[3], Tim Partridge[4], Stephen Smith[5], Julie Gough[1], Alejandro Núñez[1], Becki Lawson[6], Katharina Seilern-Moy[6]

[1] Pathology and Animal Sciences Department, Animal and Plant Health Agency, Addlestone, Surrey, United Kingdom

[2] International Zoo Veterinary Group Pathology, Keighley, United Kingdom

[3] Virology Department, Animal and Plant Health Agency, Addlestone, Surrey, United Kingdom

[4] Vale Wildlife Hospital and Rehabilitation Centre, Tewkesbury, Gloucestershire, United Kingdom

[5] Tiggywinkles Wildlife Hospital, Aston Road, Haddenham, Buckinghamshire, United Kingdom.

[6] Institute of Zoology, Zoological Society of London, Regent's Park, London, United Kingdom

[7] Department of Pathobiology and Population Sciences, Royal Veterinary College, North Mymms, Hertfordshire, United Kingdom

* Corresponding author: Fabian Z X Lean (flean22@rvc.ac.uk)

Abstract

The European hedgehog (*Erinaceus europaeus*) is a protected species of conservation concern in the UK. In recent years, there have been multiple incidents of fatal encephalitis in captive hedgehogs in wildlife rescue centers associated with molecular detection of a hedgehog arterivirus (HhAV-1). However, it remains unclear whether the virus is the causative agent of the central nervous system (CNS) lesions. In a retrospective investigation using postmortem material from seven captive hedgehogs with neurological disease, and a single hedgehog with previously identified meningoencephalitis, histologic examination was conducted in tandem with viral RNA *in situ* hybridization (ISH) to appraise tissue distribution of HhAV-1 and the colocalization with histologic lesions. ISH revealed multicellular tropism of HhAV-1 involving monocyte-macrophage and vascular endothelial cells, with viral RNA detected in multiple organs, likely due to endotheliotropism and viremia. In the CNS, encephalomyelitis was mild whilst viral RNA was abundant and widely distributed, particularly in the microglial population and localized to areas with glial nodules. Splenic lymphoid depletion was generally mild but was moderate to severe in two septicemic animals. Brain samples from thirteen control hedgehogs, found dead in the wild due to predation/trauma, were also screened for HhAV-1, of which eight tested positive by real-time RT-PCR with a low viral load. No CNS lesions or ISH labeling was observed in two of these control hedgehogs that could be examined histologically. Combined, these findings indicate that HhAV-1 infections in captive hedgehogs in English wildlife rescue centers may be associated with histopathologic alterations and clinical neurological disease.

Keywords:

Arterivirus, European hedgehog, *in situ* hybridization, neurological disease

Arteriviruses are enveloped, single-stranded positive-sense RNA viruses in the order *Nidovirales*, family *Arteriviridae*, which are classified into six subfamilies (*Crocarterivirinae*, *Equarterivirinae*, *Heroarterivirinae*, *Simarterivirinae*, *Variarterivirinae*, and *Zealarterivirinae*).[4,37] Following an investigation that excluded other viral, fungal, or protozoal etiologies, a novel hedgehog arterivirus (HhAV-1) was detected in juvenile and adult European hedgehogs (*Erinaceus europaeus*), which developed neurological clinical signs in care at a wildlife rescue center in Gloucestershire, South West England, in autumn/winter of 2019-2020.[8] Preliminary histological findings included moderate-to-severe multifocal gliosis, predominantly in the forebrain and hindbrain, and mononuclear meningitis, renal tubular proteinosis, and splenic lymphoid depletion.[8] Additionally, HhAV-1 was retrospectively detected in hedgehogs from two further incidents from wildlife rescue centers in Gloucestershire and Buckinghamshire, during the autumn-winter seasons of 2012-2013 and 2017-2018, respectively. HhAV-1 is a newly identified virus that has only been reported in England and the viral pathogenesis remains unclear.

In this retrospective case-control study, the virological and histologic investigation focused on the aforementioned three incidents in England (2012-2013, 2017-2018, and 2019-2020). This included seven captive hedgehogs from two wildlife rescue centers with histories of neurological clinical signs, and a single previously reported case[36] with histologic evidence of meningoencephalitis. Additionally, thirteen free-living hedgehogs found dead as a result of predation or trauma were included as

controls. To elucidate the potential link between HhAV-1 and the manifestation of neuropathology, and to understand the systemic virus distribution and other possible viral-associated lesions in hedgehogs, correlative histologic examination with *in situ* hybridization (ISH) using RNAScope was conducted on hedgehogs presenting with and without clinical evidence of neurological disease.

Materials and Methods

Case Selection and Postmortem Examinations

These cases were retrieved from the archives of a national scanning surveillance programme (www.gardenwildlifehealth.org), which collates reports of free-living hedgehog morbidity and mortality solicited from members of the public across Great Britain. Clinical cases comprised hedgehogs that died or were euthanized in captivity following hospitalization in wildlife rescue centers and were subsequently submitted for diagnostic investigation. These cases were further defined by the presence of neurological clinical signs or histologic evidence of meningoencephalitis of undetermined etiology, as documented in clinical and laboratory records,[18,36] and subsequently tested positive for HhAV-1 by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR). A control group of 13 free-living hedgehogs was selected for the purpose of virus surveillance and comparison of infection status by real-time RT-PCR, for which the cause of death was established as predation/trauma, and no concurrent evidence of substantial infectious disease was detected on postmortem examination.

Hedgehog carcasses were subjected to comprehensive postmortem examinations,[12,21] with systematic examination of body systems supported by

91 microbiological and parasitological examinations as routine, and histologic
92 examinations conducted in a subset of cases based on antemortem and gross
93 findings. A suite of frozen and formalin-fixed tissues was archived for future study
94 where the state of carcass preservation allowed.

95 *Next-Generation Sequencing (NGS) and Real-Time RT-PCR*

96 NGS and real time RT-PCR were conducted as described previously.[8] Briefly, RNA
97 was extracted from each sample of frozen brain tissue using TRIzol reagent (Thermo
98 Fisher Scientific) and QIAamp viral RNA kit (Qiagen) and processed individually for
99 sequencing on an Illumina NextSeq platform. NGS data assembly was performed
100 using HhAV-1 sequence (GenBank accession number MT415062.1) as referenced
101 and also through *de novo* assembly using SeqMan Pro 17.4 software of the DNASTAR
102 Lasergene Core Suite (DNASTAR, Inc., https://www.dnastar.com). QuantiFast
103 Pathogen RT-PCR kit (Qiagen) was used for the real-time RT-PCR as described
104 previously.[8]

105 *Histology and Chromogenic In Situ Hybridization (ISH) by RNAScope*

106 Histologic examination combined with ISH was conducted on HhAV-1 PCR-positive
107 hedgehogs with clinical or histologic evidence of neurological disease, using a range
108 of available organs where the state of tissue preservation permitted meaningful
109 interpretation. Where necessary to aid in the diagnosis, a selection of tissue sections
110 was also stained using the Gram Twort method for bacteria. Histologic examination,
111 combined with ISH, was conducted on brain tissue from two of the thirteen control
112 cases due to financial constraints and the state of carcass preservation. In addition,
113 brain from a single PCR-negative control case was included to screen for non-specific
114 labelling.

Three sets of twenty-pair double Z proprietary RNA probes targeting *open reading frame (ORF) 6* and *ORF7* of HhAV-1 were designed and produced by Advanced Cell Diagnostics, Inc. The RNA probes were designated as probe A (nucleotide position 12701-13707, HhAV-1 genome accession number MT415062.1) and probe B (nucleotide position 12523-13529) and probe C (nucleotide position 12523-13529) in this report. These RNAscope probes target *ORF6* and *ORF7* of HhAV-1 to maximise sensitivity of RNAScope detection, as these ORFs encoding membrane and nucleocapsid proteins, respectively, are relatively conserved, and are the most abundantly expressed amongst artervirisues.[28] Additionally, the HhAV-1 genome underwent *in silico* evaluation through alignment with the European hedgehog reference genome (mEriEur2.1, GCA_950295305.1). There was no nucleotide alignment detected between the virus genome and the host genome.

ISH was performed using the RNAScope 2.5 HD Brown Detection Kit (Advanced Cell Diagnostics, Inc) as per the manufacturer's instructions, which were previously reported.[22] Formalin-fixed, paraffin-embedded tissue sections of 4μm were collected onto positively-charged slides and were dewaxed and hydrated through xylene and alcohol, respectively. This was followed by treatment with RNAscope hydrogen peroxide for 10 min at room temperature, and heat-mediated retrieval using Target Retrieval Solution for 15 min at 100 °C and Protease Plus for 30 min at 40 °C. RNA probes were then added to sections to allow hybridization for 2 hours at 40 °C followed by 6 rounds of amplification with Hybridise Amp at 40 °C and at room temperature, alternating between 30 and 15 min incubation, in the HybEZ oven. Slides were washed with 2× wash buffer for 2 min at room temperature between incubations. Signal was detected using 3,3'-diaminobenzidine chromogen. Sections were counter-stained with Mayer's haematoxylin (Surgipath), dehydrated in ethanol and xylene, and glass

coverslips were mounted with DPX mounting medium (TCS Biosciences Ltd). Contiguous serial sections from the same tissues were also stained with hematoxylin and eosin according to standard protocols.

Results

NGS and Probe Design

Consensus HhAV-1 genome sequences obtained through NGS were utilized to develop probes for ISH (Supplemental Table S1). Three sets of probes (A, B, and C), designed to target the diversity of HhAV-1 genome sequences identified through NGS, ensured maximum nucleotide identity with their targets. The HhAV-1 *ORF6* and *ORF7* sequences, used for probe sets B and C, had nucleotide identities of 81% and 86%, respectively, with those of the published HhAV-1 sequence (accession number MT415062.1) or probe set A. The nucleotide sequences of the three ISH probes were compared to the sequences obtained from each animal to identify the highest percentage nucleotide identity. Subsequently, only the set of RNA probes with the highest nucleotide identity to the cognate virus sequence, ranging between 86 to 100% nucleotide identity, was used for each affected animal. Probe B was applied to control animals, as it has a wide range of nucleotide identity (81 to 99%) to the HhAV-1 detected in this study.

History and Clinical Findings

Eight captive hedgehogs with neurological disease that tested PCR positive for HhAV-1 were included in this study. These animals were from three morbidity and mortality

incidents reported from two wildlife rehabilitation centers in Gloucestershire (center A) and Buckinghamshire (center B) across separate years: autumn-winter 2012/2013 (incident 1, center A), 2017/2018 (incident 2, center B), and 2019/2020 (incident 3, center A)[8] (Supplemental Table S2). Neurological clinical signs were a consistent feature of affected animals in incidents 2 and 3, affecting between 100 to 200 animals, including both juveniles and adults. In incident 2, cases 3, 4, and 5 (juveniles, two female and one male; Table 1) developed neurological signs approximately four weeks following hospitalization. Clinical signs noted included paddling movements in all limbs, an inability to stand or walk, and an inability to feed. The clinical course of animal 2 (adult male) from incident 2 also involved development of paddling and spasmodic movements prior to succumbing to its condition. In incident 3, cases 6, 7, and 8 (juveniles) developed clinical disease between one week (case 7, female) and seven weeks (cases 6 and 8, male) following hospitalization, deteriorated rapidly over the course of three to four days, and subsequently developed neurological signs, including tremors, twitching, ataxia/paresis, falling to the sides, paddling when laterally recumbent, and hyperesthesia. The animals were inappetent, dehydrated, and had lost weight. In contrast, clinical signs were varied and non-specific in incident 1, from which the female juvenile hedgehog with histologic evidence of encephalitis was retrospectively identified (Supplemental Table S2). This incident involved multiple mortalities of juvenile hedgehogs, with animal 1 being euthanised after minimal improvements in its condition despite two to three months of hospitalization. Real-time RT-PCR conducted on brain samples yielded cycle threshold (Ct) values ranging from 19.13 to 24.43.

Control cases were included in this study (total n=13); nine from road traffic accidents , three with predation as the proximate cause of death, and one with an undetermined

cause of death, out of which eight tested positive for HhAV-1 by PCR with Ct values ranging from 29.8 to 36.2 (Supplemental Table S3). Histologic examination and ISH were conducted on two of the PCR-positive control cases (cases 9 and 10) where formalin-fixed brain tissue was available (Table 1).

Histologic Findings in Hedgehogs with Neurological Disease

Central nervous system. In the cerebrum (Table 2) of cases that were not complicated by bacteremia (cases 1, 2, and 6- 8; n=5), there was mild to moderate meningoencephalitis (n=5/5), characterized by multifocal glial nodules (n=4/5) (Fig. 1a), scattered lymphocytes and neutrophils in the neuropil (n=2/5), and infrequent lymphocytic perivascular cuffing (n=2/5). These changes were randomly distributed across both gray and white matter. There was no evidence of neuronal necrosis, neuronophagia, myelin degeneration, or spongiosis. Where choroid plexus and meninges were present (cases 3 and 7), the stroma was infiltrated by a small population of lymphocytes and a subset of these cells were labelled for viral RNA.

In three animals where bacteremia was suspected (case 5; no other visceral tissues were available for examination) or confirmed in other organs (cases 3 and 4; also see description within the sections of lymphoid system, kidney, and liver), occasional vascular thrombosis was observed (n=3/3) in the cerebral neuroparenchyma, with one case (animal 3) also showing intra-lesional gram-negative coccobacillary emboli. Similarly, these animals also presented with multifocal glial nodules (n=2/3) and scattered lymphocytes and neutrophils in the neuropil (n=2/3).

ISH labeling performed on the brain sections (n=7, material was exhausted for case 2) revealed multifocal scattered to abundant viral RNA labeling in both glial and

vascular endothelial cells (n=5/7), with a greater proportion of labelling present in glial than endothelial cells (Fig. 1b). The abundance of viral RNA labelling or cell tropism did not differ between bacteremic and non-bacteremic animals. In a small number of cases (n=2/7) only vascular labeling was detected, including a case that was complicated by bacteremia (case 3). Nevertheless, the viral RNA positive vascular endothelial cells were histologically unremarkable on correlative evaluation. On the other hand, glial labeling was often co-localized with areas of gliosis but was also present in areas where gliosis was not apparent on hematoxylin and eosin-stained sections.

In the cerebellum of cases that were not complicated by bacteremia, there was rare glial nodule formation (n=2/5) and peri-vascular lymphocytic cuffing (n=2/5). In the bacteremic cases, there were scattered glial nodules, neutrophilic infiltrates, or necrotic cellular debris (Fig. 1c) in the molecular layer or cerebellar peduncles (n=2/3, Fig. 1d); endothelial hypertrophy (n=1/3); and lymphocytic or neutrophilic infiltration of the meninges (n=2/3). Concurrent glial and vascular endothelium labeling was observed in majority of the cases (n=5/7), with a predominance of glial labeling over vascular endothelium (Fig. 1d). In isolated cases, there was either glial (case 6, n=1/7) or vascular endothelial (case 1, n=1/7) labeling.

Out of the four cases in which the brainstem was examined, there was only one case uncomplicated by bacteremia, in which histology revealed rare glial nodules or lymphocytic perivascular cuffing. Two of the three animals that were complicated by bacteremia also exhibited similar glial nodules and lymphocytic perivascular cuffing, and one animal was histologically unremarkable. Concurrent glial and vascular endothelial labeling was observed in these cases, with the abundance ranging between rare to multifocal and moderate.

In the four spinal cords examined, there was mild, multifocal gliosis in the gray matter, with occasional dispersed neutrophils within the neuropil (n=3/4) in both bacteremic (cases 3 and 4) and non-bacteremic (cases 2 and 5) cases. The viral RNA labelling was multifocal, with the involvement of both the gray and white matter (Fig. 1e). In one of the non-bacteremic animals (case 4), there was mild neuronal degeneration and vacuolated neuropil (Fig. 1e), which colocalized to areas with glial labelling (case 2, n=1/4; Fig. 1f). Hypertrophied vascular endothelium was infrequently detected in a bacteremic animal (n=1/4).

In summary, the brain and spinal cord consistently presented with glial nodules along with infrequent lymphocytic and neutrophilic infiltration in the neuropil, with similar labeled cell types and abundance of viral RNA, regardless of the presence of bacteremia.

Spleen. Of the spleens examined (n=7), non-bacteremic cases (n=5/7) exhibited mild to moderate lymphocytolysis (Fig. 2a). This was characterized by depletion of the white pulp, increased apoptotic bodies and tingible body macrophages in the germinal centers (Fig. 2b), and scattered fibrin deposits within the red pulp. In contrast, lymphocytolysis was marked in two bacteremic animals (cases 3 and 4; intra-lesional gram-negative coccobacillary emboli and isolation of *Klebsiella pneumoniae spp pneumoniae*, case 3). No bacteria was isolated from the other hedgehog, possibly as a consequence of antimicrobial treatment in care.

In all spleen samples, viral RNA was generally abundant, and densely labeled the marginal zone of the white pulp (n=7/7; Fig. 2a, inset). Additionally, a moderate amount of viral RNA was detected in histiocytes and dendritic cells within the parafollicular areas or scattered in the red pulp. Extramedullary haematopoiesis was observed in all

of the spleen samples. Extramedullary haematopoiesis is common in hedgehogs as an incidental finding in the spleen[45] and was not co-localized with viral RNA labeling.

Lymph node. Three lymph nodes (location not specified) were available for examination from cases 1, 3, and 4. Moderate to severe necrotizing lymphadenitis with intra-lesional gram-negative coccobacillary emboli was detected in two bacteremic cases (cases 3 and 4). Viral RNA was present in histiocytes and/or dendritic cells in both the cortex and medulla, and in the germinal centers with higher density of labeling in the mantle region. The lymph node from the non-bacteremic animal was histologically unremarkable, and viral RNA was rarely detected in capillaries of both the cortex and medulla.

Liver. Of the six livers examined, the two bacteremic animals (n=2/6, cases 3 and 4) exhibited moderate to severe, multifocal to coalescing, random, coagulative hepatic necrosis, with thrombosed sinusoids and intralesional gram-negative coccobacillary emboli. While there was a periportal labeling of Kupffer cells, there was also widespread viral RNA labeling co-localized to areas of necrosis (Fig. 2c).

In histologically unremarkable liver (n=4/6), there were small to moderate amounts of extramedullary hematopoiesis within the hepatic sinusoids (n=3/6; considered an incidental finding in hedgehogs[45]), but this was not associated with viral RNA labeling. A moderate amount of viral RNA with a periportal distribution was detected (Fig. 2d), with most labeling present in Kupffer cells and rarely within the endothelium of central veins.

Kidney. Among the six kidneys examined, four cases were generally unremarkable histologically, with mild tubular lipidosis and proteinuria. Two bacteremic cases (cases 3 and 4, n=2/6) exhibited moderate to severe, multifocal to coalescing, acute,

tubulointerstitial nephritis with cortical infarctions (Fig. 2e). Additionally, gram-negative coccobacillary emboli were detected in one of these two animals (case 3). The amount of viral RNA labeling was moderate to abundant, present extensively within and at the edge of the infarcted areas, predominantly in the interstitial vessels and infrequently in renal tubules (Fig. 2e). One of the cases had dilated renal calices and within the lumen there were moderate amounts of degenerate neutrophils, macrophages, and sloughed renal tubular epithelia, within which a subset of cells were viral RNA positive (Fig. 2f). In non-bacteremic cases, viral RNA detection was rare to scattered, present in the capillaries of the inter-tubular spaces and rarely within the glomerular tufts.

Lung. Four of the lungs were histologically unremarkable (n=4/7). The other three animals exhibited moderate to severe interstitial pneumonia with concurrent bacterial emboli in two animals (cases 3 and 4, in which bacterial emboli were also found in the spleen, lymph node, liver, and kidney), and intrabronchial nematode infestation in one animal (case 4, presumptively *Crenosoma striatum*). The interstitial lesion comprised of moderate multifocal expansion of alveolar walls with edema, fibrin deposits, degenerating neutrophils, and erythrocytes, and occasionally the alveolar spaces were lined with hyaline membranes (Fig. 3a). In cases with intra-lesional gram-negative coccobacillary emboli, there were perivenular hemorrhages, detachment of endothelial cells, expansion of the tunica media of pulmonary arterioles with edema fluid, and lymphocytic cuffing. Viral RNA labeling was detected in all seven animals with lung available for examination. This ranged from scattered to multifocal and was mostly present in the alveolar wall, with cellular morphologies suggestive of type I pneumocytes, capillary endothelium, or interstitial macrophages (Fig. 3a). Infrequently, there was labelling in the bronchial submucosal capillaries.

Heart. Most cases were histologically unremarkable (n=5/6) apart from one animal with severe multifocal necrotizing myocarditis with intra-lesional gram-negative coccobacillary emboli (case 3, Fig. 3b). These areas were colocalized with abundant viral RNA labeling of the capillaries. In other histologically normal hearts, viral RNA labeling was rare to scattered in the capillaries of the myocardium (n=4) or absent (n=1). Occasionally, bradyzoites were encountered (*Sarcocystis sp.*, considered an incidental finding) in the heart but were not associated with reactive changes or viral RNA.

Digestive tract. The stomach and tongue (Fig. 3c) from one animal (case 2) were available for examination and was histologically unremarkable but contained rare viral RNA labeling in the submucosal capillaries. In the ileum of the same animal, there was moderate lymphoid depletion of the Peyer's patches with presence of tingible body macrophages (Fig. 3d). Strong viral RNA labeling was present in the mantle and marginal zone of the Peyer's patches, in predominantly large mononuclear cells (presumptive dendritic or antigen presenting cells) and scattered labeling was also present among the cellular debris within the germinal centers. In the intestinal (case 2) and colonic (case 3) lamina propria, there was scattered viral RNA labeling of mononuclear cells. While the intestine of case 1 was not further investigated as the paraffin block was exhausted, the initial histology workup revealed localised jejunal cryptosporidiosis.

Adrenal. The adrenals evaluated (n=3) were histologically unremarkable. Viral RNA was rare to scattered and was randomly distributed in the stromal spaces of the cortex and medulla within the capillaries.

Histologic Findings in HhAV-1 PCR-positive Control Hedgehogs

Histologic examination of brain identified no substantial lesions and ISH labeling did not reveal viral RNA *in situ* in the brain of the two control hedgehogs, animals 9 and 10 (Table 2, Supplemental Table S3)

Discussion

The *in situ* detection of HhAV-1 RNA labeling within areas of gliosis in the central nervous system of multiple captive hedgehogs that developed neurological disease strongly supports an association between the virus and histologic lesions. While HhAV-1 RNA was also detected in other tissues, including the lung, liver, heart, kidney, spleen, lymph node, and digestive tract, consistent with a multisystemic viral infection, there was a lower abundance of viral RNA labeling and restricted cellular tropism in non-septicemic animals. In contrast, viral labeling in non-neural lesions in septicemic animals was more abundant, and typically more prominent in endothelium and necrotized areas. However, within the constraints of the small sample size, the potential for extra-neural lesions to be induced directly by HhAV-1 alone cannot be fully elucidated. Experimental *in vivo* studies would be required to fulfil Koch's postulates and definitively prove causation. However, the authors have currently been unable to obtain *ex-vivo* isolates of HhAV-1, and as a protected species in the United Kingdom experimental work to establish a colony of captive hedgehogs of consistent and previously known health status is unlikely to be feasible.

Primary neurological disease associated with arterivirus infection is relatively uncommon. Wobbly possum disease virus is the only virus known to consistently cause neurological disease in the Australian brushtail possum (*Trichosurus vulpecula*), both naturally and experimentally.[13,23,29] The histological lesion in the central nervous system is characterized by mononuclear perivascular cuffing, but the

359 viral pathogenesis remains unclear as the viral tropism has not yet been
360 characterized.[13,23,26,29] Lactate dehydrogenase elevating virus, another arterivirus, can
361 cause encephalomyelitis or radiculoneuritis in laboratory mice by targeting the dorsal
362 root ganglion neuronal cells.[39,40] In large animal species, arterivirus-associated
363 neurological diseases have been sporadically reported, such as highly pathogenic
364 porcine reproductive and respiratory syndrome virus (PRRSV)-associated
365 meningoencephalitis in pigs[5,16,35] and aborted equine fetuses infected with equine
366 arteritis virus (EAV).[19] PRRSV-associated meningoencephalitis in pigs demonstrates
367 viral tropism for the monocyte-macrophage cell lineage within the brain,[5,16,35] and the
368 infection of microglial cells upregulates the expression of pro-inflammatory cytokines
369 and chemokines.[6] Whilst the hedgehogs with observed disease included in this study
370 only exhibited mild meningoencephalitis associated with HhAV-1 infection, cytokine
371 release from microglial infection could potentially contribute to the bio- and/or neuro-
372 chemical dysfunction of neurons and the apparent substantial neurological clinical
373 impact.

374 Viral tropism for the monocyte-macrophage cell lineage, particularly in lymphoid
375 tissues, is a common biological feature shared across arteriviruses including PRRSV,
376 EAV, and simian hemorrhagic fever virus.[15,34,42,44] Infection of the lymphoid tissues
377 often results in viral-associated lymphoid depletion and/or necrosis.[14,16,20,24,26,42] This
378 can result in predisposition to secondary bacterial infection, which has been reported
379 in pigs naturally or experimentally infected with PRRSV,[31,38,41,46] and rhesus
380 macaques (*Macaca mulatta*) experimentally inoculated with simian hemorrhagic fever
381 virus.[20] In the current study, spleens from seven animals were examined of which all
382 presented with lymphocytolysis. Two further diseased hedgehogs (cases 3 and 4) that
383 exhibited splenic lymphocytolysis had concurrent bacteremia (*Klebsiella pneumoniae*

spp. pneumoniae was isolated from case 3), with ischemic and/or thrombotic lesions detected in the brain, heart, lung, kidney, spleen, and lymph node, consistent with septicemia. Given the small sample size in this study, definitive roles for viral or bacterial-mediated immunosuppression cannot be fully determined or excluded. Additionally, elevated corticosterone in captive hedgehogs[33] could be an additional predisposing factor to immunosuppression and/or activation of HhAV-1 infection. While the hypothesis of viral-mediated immunosuppression remains to be elucidated, the concurrent viral and bacterial infections highlight the need for strict biosecurity and sanitation measures to prevent the acquisition of other infections in the wildlife center environment.

Endotheliotropism was another common feature of HhAV-1 infection in the hedgehogs. Infected endothelial cells were generally unremarkable, rarely exhibited hypertrophy, and had no direct evidence of hemorrhage or edema. Contrastingly, in the subset of cases with concurrent bacteremia (cases 3 and 4) there was endothelial hypertrophy, thromboembolism, and infarctive coagulative necrosis in the brain, kidney, liver, spleen, and lymph node. Although the mechanism remains unclear, the prominent vascular labeling observed in bacteremic cases may be attributed to the recruitment of viral-infected immune cells or direct infection of the vascular endothelium. Based on this small number of cases, it seems unlikely that HhAV-1 contributes to a primary vasculopathy or vasculitis. Nevertheless, endotheliotropism remains an important feature in the broader pathogenesis of arteriviruses, facilitating viremia and the dissemination of the virus to various organs.

In the lung, RNA labeling was observed within the alveolar wall, with type I pneumocytes, capillary endothelial cells, and interstitial macrophages considered infected. Presumptive epitheliotropism of HhAV-1 was not detected in tissues other

than the lung. While the potential for epitheliotropism cannot be conclusively disregarded, given the precedence of PRRSV and EAV in respiratory secretions,[30,42] further confirmation will require multi-labeling to elucidate the disease pathogenesis. As for the pathologic implication of epitheliotropism, three out of seven animals demonstrated interstitial pneumonia, two of which had concurrent septicemia; therefore, such histologic lesions seem unlikely to be related to a primary HhAV-1 pulmonary infection.

In contrast to incidents 2 and 3, there were no consistent neurological signs in incident 1, with reported signs being non-specific (e.g., lethargy, inappetence) and varied (e.g. diarrhea, respiratory signs). Upon histologic examination of animal 1, non-suppurative meningoencephalitis was observed, which prompted its inclusion in the study to investigate for a potential viral etiology. ISH revealed the presence of viral RNA in the neuropil colocalized with areas of inflammation, and lymphoid depletion was observed in the spleen and lymph nodes in areas with viral RNA. Additional findings in animal 1, as well as in other hedgehogs that were investigated by gross postmortem examination from incident 1, included verminous pneumonia and intestinal cryptosporidiosis (Animal and Plant Health Agency, United Kingdom,, unpublished data), which are common findings in hedgehogs admitted for care[32,36] that may have further influenced the disease state. It was not possible to determine the extent to which HhAV-1 contributed to the ill-health of animal 1 in isolation, or whether HhAV-1 infection was a common feature affecting other hedgehogs in incident 1. No other animals from incident 1 had suitable material for inclusion in this study.

The mode of transmission of HhAV-1 remains unclear. Histologic analysis revealed the presence of viral RNA in cell exudates within the renal calyx and potentially type I pneumocytes. As virus-containing bodily fluids are known to enable transmission of

other arteriviruses, respiratory secretions or urine can be hypothesized as media for HhAV-1 transmission.[2,30] In addition, arteriviruses can cause persistent infections in some hosts;[7,11,25,43] therefore, it is possible that free-living hedgehogs may act as HhAV-1 carriers and the origin of infection. However, because the hedgehogs involved in disease outbreaks in the wildlife rescue centers were, at least on occasion, co-housed in the same air space as other species (e.g. birds, rabbits, rodents),[8] and the onset of neurological disease subsequent to casualty admission supported nosocomial infections in incidents 2 and 3, the possibility of viral origin in another species with spill-over of infection to hedgehogs in captivity cannot be excluded.

The large-scale hedgehog morbidity and mortality that occurred in the three incidents presented in this study prompted extensive investigation to determine the etiology. There are multiple differential diagnoses for neurological disease in hedgehogs, which may result from septicemia or encephalitis, such as *Salmonella* Enteritidis*, Listeria monocytogenes* or herpesvirus infection,[17,18,21] as well as the so-called wobbly hedgehog syndrome[10] or idiopathic paralysis syndromes.[27] While gliosis was consistently detected microscopically in both uncomplicated and septicemia-complicated cases across the three separate incidents, the absence of macroscopic lesions and non-specific histologic findings in non-central nervous system tissues suggests a possible scenario where previous historical infections and associated diseases were not identified. Future investigations of neurological disease in European hedgehogs should comprise a suite of ancillary diagnostic tests to identify primary infections and potential comorbidities.

For hedgehogs under care in wildlife rescue centers, establishing clinical samples to use for virus detection would be useful. Since infected hedgehogs can be presumed viremic, as evidenced by endothelial and monocyte-macrophage infection, testing of

blood samples may be considered. Additionally, in one of the HhAV-1 cases in this study, viral shedding into the renal calyx was observed. Therefore urine may be tested for HhAV-1 as has been suggested for EAV diagnosis.[9] While the possibility for viral shedding into the respiratory tract is yet to be determined, the potential of type I pneumocyte infection in hedgehogs, and understanding of PRRSV and EAV shedding in respiratory secretions, warrants the evaluation of HhAV-1 in nasopharyngeal swabs as a further diagnostic specimen. During postmortem examinations, brain, lung, liver, and spleen should be collected for virological testing to screen for HhAV-1 infection, based on the observed distribution of viral RNA by ISH in the diseased hedgehogs. Additionally, considering the potential for immunosuppression induced by HhAV-1 infection and/or concurrent bacterial infections, bacteriological testing of these tissues is recommended.

Virological testing of the control hedgehogs using real-time RT-PCR revealed a substantial proportion of animals identified as positive for HhAV-1 (n=8/13), with Ct values ≥29.8. While histologic examination of brain was only possible for two HhAV-PCR-positive control animals due to the lack of availability of well-preserved specimens, central nervous system lesions and viral RNA labeling was absent in these control animals. On the other hand, the hedgehogs with neurological disease had lower Ct values (19 to 24.4), suggesting higher viral loads in clinical cases. Although a detailed phylogenetic analysis will be reported separately, the arterivirus sequence obtained from neurological hedgehogs displayed wide nucleotide variation within *ORF6* and *ORF7* (Supplemental Table S1). Currently, it remains unclear whether the HhAV-1 detected in control hedgehogs is different from those found in hedgehogs with neurological disease. Further molecular characterization is required to address this information gap; however, the low viral RNA load in control animals presented a

significant technical challenge for next-generation sequencing and viral phylogenetic analysis. Given the occurrence of RT-PCR positive control animals, further virological investigation along with correlative microscopic investigation with virus ISH is warranted to understand the infection epidemiology.

In this study, three animals from incident 2, specifically cases 3, 4, and 5, exhibited histologic evidence of sepsis. Case 4 also had evidence of bacteriological infection, with isolation of *Klebsiella pneumoniae*. These findings may indicate that these animals acquired nosocomial infections. However, significant virus RNA labeling was detected, co-localizing with lesions within the kidney, liver, and spleen. The absence of similar lesions in other non-septicemic animals suggests that the increased viral RNA labeling may be attributed to the recruitment of infected immune cells, rather than a primary viral-mediated vascular disease.

The assessment of virus tropism represented an additional challenge in this study as the interpretation of infected cell populations relied on correlative assessment through conventional light microscopy. In cases of strong and abundant ISH signal, determining the specific cell population can be difficult. Future studies should incorporate co-labeling with host cell markers for immunological confirmation of the identified cell populations or ultrastructure examination by transmission electron microscopy to better understand virus tropism.

In summary, this study supports HhAV-1 as a potential cause of outbreaks of fatal neurological disease in hedgehogs in English wildlife rehabilitation centers. Since hedgehogs are of current conservation concern in Great Britain and are admitted to such centers in large numbers each year,[3] there is an urgent need for further research to understand the viral epidemiology and impact. To mitigate the risks of inter- and

intraspecific pathogen transmission, strict biosecurity measures (e.g. hygiene, quarantine) during rehabilitation are recommended as a routine, combined with husbandry protocols to minimize duration and stress in captive management.[1,33]

Ethical statement

Sick and injured hedgehogs were taken into care for welfare reasons, and died or were euthanized under the Veterinary Surgeons Act 1966. Samples from wild animals were found dead in the wild prior to post-mortem and, no ethical approval was required.

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Author Contributions

F.Z.X.L., M.F.S., B.L., K.S.M. for conceptualisation. J.G, A.D. for methodology. F.Z.X.L., M.F.S. K.S.M., T.P., S.S. conducted formal analysis. B.L., A.N. provided

project leadership, financial, and laboratory resources. F.Z.X.L. wrote the original draft. All authors reviewed and edited the manuscript.

Competing Interests

The authors declare that there are no conflicts of interest.

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Figure 1. Neuropathology associated with hedgehog arterivirus 1 (HhAV-1) infection. (a, b) Cerebrum. (a) Commonly, there are multifocal glial nodules in the neuroparenchyma (b) with localization of viral RNA frequently in the glial cells (solid arrow) and occasionally within the vascular endothelium (open arrow). (a) Hematoxylin and eosin (HE), (b) HhAV-1 in situ hybridization (ISH). (c, d) Cerebellum. (c) Necrosis within the molecular layer characterized by clustering of cell debris (arrow).HE. (d) Serial sections revealed presence of viral RNA in the glial cells (solid arrow), vascular endothelium (open arrow). and meningeal cells. HhAV-1 ISH. (e, f) Spinal cord. (e) There are occasional shrunken and angular neurons (solid arrow) and vacuolated neuropil (open arrow). HE. Inset: Viral RNA is multifocally distributed in both gray and white matter. (f) Viral RNA is labelled in glial cells. HhAV-1 ISH. Viral probes A (b) and B (d, e, and f) were used for labelling.

Figure 2. Splenic, hepatic, and renal lesions associated with hedgehog arterivirus 1 (HhAV-1) infection. (a, b) Spleen. (a) Lymphoid depletion of the white pulp (arrow). Hematoxylin and eosin (HE). Inset: abundant viral RNA in the marginal zone and rarely in T lymphocyte zone. HhAV-1 in situ hybridization (ISH). (b) Cells within the germinal center are sparse with widespread pyknosis and karyorrhexis (arrowheads), intermingled with large numbers of tingible body macrophages. HE. (c, d) Liver. (c) Multifocal fibrinohemorrhagic and necrotizing hepatitis (inset, HE) with dispersed viral RNA labeling in areas of necrosis (arrow) along with periportal labeling. HhAV-1 ISH. (d) Periportal viral labelling in histologically unremarkable liver. HhAV-1 ISH. (e, f) Kidney. (e) Renal cortical infarct (inset) with the edge of the infarct (dotted outline; inset, HE) exhibiting the strong presence of viral RNA within the renal interstitium. HhAV-1 ISH. (f) Presence of pale eosinophilic fluid, neutrophils,

histiocytes, and sloughed epithelium within a dilated renal calyx. HE. Inset: viral RNA localized within the cellular exudate. Viral probes A (d) and B (a, c, e, and f) were used for labelling.

Figure 3. Pulmonary, cardiac, and alimentary tract lesions and viral labelling associated with hedgehog arterivirus 1 (HhAV-1) infection. (a) Lung. Interstitial pneumonia. Hematoxylin and eosin (HE). Inset: viral RNA present in the alveolar wall. HhAV-1 in situ hybridization (ISH).(b) Heart. Necrotizing myocarditis (arrowhead) with bacterial embolus (arrow). HE. Inset: localization of viral RNA within the capillaries and venules. HhAV-1 ISH. (c) Tongue. Histologically unremarkable tissue with rare multifocal submucosal labelling. HhAV-1 ISH. (d) Ileum. Depletion of Peyer's patches (arrowheads). HE. Inset: viral RNA localized with the margin of the germinal centers and scattered labeling within the capillaries of the lamina propria. HhAV-1 ISH. Viral probe B (a, b, c, and d) was used for labelling.