REVIEW

Adenovirus: an emerging factor in red squirrel Sciurus vulgaris conservation

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ABSTRACT

1. Adenovirus is an emerging threat to red squirrel Sciurus vulgaris conservation, but confirming clinically significant adenovirus infections in red squirrels is challenging. Rapid intestinal autolysis after death in wild animals frequently obscures pathology characteristic of the disease in animals found dead.
2. We review the available literature to determine current understanding of both subclinical and clinically significant adenovirus infections in free-living wild and captive red squirrel populations.
3. Benefits of scientific testing for adenovirus incorporating both transmission electron microscopy (TEM) and polymerase chain reaction (PCR) technologies are compared and contrasted. We favour viral particle detection using TEM in animals exhibiting enteropathy at post-mortem and the use of PCR to detect subclinical cases where no enteric abnormalities are observed.
4. Adenoviral infections associated with re-introduction studies are evaluated by examination of sporadic cases in wild populations and of data from captive collections used to service such studies.
5. The paucity of data available on adenovirus infection in grey squirrel Sciurus carolinensis populations is documented, and we highlight that although subclinical virus presence is recorded in several locations in Great Britain and in Italy, no clinically significant disease cases have been detected in the species thus far.
6. Current speculation about potential interspecific infection between sciurids and other woodland rodents such as wood mice Apodemus sylvaticus is examined. Where subclinical adenovirus presence has been detected in sympatric populations using the same point food sources, husbandry methods may be used to diminish the potential for cross-infection.
7. Our findings highlight the importance of controlling disease in red squirrel populations by using clearly defined scientific methods. In addition, we propose hypothetical conservation benefits of restricting contact rates between red squirrels and sympatric grey squirrels and of limiting competition from other woodland rodent species.
INTRODUCTION

Historically, disease was not recognized as a mechanism by which red squirrels Sciurus vulgaris were replaced by grey squirrels S. carolinensis in a landscape. Indeed, it was unclear initially whether the larger grey squirrel was directly involved at all, was taking advantage of space vacated by natural fluctuations in red squirrel population or ultimately was better adapted to a larger range of habitats (Middleton 1931). Disease enzootics that were recorded in red squirrel populations were notable for encompassing areas where grey squirrels were absent (Shorten 1954). Gurnell (1987) noted ‘no evidence that grey squirrels brought with them a disease which is causing the downfall of the red’.

By the 1990s, research was focused heavily upon resource competition (Gurnell & Pepper 1993), including interspecific differences in the relative efficiency with which some tree seed is digested (Kenward & Holm 1993). Interspecific resource competition (Wauters et al. 2005), negative impacts of grey squirrels on red squirrel juvenile recruitment rates (Gurnell et al. 2004) and the effects of seed cache piracy (Wauters et al. 2002) were, and still are, recognized as major contributors to red squirrel extinction in sympatric populations.

However, progressive advances in viral research subsequently established that grey squirrels carry the squirrel pox virus (SQPV) as a subclinical infection and that interspecific infection in sympatric red squirrels leads to epizootic disease, which is a significant factor in regional population declines in the UK (Rushton et al. 2006, Sainsbury et al. 2008, Carroll et al. 2009, Bruemmer et al. 2010). More recently, adenovirus infection has been identified as a cause of mortality in free-living wild and captive red squirrel populations. An expanding geographic distribution of cases has been revealed, affecting not only wild populations, but increasingly associated with high mortality in captive collections used as both breeding stock and in wild population re-enforcement programmes. Additionally, grey squirrels have now been identified as subclinical carriers of the adenovirus among sympatric populations (Everest et al. 2009, Romeo et al. 2014).

Blood analyses, using enzyme-linked immunosorbent assay (ELISA) and tissue polymerase chain reaction (PCR) techniques, are routinely applied to determine SQPV infection in both squirrel species. Parallel transmission electron microscopy (TEM) screening of skin lesion material can be used to confirm the presence of pox viral particles in typical advanced red squirrel cases. However, in contrast to the detection of SQPV, the detection of adenovirus-associated disease or clinically significant adenovirus cases among red squirrels is challenging, due to an absence or non-specificity of external clinical signs of disease. Until relatively recently, little was known about this infection in either squirrel species, or about its significance in red squirrel declines. Due to increasing scientific activity, both as a retrospective exercise and as proactive surveillance, a wider picture is gradually emerging of the evolving impact that this virus is having on both sporadic disease cases in free-living wild squirrels in Great Britain and on red squirrel re-introduction and captive breeding programmes. However, the mere presence of adenovirus in the body does not signify disease. The virus may indeed be present as a clinically significant infection, causing the death of the animal; in this case viral particles can be detected by TEM in faecal material or viral DNA can be amplified from tissue material such as the spleen. Adenovirus can also be present as an asymptomatic infection or transient presence, causing no apparent disease signs or indications of ill health, and the animal may be outwardly healthy. Subsequent death due to an unrelated problem could then show the presence of the amplified viral DNA by PCR analysis, whereas TEM would fail to detect any viral particles.

Adenovirus infection damages the villi in the red squirrel intestinal mucosa, but autolysis within hours of death typically confounds histological examination, by precluding the detection of characteristic adenovirus inclusion bodies (Erdélyi & Duff 2012), as seen in Fig. 1. By TEM on ultrathin sections, these inclusions have been shown to contain abundant viral particles (Fig. 2, arrowed). The findings of enteropathy or diarrhoea are non-specific and are associated with several other diseases (Everest et al. 2010a). While it is difficult to obtain histologically adequate gut wall samples prior to autolysis, experience shows that gross pathological changes indicative of enteropathy, such as liquid intestinal content, correlate strongly with gut viral particle detection.

![Image 312x131 to 541x302](image)

Fig. 1. Haematoxylin and eosin histology image of a section of red squirrel Sciurus vulgaris small intestine, showing intra-nuclear virus inclusion bodies (arrowed) and extensive damage to the villi, findings consistent with adenovirus infection. ×600 magnification.
by TEM (Fig. 3). The presence of viral particles is therefore considered strongly suggestive of clinically significant infection (Everest et al. 2012b). Nonetheless, in the most autolysed wild squirrel cases, pathologists may assume intestinal material to be of such limited value that it is not retained, even though archival samples of other tissues such as liver or spleen may be. Our understanding of the temporal and spatial scope of clinical adenovirus infection (Fig. 4) has recently been improved through more frequent proactive and reactive post-mortem screening, in particular with TEM application.

We review the current understanding of infection and disease in red squirrels, grey squirrels and other small rodents such as wood mice *Apodemus sylvaticus*, with particular reference to the UK, and highlight key areas for future adenovirus infection research that have particular relevance to the applied conservation of the red squirrel.

**RED SQUIRRELS**

**The geographical distribution of adenovirus infection**

The first reports in the literature of adenovirus in free-living wild red squirrels from the UK were recorded from Suffolk (Sainsbury et al. 2001) and Cumbria, England (Duff et al. 2007), then from Wales (Everest et al. 2008), Scotland (Everest et al. 2010a) and Northern Ireland (Everest et al. 2012a), demonstrating a wide geographical distribution (Fig. 4; Table 1). Retrospective national surveillance of red squirrel mortalities throughout Great Britain, reported by Martinez-Jiménez et al. (2011), revealed that 60 (12%) of 493 cases showed enteric signs. Of these 60, 13 animals, all of which were exhibiting diarrhoea, were selected for analysis by TEM. Of these 13, two animals (15%; Table 1), one from Cumbria, the other from Lancashire, England, were confirmed as adenovirus cases by the detection by TEM of viral particles. In another retrospective study, adenovirus particles were identified by TEM in 10 (14%) of 70 free-living wild red squirrels where enteropathy was suspected, from Cumbria, Lancashire and Northumberland, England, and Anglesey, Wales (Everest et al. 2010b; Table 1). However, given the opportunistic sampling of post-mortem cases and the paucity of data from living animals, it is difficult to interpret the importance of adenovirus as an overall contributor to mortality from these studies alone.

Sainsbury et al. (2001) and Martínez-Jiménez et al. (2011) reported on a population re-enforcement study at Thetford Chase (Suffolk, England) in the late 1990s, with animals that had been translocated from Cumbria and had contracted the infection and died in 1997 (Table 1). These animals may have been under stress that could have influenced the course of the disease. Diarrhoea was associated with each of 10 adenovirus cases recorded in red squirrels, and intestinal haemorrhage or inflammation was observed in seven cases. The extant Thetford Chase wild red squirrel population at that time was judged to consist of 10–20
Fig. 4. Location of adenovirus positive free-living wild (●) and captive (□) red squirrel Sciurus vulgaris cases from Great Britain, as analysed by PCR and TEM.

Reproduced from Ordnance Survey digital map data. Copyright Northumberland Wildlife Trust OS Licence No. AL100035023. 2014.
individuals (no more than 40; Gurnell et al. 1997), and consequently adenovirus infection was a notable factor in the study. Subsequent research (Everest et al. 2012b; Table 1) has revealed adenovirus infection to be associated with a high proportion of deaths in squirrels housed in captive collections in Wales, indicating that viral epizootics can be locally significant.

Of 13 captive deaths sampled from the re-introductions on the island of Anglesey, situated off the North Wales coast, 12 (92%) were confirmed as positive for the virus (three by detecting viral particles by TEM and nine by amplifying viral DNA by PCR). Samples from 16 captive deaths at the Welsh Mountain Zoo, Colwyn Bay, Wales, revealed viral DNA amplified by PCR in 14 (88%) cases (Everest et al. 2012b). In a further 24 captive deaths originating from zoological collections in England, for which tissue, faecal or intestinal content samples were available, 20 (83%) were observed to be positive for adenovirus (Everest et al. unpublished; Table 1).

Analyses performed on 31 free-living wild red squirrels found dead on Anglesey revealed that 13 (42%) were positive for the virus. Of these positive cases, seven (54%) originated from within Newborough Forest and of these, five (71%) were identified as positive by PCR analyses, three of which also tested negative by TEM. One (14%) contained viral particles when analysed by TEM only, and one case was confirmed as positive by both tests. The remaining six cases were from other Anglesey coniferous and broad-leaved woodlands; all were detected as viral DNA carriers by PCR but negative for viral particles by TEM (Everest et al. 2012b).

In the latest published report of adenovirus in red squirrels from outside the UK, one, involving three deaths, was from a captive collection in Germany (Peters et al. 2011); in the other, 77 road traffic accident carcasses from Italy were examined by a combination of TEM and PCR analyses (Romeo et al. 2014). Twelve (16%) were positive for amplified viral DNA by PCR (Table 1). As with the outbreak in Germany (Peters et al. 2011), and unlike the situation in most of Great Britain, viral presence was detected in red squirrel populations from areas where the grey squirrel was not known to be present in the immediate landscape.

<table>
<thead>
<tr>
<th>Study location</th>
<th>Wild or captive</th>
<th>Number tested</th>
<th>Number (%) positive</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suffolk</td>
<td>Wild</td>
<td>6</td>
<td>3 (50)</td>
<td>Sainsbury et al. 2001</td>
</tr>
<tr>
<td>Cumbria</td>
<td>Wild</td>
<td>2</td>
<td>2 (100)</td>
<td>Duff et al. 2007</td>
</tr>
<tr>
<td>Anglesey</td>
<td>Captive</td>
<td>3</td>
<td>3 (100)</td>
<td>Everest et al. 2008</td>
</tr>
<tr>
<td>Scotland</td>
<td>Wild</td>
<td>1</td>
<td>1 (100)</td>
<td>Everest et al. 2010a</td>
</tr>
<tr>
<td>Great Britain</td>
<td>Wild</td>
<td>70</td>
<td>10 (14)</td>
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<tr>
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<td>Peters et al. 2011</td>
</tr>
<tr>
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<td>1 (50)</td>
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</tr>
<tr>
<td>Isle of Wight/Jersey</td>
<td>Wild</td>
<td>16</td>
<td>14 (88)</td>
<td>Everest et al. 2012b</td>
</tr>
<tr>
<td>Italy</td>
<td>Wild</td>
<td>20</td>
<td>9 (45)</td>
<td>Everest et al. 2013</td>
</tr>
<tr>
<td>England</td>
<td>Captive</td>
<td>24</td>
<td>20 (83)</td>
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Adenovirus presence determined in deaths by other causes

Traumatic deaths, such as by drowning and resulting from road traffic accidents, have revealed animals positive for amplification of viral DNA by PCR but negative for faecal viral particle detection by TEM. These cases occur in animals that lack enteric abnormalities at post-mortem examination. This suggests that subclinical infections are present and may be widespread within wild populations of red squirrels in Great Britain (Everest et al. 2012b; Table 1).
Adenovirus in red squirrel conservation

**Adenovirus strain speciation**

Phylogenetic analysis demonstrates that adenovirus sequences from squirrel samples cluster with mastadenoviruses but are distinct from other adenoviruses within the genus (Sainsbury et al. 2001, Peters et al. 2011), although squirrel adenovirus has not yet been approved as a species (King et al. 2011). Sequencing has revealed a lack of adenovirus strain variability. The identity of the adenovirus in a partial fragment of the hexon gene from the German outbreak (GU735084) described by Peters et al. (2011) was identical to the putative Suffolk strain (Sainsbury et al. 2001). In contrast, in those cases described by Everest et al. (2012b), sequences were detected that were identical to those found in Cumbrian cases from 2007 (JN205244.1).

Everest et al. (2012b) used a partial fragment of the polymerase gene, which in turn identified cases that were genetically identical to the grey squirrel cases detected on Anglesey (Everest et al. 2009). This is remarkable, as the cases were separated both spatially and temporally. It has been suggested, therefore, that the viruses involved in each of these cases are very closely related, or perhaps identical (Peters et al. 2011).

In general, the samples described above have not been randomly sourced, and case selection was influenced by carcass suitability and value in terms of post-mortem examination. This is particularly true for captive collections, where the prevailing close confinement within enclosures would have allowed for easy spread of the virus between individual animals, thus accounting for the apparently high incidence of infection in such collections.

**GREY SQUIRRELS**

Given the role that grey squirrels play in SQPV infection in red squirrel populations, it is natural to investigate whether sympatric grey squirrel populations are also a source of interspecific adenovirus infection. Romeo et al. (2014) found PCR amplified adenoviral DNA in only two (1%) of 232 grey squirrels from Italy. Screening of tissues from wild adult grey squirrels (n = 18) trapped and euthanized at the Welsh Mountain Zoo in 2011 failed to reveal viral particles in the gut by TEM (which would have suggested clinically significant infection), yet 10 of these 18 animals (56%) were positive by PCR analyses on spleen tissue (Everest et al. unpublished) and were hence determined as adenovirus carriers. Although the numbers of animals were small in this study, the PCR figure is very similar to the 60% sero-prevalence found by Greenwood and Sanchez (2002) using murine adenovirus ELISA tests for antibodies in grey squirrels from the same zoo, a location where dead captive red squirrels have been found with enteric symptoms and viral particles in the intestinal tract.

**Table 2. Positive PCR test results for adenovirus DNA from blood and spleen tissue from grey squirrels Sciurus carolinensis in North Wales. Total number tested and percentage positive are shown**

<table>
<thead>
<tr>
<th>Anglesey</th>
<th>Gwynedd (Bangor area)</th>
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<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>2007*</td>
<td>55 (0%)</td>
</tr>
<tr>
<td>2010*</td>
<td>26 (23%)</td>
</tr>
<tr>
<td>2011†</td>
<td>–</td>
</tr>
<tr>
<td>2012†</td>
<td>4 (25%)</td>
</tr>
</tbody>
</table>

*Everest et al. (2012b).†Everest et al. unpublished.†10% (n = 48); adults: 14% (n = 28) and juveniles: 5% (n = 20).

At the Newborough Forest re-introduction site on Anglesey, adenovirus DNA was detected by PCR analysis from two grey squirrels caught in 2006 (Everest et al. 2009). Wider PCR screening of archived and proactively sourced blood sampling, involving over 200 samples and thus forming a study larger than that of Romeo et al. (2014), was subsequently undertaken and reported by Everest et al. (2012b; Table 2) for both Anglesey locations and woodland in Gwynedd, North Wales, within a few kilometres of the Menai Straits. Spleen tissue collected from the Gwynedd site was examined in 2012 (Everest et al. unpublished), and amplification of DNA revealed a much higher percentage of positives (54%) than in blood (7%, see Table 2).

The 2012 Gwynedd result (Everest et al. unpublished) was further confirmed when both spleen and blood were available for analysis from each of 14 adult grey squirrels trapped at the Welsh Mountain Zoo. Adenovirus DNA was detected from spleen tissue in eight cases (57%), but there were no positive results from the 14 blood samples from the same animals (Everest et al. unpublished), thus demonstrating that source tissue type is an important consideration in adenovirus screening.

Historically, assessing infection in grey squirrels is challenging, as previously reported blood testing was serologically based. Thus exposure to the virus could result in potentially long-lasting sero-conversion, although this may wane with age. In contrast, an animal may be viraemic (and therefore PCR-positive) for only a short period, meaning that PCR analyses have only a small time window to be effective for viral diagnosis. In this context, serologically based ELISA analyses may be more sensitive in nature than PCR techniques.

Although evidence of infection has been found, no clinically significant cases of adenovirus have been identified to date in grey squirrels, and viral particles have been absent from intestinal content examined in TEM studies of grey squirrels from Cumbria (n = 36); Wales (n = 58; Everest et al. unpublished), Thetford Chase (n = 10; Martínez-Jiménez et al. 2011) and Italy (n = 3) (Romeo et al. 2014).
SMALL RODENTS

Peters et al. (2011) documented adenovirus infection by TEM in a captive red squirrel collection from Germany, and red squirrel infections have been recorded on both the Isle of Wight and Jersey (Everest et al. 2013), all of which are regions where the grey squirrel is absent. Additionally, Romeo et al. (2014) documented infections in red squirrels in areas where the grey squirrel was not known to be present. This means that alongside intra-specific and potential grey squirrel to red squirrel infections, interspecific infection from other small rodents such as wood mice is possible.

In order to investigate this potential infection route, Everest et al. (2013) examined the spleens of wood mice trapped on the Island of Anglesey for the presence of adenovirus by PCR analyses. Adenoviral DNA was amplified from three of 15 mice (20%) trapped at two woodland sites which had red squirrel feeding stations and where cases of clinically significant adenovirus infection in wild red squirrels had been recorded. Two of 24 (8%) mice trapped in north Wales within woodland enclosures housing captive red squirrels also tested positive for adenovirus by PCR. Our results therefore demonstrate the potential for adenovirus infection in sympatric communities of grey squirrel, red squirrel and wood mice.

The PCR primers used to test the wood mouse samples had been designed based on a sequence of the adenoviral DNA polymerase gene from squirrel samples (JN205244.1; Everest et al. 2012a). However, further investigations into whether these primers would detect other adenoviruses were not undertaken. It is therefore unclear at present whether the strain detected in mice is identical to that detected in squirrels, and so further molecular sequencing is required. Greenwood and Sanchez (2002) used an ELISA derived for the serological detection of murine adenoviruses to detect adenovirus in grey squirrels. Therefore, it is possible that either cross-reactivity exists between species-specific viruses from the two squirrel species, or that an identical virus infects both.

DISCUSSION

Retrospective study of archived tissue and blood samples (Everest et al. 2010a, 2012b, Martínez-Jiménez et al. 2011) has advanced our understanding of both the temporal and spatial distribution of adenovirus infection within red squirrel populations. Recent examination of trauma deaths has also revealed subclinical infections in wild individuals at the time of death, namely negative TEM results for adenovirus particles in faecal and intestinal samples but positive results for viral DNA from tissues by PCR analysis (Everest et al. 2012b, Romeo et al. 2014). Much, however, remains unclear about the epizootiology, in particular, the roles of squirrel population density and stress. Currently, much of our understanding is based upon captive collections, and so there is a need to investigate the distribution and effects of the infection among wild red and grey squirrel populations. Opportunities for the application of a qPCR technique to quantify virus load in faeces, tissues and blood in order to partition pathological from asymptomatic infections would also be beneficial.

Research in North Wales (Greenwood & Sanchez 2002, Everest et al. 2009, 2012b) and in Italy (Romeo et al. 2014) has demonstrated adenovirus infection or exposure in grey squirrels, but whether this has any clinical significance in their populations remains unknown. To this end, a controlled challenge experiment in grey and red squirrels using the same virus isolate would help to advance our understanding.

There is a paucity of data on adenovirus prevalence within regional grey squirrel populations in the UK. An annual survey combining PCR and TEM analyses was limited solely to squirrel populations in North Wales. Additional regional studies of this type would therefore be useful.

Given that grey squirrels appear to be infected with both adenovirus and SQPV, the accepted management practice of removing grey squirrel populations to control SQPV infections also mitigates the potential for adenovirus infection. Additionally, conservation managers could evolve protocols to combat potential infection pathways involving other woodland rodents such as wood mice, although there may be a significant cost implication to this approach. On Anglesey, adenovirus infection risk was highlighted as a major difficulty faced during the re-introduction of red squirrels to Newborough Forest (Shuttleworth et al. 2008). Release protocols have been modified so that animals are now housed for only a few weeks, during which time faecal and blood screening is undertaken for adenovirus (Shuttleworth 2010). More widely, it has been recommended that hygiene protocols at supplemental feeding hoppers routinely focus upon limiting adenovirus infection via faecal-oral routes (Everest et al. 2012b). Given our recent findings and because of the potential for transmission of other rodent-borne infections, protocols should include mouse control.

If wood mice act as an infection reservoir, there are obvious implications for scenarios that concentrate their activities at point food sources such as garden bird tables or supplemental feed hoppers also visited by red squirrels. It may therefore be prudent to place red squirrel supplemental feed hoppers on posts with cone-shaped baffles near the base to prevent mice from accessing them, instead of, as is common practice, fixing hoppers to tree trunks, which allows mice easy access. Accumulation of discarded shells and food remains beneath hoppers should be minimized.
Trapping protocols should include regular disinfection of all traps, not only those that have contained grey squirrels, so as to limit any potential mouse-to-red squirrel interspecific virus transmission.

CONCLUSIONS

This review of the available evidence within the published literature, coupled with recent findings, leads us to conclude that adenovirus should be regarded as a serious disease threat to red squirrel re-introduction and captive breeding programmes, and to red squirrel populations in places where grey squirrels, red squirrels and wood mice can interact at point food sources. We also conclude that TEM, while excellent at detecting clinically significant infection from intestinal samples, is not as sensitive as PCR for detecting subclinical adenovirus cases and that spleen tissue is a better material to screen by PCR than blood. The ELISA-based assay on blood samples is the only test available for live animals at present. To address practical and potentially also welfare considerations, alternative assay platforms should be investigated for live animal testing. We also recommend that disease investigation and adenoviral infection surveillance extend to all three rodent species identified in this review, and where possible, include parallel PCR and TEM sample testing of tissue and intestinal content samples, respectively.

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