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Host-pathogen dynamics of squirrelpox virus infection in red squirrels (*Sciurus vulgaris*)

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ABSTRACT

To improve our understanding of squirrelpox virus (SQPV) infection in the susceptible host, three red squirrels were challenged with wild-type SQPV via scarification of the hind-limb skin. All squirrels seroconverted to the infection by the end of the experiment (17 days post-challenge). Challenged animals suffered disease characterised by the development of multiple skin and oral lesions with rapid progression of skin lesions at the infection site by day 10 post-challenge. No internal pathological changes were found at post-mortem examination. A novel SQPV Taqman® Real-time PCR detected viral DNA from multiple organs, with the largest amounts consistently associated with the primary and secondary skin and oral lesions where viral replication was most likely occurring. Immunohistochemistry clearly detected viral antigen in the stratified squamous epithelium of the epidermis, tongue and the oropharyngeal mucosa-associated lymphoid tissue and was consistently associated with histological changes resulting from viral replication. The lack of internal pathological changes and the detection of relatively low levels of viral DNA when compared with primary and secondary skin lesions argue against systemic disease, although systemic spread of the virus cannot be ruled out. This study allowed a comprehensive investigation of the clinical manifestation and progression of SQPV infection with a quantitative and qualitative analysis of virus dissemination and shedding. These findings suggest two separate routes of SQPV transmission under natural conditions, with both skin and saliva playing key roles in infected red squirrels.

1. Introduction

The native red squirrel (*Sciurus vulgaris*) populations of the UK and Eire have declined dramatically over the last 100 years and are now heading towards extinction with just a few isolated populations remaining (Bosch and Lurz, 2012). In contrast, the grey squirrel (*Sciurus carolinensis*), first introduced from North America in 1876, has greatly expanded its range. The reasons for the decline in the red squirrel population can be attributed to many variables (Bosch and Lurz, 2012), and although an earlier report suggested disease introduced with the grey squirrel (Middleton, 1930), the connection between epidemic disease in red squirrels and the presence of the imported grey squirrels was debated throughout the 20th century (Edwards, 1962; Keymer, 1974; Vizoso, 1968). It was only latterly that a viral agent (initially described as parapoxvirus virus) responsible for disease outbreaks in red squirrels was identified by transmission electron microscopy (TEM) of the eyelid skin from a diseased red squirrel (Scott et al., 1981). Even then it took several more years before research established the role of the grey squirrel as a reservoir species for the virus (Reynolds, 1985; Sainsbury et al., 2000) and emphasised the crucial importance of this epizootic disease in the wider context of disease-mediated competition with grey squirrels (Rushton et al., 2006, 2000; Tompkins et al., 2003). Today, conservation strategies for the red squirrel take account of squirrelpox virus (SQPV) as a major contributing factor in the threat to red squirrels from the grey squirrel.

Squirrelpox virus is now classified as the sole member of an unclassified genus within the Poxviridae family (Thomas et al., 2003; McInnes et al., 2006; Darby et al., 2014). The origin of SQPV is

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still unknown, although it is thought that it was imported with grey squirrels from North America. While the virus has never been described in the USA, serological samples collected from grey squirrels in North America have tested positive for anti-SQPV antibodies (McInnes et al., 2006). Fatal cases of disease resulting from SQPV infection have been described solely in red squirrels in the UK and the Ireland (Sainsbury and Ward, 1996; Tompkins et al., 2002; Thomas et al., 2003; Sainsbury et al., 2008; McInnes et al., 2009; LaRose et al., 2010; McInnes et al., 2013; Naulty et al., 2013) with the exception of one report of a grey squirrel showing clinical signs of SQPV disease which was confirmed by TEM (Duff et al., 1996). Otherwise, grey squirrels are considered to be asymptomatic or sub-clinically affected by the virus (Atkin et al., 2010; Tompkins et al., 2002). In red squirrels, the infection causes multifocal skin lesions characterised by erythematous and ulcerative dermatitis which progress to haemorrhagic scabs and the disease causes significant mortality (Carroll et al., 2009; Chantrey et al., 2014; Duff et al., 2010; LaRose et al., 2010; McInnes et al., 2013, 2009; Sainsbury and Gurnell, 1995; Sainsbury and Ward, 1996; Tompkins et al., 2002). To confirm that the disease is clinically asymptomatic in the grey squirrel, an experimental infection with SQPV was performed (Tompkins et al., 2002). Grey and red squirrels were inoculated simultaneously via skin scarification and subcutaneous routes. All the red squirrels developed typical, severe SQPV-associated dermatitis and their health deteriorated rapidly in contrast to grey squirrels which showed no clinical signs of infection and remained healthy throughout.

Despite the disease and the potential carrier role of the grey squirrel being recognised for the last 15 years, remarkably little has been discovered about the pathogenesis or the transmission route (s) of the virus either within red or grey squirrels or between the species. To date, there have been no detailed studies on the temporal development of SQPV infection in red squirrels focusing on the incubation period, the occurrence and development of lesions or which tissues or organs support viral replication and, potentially, shedding. Furthermore, what is known has been derived primarily from epidemiological studies of field cases. As part of a wider study to investigate the feasibility of producing a vaccine against SQPV it was necessary first to establish, under experimental conditions, an infection of red squirrels resembling SQPV infection in the wild. To investigate this and provide additional information on the pathogenesis of SQPV we developed a specific and sensitive SQPV Taqman® qPCR assay to compare the viral load from a broad range of tissues from SQPV positive red squirrels both naturally and experimentally infected. We have correlated these results with those from a variety of diagnostic techniques such as post mortem examination, histopathology and a novel SQPV-specific immunohistochemical method (IHC) to determine which tissues harbour the virus and which are most likely permissive for viral replication and are therefore likely to be involved in virus transmission and shedding of virus.

2. Materials and methods

2.1. Experimentally-infected animals and related procedures

All experimental protocols involving infection of red squirrels were approved by the Moredun Research Institute Animal Experiments & Ethical Review Committee and adhered strictly to the requirements of the UK Animals (Scientific Procedures) Act 1986. Three adult red squirrels (two male, sq. 01/12 and sq. 06/12; one female, sq. 04/12) were individually housed in 1 m × 0.75 m × 0.75 m cages which were furnished with a nest box with a removable lid. Food (mixed nuts, sunflower seeds, whole corn and fruit) and water were provided *ad libitum*. All squirrels were allowed to adjust to their environment for 14 days before the start of the procedures. All handling procedures were performed under general anaesthesia. Anaesthesia was induced with gaseous 5% isoflurane (IsoFlo, Abbott Animal Health, UK) in oxygen administered within an anaesthetic chamber and maintained by 1.5–4% isoflurane in oxygen via a face mask.

2.2. Preparation of inocula, experimental infection and clinical observations

Dry skin lesions and scabs were collected from dead free-ranging red squirrels in the UK found with clinical signs typical of SQPV disease and confirmed positive by either SQPV qPCR or TEM. Scabs were ground with sterile sand in sterile PBS, approximately 6% v/v penicillin/streptomycin solution (100 units/mL and 100 mg/mL, respectively) was added and the inoculum clarified by centrifugation at 2000 × *g* for 5 min. The inoculum was dispensed into multiple aliquots and stored at −70 °C. SQPV DNA concentration was quantified by qPCR and found to be approximately 2.5×10^{10} virus genome equivalents/mL. The presence of intact SQPV virion particles was confirmed by TEM (courtesy of David Everest, Animal and Plant Health Agency, Weybridge, UK). Mock inoculum was prepared using the antibiotic solutions added to PBS.

Inocula were applied topically onto a previously shaved and scarified 2 × 2 cm area of skin on the lateral aspect of the squirrel thighs. Scarification was with the tip of a 16G needle in a cross hatched pattern with scratches approximately 0.5 cm apart. Each squirrel was challenged with 100 μL of SQPV inoculum on the right thigh and 100 μL of mock inoculum on the left thigh. Animals were monitored daily for clinical signs of disease with the skin lesions distant from the challenge sites being fully assessed and recorded at the time of *post-mortem* examination (PM). The weight of each squirrel was measured at the time of the virus challenge and estimated every day thereafter by weighing the squirrels within their nest boxes. Clinical scores were recorded daily using a modification of that used previously to assess the impact of squirrelpox disease on red squirrels (Tompkins et al., 2002). A total clinical score of 6 on three consecutive days was the designated humane end-point at which an individual animal would be removed from the experiment. All three animals were euthanised, while under general anaesthetic, by intracardiac injection of pentobarbitone sodium B.P. (approx. 200 mg/kg) 17 days post challenge (DPC) in line with this clinical scoring procedure.

2.3. Post-mortem examination and collection of samples from experimental animals

The distribution of SQPV within 34 different tissues, blood, faecal and urine samples was determined by qPCR analyses. Sterile nylon-flocked swabs (Thermo Scientific, Sterilin, Newport, UK), pre-wetted or not with sterile PBS as appropriate, were used to collect samples of oral and ocular secretions, samples from the skin surfaces associated with challenge sites and secondary lesions and the lids of the nest boxes. Immediately post-euthanasia, 3–5 mL of blood was collected by cardiac puncture using a 21G hypodermic needle. Approximately 2.5 mL was allowed to clot for serology to test for the presence of antibodies against SQPV using the enzyme-linked immunosorbent assay (ELISA) previously described (Sainsbury et al., 2000). The remainder was placed into paediatric EDTA tubes for extraction of nucleic acids. Individual sterile surgical instruments were used to harvest each tissue sample to avoid possible SQPV cross-contamination between tissues. Scarified skin samples were collected first followed by other representative skin samples (eyelid, lip, chin, nose, ear, axilla, anterior and posterior digital and mock scarified skin). Harderian glands, submandibular lymph nodes (SM LN), submandibular salivary glands (SM SG) and