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BRIEF REPORT

Detection of a novel gammaherpesvirus (genus *Rhadinovirus*) in wild muntjac deer in Northern Ireland

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Abstract This study represents the initial part of an investigation into the potential for non-native, wild, free-living muntjac deer (*Muntiacus reevesi*) to carry viruses that could be a threat to livestock. A degenerate PCR assay was used to screen a range of tissues from muntjac deer culled in Northern Ireland for the presence of herpesviral nucleic acids. This was followed by sequencing of PCR amplicons and phylogenetic analysis. We report the detection of a novel gammaherpesvirus most closely related to a type 2 ruminant rhadinovirus from mule deer. It remains to be determined if this new virus is pathogenic to deer or presents a risk to food security through the susceptibility of domestic livestock.

Keywords Muntjac deer · *Muntiacus reevesi* · Rhadinovirus · Gammaherpesvirus · Degenerate PCR

Deer, especially introduced species, are of major concern with regards to impacts on biodiversity, forestry and agriculture [1]. Deer also affect humans directly, causing fatalities and injury through deer-vehicle collisions [2]. Consequently, expansion of non-native, introduced and native deer populations will present considerable management challenges in the future [3].

The rapidity of the spread of invasive Reeves' muntjac (*Muntiacus reevesi*) Ogilby, 1839, is currently of great concern due to deliberate translocations and natural dispersal from their initial inoculation site(s) in the south-east of Great Britain [3]. The first verified record of muntjac deer in the wild in Northern Ireland was confirmed during June 2009 as a result of a road traffic accident near Newtownards, Co. Down [4]. This is the first known introduction of a new large mammal species in Northern Ireland since the introduction of sika deer (*Cervus nippon* Temminck, 1838) in 1870. The second record was a culled animal (referred to hereafter as 'animal 1') shot in the grounds of Mount Stewart, Co. Down, in June 2011. In February 2013, another animal was shot in the same locality (referred to hereafter as 'animal 2'). Furthermore, in the spring of 2013 and 2014, a camera trapping survey confirmed the presence of a small muntjac population within this area [5]. In April 2013, eleven positive detections of muntjac were made at seven separate locations in this area using camera traps.

Animal 1 was a healthy male aged 55-57 weeks, and animal 2 was a healthy female aged approximately 15 weeks of age. Carcasses were submitted to Veterinary Sciences Division, Agri-Food and Biosciences Institute for post-mortem examination. Tissues were collected from both animals and processed to produce a 10% homogenate. Tissues taken included the kidney, heart, spleen, lung, bronchial lymph node, thyroid, caecum, faeces, oesophagus, colon, small intestine, pancreas, liver, abomasum, mediastinal lymph node, mesenteric lymph node, trachea, rumen contents and blood clot. Total nucleic acids were isolated from the homogenates using a MagNA Pure LC liquid handling system and a MagNA Pure Total Nucleic

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Acid Isolation Kit (Roche Diagnostics Ltd., U.K.) according to the manufacturer's instructions. These were subjected to SYBR Green I real-time PCR using Quantitect SYBR Green PCR master mix (QIAGEN, Crawley, U.K.). PCR was carried out with nested degenerate primers designed to detect herpesviral DNA at the family level [6].

These degenerate primers were used for two purposes: 1) diagnostic PCR and 2) PCR for phylogenetic analysis. For diagnostic PCR, the first round was carried out using forward primers DFA and ILK with reverse primer KG1. The second round was carried out with forward primer ILK and reverse primer IYG; the expected amplicon size was 215-315 base pairs. For the PCR for phylogenetic analysis, the first round was carried out using forward primer DFA with reverse primer KG1. The second round was carried out with forward primer DFA and reverse primer IYG; the expected amplicon size was ~500 base pairs. A selection of samples found positive by the diagnostic PCR were subject to PCR for phylogenetic analysis. PCR reactions were subjected to agarose gel electrophoresis, and amplicons were excised and purified. Suspected herpesvirus-positive samples were sent for commercial sequencing. Resultant sequence data were first identified by BlastN analysis [7]. Sequences were trimmed and contigs generated using Geneious [8] software before alignment and generation of phylogenetic trees using MEGA6 [9]. Geneious was also used to compare percentage nucleotide identities between the muntjac viral sequences and known herpesviral sequences from GenBank.

Post-mortem examination showed no significant gross pathological lesions for either animal. Laboratory examination of animal 1 did not show the presence of any significant bacterial or parasitic infections, including bovine tuberculosis (data not shown). Amplicons of the expected size generated by the diagnostic PCR were detected in the colon, mediastinal lymph node, lung and blood clot for animal 1 and in the bronchial lymph node in animal 2. Sequencing and subsequent BlastN analysis of short 175-bp sequences determined that they were herpesviral sequences most similar to those found in a number of deer species. The sequences were identical in both animals. Amplicons of the expected size generated by the PCR for phylogenetic analysis were detected in the mediastinal lymph node of animal 1 and in the bronchial lymph node in animal 2. Sequencing and subsequent BlastN analysis of a 500-bp sequence from the bronchial lymph node of animal 2 determined it to be herpesviral sequence most closely related to the DNA-dependent DNA polymerase gene of type 2 ruminant rhadinovirus of mule deer, which belongs to the family *Gammaherpesvirinae*, genus *Rhadinovirus*. Phylogenetic analysis (Fig. 1) of this sequence compared to homologous herpesviral sequences from GenBank confirmed this relationship to type 2 rhadinovirus of mule deer. Analysis of nucleotide sequence similarity showed 82.2% sequence identity between the virus from animal 2 and the type 2 rhadinovirus of mule deer (accession no. HM014314). Other members of the family *Gammaherpesvirinae,* genus *Macavirus*, showed approximately 65% nucleotide sequence identity to the virus from animal 2, whereas human herpesvirus and bovine herpesvirus 1 (family *Alphaherpesvirinae*) showed lower similarity (Table 1).

The introduction of invasive species poses a great threat to conservation in Northern Ireland. There is a significant risk of them introducing novel pathogens to native wildlife that are immunologically naive. In addition, invasive species pose a similar threat to domesticated food animals and a zoonotic threat to human populations. Deer are known to be susceptible to bovine, caprine and ovine viruses and *vice versa*. A recent study from Japan showed native wild and American farmed sika deer to have antibodies against bovine parainfluenza virus type 3, bovine respiratory syncytial virus, bovine adenovirus type 7, bovine coronavirus, bovine viral diarrhoea virus (BVDV), and bovine herpesvirus type 1 [10]. Antibodies to the recently emerged Schmallenberg virus, which causes foetal abnormalities, have been reported in red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and muntjac deer in Austria, Belgium and England [11]. BVDV has been detected in a wide range of wild and captive deer in Europe and North America, including roe deer, red deer, fallow deer (*Dama dama*), elk (*Cervus elaphus nelsoni*), white-tailed deer (*Odocoileus virginianus*), and mule deer (*Odocoileus hemionus*) [12]. In the Republic of Ireland, an industry-led compulsory national BVDV eradication programme commenced in 2013 (http://www.bvdfree.ie), while the compulsory phase of the Northern Ireland eradication programme began in 2016 (http://www.animalhealthni.com/BVD.aspx). In order for muntjac deer to be considered a reservoir for re-introduction of BVDV to cattle after eradication efforts, the animals must be susceptible to infection and able to shed the virus, the virus must be capable of being maintained in the population, and there must be sufficient contact with domestic livestock [13]. It is not known if muntjac deer fulfil these criteria for BVDV or other pathogens, but an expanding muntjac population should be monitored closely to assess the level of risk posed.

It remains to be determined if the novel rhadinovirus detected in this study is pathogenic to deer or is a potential threat to food security if livestock are susceptible. In the animals examined, the virus was present at very low levels and was only detected after two rounds of PCR in healthy animals. A member of the genus *Rhadinovirus* known as bovine herpesvirus 4 is implicated in post-partum metritis [14] in cattle, and a number of primate viruses are

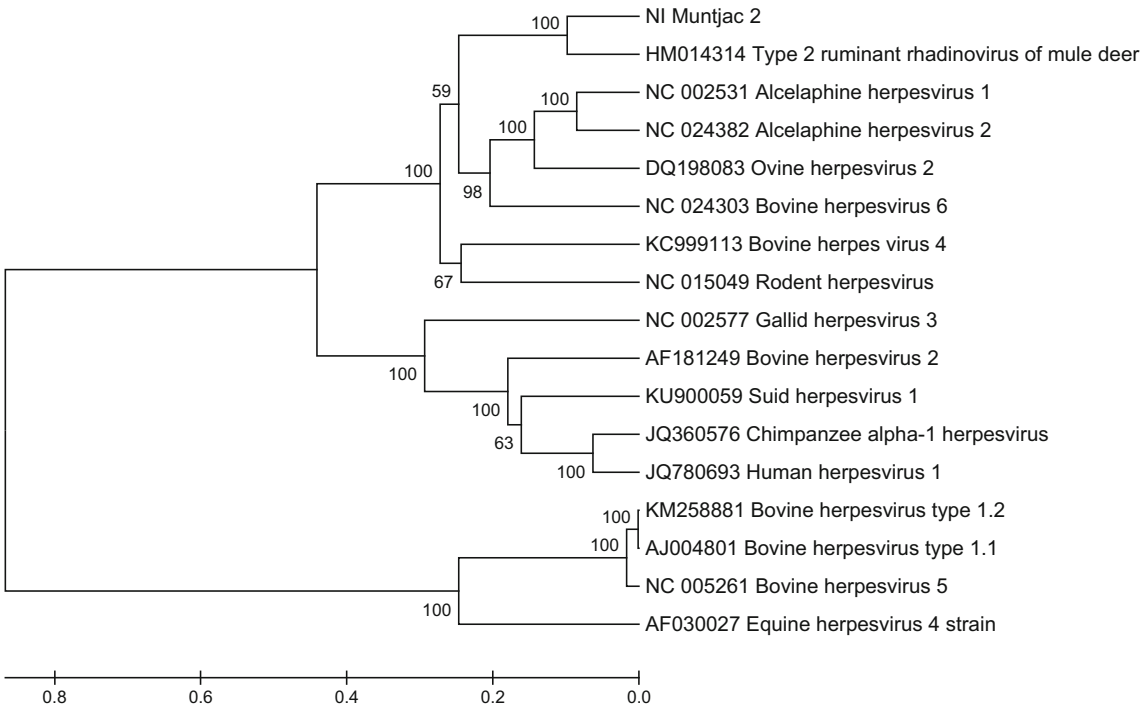

Fig. 1 Phylogenetic tree showing the relationship between NI muntjac deer herpesvirus sequence (500 base pairs of the DNA-dependent DNA polymerase gene) and homologous sequences from GenBank. The evolutionary history was inferred using the UPGMA method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. The test of phylogeny was by the bootstrap method with 500 replications. Analyses were conducted using MEGA6

Table 1 Percentage sequence identity between 500 base pairs of DNA-dependent DNA polymerase gene sequence from Northern Ireland muntjac rhadinovirus and other homologous sequences from GenBank. Type 2 ruminant rhadinovirus of mule deer, accession no. HM014314; ovine herpesvirus 2, accession no. DQ198083, a causative agent of sheep-associated malignant catarrhal fever; alcelaphine herpesvirus 1, accession no. NC002531, a causative agent of wildebeest-associated malignant catarrhal fever; bovine herpesvirus 6, accession no. NC024303; alcelaphine herpesvirus 2, accession no. NC024382; human herpesvirus 1, accession no. JQ780693; bovine herpesvirus 1.2, accession no. KM258881

	NI Muntjac 2	Type 2 ruminant rhadinovirus of mule deer	Ovine herpesvirus 2	Alcelaphine herpesvirus 1	Bovine herpesvirus 6	Alcelaphine herpesvirus 2	Human herpesvirus 1	Bovine herpesvirus type 1.2
NI Muntjac 2	-	82.2	63.1	65.4	65.7	64.4	45.9	29.6
Type 2 ruminant rhadinovirus of mule deer	82.2	-	63.1	64.2	64.1	64.2	44.5	30.9
Ovine herpesvirus 2	63.1	63.1	-	75.5	67.5	75.9	50.8	33.6
Alcelaphine herpesvirus 1	65.4	64.2	75.5	-	67.6	84.8	48	31.1
Bovine herpesvirus 6	65.7	64.1	67.5	67.6	-	68.8	48	30.9
Alcelaphine herpesvirus 2	64.4	64.2	75.9	84.8	68.8	-	46.9	29.8
Human herpesvirus 1	45.9	44.5	50.8	48	48	46.9	-	37.8
Bovine herpesvirus type 1.2	29.6	30.9	33.6	31.1	30.9	29.8	37.8	-

associated with cancer in immunosuppressed individuals [15], such as Kaposi sarcoma herpesvirus (human herpesvirus 8) [16]. The genus *Macavirus*, another genus in the subfamily *Gammaherpesvirinae* [17] includes alcelaphine herpes virus 1 and ovine herpes virus 2, which cause malignant catarrhal fever.

So while the risks associated with viruses in invasive muntjac deer in Ireland are not fully understood, if the muntjac population becomes established, it could potentially interact with livestock in terms of the evolution, spread and maintenance of viral infections. Camera trap and anecdotal evidence strongly suggest that muntjac are becoming established in Ireland and it is known that muntjac can form successful populations from a limited number of founding females [18]. As such, there is significant value in monitoring the virological status of these invasive animals. As our phylogenetic tree is based only on a small fragment of a single gene, further sequencing would be necessary to provide a definitive classification of this gammaherpesvirus, and metagenomic analysis of additional samples using next-generation sequencing would be a powerful method of further virus discovery.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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