Identification of Equid herpesvirus 2 in tissue-engineered equine tendon [version 1; referees: 2 approved with reservations]

Roisin Wardle, Jane A. Pullman, Sam Haldenby, Lorenzo Ressel, Marion Pope, Peter D. Clegg, James P. Stewart, Mohammed Al-Saadi, Philip Dyer, Mandy J. Peffers

Institute of Veterinary Science, University of Liverpool, Leahurst Campus, Chester High Road, Neston, Wirral, UK
Centre for Genomic Research, Institute of Integrative Biology, University of Liverpool, Biosciences Building, Crown Street, Liverpool, UK
Institute of Ageing and Chronic Disease, University of Liverpool, William Henry Duncan Building, West Derby Street, Liverpool, UK
Institute of Infection and Global Health, Department of Infection Biology, University of Liverpool, West Derby Street, Liverpool, UK

Abstract

Background: Incidental findings of virus-like particles were identified following electron microscopy of tissue-engineered tendon constructs (TETC) derived from equine tenocytes. We set out to determine the nature of these particles, as there are few studies which identify virus in tendons per se, and their presence could have implications for tissue-engineering using allogenic grafts.

Methods: Virus particles were identified in electron microscopy of TETCs. Virion morphology was used to initially hypothesise the virus identity. Next generation sequencing was implemented to identify the virus. A pan herpesvirus PCR was used to validate the RNASeq findings using an independent platform. Histological analysis and biochemical analysis was undertaken on the TETCs.

Results: Morphological features suggested the virus to be either a retrovirus or herpesvirus. Subsequent next generation sequencing mapped reads to Equid herpesvirus 2 (EHV2). Histological examination and biochemical testing for collagen content revealed no significant differences between virally affected TETCs and non-affected TETCs. An independent set of equine superficial digital flexor tendon tissue (n=10) examined using designed primers for specific EHV2 contigs identified at sequencing were negative. These data suggest that EHV is resident in some equine tendon.

Conclusions: EHV2 was demonstrated in equine tenocytes for the first time; likely from in vivo infection. The presence of EHV2 could have implications to both tissue-engineering and tendinopathy.
Corresponding author: Mandy J. Peffers (peffs@liv.ac.uk)

Author roles: Wardle R: Formal Analysis, Funding Acquisition, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Pullman JA: Data Curation, Formal Analysis, Methodology, Resources, Software, Writing – Original Draft Preparation, Writing – Review & Editing; Haldenby S: Data Curation, Formal Analysis, Funding Acquisition, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Ressel L: Formal Analysis, Investigation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Pope M: Formal Analysis, Investigation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Clegg PD: Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; Pullman JA: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Radford A: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Stewart JP: Formal Analysis, Investigation, Methodology, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Al-Saadi M: Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Pope M: Formal Analysis, Investigation, Writing – Original Draft Preparation; Peffers MJ: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

How to cite this article: Wardle R, Pullman JA, Haldenby S et al. Identification of Equid herpesvirus 2 in tissue-engineered equine tendon [version 1; referees: 2 approved with reservations] Wellcome Open Research 2017, 2:60 (doi: 10.12688/wellcomeopenres.12176.1)

Copyright: © 2017 Wardle R et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Grant information: This work was supported by the Wellcome Trust [107471]; the Medical Research Council (MRC) and Arthritis Research UK as part of the MRC-Arthritis Research UK Centre for Integrated research into Musculoskeletal Ageing (CIMA), and by a Wellcome Trust Summer Studentship.

First published: 03 Aug 2017, 2:60 (doi: 10.12688/wellcomeopenres.12176.1)
**Introduction**

Tendons transfer force from muscle to bone, in addition to playing a key role in positioning and reducing locomotion expenditure, such as in the equine superficial digital flexor tendon (SDFT). Their structure is hierarchical, with the predominating component being a collagen-dominated extracellular matrix (ECM). Sparsely distributed fibroblasts (tenocytes) produce ECM. Tenocytes are utilised in the production of tissue-engineered tendon constructs (TETCs), which are used within tendon research as a model for in vivo tendons, and have been explored as a potential therapeutic modality for tendinopathy.

There are few causes of musculoskeletal disease which have been attributed to viral infection, with no previous literature citing viral causes of tendinopathy in the horse. Previous viruses implicated in tendinopathy of other species include adenovirus and reovirus in tenosynovitis of broiler hens. This study suggests that a primary viral infection coupled with subsequent bacterial infection leads to catastrophic musculoskeletal infection and death.

Next generation sequencing (NGS) is frequently used as a research tool to characterise the transcriptome of eukaryotes, providing superior profiling to previously used methods such as microarrays. Wang *et al.* describe NGS as a high throughput, highly sensitive method for transcriptome analysis. We have previously used it to characterise transcriptome-wide gene expression in numerous studies, including tendon ageing and disease. Additionally, it has infrequently been used to identify viral isolates within tissues with a high sensitivity.

In this study equine TETCs were produced to report changing protein profiles with ageing. Following electron microscopy, two samples were found to contain virus-like structures. The aim of this study was to identify these particles using NGS and investigate their functional consequences on TETCs.

**Materials and methods**

All reagents were supplied by Sigma unless otherwise stated.

**Sample collection**

Equine SDFT was collected from young (n=7; age: 5 years ± 1.095 SD) and old (n=6; age: 18.5 years ± 2.429SD) donors from the equine hospital and abattoir as a by-product of the agricultural industry. The Animal (Scientific Procedures) Act 1986, Schedule 2 does not define abattoir collection as a scientific study, and hence ethical approval was not required. Samples collected from an equine hospital were subject to the University of Liverpool ethical approval and consent (VREC462).

**Tissue-engineered tendon production**

Tenocytes were digested from equine SDFT using standard collagenase protocol. Constructs were engineered as previously described by Kharaz *et al.*. In brief, tenocytes were seeded at 600,000 cells per well then scored every other day until full contraction of the construct. Constructs were harvested at 28 days and either snap frozen, collected for standard histology or transmission electron microscopy (TEM).

**Transmission electron microscopy**

TEM of tendon constructs was performed following fixation in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 8 hours, followed by buffer washing procedures and second fixation and contrast stain with 0.1% osmium tetroxide for 90 minutes. Samples were stained with 8% uranyl acetate in 0.69% maleic acid for 90 minutes, dehydrated in ascending ethanol concentrations and embedded in epoxy resin. Ultrathin cross-sections (60–90 nm) were cut with a Reichert-Jung Ultracut on an ultramicrotome (Leica Microsystems, Wetzlar, Germany) using a diamond knife. Sections were then mounted on 200 mesh copper grids and stained with ‘Reynold’s Lead citrate’ stain for 4 minutes. Images were viewed in Philips EM208S Transmission Electron Microscope (Phillips UK Limited, Guildford, UK) at 80K.

**Virion investigation**

On examination of TEM images, two TETCs were identified as containing virus-like particles (V: Y1, Y6). These particles were morphologically assessed for virion identification. Particle diameter was estimated from electron micrograph images. Particle morphological characteristics were observed for virion diameter, presence of a viral envelope, nucleocapsid shape and surface projection presence. The number of full capsids, nucleocapsids and empty capsids were counted in 22–26 TEM images from each of the infected donors. For each sample, resin sections were mounted onto copper grids and viewed in the TEM at X 44000 magnification. 25–30 successive grid squares were viewed and the first area in each grid square which was found to contain virus was photographed. The images were loaded into ImageJ (version 1.51n) and counts made of virus with:

i) envelope + nucleocapsid + DNA (full)

ii) envelope + nucleocapsid – DNA (nucleocapsid only).

iii) envelope only (empty).

TEM images were compared to current literature in order to provide a morphological reference (Supplementary File 1).

**Histological analysis**

TETCs were fixed in 4% paraformaldehyde and paraffin embedded. Sections were cut at 4µm onto polyllysine slides and subsequently stained with haematoxylin and eosin (H&E) and Mason’s Trichrome. Histology was assessed using a scoring system developed in an unpublished report by Charters and shown in Supplementary File 2.

**NGS analysis**

Two samples of TETCs were submitted for RNASeq, one virally affected (V: Y1) and one control (NV). RNA was extracted from constructs as previously described, and NGS was performed in accordance with the method described by Peffers *et al.*. Analysis was undertaken by the Centre for Genomic Research, University of Liverpool. One µg of total RNA was ribosome depleted with the RiboZero Magnetic kit (Illumina, San Diego, California, United States). NGS libraries were prepared using the ScriptSeq v2 NGS Library Preparation Kit (Illumina, San Diego, California, United States).
Figure 1A and 1B. Viral particles were identified by Transmission electron microscopy (Figure 1A and 1B). Two out of 13 samples contained virus-like particles. The virus-like particles identified were approximately 100–150nm in diameter, and had an indistinct outer border with surface protrusions and were identified both intracellularly (T) (including intranuclearly) and within the extracellular matrix.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on all samples to confirm the presence of virus-like particles. Samples were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 1 hour at room temperature. After fixation, samples were washed in the same buffer and post-fixed in 1% osmium tetroxide in the same buffer for 1 hour. Following this, samples were dehydrated in a graded series of alcohols and embedded in resin. Ultrathin sections were cut using a Reichert Ultracut S microtome and collected on 200 mesh copper grids. Grids were stained with uranyl acetate and lead citrate and imaged using a Tecnai G2 Spirit BioTwin transmission electron microscope (FEI, Hillsboro, OR).

Results

Transmission electron microscopy

Two out of 13 samples contained virus-like particles. The virus-like particles identified were approximately 100–150nm in diameter, and had an indistinct outer border with surface protrusions and a cylindrical nucleocapsid (Figure 1A and 1B). Viral particles were identified both intracellularly (T) (including intranuclearily) and within the extracellular matrix (Figure 1A). A further characteristic present in several of the images observed were ‘empty shells’.

Pan herpesvirus PCR

As RNAseq is not definitive, we used a pan-herpes PCR to confirm that the virus in question was indeed a herpes virus and there were no other herpesviruses present.

In order to validate the RNAseq findings, a pan-herpesvirus PCR was undertaken. Samples V; Y1 and Y6 (viremically affected) and a negative control (O3) were tested using a pan-herpesvirus PCR using a modified methodology described by Ehlers et al.17. This protocol uses dI-substituted primers that offer improved sensitivity and specificity than previous protocols18.

Specific EHV-2 PCR assay of equine SDFT

In order to determine the presence of EHV-2 in SDFT of a larger equine population, PCR primers were designed to amplify a region within a 37kb contig demonstrating an excellent depth of coverage (mean 4919x coverage) and 99% homology to EHV-2. The primer sequence was: V1 forward GGGCGGAGAATGTAGAGACG, V1 reverse GTTGAGCTTTAAACGGGGAGG (product size 443). DNA was extracted from 10 (mean ±SD age: 12.8±7.1) grossly normal equine SDFT collected from an abattoir. A Qiamp DNA extraction kit (Qiagen, Crawley, UK) following the manufacturer’s protocol was used. A sample of EHV-2 positive DNA (kindly gifted by Microbiology Diagnostic Laboratory, Institute of Veterinary Science, Liverpool) was used as a positive control.

PCR was performed in a final volume of 50ul consisting of 12.5ul ThermoPrime 2x ReddyMix PCR Master Mix, 1.5ul forward primer, 1.5ul reverse primer, 24.5ul water and 10ul extracted DNA using Bioanalyzer (Agilent, Santa Clara, CA, USA). Libraries were pooled in equimolar amounts and quantity and quality of each pool assessed by using Bioanalyzer (Agilent, Santa Clara, CA, USA) and subsequently by qPCR using the Illumina Library Quantification Kit from Kapa (KK4854) on a Roche Light Cycler LC480II according to manufacturer’s instructions. The template DNA was loaded at 300 PM. The sequencing was carried out on one lane of an Illumina HiSeq4000 at 2×150 bp paired-end sequencing with v1 chemistry.

Initial processing and quality assessment was undertaken prior to statistical analysis. Data was considered statistically significant at P<0.05. All normality testing and statistical analysis was undertaken using Excel (2010, Microsoft, Redmond, WA, USA) and GraphPad prism (2016, version 7, GraphPad Software Inc, CA, USA).

NGS analysis: Transcript assembly and removal of host genomic reads

Illumina sequence reads were adapter- and quality-trimmed using Cutadapt version 1.2.1 (Martin, 2011) and Sickle version 1.200. Illumina sequence reads were adapter- and quality-trimmed using Cutadapt version 1.2.1 (Martin, 2011) and Sickle version 1.200. Host sequences were removed by aligning trimmed reads to the Equus caballus genome (Equus caballus; EquCab2.56.pep) with HiSat (version 2.0.3b)19.

NGS analysis: Viral sequence determination

To taxonomically assign contigs, a BLASTN (MegaBlast, version 2.2.7+)20) search of the assembled transcripts against the NT database from NCBI was carried out and full taxon information (species, order, phylum, superkingdom) was derived based on the best hit, using the NCBI taxonomy database (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy/taxdump.tar.gz). The search was carried out with an e-value cut off of 1e-5. The results were filtered to identify all hits to the virus superkingdom.

All raw read data produced in this study has been submitted to the EBI ENA, primary accession PRJEB20552, secondary accession ERP022713.

Hydroxyproline assay

Freeze-dried samples (V (n=2) and NV (n=5) of TETC derived from the young donors were hydrolysed overnight at 60°C using papain reagent. Digests were stored at -20°C and subsequently assayed for collagen content by hydroxyproline assay21.

Statistical analysis

Data were normally tested prior to statistical analysis. Data was considered statistically significant at P≤0.05. All normality testing and statistical analysis was undertaken using Excel (2010, Microsoft, Redmond, WA, USA) and GraphPad prism (2016, version 7, GraphPad Software Inc, CA, USA).
capsids’ within cells (Figure 1C and 1D). Viral families have distinct morphological characteristics relating to size, nucleocapsid shape, and envelope present, which were used to tentatively identify the particles. The virus particles identified within the TETCs exhibited a spherical shape, with a visible envelope, contained within an icosahedral capsid shape. Virion diameter was calculated and was suggestive of a retrovirus or herpesvirus. The apparent spiked edge of the viral particles was suggestive of a retrovirus. In order to tentatively assess the level of infection within the TETCs, the number of tenocytes affected with virus particles was counted by examining the TEM images of all the donors. Whilst no virus particles were evident in images of the tenocyte-tenocyte (Figures 2A and 2B). Electron micrographs of virus-like particles found within TETCs. Tenocyte (T), extracellular matrix viral particles (black arrows). C and D: Electron micrographs showing full capsids, nucleocapsids (NC) and empty capsids (white arrows) within both of the TETCs from which virus were isolated (C; Y1, D; Y6). Scale bars are shown.

**Table 1.** Counting of full, empty and nucleocapsids in virus-infected TETC samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sum</th>
<th>Mean</th>
<th>SD</th>
<th>Sum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full capsid</td>
<td>164</td>
<td>7.5</td>
<td>4.5</td>
<td>316</td>
<td>12.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Nucleocapsid only</td>
<td>129</td>
<td>5.9</td>
<td>5.1</td>
<td>183</td>
<td>7.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Full capsid + nucleocapsid</td>
<td>293</td>
<td>13.3</td>
<td>5.5</td>
<td>499</td>
<td>19.2</td>
<td>10.6</td>
</tr>
<tr>
<td>Empty capsid</td>
<td>261</td>
<td>11.9</td>
<td>20.6</td>
<td>438</td>
<td>16.8</td>
<td>18.8</td>
</tr>
<tr>
<td>Empty + nucleocapsid</td>
<td>390</td>
<td>17.7</td>
<td>24.3</td>
<td>621</td>
<td>23.9</td>
<td>23.3</td>
</tr>
</tbody>
</table>

SD: standard deviation

NGS analysis: Viral sequence determination. Y6 had 131 transcripts that hit viral sequences, 129 were assigned to Equid herpesvirus 2, a single read was assigned Equine Infectious Anaemia (EIA) virus and a single read to Bovine viral diarrhea virus (BVDV). For Y6 84.25% reads were mapped to the Equus caballus genome and 1.32% to Equid herpesvirus 2 strain G9/92 complete genome (accession; KM924294.1); the strain that most reads mapped to in this sample. Identified gene transcripts are in Table 2.

Histological scoring
There were no significant differences in histological score between V and NV based upon the characteristics measured (Figure 2).

Hydroxyproline assay
Contrasting V and NV TETCs showed that there was no significant difference in collagen content (Figure 3).

NGS analysis
**NGS analysis: Transcript assembly and removal of host genomic reads.** Illumina sequence reads were adapter- and quality-trimmed using Cutadapt version 1.2.1 (Martin, 2011) and Sickle version 1.200. A summary of raw and trimmed sequence data is shown in Supplementary File 4, following host sequence removal, and subsequent filtering of mapped reads. Resulting paired-end reads were assembled using Trinity version r2013_08_14 for both samples. V resulted in 913,443 transcripts (including isoforms) and NV resulted in 788,076 transcripts (including isoforms).
Figure 2. Histograms of histological scoring of TETCs. Histological scoring of TETCs from virally affected (n=2) and normal (n=8) donors. Graphs A-F represent the mean scores ± standard deviation of the following characteristics; mean extracellular matrix organisation (A), cell shape (B), cellular distribution (C), cellular alignment (D), TETC cellularity (E) and mean total score (F). Where error bars are not present, scores for all donors were equal. Further details of the scoring system are available in Supplementary File 2. No significant differences were found between virally affected and normal donors (p ≤ 0.05).

Figure 3. Histogram of collagen content of young TETCs containing virus (V; n=2) and not containing virus (NV; n=5). Graphs represent mean ± standard error mean of percentage collagen normalised to dry weight. No significant difference was found in collagen content (p ≤ 0.05).
Table 2. The reads mapping to specific genes/transcripts in EHV2 (G9/92). Counts are presented in the table calculated using HTSeq-count.

<table>
<thead>
<tr>
<th>Read Count</th>
<th>Gene Name</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>E1</td>
<td>membrane protein E1</td>
</tr>
<tr>
<td>0</td>
<td>E10</td>
<td>apoptosis regulator E10</td>
</tr>
<tr>
<td>286</td>
<td>E2</td>
<td>protein E2</td>
</tr>
<tr>
<td>86</td>
<td>E3</td>
<td>membrane protein E3</td>
</tr>
<tr>
<td>867</td>
<td>E4</td>
<td>apoptosis regulator BALF1</td>
</tr>
<tr>
<td>34</td>
<td>E5A</td>
<td>protein E5A</td>
</tr>
<tr>
<td>3018</td>
<td>E6</td>
<td>membrane protein BILF1</td>
</tr>
<tr>
<td>0</td>
<td>E6A</td>
<td>protein E6A</td>
</tr>
<tr>
<td>853</td>
<td>E6C</td>
<td>protein E6C</td>
</tr>
<tr>
<td>260</td>
<td>E7</td>
<td>interleukin-10</td>
</tr>
<tr>
<td>1704</td>
<td>E7A</td>
<td>envelope glycoprotein 42</td>
</tr>
<tr>
<td>777</td>
<td>E8</td>
<td>apoptosis regulator E8</td>
</tr>
<tr>
<td>8</td>
<td>E9</td>
<td>membrane protein E9</td>
</tr>
<tr>
<td>4275</td>
<td>ORF10</td>
<td>protein G10</td>
</tr>
<tr>
<td>6163</td>
<td>ORF11</td>
<td>virion protein G11</td>
</tr>
<tr>
<td>5187</td>
<td>ORF17</td>
<td>capsid maturation protease</td>
</tr>
<tr>
<td>0</td>
<td>ORF17.5</td>
<td>capsid scaffold protein</td>
</tr>
<tr>
<td>2661</td>
<td>ORF18</td>
<td>protein UL79</td>
</tr>
<tr>
<td>6867</td>
<td>ORF19</td>
<td>DNA packaging tegument protein</td>
</tr>
<tr>
<td>3165</td>
<td>ORF20</td>
<td>nuclear protein UL24</td>
</tr>
<tr>
<td>2484</td>
<td>ORF21</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>697</td>
<td>ORF22</td>
<td>envelope glycoprotein H</td>
</tr>
<tr>
<td>5154</td>
<td>ORF23</td>
<td>tegument protein UL88</td>
</tr>
<tr>
<td>13490</td>
<td>ORF24</td>
<td>protein UL87</td>
</tr>
<tr>
<td>3629</td>
<td>ORF25</td>
<td>major capsid protein</td>
</tr>
<tr>
<td>721</td>
<td>ORF26</td>
<td>capsid triplex subunit 2</td>
</tr>
<tr>
<td>736</td>
<td>ORF27</td>
<td>envelope glycoprotein 48</td>
</tr>
<tr>
<td>157</td>
<td>ORF28</td>
<td>envelope glycoprotein 150</td>
</tr>
<tr>
<td>21228</td>
<td>ORF29</td>
<td>DNA packaging terminase subunit</td>
</tr>
<tr>
<td>4507</td>
<td>ORF3</td>
<td>G3</td>
</tr>
<tr>
<td>9</td>
<td>ORF30</td>
<td>protein UL91</td>
</tr>
<tr>
<td>286</td>
<td>ORF31</td>
<td>protein UL92</td>
</tr>
<tr>
<td>971</td>
<td>ORF32</td>
<td>DNA packaging tegument protein</td>
</tr>
<tr>
<td>1339</td>
<td>ORF33</td>
<td>tegument protein UL16</td>
</tr>
<tr>
<td>1194</td>
<td>ORF34</td>
<td>protein UL95</td>
</tr>
<tr>
<td>107</td>
<td>ORF35</td>
<td>tegument protein UL14</td>
</tr>
<tr>
<td>833</td>
<td>ORF36</td>
<td>tegument serine/threonine kinase</td>
</tr>
<tr>
<td>1325</td>
<td>ORF37</td>
<td>deoxyribonuclease</td>
</tr>
</tbody>
</table>
O3 had 7 transcripts aligned to virus sequences, all assigned Equid herpesvirus 2. A further BLASTN search against all animal ERV sequences from the NCBI database was carried out to check that the contigs suggested to be herpesvirus were not ERVs. No significant hits to known ERVS were found for contigs assigned as EHV2 in the previous search.

**NGS: Genome coverage plots of discovered viral sequence against EHV2.** Coverage plots of the samples were checked against the following genomes; Bovine herpesvirus type 1.1 (AJ004801.1), Equid herpesvirus 1 strain T953 (KM593996.1), Equid herpesvirus 2 strain G9/92 (KM924294.1), Equid herpesvirus 3 strain AR/2007/C3A (NC_024771.1), Equid herpesvirus 4 (NC_001844.1), Equid herpesvirus 5 strain 2-141/67 (NC_026421.1). From these plots for samples NV (O3) (Figure 4a) and V (Y6) (Figure 4b), it is clear that the virus isolated from TETC Y6 is likely EHV2. There does not appear to be any EHV2 in the NV sample. Occasional coverage spikes were due to mapping low complexity reads which would map well against many genomes.

The coverage plot shows that the genomic coverage of the TETC virus of EHV2 strain G9/92 was 86.26%. The virus was mapped to 3,875.605 contigs to EHV-2, compared with 2–339 from the rest of the herpesvirus panel (EHV1, 3, 4, 5).

**Pan herpesvirus PCR.** Pan herpesvirus PCR was found to be positive for EHV2 for the two V samples and negative for NV samples (Figure 5). Sequencing of PCR products was used to confirm EHV2 presence within the sample.

**Specific PCR.** Of the ten samples assessed, none were found to contain EHV2 (Figure 6).

**Phylogenic analysis.** A phylogenetic tree was produced in order to characterise the relationship between the TETC virus and currently identified EHV2s (Figure 7). Phylogenetic analysis of the TETC isolated virus glycoprotein B gene shows branching with Equid herpesvirus 2 strain 275, with further close relationships with strains G9/92, 86/87 and 86. The phylogenetic tree produced clearly
Figure 5. Pan herpesvirus gel image. Gel image of PCR fragments following restriction by ECOR 1. Virus-infected samples Y1, Y6 and virus negative sample O3 are shown. Amplicon size is 229 bp. Bands were removed and subsequently sequenced to confirm identification of herpes virus.

Figure 6. EHV2 PCR assay in an additional cohort of SDFT samples. DNA extractions from ten equine SDFT samples (1–10) were amplified with primers (V1) designed within a EHV-2 contig identified following NGS. Genomic DNA from EHV-2 was used as a PCR positive control (EHV) and water as a negative control (bl). TrackIT 1Kb Plus DNA ladder was used as a marker (1kb ladder). The positive EHV2 control demonstrates a band at 450bp.

Figure 7. Neighbour-joining trees. Trees characterised the relationship between virus isolated from V (EHV2 RJW248419) and previously isolated strains using glycoprotein B gene. Bootstrap analysis (1,000 replicates) was used to provide support for individual nodes.
demonstrates that the strain isolated from the TETCs is an EHV2, although not a strain which currently has been genome sequenced in the NCBI database.

**Discussion**

This study is the first to identify EHV2 within cells derived from equine tendons. Findings of virus particles in samples of TETCs in which TEM was undertaken were incidental. At the time in our laboratory, equine tenocytes were the only cell type being cultured. We were interested in the nature of the particles and thus set out to investigate further. As this was a post-experiment attendant result identified a number of weeks after the end of the study, we were unable to undertake culture of the virus-infected tenocytes to determine what the titre of the virus was and if this was infectious. We realise this is a limitation of our work. However, as the presence of EHV2 was previously unreported in tendons, and due to the potential use of TETCs as allogenic transplant agents, we believe the finding of EHV2 in tenocytes could have implications for future tissue-engineering studies. Additionally, it should serve as a warning that viral contamination of musculoskeletal tissues may not be appreciated if screening is not undertaken.

Initial analysis tentatively identified a herpesvirus through electron microscopy, and this was confirmed as EHV2 with NGS and a pan-herpesvirus PCR. The structure of virion particles on TEM was initially suggestive of a retrovirus or a herpesvirus. Differential features based upon morphology included glycoprotein spikes, suggestive of a retrovirus\(^2\), and undefined tegument protein, suggestive of a herpesvirus\(^3\). We demonstrated the presence of empty capsid structures within both TETCs. Previous studies have hypothesised that empty capsids are precursors of mature herpesviruses\(^4\). Previous images identifying A-capsids\(^2\) are similar to those in our study, further implicating identification of an equid herpes virus. This, coupled with measurement of virion diameter, was suggestive of a herpes or retrovirus\(^5\). Due to the non-conclusive ultrastructural features in regard to differentiating between a herpesvirus and retrovirus, further analytical methods were employed to distinguish viral identity.

NGS identified the virus within the TETCs as most similar to EHV2 strain G9/92. Phylogenetic analysis of the sequence isolated through NGS (EHV2 RJW 248419) demonstrated the close relationship between the virus in this study and the currently sequenced strains.

Swenson et al.\(^6\) studied the presence of feline herpesvirus-1 (FHV1) within the feline tendon following experimental inoculation. It has previously been described that tissue-engineered tendons can be used as allographic transplant agents\(^7\). They implied that the presence of FHV1 for application of TETCs in allogenic transplantation could lead to immune rejection of the engineered tissue and hence transplant failure. Whilst the histological structure and collagen content of the TETCs was not altered by the presence of EHV2, the ability of EHV2 within TETCs here to survive culture and multiple passages whilst retaining the ability to reproduce, may have implications for their use as a therapeutic option in terms of transplant rejection.

In the literature, isolation of primary virus within tenocytes is limited, though fibroblasts from other anatomical locations have demonstrated the ability for viral replication. Klevjer-Anderson et al.\(^8\) describe the persistent infection of equine dermal fibroblasts with EIA. Further virus isolated from fibroblasts include herpes simplex virus within avian fibroblast cells. Stulberg et al.\(^9\) discuss the growth of herpes simplex virus within cultured fibroblasts, illustrating a cytopathic effect upon cells cultured using a non-plasma technique. Such areas of focal necrosis were not evident in the TETCs here.

Viral causes of tenosynovitis have previously been described in chickens\(^10\). The study investigated outbreaks of tenosynovitis in commercial broilers using virus isolation. Tendon from affected flocks yielded isolation of reoviruses and adenoviruses. To our knowledge, viral agents have not previously been isolated from tendons in horses. Zeng et al.\(^11\) used TEM to visualise adenovirus particles. Their TEM images suggest that adenoviruses are much smaller than the particles isolated in this experiment, with the approximate diameter being 70–90nm. A similar result was evident with reoviruses\(^12\), which have an average diameter of 60–80nm.

Whilst all culturing was undertaken in sterile conditions the identified virions could have originated from a culture contaminant. Likely causes include cross-contamination between TETCs and other tissue within the laboratory or use of contaminated reagents during production. Previous studies have found viral contamination in commercially available foetal calf serum such as that used in TETC production here. However, as the virus was limited to a subset of cultured TETC this is unlikely. Viral isolates include BVDV, bovine parainfluenza virus-3 and bovine herpesvirus-1 (BoHV1)\(^13\). Membrane filtration removes many contaminants, however these may be an ineffective against viruses due to their small size\(^14\).

It is important to consider host-range and tissue specificity of viruses, which could be present as a contaminant of tissue culture reagents. Foetal calf serum was used in the tissue-engineered culture system. As a result, we compared the viral reads obtained to a variety of bovine virus\(^15\) with suggestive morphology. One read to BoHV1 was identified in virally affected TETC. As an alpha-herpesvirus BoHV1 has previously shown limited scope for crossing species-barriers\(^16\). The single read to BoHV1 when compared with EHV2 (129) suggest that BoHV-1 was not the virus isolated here. Moreover, the BoHV1 read could be a consequence of cross mapping. One read was found to EIA. This is a notifiable disease within the UK. The single read was of only 52bp hence it was suspected due to cross-mapping and further investigation was not pursued.

Unfortunately the EHV status of the donor horses was unknown. The clinical history for one of the TETC donors containing virus was available. The donor was presented at the hospital with right hind lameness and proprioceptive deficits. The horse was euthanased on humane grounds. EHV myeloencephalopathy was not suspected on presentation due to the absence of cauda equina signs\(^17\). Moreover, the equid herpesvirus most associated with
myeloencephalopathy is EHV1. NGS data in this study revealed minimal coverage of the EHV1 genome when compared with EHV-2. There was no clinical history of the TETC donor as this was derived from an abattoir and hence further conclusions cannot be drawn. Since a small population of other horses were assessed for the presence of EHV2 RJW 248419 it would appear that the presence of this virus in equine tendon is uncommon.

Finally, an interesting point to consider is the potential role of an equid herpes virus in tendinopathy. EHV2 has not previously been isolated in cases of musculoskeletal disease in the horse. Whether this is because it has not been investigated or because it has no significant role in disease has not been established. Due to the unknown status of horse from the abattoir it is difficult to draw conclusions from the single case in which hind limb pathology was described. Whilst histological examination and limited biochemical tests within this study implies that EHV2 has no significant affect upon the structure of TETCs the mechanical properties of the TETCs were not assessed. Further work is required to determine if EHV-2 has a role in tendinopathy.

Conclusions

This study identifies EHV2 in equine tendons for the first time, and describes NGS as a useful tool for virus identification. The implications of the presence of EHV2 in tendon to both tissue-engineering and tendinopathy requires further work. However, there are potential implications for the use of TETCs as allogenic transplant agents, as the presence of virus could result in transplant failure.

Data availability

All raw read data produced in this study has been submitted to the EBI ENA, primary accession PRJEB20552, secondary accession ERP022713. The data underlying this work has been uploaded to the Open Science Framework Database, and can be accessed via DOI, 10.17605/OSF.IO/WYPKQ.

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the Wellcome Trust [107471]; the Medical Research Council (MRC) and Arthritis Research UK as part of the MRC-Arthritis Research UK Centre for Integrated research into Musculoskeletal Ageing (CIMA), and by a Wellcome Trust Summer Studentship.

Acknowledgments

We thank Charlotte Charters (University of Liverpool Veterinary School) for the use of her histology scoring system and Yalda Ashraf-Kharaz, Institute of Ageing and Chronic Disease, University of Liverpool) for her assistance with the hydroxyproline assay.

Supplementary material

Supplementary File 1. Table of the characteristics of various virus families that have previously been isolated in fibroblasts or as contaminants in tissue culture.

Click here to access the data.

Supplementary File 2. Table showing the scoring system used during histological analysis of TETCs.

Click here to access the data.

Supplementary File 3. Table of the primer sequences used in the pan herpesvirus PCR.

Click here to access the data.

Supplementary File 4. Summary table of the raw and trimmed sequence data.

Click here to access the data.

References


Reference Source


Open Peer Review

Sarah Elizabeth Taylor
The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK

“This study suggests that a primary viral infection coupled with subsequent bacterial infection leads to catastrophic musculoskeletal infection and death" Please clarify that this is the study on broiler hens and not the current work.

It is not clear from the current work what statistical tests were carried out in the programmes described in the materials and methods.

Were any of the RNA seq hits for the latency associated transcript RNA?
The specific PCR for EHV2 on tendon tissue did not find any viral DNA however can this test confirm lack of presence of latency associated transcript RNA?
Was there any RNA from the virus infected tenocytes left over for analysis?
Did the authors collect these tenocytes from tissue that was known to be septic antemortem? I wonder if this statement is a jump too far?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

**Author Response 13 Oct 2017**

**Mandy Peffers, University of Liverpool**

**Reviewer**

This study suggests that a primary viral infection coupled with subsequent bacterial infection leads to catastrophic musculoskeletal infection and death* Please clarify that this is the study on broiler hens and not the current work.

**Response**

We have amended the manuscript to clarify that this was indeed in broiler hens.

**Reviewer**

It is not clear from the current work what statistical tests were carried out in the programmes described in the materials and methods.

**Response**

We have amended the manuscript to clarify that these statistical tests relate to the hydroxyproline assay.

**Reviewer**

Was there any RNA from the virus infected tenocytes left over for analysis?

**Response**

All the RNA samples were used in the studies pertaining to this manuscript.

**Reviewer**

Did the authors collect these tenocytes from tissue that was known to be septic antemortem? I wonder if this statement is a jump too far.

**Response**

All samples were grossly normal on collection. Hospital derived samples showed no signs of sepsis.

**Reviewer**

Were any of the RNA seq hits for the latency associated transcript RNA?

**Response**

Two different types of mapping were undertaken. Firstly a megablast run against the entire nt database to get taxonomic information, and secondly a blastx run against a database created of all know ERV genes in animals from NCBI. Therefore the 'latency associated transcripts’ (only appear to be sequences for these in humans in NCBI) would not have been included. We assessed the two sequences in NCBI that result from a search for 'latency associated transcript and herpes' and ran a blastx for both samples against these. Neither resulted in any significant hits. Thus whilst latency associated transcripts cannot be ruled out with data currently available this is unlikely. A more in-depth analysis than this would require further research in latency associated
transcripts linked to herpes in other animals and is beyond the scope of this paper. We have amended the manuscript accordingly.

Reviewer
The specific PCR for EHV-2 on tendon tissue did not find any viral DNA however can this test confirm lack of presence of latency associated transcript RNA?

Response
The reviewer is correct in their assumption. We have amended the manuscript.

Competing Interests: No competing interests were disclosed.

Duncan Hannant
School of Veterinary Medicine and Science, University of Nottingham, Loughborough, UK

This is a very well executed study carried out with high level of rigour to define and describe the findings of EHV-2 in equine tendon cells. There is one important aspect which potentially confounds the interpretation of the data. The authors refer to the possibilities of some contamination of samples and tissue culture reagents as a possible source of EHV-2 virus and PCR products in tendon cells and provide a good argument to discount these. However, one aspect is not fully considered in their justification of this potentially novel finding. There have been many published papers describing equine gammaherpes viruses in PBMC and several tissues/organs in both foals and adult horses. EHV-2 is ubiquitous in horses and virus and viral genes are commonly found. For example, Torfason et al. described detection of EHV-2 and EHV-5 in PBMC and a selection of tissues in about 80% of the animals they examined using co-cultivation methods to demonstrate infectious replicating virus. EHV-2 is common in foals but these observations were also made in older horses on this occasion. As with other gammaherpes viruses, EHV-2 is known to be latent in B lymphocytes (Drummer et al.) and these latent viruses were re-activated and identified by co-cultivation methods. These and other findings strongly suggest that EHV-2 virus or cells containing EHV-2 genes would have been present in a high proportion of tissue samples taken from horses for this study. Many samples were from slaughterhouse materials but even so, it would have been very difficult to remove tendon tissues from horses without significant contamination by infected (or latently-infected) cells.

These possibilities should be discussed fully in the paper. It is possible also that cell-to-cell contact of infected PBMC or other donor cells with tenocytes is the route of infection of the latter in vivo. This would be perfectly acceptable as a method by which EHV-2 genes and virus were detected in cultured tenocytes. The fact that the authors detected virus in only 2 of the 13 samples might also be worthy of note, considering that this virus is so ubiquitous.

Do the authors know if the positive samples were from old or young horses?

The considerations above do not detract from the findings that equine tendon cells may contain EHV-2 and that this might have implications for use of these cells therapeutically to treat tendinopathy. The contention is that the paper should discuss the findings in relation to published papers such as those
indicated above and the potential likelihood of this finding not being unexpected.

**References**


**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Equine viral immunology, vaccinology and diagnostics. Rapid and accurate diagnosis of infectious diseases in animals using microarray systems for detection of pathogen nucleic acids.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

**Author Response 13 Oct 2017**

**Mandy Peffers**, University of Liverpool

The authors appreciate the comments made by the reviewer. We have taken on board their concerns and amended the manuscript accordingly to include discussion of other possible reasons for the findings of EHV-2 in equine tenocytes.

**Competing Interests:** No competing interests were disclosed.