1 **Studying longitudinal neutralising antibody levels against Equid herpesvirus 1 in experimentally**
2 **infected horses using a novel pseudotype based assay**

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14 Abstract:

15 Infection with equid herpesvirus 1 (EHV-1), a DNA virus of the *Herpesviridae* family represents a
16 significant welfare issue in horses and a great impact on the equine industry. During EHV-1 infection,
17 entry of the virus into different cell types is complex due to the presence of twelve glycoproteins
18 (GPs) on the viral envelope. To investigate virus entry mechanisms, specific combinations of GPs
19 were pseudotyped onto lentiviral vectors. Pseudotyped virus (PV) particles bearing gB, gD, gH and gL
20 were able to transduce several target cell lines (HEK293T/17, RK13, CHO-K1, FHK-Tcl3, MDCK I & II),
21 demonstrating that these four EHV-1 glycoproteins are both essential and sufficient for cell entry.
22 The successful generation of an EHV-1 PV permitted development of a PV neutralisation assay
23 (PVNA). The efficacy of the PVNA was tested by measuring the level of neutralising serum antibodies
24 from EHV-1 experimentally infected horses (n=52) sampled in a longitudinal manner. The same sera
25 were assessed using a conventional EHV-1 virus neutralisation (VN) assay, exhibiting a strong
26 correlation (r=0.82) between the two assays. Furthermore, PVs routinely require -80°C for long term
27 storage and a dry ice cold-chain during transport, which can impede dissemination and utilisation in
28 other stakeholder laboratories. Consequently, lyophilisation of EHV-1 PVs was conducted to address
29 this issue. PVs were lyophilised and pellets either reconstituted immediately or stored under various
30 temperature conditions for different time periods. The recovery and functionality of these
31 lyophilised PVs was compared with standard frozen aliquots in titration and neutralisation tests.
32 Results indicated that lyophilisation could be used to stably preserve such complex herpesvirus
33 pseudotypes, even after weeks of storage at room temperature, and that reconstituted EHV-1 PVs
34 could be successfully employed in antibody neutralisation tests.

35

36 Keywords: equid herpesvirus 1; lentiviral pseudotype virus; serology; neutralisation assay.

37

38 ## 1. Introduction

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39 In the *Equidae* family, nine equid herpesviruses (EHVs) have been identified. To date, all
40 EHVs isolated belong either to the *Alphaherpesviridae* (EHV-1, EHV-3, EHV-4, EHV-6, EHV-8 and EHV-
41 9) or *Gammaherpesviridae* (EHV-2, EHV-5 and EHV-7) subfamilies according to the latest taxonomic
42 classification (Davison et al., 2009; Maclachlan Dubovi & Winton Jr, 2017). Among EHVs, EHV-1 is
43 considered the most severe EHV, as its infection is associated not only with respiratory disease but
44 can also produce abortion, perinatal death, still-birth and neurological disorders, including Equine
45 Herpesvirus Myeloencephalopathy (EHM) (Allen, 2002; Edington et al., 1986; Edington et al., 1991;
46 Paillot et al., 2008). Thus, EHV-1 infections have a significant impact on equine welfare and lead to
47 considerable economic losses within the horse industry. Latency aids virus adaption and co-
48 evolution with the natural host, allowing long-term survival and evasion of the immune system
49 (Allen et al., 2004). Primary infections occur in the respiratory epithelium with cell entry occurring
50 following interaction between specific viral envelope glycoproteins (GPs) and cell receptors (Kydd et
51 al., 1994; Patel et al., 1982).
52 Vaccination in addition to good hygiene and management measures remains an effective
53 control practice to fight EHV-1 infection and helps reduce severity of EHV-1 related clinical
54 manifestation (OIE, 2018). Nevertheless, vaccine protection against EHV-1 disease is not always
55 complete and cell-associated viremia has been identified in some animals, which subsequently led to
56 EHM (Allen et al., 2004).
57 EHV-1 infection is routinely confirmed by Polymerase Chain Reaction (PCR) testing, detecting
58 genomic DNA. This can be combined with virus isolation and assessment of viability of the circulating
59 virus via cytopathic effect (CPE) (OIE, 2018). Diagnosis of EHV-1 infection is possible by serology via
60 virus neutralisation (VN) (Thomson et al., 1976), complement fixation (CF) (Thomson et al., 1976) or
61 enzyme-linked immunosorbent assay (ELISA) (Crabb et al., 1995) to demonstrate a virus-specific
62 antibody response. However, due to cross-reactivity of antibodies among different types of EHV a
63 type-specific diagnosis is difficult to obtain, especially between EHV-1 and -4 as a result of prior
64 infections or vaccination (Balasuriya et al., 2015; Hartley et al., 2005). Nevertheless, serology has
65 been extensively employed for seroprevalence surveys (Dunowska et al., 2015; El Brini et al., 2021;
66 Gilkerson et al., 2000; Gilkerson et al., 1999; Pusterla et al., 2009), and to monitor the response to
67 vaccination (Abousenna et al., 2022; Bannai et al., 2019; Bresgen et al., 2012; Warda et al., 2021).
68 Serology can be also used as an adjunct to inconclusive PCR results, and to confirm or exclude recent
69 virus circulation during an outbreak situation, as recommended by the European Food Safety
70 Authority (EFSA, 2022). In non-vaccinated horses, EHV-1 infection can be serologically detected by
71 screening paired sera samples collected from suspected cases during the acute and convalescent
72 stages of infection against type-specific antigen able to demonstrate seroconversion, by a greater
73 than 4-fold increase in antibody titre, that is the highest dilution of serum at which
74 neutralisation/binding is detected (OIE, 2018). In the absence of a DIVA (differentiating infected
75 from vaccinated animals) test, as is the case for equine influenza serology, this approach may be
76 more complicated for herpes viruses (Galvin et al., 2013).
77 EHV-1 presents a complex array of twelve GPs on its surface envelope, and as observed for
78 some other alphaherpesviruses, four GPs (gB, gD, gH and gL) are implicated as important for EHV-1
79 entry into cells (Azab & Osterrieder, 2012; Campadelli-Fiume G, 2007; Frampton Jr et al., 2007; Kurtz
80 et al., 2010; Sasaki et al., 2011). More precisely, EHV-1 gB and gD are essential virus components for
81 EHV-1 infectivity involved in virus penetration, virus release and direct cell-to-cell spread (Csellner et
82 al., 2000; Neubauer et al., 1997). EHV-1 gH and gL, although minor components, are co-associated in
83 a heterodimer and studies suggest their requirement in viral infection, including cell-to-cell spread
84 (Azab et al., 2012; Harald et al., 2001).
85 Pseudotype viruses (PVs) offer a valuable tool to study viral entry of susceptible cells by
86 manipulation of different combinations of candidate surface GP genes, which is more difficult to

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87 achieve with native viruses or by using reverse genetics systems. PV particles usually consist of an
88 external envelope, displaying the GPs of the study virus, and internal core of another virus (e.g. a
89 retrovirus) containing a modified genome, with deletions preventing virus replication. This inability
90 to replicate allows researchers to work under low bio-containment and to focus on GP-mediated
91 entry processes to identify the virus-cell receptor interactions and to study specific aspects of the
92 viral binding mechanism (Temperton et al., 2015). The system can be employed to study individual
93 GPs (e.g. haemagglutinin, HA, for influenza virus or Spike GP for coronaviruses) (Di Genova et al.,
94 2021; Ferrara et al., 2012; Wang et al., 2004) or in combination with others (e.g. HA and
95 neuraminidase, NA, for influenza virus) (Scott et al., 2016; Temperton et al., 2007). Consequently,
96 this amenable PV system may also prove useful to investigate the contribution of EHV-1 GPs in cell
97 entry. To date, no EHV-1 PV system has been established, however there is precedence within the
98 herpesvirus family in a study by Rogalin and Heldwein (2016) in which functional herpes simplex
99 virus (HSV-1) PV particles were generated based on a vesicular stomatitis virus (VSV) core and
100 incorporating four different GPs. This work represented both the first herpesvirus, and indeed still
101 the first DNA virus successfully pseudotyped. The purpose of the current study was to generate
102 functional pseudotype particles for EHV-1, to initially investigate which of the twelve EHV-1 GPs are
103 essential for receptor attachment and cell entry in the initial stages in virus infection. In this
104 instance, a lentivirus core was employed, but in common with the HSV-1 PV, gB, gD, gH and gL
105 glycoproteins were incorporated in the viral particles. In addition, as EHV-1 gC has been noted as a
106 mediator of EHV-1 entry, driving its attachment into cells through direct envelope-plasma fusion
107 (Csellner et al., 2000; Neubauer et al., 1997; Osterrieder, 1999), the impact of its incorporation into
108 PV particles was examined via systemic GP substitution followed by target cell entry assessment.
109 PV neutralisation assays (PVNAs) offer a potential alternative to current serological tests to
110 detect the presence of serum antibodies that can neutralise virus particles. PVNAs have been
111 applied to serological screening, vaccine immunogenicity testing and study of the immune host
112 response to infection by a range of different viruses (Carnell et al., 2015; Corti et al., 2011; Ferrara et
113 al., 2015). Consequently, we utilised the functional EHV-1 PV particles generated in serological tests
114 to measure the level of neutralising antibodies in blood serum samples which had been collected
115 from horses over an extended time period following experimental infection with EHV-1 (Thieulent et
116 al., 2022). The antibody titres were then compared with results obtained from VN assays using the
117 native virus, performed using standard OIE protocols (OIE, 2018).
118 Transport of PVs between laboratories has been carried out typically using dry ice to
119 maintain the cold-chain between standard storage facilities (i.e. -80°C freezer). However, this
120 requirement may present particular issues while shipping to warm environments (i.e. summer, the
121 tropics) or where access to coolants are limited (as occurred during the COVID19 pandemic).
122 Therefore, lyophilisation has been previously investigated for stable preservation of various RNA
123 virus PVs (Mather et al., 2014; Neto et al., 2023), but not DNA virus PV particles. Retention of EHV-1
124 PV titre was assessed immediately after lyophilisation and reconstitution and following one or four
125 weeks storage under different conditions (-80°C, -20°C, +4°C and room temperature (RT).
126 Additionally the stability of EHV-1 PVs was measured following storage for one week at RT in
127 polystyrene boxes surrounded with ice packs, to reflect conditions commonly used for reagent
128 shipping. These PV samples were also tested in a PVNA, employing a small panel of EHV1-positive
129 sera, to verify the integrity and biological function of PVs to detect specific antibodies.

130 ## 2. Materials and Methods

131 ### 2.1. Cell culture

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132 Human Embryonic Kidney (HEK) 293T/17 cells were used for PV production, titration and
133 neutralisation assays. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM; PAN Biotech)
134 supplemented with 10% Foetal Bovine Serum (FBS; PAN Biotech) and 1% Penicillin/Streptomycin
135 (P/S; PAN Biotech) to make complete media.
136 Other cell lines were employed as target cells in EHV-1 PV infection experiments. Equine dermal
137 fibroblasts (E.derm; NBL-6 ATCC® CCL-57) were maintained in complete DMEM, while rabbit kidney
138 epithelial cells (RK13; ATCC® CCL-37) were grown in complete Minimum Essential Medium with
139 Earle's balanced salts solution (MEM/EBSS; HyClone™, Cytiva, Cat No. SH30024.01). Chinese hamster
140 ovary cells (CHO-K1; ATCC® CCL-61) were maintained in complete Ham's F12 medium (F-12; Gib-
141 co™, Thermo Fisher Scientific). Foetal horse kidney cells (FHK-Tcl3) were a kind gift of Dr. Maeda
142 (The National Institute of Infectious Diseases, Tokyo, Japan). FHK-Tcl3 and Madin-Darby canine
143 kidney cells (MDCK I & II; ATCC® CRL-2936 and ATCC® CRL-2936 respectively) were grown in
144 complete DMEM. All media were supplemented with 10% FBS and 1% P/S. All cell lines described
145 were maintained at 37°C in 5% CO_2 in a humidified incubator.

146 2.2. Gene synthesis

147 The full length EHV-1 gB, gD, gH and gL gene sequences (ORF 33, 72, 39 and 62 respectively) were
148 obtained from an EHV-1 strain isolated from organs of an aborted horse foetus during a significant
149 EHV-1 outbreak in Normandy (France) in 2010 (Sutton et al., 2019). The strain nomenclature is EHV-
150 1 2010.203 (year and the sample ID) and belongs to the Multi Locus Sequence Typing (MLST) group
151 10. The GP gene sequences were aligned with the respective homologues in the references EHV-1
152 strains: Ab4 (GenBank accession number: AY665713.1) (Telford et al., 1992) and strain V592
153 (GenBank accession number: AY464052.1) (Tearle et al., 2003) to verify the correct ORF length. All
154 GP genes (plus upstream Kozak sequence and terminal restriction sites) were synthesised by
155 GeneArt™ (Thermo Scientific™, Thermo Fisher Scientific) cloned into their in-house pMX plasmid
156 vectors, with the exception of gC (ORF 16) which was synthesized and supplied as a 'gene string'
157 linear DNA fragment. The EHV-1 gC sequence was obtained from EHV-1 strain Suffolk/87/2009
158 (GenBank accession number: KU206443.1), also belonging to the MLST group 10 (Bryant et al.,
159 2018).

160 2.3. Plasmid preparation

161 gB, gD, gH and gL genes were subcloned from pMX into the pCAGGS expression plasmid (Niwa et al.,
162 1991) previously used to produce functional pseudotypes representing a number of virus families (Di
163 Genova et al., 2021; Kemenesi et al., 2022; King et al., 2016). The gB gene was subcloned via a blunt
164 end strategy with *XbaI* (filled using Klenow fragment polymerase) and *XhoI* restriction enzymes and
165 T4 ligase. The gD, gH and gL genes were cloned using *KpnI* and *XhoI* and gC gene via *EcoRI* and *BglII*
166 restriction enzymes. All enzymes from Thermo Scientific™, Thermo Fisher Scientific. All plasmids
167 were purified using Monarch® Plasmid Miniprep Kit (New England Biolabs) with concentration and
168 purity determined using a Nanodrop™ 2000 Spectrophotometer (Thermo Scientific™, Thermo Fisher
169 Scientific)). Sanger sequencing was used to verify gene sequences (Eurofins Genomics, Germany)
170 using customised primers based in the pCAGGS plasmid vector arms.

171 2.4. PV generation

172 PV generation was performed using a multi plasmid transfection system adapted from protocols
173 used for various RNA virus families such as equine influenza (Scott et al., 2012). Briefly, HEK293T/17
174 were cultured in a 6-well dish the day before the DNA transfection (4 x 10^5 cells/well). 100 µL of
175 OptiMEM™ (Gibco™, Thermo Fisher Scientific) was mixed with plasmid DNAs: 250 ng each of the

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176 four GPs (EHV-1 gB, gD, gH and gL in pCAGGS), 750 ng of the reporter gene plasmid (pCSemGW or
177 pCSFLW for green fluorescent protein or firefly luciferase protein expression respectively) and 500
178 ng of the lentiviral HIV core plasmid (p8.91). Separately 100 µL of OptiMEM™ was mixed with 1
179 mg/mL of polyethyleneimine (PEI; Sigma-Aldrich®) solution transfection reagent. After 5 minutes RT
180 incubation the DNA and PEI solutions were mixed, followed by a further 20 minutes incubation, with
181 gentle flicking to mix. The transfection mix was then added dropwise to the wells, swirled then
182 incubated at 37°C for 24h. Next, the cell culture media was substituted with 2 mL of fresh complete
183 culture media. 48h post-transfection, the media containing PV was collected and passed through a
184 0.45 um syringe filter to remove cell debris, then stored at -80°C until titration or next use. An
185 additional collection at 72h post-transfection was conducted by adding 2 mL of fresh media to the
186 cells following the first supernatant harvest.

187 2.5. PV titration

188 As an initial test for successful EHV-1 PV generation, the pCSemGW was incorporated into the
189 modified genome within the particles and entry was assessed semi-quantitatively by fluorescent
190 microscopy on different target cells. If successful, a luciferase version of the EHV-1 PV was then
191 produced and titre quantified. In both cases, 1:2 fold serial dilution of the PV was performed across a
192 clear (for pCSemGW) or white (for pCSFLW) Nunc™ MicroWell™, Nunclon Delta-Treated, Flat-Bottom
193 96-well plate (Thermo Scientific™, Thermo Fisher Scientific); 100 µL PV in first well, then transfer
194 across plate into 50 µL of complete media. Next, 50 µL of target cells ($1x10^4$ cells/mL) were added
195 per well. A delta envelope (Δenv) PV bearing no envelope GPs, plus a cell only control were included
196 to define a threshold for successful PV production, and for cellular auto-fluorescence/luminescence
197 background. An equine influenza (EIV) PV bearing both the haemagglutinin (HA) and neuraminidase
198 (NA) surface GPs from the Florida clade 2 EIV strain A/equine/Richmond/1/07 (H3N8) (GenBank
199 accession number: KF559336.1) was produced as previously reported and utilised as a positive
200 control for the titration procedure (Kinsley et al., 2020; Scott et al., 2016). Plates were incubated for
201 48h at 37°C at 5% CO_2 before reading. For fluorescence evaluation, green fluorescent protein (GFP)
202 expressing cells were manually counted under a fluorescent microscope (Nikon, model: Eclipse
203 TS100). On the other hand, Bright-Glo™ luciferase assay system (BG; Promega) was employed to
204 measure the luminescence (in relative luminescence units; RLU per mL of supernatant) of PV
205 supernatant. Briefly, BG was mixed with phosphate-buffered saline (PBS; PAN Biotech) in a 50:50
206 ratio and 25 µL/well added to the 96-well plate to wells where medium had been removed. After 5
207 minutes incubation, the plate was read on a GloMax® Navigator Microplate Luminometer
208 (Promega).

209 2.6. Serum samples

210 A panel of horse serum was collected as part of an experimental EHV-1 challenge study previously
211 described by Thieulent et al., (2022). This archived serum panel was tested for EHV-1 specific
212 neutralising antibodies in the current study. The panel consisted of a total of 52 samples from four
213 10 month-old male Welsh Mountain ponies (A, B, C and D), which had been raised in a dedicated
214 specific pathogen free facility since birth and were experimentally infected by individual nebulisation
215 with the C_{2254} strain of EHV-1 (GenBank accession number: MT968035.1) (Sutton et al., 2020;
216 Thieulent et al., 2022). Sample collection occurred five days before infection (A_0, B_0, C_0, D_0) and then
217 daily from day 8 to day 18 (corresponding to sample A_8 to A_{18}, B_8 to B_{18}, C_8 to C_{18}, D_8 to D_{18}) period
218 during which a seroconversion could be recorded. Four additional negative controls were included in
219 the panel (E_0, F_0, J_0, H_0) (Thieulent et al., 2022). As a positive control, a multi-vaccinated pony serum
220 was included, which had been previously used and described in EIV PV studies (Scott et al., 2012;
221 Scott et al., 2016). The animal had been housed at the Animal Health Trust (Newmarket, UK) and

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222 vaccination records detail several influenza immunisations plus vaccination with the Duvaxyn® EHV
223 1, 4 Vaccine (Zoetis) twice in 2000. All sera were heat-inactivated at 56°C for 30 minutes prior to use.

224 2.7. PV neutralisation

225 Firstly, sera were serially diluted in a 1:2 fold in a 96-well white plate. $1x10^6$ RLU of PV (previously
226 titrated) was added to the wells. The multi-vaccinated pony serum and FBS were used as positive
227 and negative control sera respectively. The plate was incubated for 1 hour at 37°C to allow binding
228 of the antibody to the antigen. Next, $1x10^4$ HEK293T/17 cells were added to each well. PV-only and
229 cell-only controls were included in the plate to represent 0% and 100% neutralisation of the PV. The
230 plate was incubated for 48h at 37°C at 5% CO_2 before reading. Data were normalised and plotted on
231 a neutralisation percentage scale and the reciprocal of the serum dilution which induces 50%
232 neutralisation (IC_{50}) was calculated using GraphPad Prism® (Ferrara & Temperton, 2018).

233 2.8. PV lyophilisation and storage

234 In order to concentrate EHV-1 PV particles to increase usable titre, aliquots of 2 mL of both 48h and
235 72h freshly harvested PVs were low-speed centrifuged at 3000 g at +4°C for 24 hours (Jiang et al.,
236 2015) in a bench top refrigerated microcentrifuge (VWR International Ltd, model: Micro Star 17R).
237 Next, 1.95 mL of supernatant was then removed and discarded, making sure not to disrupt the
238 pelleted virus, and 100 µL of cold OptiMEM™ (kept at +4°C) were added to the tube. Samples were
239 incubated overnight at +4°C to permit particle resuspension and stored at -80°C before preparing
240 samples for lyophilisation. 100 µL aliquots of EHV-1 PV supernatant were mixed with equal volume
241 of 1 M Sucrose (Sigma-Aldrich®, Merck) solution as cryoprotectant, transferred to low retention 2mL
242 polypropylene microfuge tubes (Simport) and lyophilised in a freeze-dryer (FreeZone 2.5, Labconco,
243 model: 7670560) using the method described in (Mather et al., 2014). Next, the lyophilised pellets
244 were stored for various time periods at different temperatures: one week or four weeks at +37°C, RT
245 with or without surrounding ice blocks (in polystyrene boxes), +4°C, -20°C and -80°C. Lyophilised PVs
246 were reconstituted in complete media and titrated to assess the recovery percentage.

247 2.9. Statistical tools

248 Raw data files produced by the luminometer were analysed using Microsoft® Excel™ 365 software
249 (Microsoft® Windows). Column bar graphs and the non-linear regression curve fits were produced
250 using GraphPad Prism®. Statistical analysis was performed for comparison using a Student's t-test
251 ($p<0.005$).

252 **3. Results**

253 3.1. Optimisation of EHV-1 PV generation

254 PV generation was first attempted using equal ratios of each of the four EHV-1 GP plasmids (gB, gD,
255 gH, gL) testing three different masses (150, 250 or 500 ng), with GFP as a reporter for monitoring PV
256 transduction of HEK293T/17 target cells. Next, the firefly luciferase reporter was employed for titre
257 quantification assessed using PV supernatants harvested at 48h (Fig. 1A) and 72h (Fig. 1B) post-
258 plasmid transfection. Interestingly, the highest plasmid amount used (500 ng) did not produce the
259 highest titre for either the 48h or 72h PV harvest, with 250 ng producing the highest titre in both
260 cases. The difference was statistically significant between 250 ng and 500 ng ($p=0.0388$ and 0.0006
261 for 48h and 72h PV harvest, respectively), suggesting that increasing the amount of plasmid does not
262 lead to increased titre beyond a certain threshold. There was no significance difference in titre when
263 using 150 or 250 ng for the 48h harvest (Fig. 1A), whereas the 72h harvest shows a significant
264 difference ($p=0.0181$; Fig. 1B). No significant difference was reported when comparing titres

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265 between 150 ng and 500 ng of GP plasmids for both PV harvests. Thus, as a standard to generate
266 EHV-1 PV (and given nomenclature BDHL) 250 ng of each EHV-1 GP plasmid was employed in co-
267 transfection and PV harvested at 72h post-plasmid transfection. In attempts to further optimise
268 EHV-1 PV titres, different ratios of the four EHV-1 GP plasmids were tested (using 100 ng or 250 ng
269 amounts) (Fig. 2). It was observed that when 250 ng of only either EHV-1 gB or gH plasmid was used
270 in transfection, the titre of EHV-1 PV decreased significantly ($p=0.0377$ or $p=0.0082$ and $p<0.0001$ for
271 the 48h and 72h harvest respectively; Fig. 2A). A significant difference is also shown in Fig. 2B and
272 Fig. 2C for each combination where 250 ng of EHV-1 gH plasmid is added. Meanwhile, EHV-1 PV titre
273 neither dropped nor increased when 250 ng amounts of EHV-1 gD or gL plasmids were added
274 (1.06×10^8 RLU/mL and 7.87×10^7 RLU/mL for the 48h and 72h harvest respectively; Fig. 2A). On the
275 other hand, the titre drops when using 100 ng of gD and gL plasmids (1.14×10^6 RLU/mL and 7×10^5
276 RLU/mL for the 48h and 72h harvest respectively; Fig. 2B) suggesting using 250 ng of both plasmids
277 in transfection.
278 Further investigation was undertaken to test the contribution of gC to cell entry of EHV-1 PV, by
279 either adding gC plasmid to the BDHL combination or replacing other GP plasmids with gC plasmid in
280 turn during transfection, and measuring subsequent PV titre. Incorporating gC into PVs almost
281 eliminated the detection of viable PV particles. Therefore, the addition of gC plasmid in co-
282 transfection did not enhance EHV-1 PV transduction of target cells. Moreover, no viable PV particle
283 was detected when gC plasmid replaced one of the other GP plasmid originally employed to
284 generate EHV-1 PV, demonstrating that gB, gD, gH and gL are required for EHV-1 PV cell entry. Lastly,
285 to investigate the generic impact of increasing plasmid DNA levels in the transfection mixture (i.e.
286 increasing cell producer toxicity), an empty vector (pCAGGS) was added to the BDHL combination,
287 but no significant difference in titre was seen.

288 3.2. PV transduction of different target cells

289 The ability of the EHV-1 PV to transduce different cell lines was assessed to identify the optimal
290 target cell line for downstream application. An EIV PV and Δenv PV were included as positive and
291 negative controls respectively. RK13 and E.derm cells are routinely used for EHV-1 studies using the
292 native virus (Frampton Jr et al., 2005; Peterson & Goyal, 1988), however EHV-1 PV was not able to
293 transduce those cell lines with the same efficiency as HEK293T/17. The same was seen for the FHK
294 cell line despite its equine origin. Further testing was conducted on BHK, CHO-K1, MDCK I and II cells,
295 which have been employed as target cells with other PV types (e.g. Influenza, Ebola). Nevertheless,
296 our study shows that HEK293T/17 are the cells most efficiently transduced by EHV-1 PVs of the lines
297 tested, in addition to their role as a producer line.

298 3.3. PV Neutralisation

299 EHV-1 PV was tested in a PVNA to assess its feasibility to be used as a serological antigen. The EHV-1
300 PVNA was carried out using longitudinally collected samples of horse sera following experimental
301 infection, as described above in Section 2.6 and 2.7. Each assay included a positive and negative
302 control to verify the test. Neat sera were added (in duplicate) at a starting dilution of 1/40 in the first
303 well and the assay was repeated twice to verify reproducibility (Fig. 3). The threshold of positivity
304 corresponding to an IC_{50} value of 160 ($LogIC_{50}=2.2$) was defined by taking the average of results
305 obtained from samples collected from all eight horses prior to the experimental infection (A_0, B_0, C_0,
306 D_0, E_0, F_0, J_0, H_0). The antibody response increased steadily 8-10 days after infection, reaching a
307 plateau thereafter.

308 3.4. Correlation of Ab titres

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309 Once PVNA was successfully performed, it was deemed important to correlate the antibody titres
310 obtained with that from a conventional EHV-1 VN assay (Supplementary Table 1). Pearson
311 correlation was calculated for all samples collected from day 8 to day 18 (n=44) between the EHV-1
312 VN titres and the reciprocal PVNA IC_{50} values and revealed a strong positive correlation coefficient
313 value between the two assays (r=0.82, p<0.0001; Fig. 4) (Prion & Haerling, 2014).

314 3.5. Lyophilised PV titre

315 To simplify shipping to stakeholder laboratories, and for subsequent long term stable storage,
316 lyophilisation was employed as a method for preservation of PV supernatants. Following
317 reconstitution, the retention of biological function was assessed at several time points: immediately
318 and one or four weeks post storage under different conditions (+37°C, RT with or without adjacent
319 ice blocks, +4°C, -20°C and -80°C). Percentage retention of titre was then compared with non-
320 lyophilised control samples stored in standard -80°C freezer. Firstly, the lyophilisation process did
321 not to lead to significant loss of PV function following immediate reconstitution and titration (Fig.
322 5A). However, storage did impact retention values especially at high temperatures. After one week
323 at +37°C a significant loss of titre was observed (retention losses of 13%; Fig. 5B), while lower
324 storage temperatures reduced the impact (Fig. 5C, D and E). Importantly, storage of lyophilised PVs
325 at low temperatures (-80°C up to RT) for four weeks show no significance loss in titre while PV
326 particles subjected to +37°C lost all detectable ability to transduce susceptible target cells (Fig. 6)

327 3.6. Use of lyophilised PVs in antibody neutralisation assays

328 Having determined that the lyophilisation process did not negatively impact PV titres, the
329 antigenicity of PV particles was evaluated in antibody neutralisation assays, employing a subset
330 (n=4) of samples of the serum panel. It was observed by light microscopy that when higher volumes
331 of reconstituted PVs were employed some cyto-toxicity occurred as effect of the sucrose
332 cryoprotectant. Thus, lower volumes representing 10^5 RLU (rather than 10^6 RLU) were used in
333 neutralisation assays. IC_{50} values were obtained in a range from 987 to 246828 ($LogIC_{50}$ 2.9 to 5.4)
334 and the gradient of the neutralisation curves were not as consistent when compared to the PVNA
335 employing non lyophilised PV as depicted above in Section 3.3 (Fig. 3). A lyophilised EIV PV was
336 included as a positive control, as shown to be functional in PVNAs previously (Mather et al., 2014).
337 Both input of 10^5 and 10^6 RLUs of lyophilised EIV PV could be included in PVNAs, due to the smaller,
338 less toxic volumes added. IC_{50} values were reported for both lyophilised EIV PV input conditions with
339 little difference ($LogIC_{50}$ 4.6 to 4.9). However, using different PV input significantly affected the IC_{50}
340 results (p=0.0139).

341 **4. Discussion**

342 Pseudotyped viruses (PVs) have been shown to be useful and safe research tools to study
343 many almost exclusively RNA viruses, from fundamental *in vitro* studies (e.g. cell tropism, receptor
344 analysis), serology (e.g. antibody neutralisation assays), anti-viral screening and even as vaccine
345 antigens themselves. Due to their safe, non-replicative nature, these study viruses have included a
346 number which are classified as BSL-3 & 4 pathogens. PVs are usually easier to generate and
347 manipulate than for example reverse genetic systems, particularly with regard to altering
348 combinations or specifically mutating envelope glycoproteins (GPs) to analyse impact on infection.
349 This amenability can be particularly valuable when studying complex viruses with multiple surface
350 GPs. Herpesviruses have large DNA genomes with over a hundred genes and express a host of viral
351 proteins on their surface. In order to study the array of equid herpesvirus surface glycoproteins, and
352 with the ultimate aim of developing an effective test for the detection of infection or vaccine-

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353 mediated antibodies, we have successfully generated EHV-1 pseudotyped lentivirus particles bearing
354 four glycoproteins gB, gD, gH and gL, permitting target cell entry. To our knowledge, there has been
355 only a single report describing the pseudotyping of an herpesvirus, herpes simplex virus type 1 (HSV-
356 1), using a VSV core and the homologous glycoproteins (Rogalin & Heldwein, 2016). Additionally, no
357 lentiviral PVs have been reported bearing more than three envelope glycoproteins to date, and all
358 derived from RNA viruses. One example is an influenza PV where HA was combined with both NA
359 and M2, and was seen to increase pseudotype yields and infectivity for the PV (Wang et al., 2010).
360 Another was the human Respiratory Syncytial Virus (hRSV) small hydrophobic protein (hRSV-SH)
361 combined with the hRSV attachment glycoprotein (hRSV-G) and the hRSV fusion protein (hRSV-F) to
362 investigate cell entry (Haid et al., 2016). However, in this study we demonstrate that it is possible to
363 create a functional lentivirus PV by employing four different glycoproteins, in this case from EHV-1.
364 We also show that the precise mass of co-transfected glycoprotein-encoding plasmids affects
365 functional EHV-1 PV titre, with increasing amounts not always increasing efficiency. The current
366 study reveals that the combination of gB, gD, gH and gL envelope glycoproteins alone are needed for
367 EHV-1 PV particle entry of HEK293T target cells. Exclusion of any of these GPs abrogated cell
368 transduction. These results were in accordance with Rogalin and Heldwein (2016) where HSV-1 VSV
369 PVs were only able to enter C10 target cells when all four GP homologues were present.
370 Concentration of lentiviral particles by low-speed centrifugation increased titre by at least 1 log as
371 seen in other studies (Cepko, 1997; Darling et al., 2000).
372 To optimise EHV-1 PV titre, different ratios of co-transfected GP plasmids were tested.
373 Despite distinct differences in GP gene sequence length within the same expression vector
374 backbone, the best results were achieved using the same amount (i.e. 250 ng) for all four. When
375 examining the contribution of particular GPs it was notable that when the EHV-1 gH plasmid was
376 added in higher amounts, the titre dropped significantly. Nevertheless, gH is known to be required
377 for EHV-1 virion entry by complexing with gL, to regulate viral fusion by interaction with gB (Azab et
378 al., 2013). In addition, EHV-1 gC is often mentioned as a mediator of EHV-1 entry into cells through
379 direct envelope plasma membrane fusion (Csellner et al., 2000; Neubauer et al., 1997; Osterrieder,
380 1999). Consequently, we investigated the inclusion of gC in our EHV-1 PV particles, and whether it
381 would enhance target cell entry. However, the incorporation of gC plasmid actually resulted in a
382 significant decrease in EHV-1 PV titre. Additionally, sequential swapping of gC for another GP
383 plasmid in the four plasmid sets (i.e. gB, gD, gH or gL) was tested. In each case, no measurable EHV-1
384 PV titre was obtained in target cell transduction experiments. Thus, despite the known role of gC in
385 early steps of EHV-1 infection, by attaching to cell surface heparan sulphate-containing
386 glycosaminoglycan receptor molecules, this glycoprotein was not found to be essential for EHV-1 PV
387 entry of HEK293T cells. Despite EHV-1 having a tropism for epithelial and endothelial cells, its
388 infectivity is not restricted to these cell types. Indeed, EHV-1 can enter permissive cells either
389 through fusion of its viral envelope with the host cell membrane or through endocytosis (Frampton
390 Jr et al., 2007). In addition, the host range of cell lines which EHV-1 is capable of infecting *in vitro* is
391 much wider compared to other EHVs or to HSV (Whalley et al., 2007). We demonstrated that
392 HEK293T cells were the most transducible with EHV-1 PVs suggesting that other minor EHV-1 GPs
393 might be involved in entry of other cells, which could be further investigated using the PV system
394 and different target cells.
395 Following optimisation, we were able to successfully generate EHV-1 PVs of sufficient titre
396 for downstream use, specifically in antibody neutralisation tests. This involved utilising sera from
397 horses experimentally challenged with EHV-1, sampled at various time intervals post infection. The
398 results demonstrated expected patterns of neutralising antibody responses. Gradual increase of
399 antibodies specifically neutralising EHV-1 PVs were observed from day 8, peaking around day 13
400 before stabilising in a plateau phase (Fig. 3). It was necessary to delineate a threshold to distinguish

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401 positive from negative samples. This cut-off was defined as an IC_{50} value of 160, obtained from the
402 mean value of the negative samples (n=8; samples collected on day 0). These samples were
403 confirmed negative in native virus neutralisation (VN) assays also. This approach was necessary as
404 naïve horses which have never been exposed to EHV-1 are rare. EHV-1 is remarkably ubiquitous in
405 the horse population and it has been estimated that by two years of age, 80-90% are infected (Allen,
406 2002). The difficulty of preventing the spread of infection to unexposed subjects is mostly due to
407 asymptomatic horses after primo-infection or reactivation from latency (Allen et al., 2004; Paillot et
408 al., 2008). So far, the results obtained with PVNA for EHV-1 are very promising, including when
409 correlating the neutralising antibody titres with VN ($r=0.82$, $p<0.0001$) (Fig. 4), noting that these
410 EHV-1 PVs are displaying only four of the total twelve EHV-1 GPs. PVs could also provide an
411 amenable tool to investigate the roles of these GPs in various combinations. For instance, it has been
412 demonstrated that gG enables differentiation between antibodies present in polyclonal sera from
413 mixed cases of infection involving both EHV-1 and EHV-4, by eliciting a type-specific serological
414 response to EHV-4 (Crabb et al., 1995; Crabb et al., 1992; Crabb & Studdert, 1993). Existing assays
415 have shown a strong cross-reactivity in polyclonal sera due to the close antigenic similarity between
416 EHV-1 and EHV-4 (Allen et al., 2004). Inclusion of EHV-1 gG in PV particles may be an avenue worth
417 pursuing to develop a more type-specific antibody test. EHV-1 gG is also highly immunogenic, thus
418 incorporating it in the PV system could give a better representation of the neutralising antibodies in
419 sample sera.
420 Lyophilisation of PVs was investigated as an alternative to dry-ice shipments (and associated
421 costs), potential customs delays, and for downstream storage. Thus, a stability study was conducted
422 on lyophilised PVs, exposing samples to varying temperatures for various time periods, to reflect
423 shipping conditions and subsequent short-term storage. Firstly, PV titre retention following
424 reconstitution of lyophilised PV pellets was assessed, secondly performance in PVNAs was
425 measured, testing different aspects of biological functionality dependent of GP integrity. Sucrose
426 was employed as cryoprotectant as we have previously shown this to be an effective excipient
427 (Mather et al., 2014). Stability of lyophilised PV was also assessed by measuring the titre after
428 immediate reconstitution of PV pellets, or one-week (short-term) storage to mimic a shipment time
429 frame scenario and after four weeks to reflect possible shipping delays, after exposure to different
430 temperatures (Fig. 5). Excellent recovery was observed when lyophilised PV pellets were
431 immediately reconstituted and tested (overall no significant difference between the lyophilised and
432 non-lyophilised PVs). Following storage in different conditions, higher temperatures (+37℃) were
433 most deleterious to functionality, as noted previously (Mather et al., 2014). Nevertheless, the
434 lyophilised PVs were able to retain more than 87% of their original titres respectively after one week
435 storage at 37℃, a useful attribute if shipping at high temperatures (i.e. summer, hot countries). At
436 lower temperatures (+4℃ and -20℃) lyophilised PVs completely retained titres compared with -80℃
437 storage (non lyophilised PVs dropped to 86%). The addition of ice packs to mimic a shipping
438 condition was able to slightly increase titre retention under RT conditions. Storage was also
439 increased to four weeks with PVs retaining more than 97% of their initial titres for both RT and lower
440 temperatures (Fig. 6). By contrast, when stored at +37℃ for four weeks, no viable titre was
441 detectable. A high functional titre is essential for the correct performance of a PVNA. Since EHV-1 PV
442 was found to have lower titres when compared to many other PVs (e.g. influenza), the volume of
443 reconstituted lyophilised PV which needs to be used in an assay is higher to provide a suitable
444 amount of viral particles as antibody targets. Reconstituted, lyophilised PV samples contain
445 cryoprotectant which can impact target cell viability. Thus, it is advisable to optimise PV titre in order
446 to reduce input volumes and incorporate a suitable serial dilution across the assay plate.

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447 Taking these optimisations into account, we have developed a robust and amenable system with
448 wide utility in fundamental virological research (e.g. GP-mediated cell entry mechanisms) and an
449 effective alternative to traditional native EHV-1 VN assays applicable to quantitative serology to
450 investigate experimental and natural infection or vaccine efficacy.

451 **Author Contributions**

452 **Cecilia Di Genova:** Conceptualisation, Data curation, Formal analysis, Investigation,
453 Methodology, Project administration, Validation, Visualisation, Writing – original draft, Writing –
454 review & editing. **Gabrielle Sutton:** Data curation, Formal analysis, Investigation, Writing – review &
455 editing. **Romain Paillot:** Conceptualisation, Funding acquisition, Supervision, Writing – review &
456 editing. **Nigel Temperton:** Supervision, Writing – review & editing. **Stéphane Pronost**:
457 Conceptualisation, Funding acquisition, Supervision, Writing – review & editing. **Simon Scott**:
458 Conceptualisation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing –
459 review & editing.
460
461 **Ethical Approval**

462 No animal was used for this study. The archived serum panel was collected as part of the
463 Thieulent et al.(2022) study with ethical approval and the use of archived material from this study
464 was authorised.
465
466 **Declaration of Competing Interest**

467 The authors declare that they have no known competing financial interests or personal
468 relationships that could have appeared to influence the work reported in this paper.
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475
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