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1 **Detection of equine herpesviruses in aborted fetuses by consensus PCR**

2

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20 **Abstract**

21 The major role of EHV-1 in equine abortion is widely reported in the literature but the
22 contribution of EHV-2, EHV-3, EHV-4 or EHV-5 remains less well documented. The
23 objective of this study is to evaluate the contribution of these five different EHV-1 to equine
24 abortion in a variety of biological tissues using a consensus polymerase chain reaction (PCR).
25 The test was validated for specificity and sensitivity in horses before screening specimens
26 from 407 fetuses, stillbirths and premature foals collected over a 2½-year interval. Positive
27 results obtained with this assay were compared to other EHV type-specific PCR or by
28 sequencing. EHV-1 was identified as the major cause of abortion in French mares (59/407
29 cases). However, there was evidence to suggest some variation in the potential of EHV-1
30 strains to induce abortion. Indeed, DNA samples from EHV-2 (in three cases) and EHV-5 (in
31 one case) inferred a role of these viruses in abortion. The presence of viral DNA from EHV-3
32 or EHV-4 strains was not detected in the specimens studied. The data obtained suggest that
33 the consensus herpesvirus PCR is an efficient screening tool. In association with a specific
34 PCR, the test provides a rapid identification of the type of herpesvirus involved in abortion
35 and is useful for routine diagnostic tests as it allows the identification of herpesviruses other
36 than the EHV-1 strain.

37

38 **Keywords:** horse, abortion, herpesviruses, PCR, diagnosis.

39 **1. Introduction**

40 The family *Herpesviridae* is divided into three subfamilies: *Alphaherpesvirinae*, which
41 include equine herpesvirus type 1 (EHV-1), EHV-4 and EHV-3; *Gammaherpesvirinae* that
42 include EHV-2 and EHV-5 and *Betaherpesvirinae* (Smith, 1997). The important role of EHV-
43 1 in equine abortion is well-known whereas, the involvement of EHV-4 is much debated and
44 the presence of EHV-2, EHV-3 and EHV-5 in tissues from aborted foetuses remains poorly
45 documented (van Maanen, 2002).

46 EHV-1 is the most common viral cause of abortion in mares resulting in major financial
47 losses to the horse-breeding industry. Over the last 20 years, research into the role of EHV-1
48 in equine abortion revealed that the incidence of this virus varies between 4.5% and 8.9%
49 (data obtained in the U.K. and the U.S.A.).

50 EHV-4 causes respiratory disease and is known provoke sporadic abortion in horses (Allen
51 and Bryans, 1986; Crabb and Studdert, 1995). However, the incidence of EHV-4 related
52 abortion is negligible with <1% of cases being reported in Kentucky broodmares between
53 1983 and 1992 (Ostlund, 1993). O'Keefe et al. (1995) were the first to identify the presence of
54 EHV-4 in a foal that died during the neonatal period.

55 EHV-3 is a rapidly growing, antigenically and genomically distinct alphaherpesvirus that
56 causes equine coital exanthema (Blanchard et al., 1992). Since the study of Feilen et al.
57 (1979), there was no evidence to suggest that the virus is abortifacient in natural infections
58 although experimentally, following *in utero* infection, EHV-3 can induce abortion (Gleeson et
59 al., 1976).

60 EHV-2 is a slowly developing cytopathic virus that is ubiquitous in the equine population
61 (Agius and Studdert, 1994; Schlocker et al., 1995). However, the precise role of EHV-2 as a
62 pathogen and its tissue tropism remain uncertain although there is evidence that EHV-2 is
63 latent in B-lymphocytes (Drummer et al., 1996).

64 Information about horses infected with EHV-5 is scarce. The initial isolations of EHV-5 were
65 from four horses with an upper respiratory tract disease (Agius et al., 1992). An Australian
66 study (PCR on leukocytes) reported detectable levels of EHV-5 DNA in approximately 16%
67 of horses tested (Reubel et al., 1995). More recently, EHV-5 infection (identified in
68 nasopharyngeal secretions) was estimated at 64% in a group of young racehorses (Bell et al.,
69 2006b). These findings suggest that EHV-5 is widespread in the equine population. To date, it
70 has not been demonstrated that EHV-5 plays a significant role in equine abortion.

71 Diagnosis of EHV infection is possible through indirect serological methods (virus
72 neutralization, complement fixation, EHV-1/EHV-4 type-specific antibody detection ELISA)
73 (Hartley et al., 2005), or by direct assays. Direct detection of EHV in biological tissues has
74 evolved from virus isolation in cell cultures or antigen detection methods, to the development
75 of more rapid and type-specific PCRs which detect viral DNA (Dynon et al., 2001; Galosi et
76 al., 2001; Varrasso et al., 2001; Szeredi et al., 2003; Allen and Breathnach, 2006; Bell et al.,
77 2006a; Diallo et al., 2006; Elia et al., 2006; Hussey et al., 2006; Diallo et al., 2007).

78 A PCR test developed for the general screening of herpesviruses was originally described by
79 VanDevanter et al. (1996). The consensus herpesvirus PCR, which is based on the DNA
80 polymerase gene, was principally used on human herpesviruses (type 1 to 8) and on some
81 strains of animal herpesviruses (dog, cat, rabbit or monkey). These authors demonstrated that
82 the test can detect one equine herpesvirus, namely EHV-2. More recently, this consensus
83 DNA polymerase PCR was used to identify EHV-3 sequences in ulcerative and pustular skin
84 lesions of two mares and one stallion (Kleiboeker and Chapman, 2004).

85 The five distinct equine herpesviruses (types 1, 2, 3, 4 and 5) have not been studied under
86 comparable conditions or in a large set of foetal tissues. The aim of this study was to
87 characterize the role of these five equine herpesviruses in abortion. To this end, we used a

88 consensus PCR (based on the herpesvirus DNA polymerase gene) to test 407 foetuses,
89 stillbirths and premature foals, collected in France over a 2½-year interval.

90 **2. Materials and methods**

91 **2.1. Population**

92 A total of 407 equine samples comprising 279 (69%) foetuses, 49 (12%) stillbirths and 77
93 (19%) premature (up to seven-day old) foals, were used in this study. The samples were
94 obtained from 199 (49%) males and 208 (51%) females of which 182 (45%) were English
95 thoroughbred horses, 160 (39%) French standardbred horses, 46 (11%) French saddle horses
96 and 19 (5%) other races.

97 All foetuses, stillbirths and premature foals were collected from 225 farms in the West of
98 France and submitted to the Equine Pathology and Diseases Research Laboratory (AFSSA,
99 Dozulé, France) during October 2002 through to June 2005.

100 The specimens were collected in accordance with a standard protocol (Rooney, 1970).
101 Samples of lung and placenta (when available) were collected for routine bacteriological
102 cultures, histopathological analysis or for DNA extraction and subsequent equine herpesvirus
103 detection by PCR.

104 **2.2. Polymerase chain reaction**

105 DNA was extracted from 30 mg of lung and of placenta and tested using the QIAamp DNA
106 mini kit[®], according to the manufacturer's instructions. Concentrated DNA was stored at -
107 80°C until used. Only DNA extracts from the lung and placenta were used for the study and
108 all extracts were known to be free of PCR inhibitors (Leon et al., 2006).

109 PCR conditions are summarized in Table 1. PCRs were performed with pure Taq Ready to Go
110 PCR beads. The final concentration of each dNTP was 200 µM in 10 mM Tris-HCl (pH=9 at
111 room temperature), 50 mM KCl and 1.5 mM MgCl₂. All amplifications were performed on a
112 GeneAmp PCR system 9700[®].

113 The consensus herpesvirus PCR method as described by VanDevanter et al. (1996) was used
114 in this study. The assay employed a nested format in which primary and secondary PCRs
115 were performed with degenerate PCR primers targeted to highly conserved amino acid motifs
116 directed to the DNA polymerase gene. Primary PCR mixtures contained two upstream
117 primers (DFA and ILK) and one downstream primer (KG1) in a multiplex format. A
118 secondary PCR was performed on 2.5 μ L of the primary PCR mixture in a 25 μ L volume with
119 upstream primer TGV and downstream primer IYG under the same thermal conditions as
120 those used for the primary reaction.

121 The size of the amplified fragment was between 215 and 315 bp. DNA from a Kentucky
122 reference strain (Ky A) obtained from ATCC was used as a control for EHV-1. Other DNA
123 controls (EHV-2, EHV-3 and EHV-4) came from our laboratory collection. The typing of all
124 the different strains was based on sequencing. The specificity of the consensus herpesvirus
125 PCR for EHV was assessed by testing DNA extracts of various pathogens implicated in
126 diseases of the horse: *Streptococcus equi subsp. zooepidemicus*, *Streptococcus equi subsp.*
127 *equi*, *Listeria monocytogenes*, *Listeria innocua*, *Leptospira interrogans serovar australis*,
128 *Leptospira biflexa*, *Coxiella burnetii*, *Chlamydophila abortus*, *Rhodococcus equi*, *Taylorella*
129 *equigenitalis*, equine arteritis virus and *Neospora caninum*.

130 Analytical sensitivity of the assay was tested using a suspension of EHV-1 grown in cell
131 culture (RK13). The viral suspension was diluted 10-fold (10^{-1} to 10^{-8}) and titrated on RK13
132 cells. Simultaneously, DNA was extracted from these dilutions and used for detection of virus
133 in consensus and specific PCR. The results were obtained in triplicate and the minimum
134 quantity necessary to allow detection of the virus was determined (expressed as the TCID₅₀).

135 Specific PCRs for EHV-1 or EHV-4 detection were adapted from a previously described
136 method (Kirisawa et al., 1993). Primers R1 and FC3 were used for amplification of an 188 bp

137 fragment internal to the glycoprotein B gene of EHV-1 and primers R4 and FC3 for
138 amplification of a 677 bp fragment of glycoprotein B gene of EHV-4.

139 EHV-2 was detected by amplification of a sequence upstream from the ORF coding for a
140 product homologous to human Interleukin-10, within the *Eco*R1 N fragment of EHV-2, as
141 previously described (Borchers et al., 1997). This PCR test was intended to amplify fragments
142 between 521 and 721 bp. Primary PCR mixtures contained an upstream primer ALM1 and
143 one downstream primer ALM2. Secondary PCR was performed on 2.5 μ L of the primary
144 PCR mixture in 25 μ L with upstream primer ALM3 and downstream primer ALM4 under the
145 same conditions used for the primary reaction.

146 For specific detection of EHV-3, we developed a one-step PCR test using primers HSP3F and
147 HSP3R to amplify a fragment of 100 bp in the DNA polymerase gene.

148 All PCR tests included a negative control where DNA was replaced with distilled water. The
149 amplified products were analyzed by electrophoresis (2% agarose gel) and their size estimated
150 by comparison with molecular weight markers (SmartLadder SF) run in parallel.
151 Visualization was performed under UV transillumination after soaking the gels in ethidium
152 bromide (50 ng / μ L).

153 Only positive samples, as determined by consensus herpesvirus PCR and negative by specific
154 EHV-1 PCR, were sequenced after extraction of the PCR product from agarose gel through
155 the use of a QIAquick extraction kit[®]. Nucleotide and amino-acid sequences were analyzed
156 using software available over the Internet at the National Center for Biotechnology
157 Information website (<http://www.ncbi.nlm.nih.gov/>) and MultAlin software
158 (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>)

159 **2.3. Virus isolation**

160 Virus isolation was performed on foetal and neonatal lung tissue. Tissue samples were
161 homogenized in minimum essential medium (MEM) supplemented with 100 U penicillin, 100

162 $\mu\text{g/mL}$ streptomycin and $0.25 \mu\text{g/mL}$ amphotericin B to produce approximately 10% tissue
163 suspension that was centrifuged at 3000 g for 30 minutes at 4°C . The supernatant fluid was
164 filtered through a $0.45 \mu\text{m}$ membrane and $100 \mu\text{L}$ was inoculated onto subconfluent
165 monolayers of rabbit kidney cells (RK13) in a 25 cm^2 flask. To prevent any possible effects of
166 cytotoxicity, the inoculums were replaced, after adsorption during 1 h 15 min at 37°C , by 7
167 mL of culture medium (MEM supplemented with 10% of foetal calf serum, 1% of L-
168 glutamine, 100 U penicillin, $100 \mu\text{g/mL}$ streptomycin and $0.25 \mu\text{g/mL}$ of amphotericin B).
169 The cultures were incubated at 37°C for up to one week and the cytopathic effect (CPE) was
170 checked daily. Monolayers with no CPE at the end of seven days were seeded once more on
171 RK13 cells and the CPE was checked daily over a three-week interval.

172 **2.4. Histopathology**

173 Tissues samples were taken from lung and placenta and fixed in 10% formalin solution. The
174 samples were embedded in paraffin and $4 \mu\text{m}$ thick sections were cut and stained with
175 haematoxylin and eosin.

176 **2.5. Sequencing**

177 Sequencing was performed on the amplified fragment from the secondary PCR product of the
178 consensus herpesvirus PCR.

179 **3. Results**

180 **3.1. Validation of the consensus herpesvirus PCR for each equine virus**

181 Concerning specificity of the consensus herpesvirus PCR, no product was amplified from
182 DNA of various micro-organisms implicated in diseases of the horse (see 2.2 above). DNA
183 fragments of the expected size were amplified from DNA extracts of EHV-1, EHV-2, EHV-3
184 and EHV-4. Specificity of each fragment was confirmed by sequencing.

185 The limits of detection for the assays were 10^{-5} and 10^{-6} TCID₅₀/50 μL for specific EHV-1
186 and consensus herpesvirus PCR, respectively and with an EHV-1 (Ky A) viral suspension

187 with a known titre of $10^{4.8}$ TCID₅₀/50 μ L. Consensus herpesvirus PCR shows a sensitivity that
188 is one log higher than the specific EHV-1 PCR.

189 **3.2. Detection of EHV**

190 Firstly, for each animal, the lung and placenta were analysed by the consensus herpesvirus
191 PCR. A positive PCR signal was obtained in 54/406 (13%) lungs (one lung was not available)
192 and 44/283 (16%) placentas; the data represent 67 individual cases in which the herpesviruses
193 that were detected are of a sporadic nature.

194 Secondly, the same samples were also tested by the specific EHV-1 PCR and a correlation
195 was found between the results obtained with both methods. All viruses detected by specific
196 EHV-1 PCR were also positive by consensus herpesvirus PCR (44 cases). In contrast, among
197 the 54 lungs and 44 placentas that were positive by consensus herpesvirus PCR, only 35
198 (65%) lungs and 31 (71%) placentas were positive by the specific EHV-1 PCR. It is
199 noteworthy that, 19 EHV-1 strains could be isolated and identified by cell culture techniques
200 for all EHV-1 positive lungs.

201 DNA fragments were purified and sequenced from 23 cases in which a positive PCR signal
202 was obtained by consensus PCR (but not by specific PCR). Fifteen sequences (bankit897496)
203 of amplified fragments were in complete concordance with that for EHV-1 reference strains
204 Ab4 (GenBank accession number AY665713) and V592 (GenBank accession number
205 AY464052). Three additional sequences (bankit897502) were in concordance with that for
206 EHV-2 (Genbank accession number EHVU20824). Another DNA sequence (bankit895337)
207 was in concordance with that for EHV-5 (GenBank accession number AF141886). The
208 seasonal distribution of this data is shown in table 2.

209 Using specific EHV-1 PCR, we also analysed 49 samples for which lung and placenta were
210 both available. Twenty-two cases (45%) were positive for both organs, 3 cases (6%) were
211 positive only for lung, 8 cases (16%) were positive only for placenta and 16 cases (33%) were

212 negative. The results indicate that the placenta should be systematically analysed for EHV-1
213 in investigations of equine abortion.

214 ***3.3. Correlations between molecular detection, virus culture and histopathology***

215 The use of the consensus herpesvirus PCR in conditions of equine abortion and neonatal death
216 can elucidate the presence of two groups of EHV-1 infection.

217 Group 1: nineteen cases were positive by consensus herpesvirus PCR, specific EHV-1 PCR
218 and by culture of lung tissues on RK13 cells. Most specimens had characteristic macroscopic
219 lesions at post-mortem examination. Histopathological lesions and eosinophilic intranuclear
220 inclusion bodies were clearly demonstrated. Thus, we consider that EHV-1 infection is
221 implicated in abortion in these 19 cases.

222 Group 2: in 40 other cases, specific EHV-1 PCR and/or sequencing of the fragment amplified
223 by consensus PCR, demonstrated the presence of EHV-1 genome, in either lung or placenta.
224 However, at autopsy, there was no direct evidence for EHV-1 infection and subsequent
225 culture of lung tissues did not allow isolation of the virus. In all cases (10/10),
226 histopathological examination did not have lesions to support a diagnosis of EHV-1 abortion.
227 Nevertheless, the evidence suggests the existence of two subgroups: in the first subgroup (25
228 cases), PCR signals were observed in DNA from lung or placenta by the two PCRs
229 (consensus and specific EHV-1 PCR), in the second subgroup (15 cases), only consensus
230 PCR showed a positive signal and confirmed the presence of herpesvirus. Thus, although
231 herpesviral DNA was present in lung and placenta in these samples, EHV-1 was not
232 confirmed by specific PCR or culture.

233 The application of the consensus herpesvirus PCR to tissues from a large population of equine
234 abortion and neonatal deaths allowed us to detect three positive cases of EHV-2 confirmed by
235 specific EHV-2 PCR. However, the viruses could not be isolated and histopathological lesions
236 were not observed. The sequencing of a positive consensus herpesvirus PCR product also

237 allowed us to detect a sequence that had 100% homology with a sequence of EHV-5 but once
238 again, the virus could not be isolated and histopathological lesions were absent. As such, there
239 is no evidence to implicate EHV-5 in abortion. Furthermore, the samples studied were not
240 positive for EHV-3 or EHV-4.

241 **4. Discussion**

242 This is the first French study to present molecular biology data relative to abortion in mares
243 due to herpesvirus.. The incidence 15% (67/407) of herpesviruses is not in agreement with
244 that reported in the literature. Two retrospective studies showed lower rates of EHV in
245 abortion in the UK (7% by Smith et al., 2003) and in the USA (4% by Giles et al., 1993) as
246 detected by virus isolation and a direct fluorescent antibody test coupled with cell culture,
247 respectively. The use of a nested consensus PCR followed by specific PCR, or sequencing,
248 might have increased the sensitivity of the present method. The limit of detection (10^{-6}
249 TCID₅₀/50 μ L) is comparable with other methods (Elia et al., 2006; Diallo et al., 2007).
250 Moreover, the extraction protocol was optimal (high quality of samples) and the presence of
251 inhibitors was not observed. The findings suggest that, at least during the 2½ years of the
252 study, the prevalence of EHV-1 in abortion is greater than that mentioned in previous reports.
253 Analysis of the placenta confirmed recent data reported by Gerst et al. (2003) and Smith et al.
254 (2004) and stresses the importance of including this tissue in the diagnosis of abortion.
255 Concerning EHV-1, 15 viruses were detected by consensus PCR. In fact, an absence of signal
256 with specific PCR could be related to lower efficiency of the one-step test, or to heterogeneity
257 of the glycoprotein B gene which could alter the annealing of the specific primers. Moreover,
258 the nucleotide sequencing of DNA polymerase gene (samples that were shown to be positive
259 with a consensus herpesvirus PCR and negative with the specific EHV-1 PCR) revealed no
260 difference with the EHV-1 strains Ab4 and V592. However, recent studies demonstrated
261 differences between the two strains Ab4 and V592 (Allen and Breathnach, 2006; Kydd et al.,

262 2006; Nugent et al., 2006; Slater et al., 2006) and particularly in a specific area of the
263 polymerase gene (ORF30 coding for the catalytic subunit of the DNA polymerase). The Ab4
264 strain was identified as an endotheliotropic, abortigenic and paralytic virus that causes a high
265 level viremia, whereas the V592 strain was identified as a less abortigenic virus that produces
266 no neurological damage (Allen and Breathnach, 2006).

267 The presence of EHV-4 was not detected but this strain is not normally related with abortion;
268 some reports suggest a putative role in abortion only in a minority of isolates studied (Ostlund
269 et al., 1993; Smith et al., 2003). Indeed, we detected EHV-4 only once in a foetus in 1999.
270 The absence of the EHV-3 gene in the population studied suggests that this virus is minimally
271 involved in cases of abortion even if its tropism is in the genital tract. Only a few studies
272 looked for the presence of EHV-3 and none reported its presence in naturally occurring
273 abortion over the last 27 years (Feilen et al., 1979).

274 EHV-2 was detected in three specimens which represents 0.74% of the samples studied and
275 confirms the recent data of Galosi et al., (2005). The role of this virus in abortion is still not
276 clear.

277 The only EHV-5 genome detected in our study was confirmed by sequencing. Studies
278 involving isolated cultures are currently ongoing but the data are not yet available.
279 Nevertheless, the 100% identity of the sequence with that of the reference strain (AF141886)
280 confirms the presence of EHV-5 in our specimens.

281 In the present 2½-year study, we demonstrated that the consensus herpesvirus DNA
282 polymerase PCR is appropriate for the detection of all known equine herpesviruses (type 1 to
283 5) present in placenta or lung tissue (from aborted or neonatal foal deaths). We have validated
284 this assay to show the involvement of herpesvirus in abortion. Moreover, since 1998, this test
285 has been successfully used in our laboratory on different DNA extracts from spinal, broncho-
286 alveolar or trans-tracheal fluid. The use of this consensus herpesvirus PCR appears to be a

287 good rapid diagnostic tool for use in veterinary laboratories. This approach is sensitive and
288 broadly applicable to the detection and identification of previously characterized
289 herpesviruses present in equine tissues and can also provide valuable initial sequence
290 information for previously unreported equine herpesviruses. This test has already allowed us
291 to detect one case of asinine herpesvirus on an aborted foetus issued from a she-ass (AHV4
292 with the GenBank accession number AY054992).

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300 **References**

- 301 Agius, C.T., Nagesha, H.S., Studdert, M.J., 1992. Equine herpesvirus 5: comparisons with
302 EHV-2 (equine cytomegalovirus), cloning, and mapping of a new equine herpesvirus with
303 a novel genome structure. *Virology*. 191, 176-186.
- 304 Agius, C.T., Studdert, M.J., 1994. Equine herpesviruses 2 and 5: comparisons with other
305 members of the subfamily gammaherpesvirinae. *Adv. Virus Res.* 44, 357-379.
- 306 Allen, G.P., Bryans, J.T., 1986. Molecular epizootiology, pathogenesis, and prophylaxis of
307 equine herpesvirus-1 infections. *Prog. Vet. Microbiol. Immunol.* 2, 78-144.
- 308 Allen, G.P., Breathnach, C.C., 2006. Quantification by real-time PCR of the magnitude and
309 duration of leucocyte-associated viraemia in horses infected with neuropathogenic vs. non-
310 neuropathogenic strains of EHV-1. *Equine Vet. J.* 38, 252-257.
- 311 Bell, S.A., Balasuriya, U.B., Gardner, I.A., Barry, P.A., Wilson, W.D., Ferraro, G.L., 2006a.
312 Temporal detection of equine herpesvirus infections of a cohort of mares and their foals.
313 *Vet. Microbiol.* 10, 249-257.
- 314 Bell, S.A., Balasuriya, U.B., Nordhausen, R.W., MacLachlan, N.J., 2006b. Isolation of equine
315 herpesvirus-5 from blood mononuclear cells of a gelding. *J. Vet. Diagn. Invest.* 18, 472-
316 475.

- 317 Blanchard, T.L., Kenney, R.M., Timoney, P.J., 1992. Venereal disease. *Vet. Clin. North Am.*
318 *Equine Pract.* 8, 191-203.
- 319 Borchers, K., Wolfinger, U., Goltz, M., Broll, H., Ludwig, H., 1997. Distribution and
320 relevance of equine herpesvirus type 2 (EHV-2) infections. *Arch. Virol.* 142, 917-928.
- 321 Crabb, B.S., Studdert, M.J., 1995. Equine herpesviruses 4 (equine rhinopneumonitis virus)
322 and 1 (equine abortion virus). *Adv. Virus Res.* 45, 153-190.
- 323 Diallo, I.S., Hewitson, G., Wright, L.L., Kelly, M.A., Rodwell, B.J., Corney, B.G., 2007.
324 Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-
325 1) and equid herpesvirus 4 (EHV-4). *Vet. Microbiol.* 9.
- 326 Diallo, I.S., Hewitson, G., Wright, L., Rodwell, B.J., Corney, B.G. 2006. Detection of equine
327 herpesvirus type 1 using a real-time polymerase chain reaction. *J. Virol. Methods.* 131, 92-
328 98.
- 329 Drummer, H.E., Reubel G.H. and Studdert, M.J. 1996. Equine gammaherpesvirus 2 is latent
330 in B lymphocytes. *Arch. Virol.* 141, 495-504.
- 331 Dyonon, K., Varrasso, A., Ficorilli, N., Holloway, S., Reubel, G., Li, F., Hartley, C., Studdert,
332 M., Drummer, H., 2001. Identification of equine herpesvirus 3 (equine coital exanthema
333 virus), equine gammaherpesviruses 2 and 5, equine adenoviruses 1 and 2, equine arteritis
334 virus and equine rhinitis A virus by polymerase chain reaction. *Aust. Vet. J.* 79, 695-702.
- 335 Elia, G., Decaro, N., Martella, V., Campolo, M., Desario, C., Lorusso, E., Cirone, F.,
336 Buonavoglia, C., 2006. Detection of equine herpesvirus type 1 by real time PCR. *J. Virol.*
337 *Methods.* 133, 70-75.
- 338 Feilen, C.P., Walker, S.T., Studdert, M.J., 1979. Equine herpesvirus type 3 (equine coital
339 exanthema) in New South Wales. *Aust. Vet. J.* 55, 443-444.
- 340 Galosi, C.M., Vila Roza, M.V., Oliva, G.A., Pecoraro, M.R., Echeverria, M.G., Corva, S.,
341 Etcheverrigaray, M.E., 2001. A polymerase chain reaction for detection of equine
342 herpesvirus-1 in routine diagnostic submissions of tissues from aborted fetuses. *J. Vet.*
343 *Med. B Infect. Dis. Vet. Public Health.* 48, 341-346.
- 344 Galosi, C.M., De La Paz, V., Fernandez, L.C., Martinez, J.P., Craig, M.I., Barrandeguy, M.,
345 Etcheverrigaray, M.E., 2005. Isolation of equine herpesvirus-2 from the lung of an
346 aborted fetus. *J. Vet. Diagn. Invest.* 17, 500-502.
- 347 Gerst, S., Borchers, K., Gower, S.M., Smith, K.C., 2003. Detection of EHV-1 and EHV-4 in
348 placental sections of naturally occurring EHV-1- and EHV-4-related abortions in the UK:
349 use of the placenta in diagnosis. *Equine Vet. J.* 35, 430-433.
- 350 Giles, R.C., Donahue, J.M., Hong, C.B., Tuttle, P.A., Petrites-Murphy, M.B., Poonacha, K.B.,
351 Roberts, A.W., Tramontin, R.R., Smith, B., Swerczek, T.W., 1993. Causes of abortion,
352 stillbirth, and perinatal death in horses: 3,527 cases (1986-1991). *J. Am. Vet. Med. Assoc.*
353 203, 1170-1175.
- 354 Gleeson, L.J., Sullivan, N.D., Studdert, M.J., 1976. Equine herpesviruses: type 3 as an
355 abortigenic agent. *Aust. Vet. J.* 52, 349-354.

- 356 Hartley, C.A., Wilks, C.R., Studdert, M.J., Gilkerson, J.R., 2005. Comparison of antibody
357 detection assays for the diagnosis of equine herpesvirus 1 and 4 infections in horses. *Am. J.*
358 *Vet. Res.* 66, 921-928.
- 359 Hussey, S.B., Clark, R., Lunn, K.F., Breathnach, C., Soboll, G., Whalley, J.M., Lunn, D.P.,
360 2006. Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by
361 real-time polymerase chain reaction. *J. Vet. Diagn. Invest.* 18, 335-342.
- 362 Kirisawa, R., Endo, A., Iwai, H., Kawakami, Y., 1993. Detection and identification of equine
363 herpesvirus-1 and -4 by polymerase chain reaction. *Vet. Microbiol.* 36, 57-67.
- 364 Kleiboeker, S.B., Chapman, R.K., 2004. Detection of equine herpesvirus 3 in equine skin
365 lesions by polymerase chain reaction. *J. Vet. Diagn. Invest.* 16, 74-79.
- 366 Kydd, J.H., Townsend, H.G., Hannant, D., 2006. The equine immune response to equine
367 herpesvirus-1: The virus and its vaccines. *Vet. Immunol. Immunopathol.* 111, 15-30.
- 368 Leon, A., Pronost, S., Tapprest, J., Foucher, N., Blanchard, B., Andre-Fontaine, G., Laugier,
369 C., Fortier, G., Leclercq, R., 2006. Identification of pathogenic *Leptospira* strains in tissues
370 of a premature foal by use of polymerase chain reaction analysis. *J. Vet. Diagn. Invest.* 18,
371 218-21.
- 372 Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden,
373 R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equine herpesvirus 1 strain variation
374 reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic
375 versus nonneuropathogenic disease outbreaks. *J. Virol.* 80, 4047-4060.
- 376 O'Keefe, J.S., Alley, M.R., Jones, D., Wilks, C.R., 1995. Neonatal mortality due to equine
377 herpesvirus 4 (EHV-4) in a foal. *Aust. Vet. J.* 72, 353-354.
- 378 Ostlund, E.N., 1993. The equine herpesviruses. *Vet. Clin. North Am. Equine Pract.* 9, 283-
379 294.
- 380 Reubel, G.H., Crabb, B.S., Studdert, M.J., 1995. Diagnosis of equine gammaherpesvirus 2
381 and 5 infections by polymerase chain reaction. *Arch. Virol.* 140, 1049-1060.
- 382 Rooney J.R., 1970. *Autopsy of the horse.* The William & Wilkins Company, Baltimore.
- 383 Schlocker, N., Gerber-Bretscher, R., von Fellenberg, R., 1995. Equine herpesvirus 2 in
384 pulmonary macrophages of horses. *Am. J. Vet. Res.* 56, 749-754.
- 385 Slater, J.D., Lunn, D.P., Horohov, D.W., Antczak, D.F., Babiuk, L., Breathnach, C., Chang,
386 Y.W., Davis-Poynter, N., Edington, N., Ellis, S., Foote, C., Goehring, L., Kohn, C.W.,
387 Kydd, J., Matsumura, T., Minke, J., Morley, P., Mumford, J., Neubauer, T., O'callaghan,
388 D., Osterrieder, K., Reed, S., Smith, K., Townsend, H., van der Meulen, M.K., Whalley,
389 M., Wilson, W.D., 2006. Report of the equine herpesvirus-1 Havermeier Workshop, San
390 Gimignano, Tuscany, June 2004. *Vet. Immunol. Immunopathol.* 111, 3-13.
- 391 Smith, K.C., 1997. Herpesviral abortion in domestic animals. *Vet. J.* 153, 253-268.

- 392 Smith, K.C., Blunden, A.S., Whitwell, K.E., Dunn, K.A., Wales, A.D., 2003. A survey of
393 equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997. *Equine Vet. J.*
394 35, 496-501.
- 395 Smith, K.C., Whitwell, K.E., Blunden, A.S., Bestbier, M.E., Scase, T.J., Geraghty, R.J.,
396 Nugent, J., Davis-Poynter, N.J., Cardwell, J.M., 2004. Equine herpesvirus-1 abortion:
397 atypical cases with lesions largely or wholly restricted to the placenta. *Equine Vet. J.* 36,
398 79-82.
- 399 Szeredi, L., Palfi, V., Molnar, T., 2003. Comparison of methods for the diagnosis of equine
400 herpesvirus type 1 infection. *Acta Vet. Hung.* 51, 153-163.
- 401 van Maanen, C., 2002. Equine herpesvirus 1 and 4 infections: an update. *Vet. Q.* 24, 58-78.
- 402 VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose,
403 T.M., 1996. Detection and analysis of diverse herpesviral species by consensus primer
404 PCR. *J. Clin. Microbiol.* 34, 1666-1671.
- 405 Varrasso, A., Dynon, K., Ficorilli, N., Hartley, C. A., Studdert, M. J. and Drummer, H. E.
406 2001. Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. *Aust.*
407 *vet. J.* 79, 563-569.

408 **Table 1:** Characteristics of the different PCR assays used for the detection of equine herpesviruses.

PCR assay	Target gene	Primers sequence (5'-3')	Amplification program	Product size (bp)	PCR type	References
Consensus herpes	DNA polymerase	DFA: + GAYTTYGCNAGYYTNTAYCC ILK: + TCCTGGACAAGCAGCARNYSGCNMTNAA KG1: - GTCTTGCTCACCAGNTCNACNCCYTT TGV: + TGTAACTCGGTGTAYGGNTTYACNGGNGT* IYG: - CACAGAGTCCGTRTCNCCRTADAT*	94°C 5 min 94°C 30s 46°C 1 min x 45 72°C 1 min 72°C 10 min	215-315	Nested	Van Devanter et al. (1996)
EHV-1	<i>Glycoprotein B</i>	R1: + GCGTTATAGCTATCACGTCC FC3: - ATACGATCACATCCAATCCC	94°C 5 min 94°C 30s 64°C 30s x 40 72°C 1 min 30 72°C 10 min	188	One-step	Kirisawa et al. (1993)
EHV-2	"human Interleukin 10 homologous sequence"	ALM1: + GGC ACTGAAACCCCATACTG ALM2: - AAAACCATCCTGTCCAACCA ALM3: + CCACTAACCCCAACCTT* ALM4: - CCTCTATCCTCACAAACAG*	94°C 5 min 94°C 30s 64°C 30s x 40 72°C 1 min 72°C 10 min	521-721	Nested	Borchers et al. (1997)
EHV-3	<i>DNA polymerase</i>	HSP3F: + GACGGTCACCACGATCG HSP3R: - CTCGGGAAAGTTGGCC	94°C 5 min 94°C 30s 62°C 30s x 40 72°C 1 min 72°C 10 min	100	One-step	Pronost (unpublished)
EHV-4	<i>Glycoprotein B</i>	R4: + CCTGCATAATGACAGCAGTG FC3: - ATACGATCACATCCAATCCC	94°C 5 min 94°C 30s 64°C 30s x 40 72°C 1 min 30 72°C 10 min	677	One-step	Kirisawa et al. (1993)

409 (+: forward primer; -: reverse primer; * second round primers of nested PCR)

410 **Table 2:** Seasonal distribution of herpesviruses in 407 aborted foetuses from October 2002 to June 2005.
 411

Herpesvirus type	Breeding seasons			Total in 407 samples
	2002/2003	2003/2004	2004/2005	
EHV-1	19	30	10	59 (14.5%)
EHV-2	1	2	0	3 (0.75%)
EHV-4	0	0	0	0
EHV-5	0	0	1	1 (0.25%)
Undetermined	1	2	1	4 (1%)

412
 413 (Each breeding season covers October to June.)
 414

415 **Legend to Figure**

416

417 **Figure 1:**

418 Alignment of nucleotide sequences of 19 FR-EHV (French equine herpesvirus) isolates with
419 representatives of three known genetic lineages of EHV a/ EHV-1 (AY665713, AY464052).

420 Only nucleotides that differ from isolates (AY665713, AY464052) are shown; b/ EHV-2
421 (EHVU20824); c/ EHV-5 (AF141886). Identical nucleotides are represented by dots. Dashes
422 indicate gaps introduced to maximize the alignment of corresponding nucleotides.

423

a

1 140

FR2002-1 GTGGCAAACG GCCTGTTGCC GTGTCTGAGG ATAGC---GG CTACCGTTAC CACGATAGGA CGCGACATGC TCCTCAAGAC CCGAGATTAC GTTCACTCTC GTTGGGCGAC GCGCGAGCTG CTGGAGGACA ATTTTCCAGG

FR2002-5

FR2002-6

FR2002-9

FR2002-11

FR2003-1

FR2003-2

FR2003-9

FR2004-6

FR2004-7

FR2004-17

FR2004-11

FR2004-14

FR2004-28

FR2005-1

AY665713

AY464052

FR2005-5 . . .CTCG. . .A.CC. . . .C. .A.A. . . .---A. AG. .G. .C. .TTTCAG. .G A.GAGG. . . .AGAG.G.T. .AAGCGC. . .A.AG.GG.-. . .----- . . .C.C. . .GG. T. . .CT.C. .TC. .G.AGA.

FR2003-5 .A.T.CGTGT C. .C. .A.AT .ACCT.A.AA C.G. .ATC. .GGG.GCACT. . .CCGCCTG .C.A.T. . .T CTGC. .GCC. .TCG.GGGT. ACC.C. . . .A .GTA. . .CT TG.A.TT. .C .A.C.T.CGC C.GCC.TG.A

Consensus gt.g.aaacg g.ctggt.cc .tgtc.g.gg a.a.g. cta.cgtta .acgatagga cgcgac. . .c tcctc.aga. .cgagatta. gttcact.tc g.tgggc.ac gcgc.agctg ct.gag.aca atttt.cagg

141 190

FR2002-1 GGCTATAGGT TTCCGAAACC -----ACAAG CCTTACTCCG TCAGGGTTAT

FR2002-5

FR2002-6

FR2002-9

FR2002-11

FR2003-1

FR2003-2

FR2003-9

FR2004-6

FR2004-7

FR2004-17

FR2004-11

FR2004-14

FR2004-28

FR2005-1

AY665713

AY464052

FR2005-5 . .C.CG.G.CG GC.T.CG. . .---C.G. .G.CAG. .--- .T.A. . .C

FR2003-5 A.G.GACC. .C. .G.CT.T. TTGAG. . .G. G.AGGA.G.- C.GA. .CC.C

Consensus ggctataggt tt.c.aa.c.a.aa. c.ttac.ccg tcag. .ttat

b

FR2003-5 1 GAGTCCGTGT CCCCGTAGAT GACCTTAAAA CTGGCATCGG GGGCGCACTC CACCCGCCTG CCCAATATGT CTGCCAGCCC CTCGGGGGTC ACCCCCTCTA TGTAGCGTT GGAGTTCTCC AGCATGCGCC TGCCCTGGAA 140
 FR2004-1
 FR2003-11
 EHVU20824
 Consensus

FR2003-5 141 GGTGACCGTC TCGGCTATCT TGAGACAGGG CAGGATGCC CAGGCCAC 188
 FR2004-1
 FR2003-11
 EHVU20824
 Consensus

c

FR2005-5 1 GGCTCGGGC ATCCTGCCGT GCCTAAAGAT AGCAGAGACG GTCACCTTC AGGGGAGGAG GATGCTAGAG AGGTCCAAGC GCTACATAGA GGCCGTGACC CCCGAGGGGT TGGCTGCCAT CTTGCAGAGG CCCGTGGCGG 140
 AF141886
 Consensus

FR2005-5 141 CCTGCGACCC CGAGGCCAGC TTTAAGGTC 169
 AF141886
 Consensus