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HERPESVIRUSES IN RESPIRATORY LIQUIDS OF HORSES: PUTATIVE IMPLICATION IN AIRWAY INFLAMMATION AND ASSOCIATION WITH CYTOLOGICAL FEATURES

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Abstract

The objectives of this study were to estimate the prevalence and the potential role of equine herpesviruses (EHVs) detection in both bronchoalveolar lavage (BAL) and tracheal wash (TW). The population included a control group (CTL; 37 TW and 25 BAL) and a pathological group (PAT; 259 TW and 387 BAL), including horses either suffering from respiratory diseases including syndrome of tracheal inflammation, inflammatory airway disease, recurrent airway obstruction, or submitted to respiratory investigation because of exercise intolerance or poor performance. Each respiratory liquid was submitted to a standardised cytological analysis, mentioning the morphological abnormalities of exfoliated epithelial cells (ECAb) and ciliocytophthoria (CCPh) as markers of potential viral infection, as well as PCR assays including a consensus PCR and virus-specific PCR for both equine alphaherpesviruses (EHV-

1; EHV-4) and gammaherpesviruses (EHV-2; EHV-5). The EHV infections were more prevalent in the TW of PAT group ($P = 0.004$), with the highest prevalence being for EHV-2 ($P = 0.006$). The EHV detection in BALs was not significantly different between groups. The EHV detection in TW was correlated to the polymorphonuclear neutrophil (PMN) counts in the respiratory liquid but not with CCPh or ECAB. CCPh or ECAB were associated with both consensus PCR and EHV-2 and EHV-5 virus-type PCR in the BAL only. The significant detection of EHV in the TW of PAT group in association with the PMN increased counts could lead to further investigations about their putative role in equine syndrome of tracheal inflammation.

Keywords: ciliocytophthoria, respiratory disease, horse, herpesvirus, PCR, gammaherpesvirus

1 **1. Introduction**

2 Respiratory diseases are a consensually recognised cause of wastage in the horse industry.
3 Among respiratory diseases, airway inflammation is the most prevalent clinical feature and is
4 largely encountered in the field. Recently, inflammatory airway disease (IAD) was redefined
5 as a clinical syndrome causing a significant elevation of polymorphonuclear neutrophil
6 (PMN) count in the bronchoalveolar lavage fluid (Couetil et al., 2007). Less is however
7 known about the syndrome of tracheal inflammation (STI) even if viruses, bacteria or fungi
8 are suspected as potential actors of this local neutrophilia (Couetil et al., 2007). Modifications
9 in the cytological profiles of BAL or TW have sometimes been evoked in case of viral
10 diseases but without clear demonstration (Whitwell and Greet, 1984; Viel, 2002). Transient
11 neutrophilia in respiratory liquids has been also described after viral exposure but cannot be
12 interpreted as a specific profile (McGorum and Dixon, 1994; Sutton et al., 1998). On the other
13 hand, ciliocytophthoria is a criteria used for the differential diagnosis of viral disease in
14 human pathology, especially for respiratory or genital cytology (Papanicolaou et al., 1961).
15 Whitwell and Greet (1984) also described this anomaly in cases of viral diseases and more
16 particularly in those caused by herpesvirus in horses with tracheal inflammation and mucosal
17 erosion. Pointing out the putative role of viruses in inflammatory airway diseases, Viel (2002)
18 emphasized the need of incorporation of exfoliated epithelial cells (EEC) in the BAL
19 cytological analysis.

20 Due to their high prevalence and their capability of latency in various sites, viruses and
21 particularly herpesviruses are frequently incriminated in equine poor performance syndromes
22 (Wood et al., 2005). However, alphaherpesviruses such as equid herpesvirus 1 (EHV-1) or
23 equid herpesvirus 4 (EHV-4) do not seem to be clearly associated with such syndromes
24 (Newton et al., 2003; Wood et al., 2005). The alphaherpesviruses are recognised as causative
25 agents of various epidemic diseases in the horse population (i.e; abortion,

26 myeloencephalopathy) and as well as of upper respiratory tract infections, but equine
27 gammaherpesviruses have been less studied and have been characterized (Agius and Studdert,
28 1994) and reclassified (Davison et al., 2009) only recently. Their real pathogenic significance
29 remains unclear because of their frequent isolation in healthy horse populations and their high
30 seroprevalence worldwide (Bell et al., 2006). In mice, equid herpesvirus 2 (EHV-2) DNA can
31 be retrieved from the lungs 30 days after experimental exposure and infection (Borchers et al.,
32 2002). In foals or young race horses, this virus has been shown to induce conjunctivitis and
33 mild respiratory diseases, (Bell et al., 2006). In addition, equid herpesvirus 5 (EHV-5) has
34 been recently associated with equine pulmonary multinodular syndrome (Williams et al.,
35 2007).

36 Performing both consensus PCR and virus-specific PCR assays, the purposes of this study
37 were (1) to precise the DNA prevalence of EHV-1, 2, 4 and 5 in respiratory liquids of horses
38 suffering from various respiratory diseases and compare it to a control group, and (2) to
39 determine if particular cytological profiles of the sampled liquids could be associated with
40 herpesviruses detection and to investigate within the different groups the correlation between
41 EHV's detection and neutrophilia or epithelial cells abnormalities in both the TW and the
42 BAL.

43

44 **2. Materials and methods**

45 2.1. Horse population and respiratory liquids

46 The study included 708 samples (647 horses aged 18 months - 13 years; Table 1) of various
47 breeds and/or disciplines (Standardbred, Thoroughbred, Endurance and French saddle horses)
48 harvested between October 2006 and February 2008. Horses from the control group (CTL)
49 were referred to a sports medicine facility (CIRALE, Goustranville, France) for routine
50 clinical and fitness follow-up using standardised protocols. Thorough clinical and

51 complementary exams were systematically performed, including haematological and
52 biochemical evaluation at rest and post-exercise, Doppler echocardiography, standardised
53 exercise tests on both treadmill and racetrack, locomotor evaluation, high-speed treadmill
54 video-endoscopy of the upper respiratory tract, and collection of respiratory liquids including
55 TW and BAL. Each CTL horse was free of any abnormality for all these clinical
56 investigations, and particularly a scoring mucus ≤ 2 (Gerber et al., 2004), no signs of
57 infection/inflammation at the haemato-biochemical evaluation (Couetil et al., 2007) and both
58 TW and BAL cytological values within proposed references (Robinson, 2003). The
59 pathological group (PAT) was composed of horses with signs of various respiratory disorders
60 or “poor performance syndrome” and originated from 28 veterinary practices located close to
61 training yards or race tracks in the Normandy or Ile de France regions. To be included, at least
62 one of the following criteria had to be detected by the clinician: coughing, scoring mucus >2 ,
63 TW or BAL neutrophilia, or poor performance suspected by the practitioner to be due at least
64 partly to respiratory dysfunctions. Any horse exhibiting dyspnoea at the moment of sampling
65 or clinical signs of either acute viral (i.e. serous nasal discharge, hyperthermia, ...) or
66 bacterial infection (i.e. purulent nasal discharge, leukocytosis, ...), were excluded from the
67 study.

68 2.2. Samples processing and cytological analysis

69 Each practice or referring veterinarian was expected to follow the main recommendations
70 regarding sampling and shipment of TW or BAL (Viel, 2002; Pickles et al., 2002; Malikides
71 et al., 2007). Samples of each group were excluded if a set of quality criteria was not met (i.e
72 presence of squamous pharyngeal cells, cell degradation, contaminating bacterial flora or lack
73 of historical information). Aliquots of respiratory liquids were placed in EDTA- and serum
74 tubes and respectively submitted for cytology and virology. May Grunwald Giemsa stain was
75 applied to slides previously prepared by smooth cytocentrifugation (75 x g, 10 min) using a

76 Cytospin (Shandon-Thermoscientific, Waltham, MA, USA). Differential cell counts were
77 performed by counting an average of 300 cells and the results of each cell type were
78 expressed as a percentage of the total nucleated cells. Presence of exfoliated epithelial cells
79 (EEC) were noted and defined as either normal or presenting morphological abnormalities
80 (ECAb) when free cilia, ciliated plates or detached ciliated tufts were found in the liquid
81 (Sutton et al., 1997; Viel, 2002) or ciliocytophthoria (CCPh) when the previous criteria was
82 accompanied by cytoplasmic or nuclear modifications (Whitwell and Greet, 1984; Freeman et
83 al., 1985). The EEC were initially included in the sample cell counts to determine if there
84 were any significant differences in proportion between the CTL and the PAT group.
85 Thereafter, EEC were excluded from the differential cell counts but ECAb and CCPh were
86 taken into account in the statistical analysis as “present” or “absent”. Hematological and
87 biochemical tests were performed with an Advia 120 (Bayer) and a Rx Imola (Randox,)

88 2.3 Nucleic acids extraction and PCR assays

89 Respiratory liquids were centrifuged at 2000 x g, 4 °C, during 15 min, and pellets were
90 homogenised with 500 µl of supernatant. DNA was extracted from 140 µl of each sample,
91 using the QIAamp DNA Mini Kit (Qiagen, France) according to the manufacturer’s
92 instructions, and eluted in a 50 µl final volume. Concentrated DNA was stored at -80 °C until
93 used. All extraction included a negative control where respiratory liquid was replaced with
94 distilled water and a positive control.

95 The five PCR assays systematically carried out on each sample for this study are summarized
96 in table 2. A consensus herpesvirus PCR assay was carried out as previously described by
97 Leon et al. (2008). EHV-2 was detected by amplification of a sequence encoding a product
98 homologous to human interleukin-10 (Leon, personal communication 2008; adapted from
99 Borchers and Frolich, 1997) whereas EHV-5 was detected by a virus-specific EHV-5 PCR
100 assay described by Dynon et al. (2001). Classical PCRs (consensus PCR and virus-specific

101 EHV-2 and EHV-5 PCR) were performed with pure Taq Ready to Go PCR beads (Amersham
102 Biosciences, Europe GmbH, France); the final concentrations of each primer were 1 μ M
103 consensus PCR, 1 μ M EHV-2 and 800 nM EHV-5. Classical PCRs were performed on
104 GeneAmp PCR System 9700 (Applied Biosystems). Virus-specific real-time PCRs were
105 performed as described by Diallo et al. (2007). Each test was used as a simplex assay in a
106 total volume of 25 μ l containing 2x Taqman Universal PCR Master Mix (Applied
107 Biosystems, France), 1 μ M of each primer and the optimum concentration of probe (between
108 100 and 400 nM as determined after titration). Real time amplifications were performed on
109 Step One Real-Time PCR system (Applied Biosystems). Each different PCR test included a
110 negative control where DNA was replaced with distilled water and a specific positive control
111 where DNA from each virus was amplified. DNA from American Type Culture Collection
112 reference strains for EHV-1 (Kentucky reference strain, KyA), EHV-2 (LK strain) and EHV-4
113 strain 405/76 and DNA from our laboratory for EHV-5 (Fortier et al. 2008, *in press*).
114 Positive samples by consensual PCR which were negative with each virus-specific PCR
115 assays (EHV-1, 2, 4 and 5), were sequenced after extraction of the PCR product from agarose
116 using the QIAquick gel extraction kit (Qiagen, France). Both sense and anti-sense strands of
117 the PCR products were sequenced (Biofidal, France). The nucleotide sequences were
118 assembled and manually edited (CodonCode Aligner), and consensus PCR sequences were
119 analysed for similarities on the National Center for Biotechnology Information (NCBI)
120 website using databases and the Basic Local Alignment Search Tool program (BLASTn).

121

122 2.4 Statistical analysis

123 Differences in the results between the groups were compared using a Fisher's exact test. A
124 Cochran Armitage trend test was used to evaluate the trend between PMN cell counts and
125 herpesviruses DNA detection in the samples. According to the percentage of PMNs in the

126 liquids, PMN results were conventionally distributed into 6 different classes, from 0 to 20%,
127 from 21 to 40%, from 41 to 60%, 61 to 80% and > 80%.Odds ratio (OR) were performed to
128 evaluate the risk of being in the PAT group when herpesvirus DNA was detected. All tests
129 were performed on SAS System (Cary, NC, USA). *P* values < 0.05 were considered
130 significant.

131

132 3. Results

133 3.1. PCR assays in the respiratory fluids

134 In the TW of the CTL group, EHV-2 and EHV-4 were detected by virus-specific PCR
135 whereas EHV-1 and EHV-5 were not. In this group, two samples were positive using the
136 consensus PCR but were not confirmed by the specific tests and no clear identification was
137 achieved using the sequencing. In the PAT group, all the targeted viruses were detected.
138 Using PCR, 114 and 112 PAT TW were positive by virus-specific and consensus PCRs
139 respectively (Table 3); 2 samples were detected positive for both EHV-2 and EHV-5. The
140 prevalence of herpesviruses in the TW was significantly higher in the PAT group ($P=0.004$).
141 Detection of EHV-2 was also higher in the PAT group compared to the CTL group
142 ($P=0.006$). The highest level of detection for both groups was found for EHV-2, with 11%
143 and 33% for the CTL group and PAT group respectively. In the BAL, only EHV-2 and EHV-
144 4 were detected in the CTL group (Table 3), whereas in the PAT group all viruses were
145 detected, as in the TW. The prevalence of these viruses in BAL was not significantly different
146 between groups. The highest level of detection for both groups was found for EHV-2, with
147 36% and 21% for the CTL group and PAT group respectively. Whatever the group and
148 sample, EHV-2 was the most frequently detected strain. A double detection (EHV-2 and
149 EHV-4) was found in two BAL samples of the PAT group. Among the 708 samples, Asinine
150 herpesvirus 5 (AHV-5) was identified in 24 PAT liquids (16 in the BAL and 8 in the TW of

151 by sequencing the amplified fragment of the consensus PCR (Table 3 and 4). Among them, a
152 mild positive result was obtained in 8 samples at the second run using the virus-specific EHV-
153 5 PCR. Only consensus PCR positive results were taken into account for the statistical
154 analysis but not virus type specific results. For the two real-time PCR (EHV-1, EHV-4) the
155 cycle threshold (C_t) was mentioned for each analysis. In the TW of PAT group, the C_t range
156 was 22 - 42 for EHV-4 and 36.5 - 38.4 for EHV-1, whereas the C_t was 38 for the only EHV-4
157 positive sample in the CTL group.

158 In the BAL of the PAT group, the C_t range was 34 - 44 for EHV-4 and 35 - 40.5 for EHV-1,
159 whereas the C_t was 39 for the only EHV-4 positive sample in the CTL group.

160 Inclusion in the PAT group was positively and significantly associated with the detection of
161 herpesviruses by both consensus PCR (odds ratio= 3.26, 95% C.I = 1.38-7.04) and EHV-2
162 specific PCR assay (odds ratio= 4.03, 95% C.I = 1.37-11.74). No significant association was
163 observed for any other herpesvirus neither in the TW nor in the BAL.

164

165 3.2. Association between groups, viral detection and cytology of the liquids

166 The proportion of EEC in the BAL (0.7 % vs 2.7 %) and TW (15.6 % vs 12.8 %) were not
167 significantly different between the CTL group and the PAT group and ranged with normal
168 values (Derksen et al., 1989). Percentages of CCPH or ECAB were not significantly associated
169 with groups (Table 4). No morphological abnormality was detected among the BAL epithelial
170 cells (ECAB and CCPH) in the CTL group. The proportion of CCPH was increased in the PAT
171 group, but not significantly ($P = 0.32$).

172 As described in Table 5, the presence of one of the two positive criteria for epithelial cell
173 alterations (ECAB, CCPH) was significantly associated with herpesvirus DNA detection for
174 EHV-2 and EHV-5, but in the BAL only. No other significant association was found for
175 EHV-4 in either liquid neither for any other herpesvirus in the TW. No significant association

176 were found between herpesviruses detection (in TW or BAL) and either lymphocytes, giant
177 cells, mast cells, eosinophils, macrophages, or haemosiderophages. In the TW only, a
178 significant association between PMN counts level and DNA detection with the consensus
179 PCR or the EHV-2 virus-type specific PCR was observed (Table 6), whereas a tendency, at
180 the limit of significance, was found for EHV-4 detection ($P = 0.05$). In the TW, the highest
181 percentage of EHV-2 detection was in the sub-group of 40-60% of PMN and in the sub-group
182 80-100% of PMN for EHV-2 with the consensus PCR. The mean level of PMN was higher
183 when herpesvirus DNA was detected with consensus PCR ($P= 0.003$) or EHV-2 specific PCR
184 ($P= 0.04$). The results were not significant using EHV-5, EHV-1 and EHV-4. The trend test
185 was not significant for any herpesvirus in the BAL.

186 No significant trend was found between ciliocytophthoria and the PMN cell counts in either
187 BAL or TW, meanwhile the highest percentage of CCPh was found in the 40-60 % PMN
188 class for TW.

189

190 **Discussion**

191 The precise role of equine herpesviruses in subclinical respiratory diseases has been
192 documented mainly through the results of serologic assays or naso-pharyngeal sampling
193 studies in young racing horses (Wood et al., 2005).

194 Previous studies evaluating viral causes of respiratory diseases in young horses in training
195 mainly focused on alphaherpesviruses, which remain the most worldwide studied
196 herpesviruses in the equine population (Newton et al., 2003; Wood et al., 2005).
197 Gammaherpesviruses were seldom included and information lacked regarding their
198 implication in larger populations of horses suffering from various respiratory disorders. Our
199 study aimed at investigating this missing information by undertaking a systematic DNA
200 detection of herpesviruses in TW and BAL respiratory liquids of horses suspected of

201 respiratory affections. Serologic tools were not implemented for EHV detection as they may
202 not be a reliable method for investigating viral reactivation because of cross-reaction, poor
203 humoral response and lack of seroconversion due to repeated exposures and vaccination. One
204 of the limits in the PCR strategy used in the present study is that it could not allow
205 discrimination between latent, non replicating and lytic states of the detected herpesviruses
206 (Pusterla et al., 2008).

207 Among the alphaherpesviruses detected in the present study, EHV-4 was much more
208 prevalent than EHV-1, and this can be related to its particular role in clinical respiratory
209 disorders in the field. Even if the C_t values obtained for EHV-1 and EHV-4 were in
210 previously described ranges (Diallo et al., 2007), taking into account the artificial dilution due
211 to the sampling method for respiratory liquids, the detection of latent viruses could not be
212 avoided. EHV-4 were sometimes retrieved from liquids with low C_t (high viral load) when a
213 history of infectious disease of suspected viral origin was mentioned by the practitioner.

214 Equine herpesvirus DNA detection was clearly different in the two groups of horses,
215 especially in the TW.

216 The high prevalence of EHV-2 and EHV-5 DNA shown in our study's pathological group is
217 in accordance with previous observations, indicating their frequent detection in nasal swabs,
218 yet from horses without clear clinical signs (Bell et al., 2006). Only few reports exist on
219 EHV-2 and EHV-5 and airway diseases of horses (Allen and Murray, 2004) and most reports
220 concern alphaherpesviruses and relate experimental viral infections with these strains in a
221 lytic phase (Sutton et al., 1998).

222 EHV-2 is known to be latent in lymphocytes (Drummer et al., 1996) or macrophages
223 (Borchers et al., 2002), however no correlation was found between DNA detection and
224 lymphocyte or macrophage counts in both TW and BAL cytological analysis (data not
225 shown). Detection of EHV-2 in the BAL of the CTL group, without EEC abnormalities

226 (ECAb or CCPh) could be due to latency states of the virus in the lymphocytes or
227 macrophages of these liquids. Identification of AHV-5 by sequencing the PCR product of the
228 consensus PCR, with only a low percentage of confirmation by virus-specific PCR tests
229 (EHV-5) was also surprising. No history of concomitant grazing or neighbouring between
230 horses and donkeys could be proven retrospectively. We hypothesised that the difference in
231 detection between the consensus PCR and virus-type specific EHV-5 PCR could have been a
232 consequence of the two different PCR methods, a nested PCR assay versus a standard PCR
233 assay for EHV-5. The AHV-5 matching assessed with Genbank could be due to a lack of
234 reported sequences for the EHV-5 virus. The EHV-5 DNA detection was therefore probably
235 underestimated in the present work, mainly in the BAL samples.

236 Herpesvirus DNA detection was then considered in the light of other potential criteria of viral
237 infection such as clinical suspicion or expression (group), epithelial cell morphological
238 criteria or cytological neutrophilic profiles in the respiratory samples. The interpretation of
239 high exfoliated epithelial cell counts or abnormal morphology in TW or BAL analysis is a
240 recurrent preoccupation of clinicians and searchers and is often considered as a result of
241 mechanical trauma during sampling procedures, leading to a wide range of published
242 epithelial cell percentages between studies (Derksen et al., 1989). More interesting is the
243 analysis of cell morphology during cytological examination, which takes into account the
244 presence and integrity of the *ciliae*, nuclear damage (Whitwell and Greet, 1984; Viel, 2002),
245 or the better defined ciliocytophthoria (Papanicolaou et al., 1961; Freeman et al., 1985).

246 The disparity between results of the TW and BAL in investigations of respiratory disorders
247 and perhaps in virus DNA detection could be attributed to the intrinsic differences of these
248 two liquids. The *trachea* is a “crossroad” connecting the upper and lower respiratory tracts
249 and the TW is therefore recognised as potentially contaminated and of variable cytological
250 quality compared to BAL, even in a healthy population. For this reason, IAD or RAO are

251 exclusively based on a BAL cytological diagnosis. This was recently confirmed by Couetil et
252 al.(2007) who suggested that TW should be studied more in depth in terms of the mechanisms
253 inducing tracheal inflammation (i.e; viruses, bacteria, fungi...). In the present study,
254 ciliocytophthoria (CCPh) or epithelial cell abnormalities (ECAb) did not seem to be sensitive
255 nonspecific “herpesvirus markers” in TW, whereas in the BAL theses epithelial alterations
256 were significantly linked to the presence of both EHV-2 and EHV-5 whatever the group
257 considered.

258 Detection of other viruses such as rhinovirus or influenza virus was not achieved in this study.
259 However these viruses are known to potentially induce mild to severe airway inflammation in
260 horses even a long time after an initial exposure (Willoughby et al., 1992; Sutton et al., 1997).
261 Epithelial cell erosion in the *trachea* (CCPh or ECAb) may also be induced by these viruses
262 explaining the lack of specificity the chosen criteria for positives samples with herpesviruses
263 specifically. The tracheobronchial lymphoid associated tissues represent major latency sites
264 (Allen and Murray, 2004), almost exclusive for herpesviruses and less exposed to other
265 viruses. Epithelial cell damage is therefore more likely to be associated to herpesviruses
266 pathogenicity. During an EHV-2 experimental infection, Borchers et al. (1998) retrieved
267 infectious viruses in nasal or ocular swabs, and viral DNA in the lung and lymphoid tissues
268 after an induced reactivation with dexamethasone (30 days post infection). One could also
269 hypothesize that reactivation can take place after various stressful conditions (intense
270 exercise, competition, transport,...) or corticotherapy and lead to viral shedding in the
271 respiratory tract.

272 Conversely, in the TW, herpesvirus DNA detection was significantly associated with
273 increased neutrophil counts and group. The causative effect of herpesviruses on inflammation
274 after viral exposure remains to be elucidated, even being experimentally showed with EHV-1
275 (Smith et al., 2000). One of the EHV-2 G protein coupled receptor (GPCR) is a receptor for

276 interleukine 8 (IL-8) and the EHV-2 open reading frame E7 codes for IL-10, which is an
277 immunosuppressive cytokine. The chemo-attractant effect of IL-8 for PMNs has previously
278 been described in several clinical or experimental situations (Franchini et al., 2000). EHV-2
279 can be retrieved in the lung several weeks post infection in mice (Borchers et al., 2002). As
280 EHV-2 replication occurs following the delivery of virus or virus genome by a cell-to-cell
281 contact mechanism from lymphocytes to fully permissive epithelial cells, the detection of
282 viral DNA could be associated with immunomodulation of the host pathway, epithelial cells
283 modifications and neutrophilic recruitment a long time after natural exposure (Drummer et al.,
284 1996). Schlocker et al. (1995) suggested early on that EHV-2 could play a role in equine
285 chronic pulmonary disease. The significant association between CCPH or ECAB and DNA
286 detection could also be explained by such a particular viral physiopathology.

287 Genomic variations (Browning and Studdert, 1987; Sharp et al., 2007) and concomitant
288 infections with several field strains of EHV-2 have been demonstrated (Bell et al., 2006) and
289 may determine a graduation in the clinical expression of the disease.

290 **Conclusions**

291 The purposes of this study were (1) to precise the DNA prevalence of four EHV-1s in
292 respiratory liquids of horses suffering from various respiratory diseases in comparison with a
293 control group, (2) to determine if particular cytological profiles of the liquids were associated
294 with EHV-1s detection, by investigating, in the different groups, the correlation between EHV-1s
295 detection and neutrophilia or epithelial cells abnormalities and/or ciliocytophthoria.
296 Herpesviruses DNA was more frequently detected in the TW of the PAT group and associated
297 with liquid neutrophilia. Among these viruses, EHV-2 was the most prevalent. No specific
298 epithelial cells profile could be found in association with viral DNA detection in the TW but
299 ciliocytophthoria or epithelial cells abnormalities were interesting features of the BAL
300 significantly associated with the detection of EHV-2 and EHV-5. As respiratory tract

301 inflammation has already been demonstrated during experimental infection with EHV-1 or
302 influenza virus in the horse, equine herpesviruses and particularly gammaherpesviruses may
303 be implicated in the syndrome of tracheal inflammation.

304 Even if the precise status of the viral infection (lytic, non replicating, latent) could not be
305 elucidated through the PCR assays strategy used in this study, an experimental infection with
306 EHV-2 in “naïve” horses or induced reactivation should give more information about the
307 potential role of this gammaherpesvirus in the horse. Other tools, such as interferon gamma
308 detection or cytokine profile of the liquids could be useful to better understand or clarify the
309 association with virus DNA detection and cytology of the liquids, particularly the TW.

310

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315

316

317

318

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Table 1

Description of the studied population

	Control group (CTL)		Pathological group (PAT)	
	Tracheal washes (TW)	Bronchoalveolar lavages (BAL)	Tracheal washes (TW)	Bronchoalveolar lavages (BAL)
Number of samples	37	25	259	387
Age (years) (mean \pm s.d.)	5.6 \pm 2.9	6.3 \pm 3.05	5.8 \pm 2.2	5.4 \pm 3.4
PMNs count (%) (mean \pm s.d.)	10.4 \pm 6.9	5.7 \pm 2.9	57.1 \pm 34.7*	32.6 \pm 29.1*

PMN: Polymorphonuclear neutrophils

* Statistically different from the corresponding CTL sample ($P < 0.0001$)

Table 2

Characteristics of the PCR assays used for the detection of equine herpesviruses.

PCR assay	Target gene	Primer and probe sequences (5'-3')	amplification program		Product size (bp)	PCR type	References	
Consensual herpes	DNA pol	DFA: + GAYTTYGCNAGYYTNTAYCC	94°C	5min	x45	215-315	Classical Nested	Van Devanter et al. (1996)
		ILK: + TCCTGGACAAGCAGCARNYSGCNMTNAA	94°C	30sec				
		KG1: - GTCTTGCTCACCAGNTCNACNCCYTT	46°C	1min				
		TGV: + TGTAACCTCGGTGTAYGGNTTYACNGGNGT*	72°C	1min				
		IYG: - CACAGAGTCCGTRTCNCRTADAT*	72°C	10min				
EHV1	gB	EHV1F1: + CATGTCAACGCACTCCCA	95°C	10min	x50	63	Real-time	Diallo et al.(2006)
		EHV1R1: - GGGTCGGGCGTTTCTGT	95°C	15sec				
		EHV1MGBPROBE: 6 FAM-CCCTACGCTGCTCC-MGB-NFQ	64°C	1min				
EHV2	« human interleukin 10 homologous sequence »	EHV2pS: + AACTCCCATCCCCAGAAGAC	95°C	15min	x45	245	Classical One step	Leon (personal communication 2008)
		EHV2pAS: - TGTTTCCTCGCTAACCAACC	94°C	30sec				
			60°C	1min30				
			72°C	1min30				
			72°C	10min				
EHV4	gB	EHV4F1: + GGGCTATTGGATTACAGCGAGAT	95°C	10min	x50	59	Real-time	Diallo et al. (2007)
		EHV4R1: - TAGAATCGGAGGGCGTGAAG	95°C	15sec				
		EHV4MGBPROBE: VIC-CAGCGCCGTAACCAG-MGB-NFQ	64°C	1min				
EHV5	gB	EHV5-gB-S: + ATGAACCTGACAGATGTGCC	94°C	5min	x50	293	Classical One step	Dynon et al.(2001)
		EHV5-gB-AS: - CACGTTCATATCACGTCGC	94°C	30sec				
			60°C	45sec				
			72°C	1min30				
			72°C	8min				

+: forward primer, - : reverse primer

* second round primers of nested PCR

Table 3

PCR detections of herpesviruses in the tracheal washes (TW) and bronchoalveolar lavages (BAL) of the control (CTL) and pathological (PAT) groups.

PCR target	TW			BAL		
	CTL (n=37)	PAT (n=259)	<i>P</i> value ^a	CTL (n=25)	PAT (n=387)	<i>P</i> value ^a
	+	+		+	+	
EHV-1	0 [0%]	4 [2%] ^b	> 0.05	0 [0%]	4 [1%] ^c	>0.05
EHV-2	4 [11%]	85 [33%]	0.006 *	9 [36%]	82 [21%]	> 0.05
EHV-4	1 [3%]	17 [7%]	> 0.05	1 [4%]	16 [4%]	> 0.05
EHV-5	0 [0%]	8 [3%]	> 0.05	0 [0%]	16 [4%]	>0.05
EHV consensus PCR	7 [19%]	112 ^d [43%]	0.004 *	10 [40%]	133 ^e [34%]	>0.05

^a Fisher's exact test

^b 11 tests were not performed on this run for EHV-1 due to insufficient quantity of biological material.

^c 2 tests were not performed on this run for EHV-1 due to insufficient quantity of biological material.

* Statistically significant result

^d 8 samples were sequenced as Asinine herpesvirus 5 in the PAT group.

^e 16 samples were sequenced as Asinine herpesvirus 5 in the PAT group.

Table 4

Association between ciliocytophthoria or epithelial cells abnormalities with the status of horses and sample type (TW or BAL)

		CTL		PAT		<i>P</i> value ^a
		+	-	+	-	
BAL	ECAb	0/25 [0%]	25/25 [100%]	15/387 [4%]	372/387 [96%]	0.6
	CCPh	0/25 [0%]	25/25 [100%]	31/387 [8%]	356/387 [92%]	0.2
TW	ECAb	9/37 [24%]	28/37 [76%]	61/259 [23%]	198/259 [76%]	1
	CCPh	7/37 [19%]	30/37 [81%]	72/259 [27%]	187/259 [73%]	0.3

CTL = control group; PAT = pathological group; CCPh = ciliocytophthoria; ECAb = exfoliated epithelial cells abnormalities

^a Fisher's exact test

Note: a combination of the two criteria (CCPh and/or ECAb) is not significant in TW or BAL (0.24 and 0.70 respectively)

Table 5

Herpesvirus DNA detection by ciliocytophthoria or epithelial cells abnormalities in the BAL and TW

<i>P</i> values ^a	EHVs		EHV-2		EHV-5		EHV-4	
	TW	BAL	TW	BAL	TW	BAL	TW	BAL
Ciliocytophthoria	0.28	0.04*	0.39	0.17	0.31	0.02*	0.41	1
Epithelial cells abnormalities	0.2	0.01*	0.5	0.007*	0.16	0.45	1	0.47
CCPh or ECAb	0.30	0.06	0.33	0.13	0.52	0.034*	0.59	1

* Significant results ($P < 0.05$)^a Fisher's exact test

Table 6

Association between herpesvirus DNA detection and polymorphonuclear neutrophils cells (PMN) percentage in the TW for the control group (CTL) and the pathological group (PAT).

Herpesvirus		EHVs		EHV-2		EHV-4	
	PMN (%)	+	-	+	-	+	-
CTL	0-20	18.92	81.08	10.81	89.19	2.86	97.14
PAT	0-20	37.74	62.26	30.19	69.81	3.77	96.23
PAT	21-40	40.91	59.09	25.00	75.00	4.55	95.45
PAT	41-60	45.45	54.55	40.91	59.09	0.00	100
PAT	61-80	40.54	59.46	35.14	64.86	8.11	91.89
PAT	81-100	47.57	52.43	34.95	65.05	9.71	90.29
Cochran –Armitage trend test / two-sided $P > Z$		0.009		0.0165		0.0542	

Results for EHV-1 and EHV-5 were not significant