

1 Cerebrospinal fluid parameters of horses with West Nile virus neuroinvasive disease

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20 Conflict of interest

21 The authors declare no conflict of interest.

## 22 **Abstract**

23 Objective: To compare biochemical and cytological findings of cerebrospinal fluid (CSF)  
24 samples in horses with acute neuroinvasive West Nile virus (WNV) infections with those of  
25 control healthy horses.

26 Design: Retrospective case-control study.

27 Samples: Fifteen CSF samples from horses with acute WNV neuroinvasive disease (WNVND)  
28 and twenty from healthy horses.

29 Procedures: WNVND was diagnosed based on acute neurologic symptoms and positive IgM  
30 ELISA results. CSF samples were collected either from the atlanto-occipital or the lumbosacral  
31 sites.

32 Results: CSF results of the WNV affected group did not follow normal distribution. Protein,  
33 creatine-kinase, aspartate-aminotransferase, lactate-dehydrogenase, alkaline-phosphatase,  
34 magnesium, glucose, and lactate concentrations showed abnormal levels in a number of WNV  
35 cases. None of the 6 horses with elevated glucose concentrations survived ( $\leq 0.36$ , modified  
36 Wald method). Opposite to previous equine studies we have found neutrophilic pleocytosis in  
37 54% of cases. Measured data also indicates that CSF neutrophilia is more likely to be found  
38 parallel with high protein content (Fisher exact test,  $p = 0.1026$ ).

39 Conclusions and clinical relevance: The CSF findings with WNVND are nonspecific and  
40 variable. Neutrophils likely play a role in the development of inflammatory response and brain  
41 damage. Increased enzyme activities and changes in the electrolyte concentrations reflect CNS  
42 cellular injury rather than blood-brain barrier leakage. Although elevated glucose levels reliably  
43 predicted outcome, these results might be the consequences of increased plasma levels and reflect

44 general stress rather than any CNS pathophysiology. Examination of CSF is most useful when the  
45 results are correlated with history, clinical findings and ancillary laboratory studies.

#### 46 **Abbreviations**

47 CSF cerebrospinal fluid

48 WNV West Nile virus

49 WNVND West Nile Virus Neuroinvasive Disease

50 CNS central nervous system

51 AST aspartate-aminotransferase

52 ALP alkaline-phosphatase

53 CK creatine-kinase

54 LDH lactate-dehydrogenase

55 RT-PCR reverse transcriptase polymerase chain reaction

56 CI confidence interval

#### 57 **Introduction**

58 West Nile virus (WNV) is a mosquito-borne zoonotic arbovirus belonging to the genus *Flavivirus*  
59 in the family *Flaviviridae*<sup>1</sup>, and transmitted in natural cycles between mosquitoes, (mainly the  
60 genus *Culex*), and wild birds<sup>2,3</sup>. Horses and human beings are incidental and dead-end hosts, but  
61 severe neurological disorders can develop in horses<sup>4,5</sup>. Phylogenetic studies have identified 2  
62 main lineages of WNV strains. The Hungarian equine WNV outbreak reported in 2008 was the  
63 first to be caused by a lineage 2 sub-Saharan strain in Europe. The pathogenicity of this lineage 2

64 strain resembled that of lineage 1 strains, and its sudden spread was unpredictable<sup>2,4</sup>. During the  
65 last decade, the epidemiology of WNV in human beings has changed in the southern regions of  
66 Europe, with high incidence of West Nile fever cases, but also of WNVND<sup>6</sup>.

67 Depending on the level of viremia WNV can cross the blood-brain barrier into the brain and  
68 cause meningo-encephalitis<sup>5</sup>. Due to its close contact with the extracellular fluid of the brain,  
69 analysis of cerebrospinal fluid (CSF) composition can reflect biological central nervous system  
70 (CNS) impairments enabling the diagnosis and understanding of various neurodegenerative CNS  
71 disorders<sup>7</sup>. To obtain accurate results when evaluating equine CSF samples, apart from the  
72 precise sampling technique, the application of the correct laboratory analytical methods is also  
73 important. However, due to the varied methods that different laboratories use today, it has  
74 become imperative for each laboratory to establish its own reference ranges based on the  
75 calibrated measurement techniques<sup>8</sup>. Until now, few studies investigated the association between  
76 the imbalance of CSF elements and the severity of WNV infection. The aim of the present study  
77 was to compare biochemical and cytological findings of CSF in horses with acute neuroinvasive  
78 WNV infections with those of control healthy horses.

## 79 **Materials and methods**

80 The data were obtained performing an observational retrospective case-control study between  
81 2008 and 2014. The study was permitted by the Animal Health and Welfare Directorate of  
82 National Food Chain Safety Office (22.1./1606/003/2009).

83 West Nile virus neuroinvasive cases were defined based on seasonality (August-November),  
84 acute neurologic clinical signs (less than 5 days), positive serum IgM ELISA test<sup>a</sup> and the  
85 absence of any WNV vaccination in their history. Only clinically healthy horses without any

86 neurologic signs and with hematological and biochemistry parameters within reference interval  
87 were included in the control group. Age, breed, gender characteristics and sampling sites are  
88 described in table 1.

89 All WNV cases were sampled within 36 h of clinical admission, sampling site was determined  
90 based on the clinical signs. Horses with characteristics of more pronounced brainstem and  
91 cerebral involvement were sampled by atlanto-occipital puncture during general anesthesia (1  
92 mg/kg [2.2 mg/lb] xylazine iv., 0.02 mg/kg [0.045 mg/lb] butorphanol iv. and 1 mg/kg [2.2 mg/lb]  
93 ketamine iv.) and horses with spinal cord involvement and 12 control horses were sampled in  
94 sedation (0.3-0.4 mg/kg xylazine [0.66-0.88 mg/lb] iv. and 0.02 mg/kg [0.045 mg/lb] butorphanol  
95 iv.) with local anesthesia (lidocaine) on the lumbosacral site as previously described<sup>9</sup>. In case of  
96 diffuse CNS involvement both locations were sampled under general anesthesia. Altogether  
97 fifteen samples were collected and 2 horses were sampled both by lumbosacral and atlanto-  
98 occipital punctures.

99 CSF was first analyzed macroscopically for color and turbidity in front of a white paper.  
100 Cytological analysis was performed within 6 h of sampling after cytocentrifugation and Wright-  
101 Giemsa staining. Protein content was measured with spectrophotometry<sup>b</sup> and other biochemical  
102 parameters like aspartate-aminotransferase (AST), alkaline-phosphatase (ALP), glucose, lactate,  
103 urea, creatine-kinase (CK), lactate-dehydrogenase (LDH), sodium, potassium, calcium, chloride,  
104 anorganic phosphate, and magnesium were determined spectrophotometrically using  
105 commercially available test kits on an a chemistry analyzer<sup>c</sup>.

106 In each case we attempted to characterize the virus from peripheral blood leukocytes or brain and  
107 spinal cord samples by virus isolation, nested reverse transcriptase polymerase chain reaction  
108 (RT-PCR), real-time RT-PCR, and sequencing techniques as described previously<sup>4</sup>.

109 When analyzing our results, first we evaluated normality of our data, whether parametric or non-  
110 parametric statistics should be used. We used Wald method and Fisher exact test to evaluate  
111 which measured variables are predictors of the outcome. Finally Fisher exact test was applied to  
112 establish relationships between the measured parameters<sup>d</sup>.

### 113 **Results**

114 All CSF sampling procedures were performed without any complications. Horses of the control  
115 group recovered quickly from the anesthesia without having any sequela. Three horses from the  
116 WNV group were euthanized right after the sampling procedure and three other were euthanized  
117 on human grounds within the next 5 days. Seven horses survived the neuroinvasive disease, five  
118 without any residual symptoms. In all 6 cases where it was identified with PCR or virus isolation,  
119 lineage 2 strain was responsible for the infection.

120 On macroscopical examination the CSF was transparent and non-turbid in all control animals and  
121 in 9 WNV cases and slightly hazy in 6 WNV cases. Cytological analysis revealed normal cell  
122 counts within reference intervals with exclusively small and large mononuclear cells on all  
123 control samples and on three WNV samples. There were 4/15 cases with mononuclear and 8/15  
124 cases with neutrophilic pleocytosis in the diseased group.

125 Most of the data obtained from the WNV neuroinvasive cases did not seem to follow a normal  
126 distribution, their mean, mode and median were not close to being equal, hence t-test based  
127 comparison of the means with the control group was not feasible. Instead we opted to establish  
128 reference ranges (95% prediction intervals) based on the control groups and count the number of  
129 cases for each variable that fall outside of this range. We have found that protein, CK, AST,

130 LDH, ALP, magnesium, glucose, and lactate concentrations showed abnormal levels in a number  
131 of cases. The following table (2. table) describes our results.

132 We also studied if any of the measured variables are a good predictor of the outcome  
133 (death/survival) of the disease. Most noteworthy was that none of the 6 horses with elevated  
134 glucose levels survived the disease (0/6,  $\leq 0.36$ , modified Wald method with 90% CI) and all of  
135 the 6 horses with normal glucose levels have survived (6/6,  $\geq 0.64$ , modified Wald method with  
136 90% CI). The dependence of the outcome on the glucose level was also verified with a Fisher  
137 exact test (two-tailed,  $p=0.0022$ ).

138 Measured data also indicated that neutrophilic pleocytosis in CSF was more likely in the cases  
139 with high total protein content (Fisher exact test, two tailed,  $p = 0.1026$ ).

140 In the two WNV cases where both samples were collected, results differed based on the location.  
141 In one case atlanto-occipital sample cytology was negative, while lymphocytic pleocytosis was  
142 found on lumbosacral puncture also showing higher protein, glucose and lactate levels. In the  
143 other case lymphocytic pleocytosis was found in the lumbosacral sample, and more neutrophils  
144 with higher protein content, CK, LDH, AST and lower urea were identified in the atlantooccipital  
145 one, while glucose levels were similar. None of these horses survived.

146

## 147 **Discussion**

148 Limitations of the study were the relative low sample number according to sampling sites and  
149 that blood biochemistry and hematology parameters were not measured and evaluated parallel.

150 The reference ranges set up by our control group were in concordance with previous  
151 findings<sup>10,11,12</sup>, except that lactate concentration being slightly and LDH value being moderately

152 higher in our reference group. This could be attributed to different methodology used by our  
153 laboratory.

154 According to previous studies in human beings, the CSF findings in patients with WNVND are  
155 nonspecific and include pleocytosis (neutrophil or lymphocyte predominance) with elevated  
156 protein and normal glucose levels<sup>13</sup>. Our findings are very similar except we have found high  
157 glucose levels in nonsurvival patients. On the other hand previous reports in horses most  
158 commonly described mononuclear pleocytosis with lymphocytic predominance<sup>14</sup>. Although  
159 WNV disease was caused by lineage 1 strains in those cases and lineage 2 strains were  
160 responsible in the present report, lineage differences are not likely to be the reasons for these  
161 discrepancies. Other studies on human CSF also resulted variable data, where patients did not  
162 present with the typical lymphocytic pleocytosis often quoted when discussing a viral  
163 meningitis/encephalitis; rather most presented with a cerebrospinal fluid neutrophilia<sup>13,15,16</sup>.  
164 Previous results in mice suggest that neutrophils are the predominant immune cells that are  
165 initially and rapidly recruited to sites of infection with WNV<sup>5</sup>. According to a study in human  
166 patients<sup>15</sup> mean total leukocyte counts and mean neutrophil fractions were greater in individuals  
167 sampled within the first 3 days of symptoms than in those sampled beyond day 3. Sampling time  
168 might also be responsible for the different findings. Most of our horses were sampled relative  
169 early (all horses within 5 days and 8 within 3 days of onset of clinical signs) in the disease  
170 process. In another study it was found that older WNV patients were more likely to have  
171 neutrophils in their CSF<sup>17</sup>. The average age of our patients with high neutrophil numbers was 8  
172 years (4-13 years) and with mononuclear pleocytosis it was 6.6 years (4-9 years). Furthermore,  
173 neutrophil-related proteins were found at higher levels in CSF of WNVND patients, underlining  
174 the likely key role played by neutrophils in the development of the inflammatory response and



175 brain damage<sup>6</sup>. We have also found that CSF neutrophilia is more likely to be found parallel with  
176 high protein content. Albumin was lower in most cases and total protein was increased suggesting  
177 the presence of increased inflammatory proteins like globulins in the CSF of diseased animals.  
178 Opposite to some previous data, we have detected normal or high CSF glucose concentration  
179 similarly to a study of seasonal human epidemic West Nile Virus meningo-encephalitis<sup>15</sup>.  
180 Cerebrospinal glucose concentrations might reflect changes of blood glucose which could be  
181 increased because of critical illness causing a stress response<sup>11,18</sup>. Human and animal studies  
182 suggest that this is not benign, and that stress-induced hyperglycemia is associated with a high  
183 risk of mortality<sup>19,20</sup>. Increased lactate levels were found in most of the cases as well increased  
184 LDH levels in half of the samples in the WNV affected group. L-lactate is formed during normal  
185 anaerobic glycolysis by interconversion from pyruvate via the actions of LDH<sup>21</sup>. Lactate  
186 concentrations in the CSF largely represent its production by the brain but it is also increased in  
187 case of low glucose concentrations to meet the energy requirements by the anaerobic pathway<sup>21</sup>.  
188 Hypoglycemia was not present in our cases. Increases in CSF lactate concentration reportedly  
189 occur with bacterial infections but not with nonseptic meningitis<sup>9</sup>. On the other hand CSF lactate  
190 increases also occur with any condition that results in reduced brain oxygenation and/or increased  
191 intracranial pressure. CNS tissue hypoxia could be the result of inflammatory processes  
192 secondary to the WNV infection.

193 As most enzymes are relatively large molecules, there is very little diffusion across the intact and  
194 normal blood-CSF barrier and increased activities of the enzymes in the CSF are assumed arise  
195 from cells within the CNS<sup>22</sup>. Potential sources of the increased enzyme activity in the WNV  
196 group horses are the release of these enzymes from the inflammatory cells or directly from the  
197 damaged nerve cells and myelin. Recently, it has been shown that WNV induced the expression

198 of interleukin-1 $\beta$ , -6, -8, and tumor necrosis factor- $\alpha$ , where neurons were one of the potential  
199 sources of pro-inflammatory cytokines, and these pro-inflammatory mediators were one of the  
200 main factors driving WNV-induced neurotoxicity, cell death and CNS tissue damage<sup>5</sup>. Based on  
201 previous histologic findings of WNV encephalitis including perivascular inflammation,  
202 microgliosis, variable degree of necrosis, and loss of neurons it is less likely that the source of  
203 increased enzymes is secondary to a damaged blood-brain barrier or blood-CSF barrier and  
204 increased leakage from the plasma.

205 The most reliable increase was demonstrated by the alkaline phosphatase enzyme. The CSF of  
206 patients without neurological disorders contains little or no activity as it was also shown in our  
207 study based on our control group<sup>23</sup>. According to a previous human study the CSF alkaline  
208 phosphatase activities of patients with meningitis and other neurological disorders varied directly  
209 with the number of polymorphonuclear leukocytes present and with the protein concentration<sup>23</sup>.  
210 We could not demonstrate a clear relationship between the number of neutrophils, the level of  
211 protein and the alkaline phosphatase activity. The reason for this might have been the low sample  
212 number.

213 There is surprisingly little information about urea levels in the normal CSF, although an increase  
214 would have significance in differentiating uremic encephalopathy. Since urea is readily  
215 diffusible, therefore urea levels should be parallel that found in the serum<sup>21</sup>. Decreased urea  
216 levels in some of our patients' sample could be secondary to reduced hepatic synthesis of urea  
217 from ammonia in case of severe systemic disease. None of our patients had increased urea  
218 concentration in their CSF.

219 Electrolyte composition of the CSF has been only sparsely reported, but in general the CSF  
220 sodium, chloride are similar or slightly higher, potassium concentrations are similar or slightly

221 lower and magnesium concentrations are slightly higher than those in the serum<sup>10,12</sup>. Our  
222 reference ranges based on the control group were concordant with these results previously  
223 published. In some of the WNV affected horses low sodium and increased magnesium  
224 concentration could be detected. These electrolyte abnormalities can originate from the cellular  
225 damage in the CNS, where intracellular solutes may leak out of the cell because of an increased  
226 membrane permeability and may lead to redistribution of sodium and an increased magnesium.  
227 Based on studies in humans, phosphate is found in normal CSF at levels of 50-60% of expected  
228 serum concentration and it has also previously been observed that CSF anorganic phosphate  
229 concentrations increase in direct proportion to total CSF protein levels<sup>21</sup>. Although we have  
230 measured increased anorganic phosphate concentration in three cases, similar relationship could  
231 not be demonstrated.

232 When we collected both lumbosacral and atlanto-occipital samples from the same patient, we got  
233 different results. This can be attributed to the different locations of the more severe CNS damage  
234 causing more significant alterations in the sample collected from the closer site. Although there is  
235 a synchronous appearance of WNV at many sites in the brain and spinal cord and pathological  
236 alterations can be detected in many parts of the CNS, but the severity of these damages can differ  
237 which is also reflected in the detectable clinical signs and disease progression. On the other hand  
238 values of certain parameters differ depending on sampling sites even in the healthy horses  
239 because of different blood-CSF permeability and flow rates between the atlanto-occipital and the  
240 lumbar regions<sup>8</sup>.

241 CSF analysis is a general index of neurological health and often provides evidence of the  
242 presence of a disease. Similar to a complete blood count, CSF analysis has reasonable sensitivity  
243 but low specificity. The CSF findings with WNVND are nonspecific and variable and possibly

244 depend on the age of the patient, the sampling time, the site of sampling in relation to the location  
245 of the most severe lesions and also on previous treatments. Neutrophils likely play a role in the  
246 development of inflammatory response and brain damage but further examinations would be  
247 required to fully elucidate their role in the pathogenesis of WNVND. Increased enzyme activities  
248 and changes in the electrolyte concentrations reflect CNS cellular injury rather than blood-brain  
249 barrier damage. Higher sample number would be required to demonstrate relationships between  
250 inflammation, CNS damage and changes of the CSF parameters. Although elevated glucose  
251 levels reliably predicted the outcome, these results might be secondary to increased plasma levels  
252 and reflect general stress to serious illness rather than any CNS pathophysiology. Based on all  
253 these findings, examination of CSF is most useful when the results are correlated with history,  
254 clinical findings and ancillary laboratory studies.

255

## 256 **Footnotes**

257 <sup>a</sup> IDEXX IgM WNV Ab Test, Hoofddorp, The Netherlands

258 <sup>b</sup> Olympus AU400, Beckman Coulter, Hamburg, Germany

259 <sup>c</sup> Olympus AU640, Beckman Coulter, Hamburg, Germany

260 <sup>d</sup> IBM SPSS Statistics 20 Documentation, United States

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320 Table 1: Age, breed, gender characteristics and sampling sites

Group	Age	Breed	Gender	Sampling site
WNV affected	M: 7.53 years, SD: 2.84	12 warmbloods 1 pony	9 mares 4 geldings	8 lumbosacral 7 atlanto-occipital
Control	M: 8.94 years, SD: 3.57	16 warmbloods 3 draught horses 1 thoroughbred	10 mares 10 geldings	12 lumbosacral 8 atlanto-occipital

321 M: mean, SD: standard deviation

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Table 2: Results of cerebrospinal fluid analysis compared to previously published data<sup>10,11,12</sup>.

	inflammatory proteins (total-albumin)	albumin (mg/l)	total protein (mg/l)	AST IU/l	ALP IU/l	GGT IU/l	glucose mmol/l	lactate mmol/l	CK IU/l	LDH IU/l	urea mmol/l	Na mmol/l	K mmol/l	Ca mmol/l	Cl mmol/l	P mmol/l	Mg mmol/l
control reference	0-56	10-50	32,16-75,55	6,0-14,0	0,1-3,5	0-4,0	2,54-3,81	1,89-3,07	0-4,6	14,7-44,1	4,6-8,8	140-151,8	2,8-3,1	1,13-1,41	113-128,2	0,02-0,38	0,53-1,34
horses with norm values	4	3	6	6	1	12	6	3	5	6	9	8	11	6	7	9	7
horses with abnorm values	8	9	7	6	11	0	6	7	7	6	3	4	1	0	5	3	5
mean WNV group	59	11	98,36	12,85	8,16	1,10	3,89	3,81	20,07	46,57	4,80	136,96	2,92	1,24	120,53	0,43	1,15
sd	0,32	0,10	54,76	5,45	4,80	0,93	1,54	1,88	24,32	31,20	1,61	40,25	0,85	0,51	36,22	0,24	0,55
mean control group	27	26	50,32	9,9235	1,9	1,62	2,98	2,45	2,17	29,49	6,73	145,08	2,95	1,26	121,575	0,3095	0,89
sd	15	12	11,85	2,22	1,01	1,65	0,32	0,30	1,46	13,02	1,29	3,10	0,08	0,07	3,49	0,24	0,19
other references			5-100 <sup>10</sup> 20-124 <sup>11</sup> 20-80 <sup>12</sup>	15-50 <sup>10</sup> 0-16 <sup>11</sup> 7-24 <sup>12</sup>			1,67-3,89 <sup>10,12</sup> 30-70% of blood glucose <sup>11</sup>	1,92-2,3 <sup>11</sup>	0-8 <sup>10,11,12</sup>	0-8 <sup>10,11</sup>		140-150 <sup>10,12</sup>	2,5-3,5 <sup>10,12</sup>		95-123 <sup>10,12</sup>		

Note that inflammatory protein is a calculated value based on the total protein and albumin levels.

Na: sodium, K: potassium, Ca: calcium, Cl: chloride, P: phosphate, Mg: magnesium, sd: standard deviation