Cerebrospinal fluid parameters of horses with West Nile virus neuroinvasive disease

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Conflict of interest

The authors declare no conflict of interest.

Abstract

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

Design: Retrospective case-control study.

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

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

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

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

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

Abbreviations

CSF cerebrospinal fluid

WNV West Nile virus

WNVND West Nile Virus Neuroinvasive Disease

CNS central nervous system

AST aspartate-aminotransferase

ALP alkaline-phosphatase

CK creatine-kinase

LDH lactate-dehydrogenase

RT-PCR reverse transcriptase polymerase chain reaction

CI confidence interval

Introduction

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

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

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

Materials and methods

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

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

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

All WNV cases were sampled within 36 h of clinical admission, sampling site was determined based on the clinical signs. Horses with characteristics of more pronounced brainstem and cerebral involvement were sampled by atlanto-occipital puncture during general anesthesia (1 mg/kg [2.2 mg/lb] xylazine iv., 0.02 mg/kg [0.045 mg/lb] butophanol iv. and 1 mg/kg [2.2 mg/lb] ketamine iv.) and horses with spinal cord involvement and 12 control horses were sampled in 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 iv.) with local anesthesia (lidocaine) on the lumbosacral site as previously described[9]. In case of diffuse CNS involvement both locations were sampled under general anesthesia. Altogether fifteen samples were collected and 2 horses were sampled both by lumbosacral and atlanto-occipital punctures.

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

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

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

Results

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

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

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

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

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

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

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

Discussion

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

The reference ranges set up by our control group were in concordance with previous findings[10,11,12], except that lactate concentration being slightly and LDH value being moderately

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

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

brain damage[6]. We have also found that CSF neutrophilia is more likely to be found parallel with high protein content. Albumin was lower in most cases and total protein was increased suggesting the presence of increased inflammatory proteins like globulins in the CSF of diseased animals.

Opposite to some previous data, we have detected normal or high CSF glucose concentration similarly to a study of seasonal human epidemic West Nile Virus meningo-encephalitis[15]. Cerebrospinal glucose concentrations might reflect changes of blood glucose which could be increased because of critical illness causing a stress response[11,18]. Human and animal studies suggest that this is not benign, and that stress-induced hyperglycemia is associated with a high risk of mortality[19,20]. Increased lactate levels were found in most of the cases as well increased LDH levels in half of the samples in the WNV affected group. L-lactate is formed during normal anaerobic glycolysis by interconversion from pyruvate via the actions of LDH[21]. Lactate concentrations in the CSF largely represent its production by the brain but it is also increased in case of low glucose concentrations to meet the energy requirements by the anaerobic pathway[21]. Hypoglycemia was not present in our cases. Increases in CSF lactate concentration reportedly occur with bacterial infections but not with nonseptic meningitis[9]. On the other hand CSF lactate increases also occur with any condition that results in reduced brain oxygenation and/or increased intracranial pressure. CNS tissue hypoxia could be the result of inflammatory processes secondary to the WNV infection.

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

of interleukin-1β, -6, -8, and tumor necrosis factor-α, where neurons were one of the potential sources of pro-inflammatory cytokines, and these pro-inflammatory mediators were one of the main factors driving WNV-induced neurotoxicity, cell death and CNS tissue damage[5]. Based on previous histologic findings of WNV encephalitis including perivascular inflammation, microgliosis, variable degree of necrosis, and loss of neurons it is less likely that the source of increased enzymes is secondary to a damaged blood-brain barrier or blood-CSF barrier and increased leakage from the plasma.

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

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

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

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

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

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

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

Footnotes

[a] IDEXX IgM WNV Ab Test, Hoofddorp, The Netherlands

[b] Olympus AU400, Beckman Coulter, Hamburg, Germany

[c] Olympus AU640, Beckman Coulter, Hamburg, Germany

[d] IBM SPSS Statistics 20 Documentation, United States

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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

M: mean, SD: standard deviation

Table 2: Results of cerebrospinal fluid analysis compared to previously published data[10,11,12].

	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[10] 20-124[11] 20-80[12]	15-50[10] 0-16[11] 7-24[12]			1,67-3,89[10,12] 30-70% of blood glucose[11]	1,92-2,3[11]	0-8[10,11,12]	0-8[10,11]		140-150[10,12]	2,5-3,5[10,12]		95-123[10,12]		

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