Serum trace element levels in Equine Herpesvirus 1 infected horses

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SUMMARY

Equine herpesvirus 1 (EHV-1) is responsible for several syndromes, such as respiratory disease, abortion, fatal viral pneumonia in neonatal foals, and a neurological syndrome, currently referred to as EHV-1 myeloencephalopathy. The analysis of trace element levels in the sera of EHV-1 infected horses could be useful in clarifying the pathogenesis or pathophysiology of these EHV-induced clinical syndromes. Previous studies have shown significant alterations for some serum trace elements (zinc, iron, copper) in EHV-1 infected horses, and this could be justified by the putative role of these elements in many immunological pathways or by their antiviral activity. The aim of the present study was to perform a comparison by retrospective serological study of 52 EHV-1 infected and non-infected horses, both healthy and ill, to establish whether there were possible alterations in serum levels of arsenic, copper, boron, zinc, iron, chromium, magnesium, manganese, selenium, and silicium. Horses were categorized based on the type of syndrome (respiratory disease, abortion, or neurological disease) and the presence of seroconversion (by virus neutralization) and the result of polymerase chain reaction (PCR) for EHV-1. Levels of serum chromium, copper, selenium, boron, and silicium were significantly different among different groups of EHV-1 infected or non-infected horses. Serum chromium levels were higher in infected horses compared to non-infected individuals (p=0.0001). Levels of serum copper (p=0.001), magnesium (p=0.05), selenium (p=0.004), and silicium (p=0.004) were significantly lower in the horses with neurological disease. While levels of serum chromium (p=0.005) were higher, those of boron (p=0.002) were significantly lower in cases of EHV-1 abortion. Overall, the present study revealed alterations in the serum levels of some trace elements between EHV-1 non-infected and infected horses, such as those that aborted or developed neurological signs. However, the relationship between the trace elements and the outcomes of the infection could not be established. Further research is needed to enlighten the effects of trace element alterations on the equine herpesvirus-1 infection pathogenesis in horses.

KEY WORDS

Serum trace element levels; iron; zinc; selenium; Equine Herpesvirus-1; horse.

INTRODUCTION

Equine herpesvirus 1 (EHV-1) is a virus of considerable importance in equine medicine from an epidemiological, clinical, and economic viewpoint[1]. It is primarily responsible for causing respiratory disease, abortion, fatal viral pneumonitis in neonatal foals, and a neurological syndrome, currently referred to as EHV-1 myeloencephalopathy (EHM). This last syndrome has been associated with EHV-1 strains of different genotypes. Earlier studies had shown that outbreaks of this clinical disease were commonly associated with strains of the virus termed as mutant strains, because they had substitution of nucleotide A with G at position 2254. This resulted in an amino acid change of N for D at position 752 within the DNA Polgene[2]. These strains were also associated with cases of abortion[3,4,5]. Although numerous studies have been published to identify specific strains with neuropathogenic potential this issue have not been elucidated yet[3,6,7,8,9]. A recent research employed a case-control design to investigate possible risk factors associated with an extensive series of related outbreaks of EHM and dietary zinc supplementation was found to decrease the risk of this syndrome[10]. Moreover, the results of an earlier study, that investigated levels of serum copper, zinc, iron, and cobalt in EHV-1 infected horses, found significant alterations in iron,

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copper and zinc levels when compared with those of a non-infected control group[11]. Previous studies have shown that trace element levels possessed a dynamic mechanism in the course of infectious disease occurrences[12,13,14]; this may possibly be linked to their putative role in different immunological pathways[15,16,17,18,19,20,21]. Furthermore, antiviral activity has also been associated with certain trace elements; this could also have potential therapeutic value[15,17].

The aim of the present study was to perform a retrospective serological study to compare EHV-1 infected and non-infected horses, either clinically ill or healthy, to determine whether there were possible alterations in serum levels of specific trace elements associated with this virus infection.

MATERIALS AND METHODS

Study design

A retrospective serological study was carried out using sera collected from 52 horses, to compare serum levels of arsenic (As), copper (Cu), boron (B), zinc (Zn), iron (Fe), chromium (Cr), magnesium (Mg), manganese (Mn), selenium (Se), and silicium (Si). The horses comprised those uninfected with EHV-1 and those infected with the virus. Each horse in the infected group was categorized based on whether they represented a case of respiratory disease, abortion, or neurological disease. The horses were all adult and infected horses were from 5 different outbreaks that occurred from 2004-2013, involving especially Thoroughbreds, Standardbreds, Warmbloods as previously described[5]. The horses were considered EHV-1 infected based on the presence of seroconversion (development of antibodies) or a 4-fold or greater increase in antibody levels based on testing paired sera. Blood samples were collected in the acute phase and after 21 days and tested by virus neutralization (VN). Infection was also based on a positive virus result using the polymerase chain reaction (PCR) assay.

The groups used for statistical analysis included the following: controls (horses without clinical signs and negative on both VN and PCR tests for evidence of EHV-1 infection); cases of symptomatic or subclinical EHV-1 infection as confirmed on VN or PCR testing (horses with respiratory signs, mares that aborted, neurological cases); EHV-1 negative cases (clinical cases or healthy horses with an EHV-1 negative result); neurological cases (all the cases with neurological signs); non-neurological cases (all clinical cases, both healthy horses and those exhibiting signs other than those of a neurological nature); EHM cases (confirmed neurological cases positive for EHV-1); cases of EHV-1 positive abortion; cases of EHV-1 negative abortion. The same animal could be classified in different categories based on the specific statistical analysis.

Clinical presentation

Abortion was defined as fetal loss before 300 days of gestation, diagnosis of pregnancy was determined by ultrasound examination.

The presence and nature of acute neurological signs were determined on neurological examination. Cases with acute neurological signs suggestive of EHM and associated with a positive test result for EHV-1 (VN or EHV-1 nucleic acid detection by PCR on blood or nasal swab or cerebrospinal fluid or tissues) were considered EHM cases. EHV-1 negative neurological cases were classified based on a negative result for EHV-1 (either absence of VN antibodies or negative PCR result for virus on blood or nasal swab or cerebrospinal fluid or tissues). In the absence of a diagnosis of EHM, most of these cases were confirmed to have other diseases characterized by neurological signs.

Respiratory signs included nasal discharge, cough, with or without fever.

Sample collection

Blood samples from clinically affected and healthy horses, were collected into sterile, non-additive Vacutainer tubes (Becton Dickinson, Milan, Italy), and allowed to clot. Serum was drawn off after centrifugation and stored in sterile 2 ml tubes at -20 °C until analysis.

Serum samples used to perform measurement of trace elements were collected at time of clinical illness for diagnostic testing or during routine sanitary monitoring of healthy horses.

Testing for EHV-1 infection

The PCR and VN test were carried out in accordance with the respective test procedures described in the OIE Terrestrial Manual[5,22]. Serum samples were also tested for VN antibodies to EHV-4 to confirm that seroreactivity to any positive sample was due to EHV-1 and not EHV-4, taking into consideration the cross-reactivity between the two viruses.

Cases of EHV-1 abortion were generally diagnosed by PCR examination of the tissues of the fetus. Respiratory, neurological, and subclinical cases of EHV-1 infection were diagnosed by PCR and/or VN testing within outbreaks of EHV-1 infection[5].

Where possible, strains of EHV-1 were typed as neuropathogenic or non-neuropathogenic according to a method previously described[23].

Measurement of trace elements

Levels of As, Cu, B, Zn, Fe, Cr, Mg, Mn, Se and Si in serum were analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Thermo iCAP 6000 series at the Trace Element Analysis Laboratory, Biophysics Department, Istanbul University-Cerrahpasa Medical Faculty, Istanbul, Turkey. The wavelengths used for the detection of each element are shown in Table 1. The plasma operating conditions for the ICP-OES system are provided in Table 2. The peristaltic pump speed was

Table 1 - Wavelengths used for analyzed trace elements with ICP-OES. These lines were selected based on their sensitivity and freedom from spectral interferences.

Elements	Wavelengths (nm)
Arsenic	189.04
Copper	324.75
Boron	249.77
Zinc	206.20
Iron	259.94
Chromium	267.72
Magnesium	285.21
Manganese	257.61
Selenium	196.09
Silicium	251.61

Table 2 - Instrumental conditions of ICP-OES.

Parameter	Value
RF incident power	1.3 kW
Plasma argon flow rate	15 L / min
Auxiliary argon flow rate	0.5 L / min
Nebulizer argon flow rate	0.7 L / min
Mist chamber	Stumar-master
Nebulizer	V-groove

100 rpm, and 1.25-mm-i.d. polytetrafluoroethylene tubing was used as transport lines. The standard concentrations used in development of calibration curves were prepared from standard stock solutions of 1000 µg/ml for each analyzed trace element[24,25,26]. Internal quality controls (inorganic solutions traceable to NIST certified reference material) for each analyte, were obtained from Chem-Lab NV (Belgium). The serum samples from all the groups were analyzed and compared. Each analysis was repeated three times and element levels averaged. Trace element levels were expressed as micrograms per milliliter of serum (µg/mL).

Statistical analysis

Considering the limited numbers of animals comprising each of the groups, non-parametric tests were used to investigate possible differences among the groups. The serum levels of each trace element were compared among the different categories of horses, whether based on the presence or absence of clinical signs of EHV-1 infection, and the results of tests used to establish evidence of infection.

Statistical association between serum levels of the selected trace elements and whether neuropathogenic or non-neuropathogenic strains of EHV-1 were detected, was also attempted. The Mann-Whitney or Kruskal Wallis test was used for comparison. A p-value ≤ 0.05 was considered significant in conducting the analysis. Data were analyzed by commercial software R, version 2.8.1 (R, Development Core Team 2007).

RESULTS

The results with respect to the different categories of horses are summarized in Table 3. Only two cases of neurologic disease met the criteria for EHM. The other neurological cases included horses with ataxia (n= 7), paresis of the hind limbs (n=5), suspicion of encephalitis (n=3), epilepsy (n=1). Because of the limited number of cases, no further analyses were performed on the EHM group. Respiratory signs were observed in 4 cases and so they were limited to a slight nasal discharge lasting a day or so, the cases were considered subclinical.

Levels of serum chromium, copper, selenium, boron, and silicon were found to be significantly different among the five different groups. Trace element levels in the serum of the EHV-1 uninfected healthy horses (controls) are provided in Table 4. Medians and minimum-maximum serum trace element levels in EHV-1 infected and non-infected horses are presented in Table 5. On statistical analysis, serum chromium levels were higher in infected horses compared to non-infected animals (p=0.0001).

Medians and minimum-maximum of serum trace element levels in horses with or without neurological signs are presented in Table 6. Serum copper (p=0.001), magnesium (p=0.05), selenium (p=0.004) and silicium levels (p=0.004) were found to be significantly lower in the horses displaying neurological signs.

Serum trace element levels in EHV-1 abortion positive and negative mares are presented in Table 7. Whereas the levels of serum chromium (p=0.005) were significantly higher, those of boron (p=0.002) were significantly lower in mares positive for EHV-1 abortion.

Three cases of neuropathogenic strain infection, one case of EHM, two cases of abortion and five cases of infection with non-neuropathogenic strains of EHV-1, all had similar levels of the trace elements under investigation.

DISCUSSION

Based on the outcomes of the present study, significant alterations of serum trace element levels were observed in EHV-1 infection. However, there is a need to define whether these alterations were related to EHV-1 infection or to the particular clinical outcome (respiratory, neurological or abortion) recorded in the mixed population of horses used in this study. There may be some relationship between serum trace element levels and the pathophysiology of the mentioned clinical syndromes.

In a previous study[11], serum copper and zinc levels were lower in cases of EHV-1 infection. By comparison, the findings of the current study indicated that serum copper levels were lower (p=0.001), and serum zinc levels had no significancy among the various groups. Zinc appeared to have a relevant role in EHV-1 and certain other herpesvirus infections such as bovine herpesvirus type 1[10,11,12]. Traub-Dargatz et al. in 2013[10] found fewer cases of EHM in horses receiving a zinc dietary supplementation. In another study, levels of serum zinc were decreased in EHV-1 infected horses[11].

However, this finding should be interpreted with caution since the number of cases and controls (9 infected horses and 9 con-

Table 3 - Grouping of horses based on clinical outcome and EHV-1 test results.

Clinical category	EHV-1 test results		
	Positive	Negative	Total
Abortion	6	7	13*
Neurological disease	2	13	15
Healthy	8 (subclinical cases)	16 (controls)	24*
Total	16	36	52

*37 cases were non neurological cases.

Table 4 - Parameters of the serum trace element levels, expressed in µg/mL, in the control horses.

Trace elements (µg/mL)	Controls (n= 16)
Arsenic	0.12 (0.80-0.18)
Copper	1.29 (0.99-2.23
Boron	0.08 (0.03-0.19)
Zinc	0.92 (0.51-1.44)
Iron	2.63 (0.78-4.8)
Chromium	0.01 (0-0.032)
Magnesium	18.3 (10.65-28.65)
Manganese	0.004 (0-0.27)
Selenium	1.005 (0.71-1.29)
Silicium	3.17 (0.15-7.96)

trols) was limited. Moreover, the study classified EHV-1 infected and control horses based on the outcome of VN testing and clinical examination, without providing a description of the observed clinical signs, nor information about the origin of the animals, their possible genetic relationship, feed, environment, or other factors[11].

Serum chromium levels were found to be significantly higher in horses infected with EHV-1 (p=0.0001) and mares that aborted due to EHV-1 (p=0.005). There is a lack of information in the literature about chromium levels in cases of infectious diseases in horses. Based on the findings of the current study, chromium would be worth investigating further to determine if the element might be associated with abortion in mares caused by EHV-1 or whether it was related to the immune response to infection. Chromium has been reported as beneficial for reducing exercise stress[27]. If preliminary findings are confirmed, chromium supplements could be easily added to the daily ration of horses. However, additional research on this trace element is needed in horses, taking into consideration parameters such as breed, age, nutritional diet, season of the animals under study.

Serum magnesium (p=0.05), selenium (p=0.004), and silicium (p=0.004) were found to be significantly lower in neurological cases. It is possible that these elements could be involved in the pathophysiology of neurological disease[28].

No statistical differences were found in serum iron levels in the present study. This contrasts with the finding of a previous study[11], in which an increase in serum iron was reported in EHV-1 infected compared to non-infected horses. However, yet a further study[20] found transitory differences in serum iron levels only during the acute phase of experimental EHV-1 infection,

Table 5 - Comparison among medians, expressed in µg/mL (minimum-maximum values in brackets) of analyzed serum trace elements in EHV-1 infected and non-infected horses and their corresponding p-values.

Trace elements (µg/mL)	EHV-1 infected cases group (n= 16)	EHV-1 non-infected cases group (n=36)	p-value
Arsenic	0.14 (0.03-0.19)	0.13 (0.05-0.22)	0.76
Copper	1.27 (0.50-1.62)	1.20 (0.81-2.23)	0.98
Boron	0.07 (0.02-0.12)	0.97 (0.03-0.47)	0.07
Zinc	0.90 (0.03-1.80)	0.86 (0.51-1.98)	0.82
Iron	2.36 (1.55-8.90)	2.61 (0.78-4.8)	0.76
Chromium	0.02 (0.004-0.04)	0.01 (0.0-0.03)	0.0001
Manganese	0.003 (0.001-0.03)	0.004 (0-0.03)	0.76
Magnesium	18.34 (7.17-24)	17.78 (5.55-28.65)	0.57
Selenium	0.83 (0.3-1.29)	0.97 (0.65-1.29)	0.37
Silicium	1.25 (0.28-3.61)	1.78 (0.09-7.96)	0.53

Table 6 - Comparison among medians, expressed in µg/mL (minimum-maximum values in brackets) of analyzed serum trace elements in horses with and without neurological signs and their corresponding p-values.

Trace elements (µg/mL)	Neurological cases group (n=15)	Non-neurological cases group (n=37)	p-value
Arsenic	0.13 (0.05-0.2)	0.14 (0.03-0.22)	0.16
Copper	1.13 (0.5-1.54)	1.29 (0.87-2.23)	0.001
Boron	0.69 (0.03-0.47)	0.81 (0.02-0.32)	0.12
Zinc	0.86 (0.51-1.8)	0.83 (0.03-1.98)	0.56
Iron	2.67 (1.13-8.90)	2.41 (0.78-5.66)	0.99
Chromium	0.007 (0-0.21)	0.01 (0-0.04)	0.43
Manganese	0.003 (0.001-0.02)	0.004 (0-0.03)	0.26
Magnesium	16.66 (5.55-22.95)	18.3 (7.17-28.65)	0.05
Selenium	0.81 (0.3-1.16)	0.98 (0.39-1.29)	0.004
Silicium	0.59 (0.09-2.70)	2.06 (0.15-7.96)	0.004

Table 7 - Comparison among medians, expressed in µg/mL (minimum-maximum values in brackets) of analyzed serum trace elements in EHV-1 abortion positive and negative mares and their corresponding *p*-values.

Trace elements (µg/mL)	EHV-1 positive aborted mares group (n=6)	EHV-1 negative aborted mares group (n=7)	*p*-value
Arsenic	0.14 (0.03-0.17)	0.15 (0.12-0.22)	0.63
Copper	1.45 (0.87-1.62)	1.40 (1.08-1.77)	0.84
Boron	0.07 (0.02-0.12)	0.17 (0.09-0.32)	0.002
Zinc	0.89 (0.62-1.8)	0.73 (0.52-1.50)	0.30
Iron	2.17 (1.55-4.05)	1.77 (1.43-3.75)	0.63
Chromium	0.02 (0.06-0.04)	0.001 (0-0.01)	0.005
Manganese	0.002 (0.001-0.03)	0.004 (0.003-0.01)	0.30
Magnesium	17.39 (7.17-18.53)	19.95 (15.15-25.9)	0.53
Selenium	0.79 (0.39-1.2)	1.06 (0.72-1.22)	0.18
Silicium	0.64 (0.28-3.61)	3.88 (0.18-5.77)	0.53

but not in the recovery phase. Moreover, both studies were carried out on a limited number of cases. Further studies are needed to understand the dynamics of iron in EHV-1 infection. Iron is well recognized as a relevant element in various infections[11,20,21]. In some of them, iron levels remain low beyond the acute phase of the infection, for instance in the case of *Streptococcus zooepidemicus* infection[21]. Serum iron levels were previously investigated also in mares that aborted. Iron storage has been reported during pregnancy of horses[29]. However, no significancy in serum iron levels was observed between the different groups in the present study.

Because the number of cases analyzed in particular categories of this study (EHM cases or cases infected with neuropathogenic or non-neuropathogenic strains of EHV-1) was very limited, possible alterations in serum trace element levels may not have been detected. It should be noted that all the studies carried out on trace element levels in EHV-1 infected or non-infected horses[11,20,30] involved relatively few animals. However, based on a limited contribution, it could be worthwhile to increase the number of analyses undertaken from the perspective of cumulative analysis (meta-analysis).

CONCLUSIONS

The variations of priorly published studies related with trace element levels should be taken into consideration. More studies of specific systemic diseases might improve our knowledge of the pathogenesis of infectious diseases. Further studies are needed to better understand trace element involvement in EHV-1 infections and whether the role(s) of these elements might have potential for prophylactic or therapeutic interventions in this infection.

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