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Original Article

Oxidative stress and DNA damage in horses naturally infected with *Theileria equi*

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Highlights

- Horses infected with *T. equi* showed clear changes in blood markers of oxidative stress.
- DNA damage was significantly increased in the presence of *T. equi*.
- *T. equi* infection was associated with oxidative stress.

Abstract

The aim of this study was to determine the concentrations of oxidative stress parameters and DNA damage in horses infected by *Theileria equi*. Initial screening of 110 horses with duplex PCR enabled the selection of 30 infected horses with *T. equi* and 30 free of infection (control). Specimens from the 60 horses were further analysed by determining the following oxidative stress parameters: extent of haemolysis (EH), plasma free haemoglobin (PHb), catalase (CAT), Cu,Zn superoxide dismutase (SOD1), paraoxonase (PON1), nitrite (NO_2^-), total nitrate and nitrite (NOx), malondialdehyde (MDA) and free thiol groups (-SH). In addition, relative distribution of lactate dehydrogenase (LDH_1–LDH_5) activity and the DNA-damaging effects of *T. equi* infection were evaluated.

Compared to control horses, horses infected with *T. equi* had significantly higher SOD1 activities ($P < 0.05$) and PHb ($P < 0.01$), NO_2^- ($P < 0.001$), NOx ($P < 0.05$) and MDA concentrations ($P < 0.001$), and significantly lower EH ($P < 0.001$), CAT ($P < 0.01$) and PON1 ($P < 0.001$) activities, and thiol group concentrations ($P < 0.05$). The comet assay demonstrated significantly increased DNA damage in *T. equi* infected cells compared to non-infected cells ($P < 0.001$). Infected horses had significantly increased LDH_5 isoenzyme activities ($P < 0.05$). There was higher production of ROS/RNS in *T. equi*-infected horses, which resulted in changes in osmotic fragility, damage to lipids, proteins and DNA, haemolysis and hepatocellular damage. Oxidative stress in horses naturally infected with *T. equi* could contribute to the pathogenesis of the infection.

Keywords: DNA damage; Horses; Duplex PCR; Oxidative stress; *Theileria equi*

Introduction

Equine piroplasmosis is an important tick-borne disease caused by the intraerythrocytic haemoprotozoan parasites *Theileria equi* and *Babesia caballi* (Wise et al., 2013) in horses, donkeys, mules and zebras. It has a worldwide distribution and is of great concern to the global horse industry (De Waal, 1992). Seroprevalences vary widely in Europe; there have been published reports of 8-35% for *B. caballi* and 20-40% for *T. equi* (Camacho et al., 2005; Hornok et al., 2007; Sevinc et al., 2008). Mortality due to piroplasmosis ranges from 5-10% in horses native to endemic regions, depending on the strain of pathogen, the clinical health of the host, and the treatment used (Rothschild, 2013). Clinical signs include fever, anaemia, inappetance, icterus, haemoglobinuria and poor exercise tolerance. Sudden death has also been reported. Horses infected with *B. caballi* and *T. equi* can show similar clinical signs, although those produced by the former tend to be milder or inapparent (Rothschild, 2013). Following recovery, horses can become asymptomatic carriers for life, but act as reservoirs for subsequent tick infection (De Waal, 1992). In recent years, molecular methods have been used to identify unicellular parasite species (Stevanovic et al., 2013; Davitkov et al., 2015; Laus et al., 2015) and have enabled accurate diagnosis.

Parasitic diseases can cause oxidative stress since hosts produce reactive oxygen species (ROS), primarily to attack invading pathogens (Dimitrijević et al., 2012; Esmaeilnejad et al., 2014). Despite their protective role, these reactive molecules cannot distinguish between host cells and infectious agents (Sorci and Faivre, 2009). Under these circumstances, the host activates protective mechanisms including antioxidative defences. If the host is overwhelmed by ROS, oxidative stress may occur (Rahal et al., 2014).

The role of oxidative stress in the pathogenesis of equine diseases has previously been described (de la Calle et al., 2002; McFarlane et al., 2005; Lykkesfeldt and Svendsen, 2007; Soffler, 2007; Yin et al., 2009). Overproduction of ROS can induce oxidative modifications in cellular macromolecules such as lipids, proteins and DNA (Trachootham et al., 2008). Lipid peroxidation of cell membranes by ROS leads to progressive damage and loss of selective permeability. Oxidative damage of proteins can result in loss of biochemical functions, while oxidative DNA damage can cause DNA mutations, replication errors, genomic instability and cell death (Klaunig et al., 2010).

Several investigations have revealed elevated concentrations of oxidative stress parameters and reduced concentrations of antioxidants in ruminants with babesiosis (Saleh 2009; Esmaeilnejad et al., 2014; Kucukkurt et al., 2014). In horses naturally infected with *T. equi* only

a few oxidative stress parameters have been evaluated, so knowledge of the pathology of *T. equi* infection is limited (Cingi et al., 2012; Salem and El-Sherif, 2015). Therefore, the aim of this study was to determine the concentrations of oxidative stress parameters and DNA damage in horses naturally infected with *T. equi*. The importance of oxidative stress as a serious consequence of the pathogenic action of this parasite is also discussed.

Materials and methods

Horses and blood sampling

One hundred and ten Serbian Mountain ponies from South-East Serbia were examined for potential enrollment in this study. The Serbian Mountain pony is highly endangered and its breeding is subsidized by the Serbian government. All the horses investigated lived in herds of 20-30 females and one male, grazing most of the year. Physical examinations were performed and other relevant data (age, gender and clinical history) were obtained from the horse owners. Blood specimens were collected by jugular venipuncture into sterile EDTA tubes (2 mL; Demophorius Healthcare) for duplex PCR, and plain vacutainers (8 mL; Demophorius Healthcare) for analysis of biochemical parameters (blood urea nitrogen, creatinine, total protein, glucose, creatine kinase and alkaline phosphatase). Based on physical examination, biochemical analyses and PCR results, 60 mares (aged 3-4 years) were selected for investigation of oxidative stress parameters and DNA damage. Horses were selected on the basis of lack of health problems in the previous 6 months, normal physical examination and serum biochemistry results in the reference range (Cynthia et al., 2011; Pađen et al., 2014). *B. caballi* DNA was not detected in any of the analysed specimens; *T. equi* DNA was detected in 30 specimens. The selected horses were divided into two groups: infected (*T. equi* group; *n*=30) and uninfected (control group; *n*=30). Venous blood specimens (10 mL) from all 60 horses were collected into lithium heparin tubes (Demophorius Healthcare) for analyses of oxidative stress parameters and comet assay.

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine University of Belgrade and Ministry of Agriculture and Environment in accordance to Serbian law for protection of animal welfare and EU declaration 63/2010 (Ref. No. 323-07-05566/2015-05/2; 30 June 2015).

Specimen processing and cell preparation

Plasma was obtained from whole blood collected into lithium heparin by centrifugation for 10 min at 3,000 rpm. Erythrocytes were rinsed three times in physiological saline solution. Blood plasma and erythrocytes were frozen at -20 °C until further analysis. Plasma was used for

determination of PON1 activity, LDH isoenzyme activity, and concentrations of NO_2^-, NOx and thiol groups. CAT and SOD activities and MDA concentrations were measured in the erythrocyte suspension. For the evaluation of damage to DNA, lymphocytes were isolated from whole blood with Ficoll-Paque medium (Radakovic et al., 2013). Viability of cells after isolation was determined with the trypan blue exclusion assay.

PCR detection of parasite DNA

DNA was isolated from the blood specimens using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's recommendations. Extracted DNA specimens were stored at -20 °C until the assay was performed. A duplex PCR method for the simultaneous detection and differentiation of *T. equi* and *B. caballi* with the following set of primers was used: Bec-UF2 (5'-TCGAAGACGATCAGATACCGTCG-3') as a universal forward primer and Cab-R (5'-CTCGTTCATGATTTAGAATTGCT-3') and Equi-R (5'-TGCCTTAAACTTCCTTGCGAT-3') as reverse primers specific for *B. caballi* and *T. equi*, respectively (Alhassan et al., 2005).

All PCR assays were performed in a MultiGene Gradient Thermal Cycler (Labnet International) in 25 μL volumes containing 12.5 μL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, PN KK7152), 1.25 μL of each primer (10 μM) and 10 μL (10 ng/μL) DNA specimen. The PCR parameters for amplification were: initial DNA denaturation over 3 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 15 s at 58 °C and 15 s at 72 °C, and terminated with a final extension step at 72 °C for 8 min. Amplification products were separated on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Oxidative stress parameters

The activity of Cu, Zn superoxide dismutase (SOD1) in erythrocytes was determined by spectrophotometer (Misra and Fridovich,1972), and expressed in Units/gram of haemoglobin (U/g Hb). Isoenzymes SOD1a and SOD1b bands were detected by vertical 10% polyacrylamide gel electrophoresis (PAGE; Hoeffer miniVE) with nitro blue tetrazolium – NBT (Beauchamp and Fridovich, 1971). The relative activity of each isoenzyme was estimated by Scion Image software, version beta 4.0.2 (Scion Corporation, 2000) and expressed in arbitrary U/g Hb.

The osmotic resistance of erythrocytes was assessed as described by Beutler (1983). The extent of haemolysis (EH) in each specimen was expressed as a percentage of the absorbance in

NaCl, which was measured at 540 nm. The cumulative osmotic fragility curve was plotted from the EH values obtained in serial dilutions of saline by Boltzmann sigmoidal function (Luzzatto and Roper, 1995).

Catalase activity (CAT) in erythrocytes was assayed in the presence of H_2O_2 (Aebi, 1984). Decomposition of H_2O_2 was evaluated directly by monitoring the decrease of absorbance at 240 nm. The activity was expressed in U/g Hb.

Paraoxonase activity (PON1) in the plasma was assayed using synthetic paraoxon (diethyl-p-nitrophenyl phosphate) as a substrate. The enzyme activity was expressed in U/L (Alapati and Mihas, 1999).

Nitrite concentrations (NO_2^-) and total nitrate and nitrite concentrations (NOx) in plasma were evaluated using vanadium trichloride (VCl_3) with Griess reagent (Guevara et al., 1998; Miranda et al., 2001). Absorbance was measured on a micro plate reader (Plate reader Mod.A1; Nubenco Enterprises) at a wavelength of 540 nm. The results were expressed in µmol/L.

The concentration of malondialdehyde (MDA) in erythrocytes was determined by measuring the formation of thiobarbituric acid-reactive substances (TBARS; Gutteridge, 1995; Traverso et al., 2004). The absorbance of the colored MDA-TBARS complex was measured by spectrophotometry at a wavelength of 535 nm and expressed in nmol/g Hb.

Free thiol groups were assayed by spectrophotometer according to a modified method of Ellman (1959). The interaction of thiols with 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB), formed a colored anion with an absorption peak at 412 nm. The results obtained were expressed in µmol/L.

Lactate dehydrogenase isoenzymes (LDH_1–LDH_5) were detected by PAGE technique using Tris–glycine buffer (25 mM Tris, 192 mM glycine pH 8.3) and sodium lactate as a substrate in the presence of nitroblue tetrazolium chloride (Yoshida and Takakuwa, 1997). LDH isoenzyme bands were analysed using Scion Image software, version beta 4.0.2 (Scion, 2000) and the relative activity of each isoenzyme was expressed as a percentage of total LDH band intensity.

All spectrophotometric measurements were performed using a Cecil CE 2021 UV/VIS spectrophotometer. Haemoglobin concentrations in erythrocyte haemolysates and plasma were also determined (Tentori and Salvati, 1981).

DNA damage estimation with the alkaline comet assay

Alkaline comet assay was performed (Singh et al., 1988 ; Tice et al., 2000) with slight modifications as described by Radakovic et al. (2013). Before analysis, slides were stained with ethidium bromide and analyzed under a fluorescence microscope (AxioImager Z1, Carl Zeiss; excitation filter, 515–560 nm; emission filter, 590 nm). Comets were scored visually and classified into five categories corresponding to the amounts of DNA in the tails (Anderson et al., 1994). Comet scores were calculated by multiplying the number of damaged cells per specimen with the value of the respective comet class (0-4) and expressed as the total comet score (TCS).

Statistical analysis

Shapiro-Wilk tests were performed to determine the normality of data distribution. Two-tailed unpaired Student *t*-tests were used to compare data from control and *T. equi*-infected groups (Graph Pad Prism 5.0). Data were expressed as mean ± standard deviation (SD) and statistical significance was set at $P < 0.05$.

Results

Duplex PCR demonstrated *T. equi* DNA in 30 horses (*T. equi* group), but *B. caballi* DNA was not present in any specimens (Fig. 1). The EH curves for the *T. equi* infected group (EH_{50} 0.578 ± 0.045) and the control group (EH_{50} 0.501 ± 0.045) are presented in Fig. 2a. Infected animals had significantly lower ($P < 0.001$) resistance to hemolysis (Fig. 2a) and significantly higher PHb ($P < 0.01$) than control horses (4.81± 0.73 vs. 0.99±0.1; Fig. 2b).

The activity of antioxidant enzyme SOD1 in infected horses was significantly increased (63.64%; $P < 0.01$) compared to control horses (Fig. 3a). The results of spectrophotometric measurements were confirmed with staining for SOD1 activity on PAGE. The appearance of two distinct bands demonstrated the presence of different isoenzymes, SOD1a and SOD1b, in both the control and the infected group (Fig. 3b). The intensities of isoenzyme bands in *T. equi*-infected horses (specimens 6-10) were higher than those of control horses (specimens 1-5) and

densitometry analysis confirmed that this difference was statistically significant ($P < 0.05$; Fig. 3c).

CAT activity was significantly lower (21.22%; $P < 0.01$) in horses with theileriosis than in the control group (Fig. 3d). Additionally, PON1 activity was 45% lower in the infected group than in and control group ($P < 0.001$; Fig. 3e). Horses with theileriosis had significantly higher concentrations of NO_2^- ($P < 0.001$) and NO_x ($P < 0.05$) than those in the control group. This was particularly noticeable in the NO_2^- concentration results (Fig. 4a).

MDA concentration in *T. equi* group was increased by 80%, which was significantly higher ($P < 0.001$) than in control horses (Fig. 5a). However, the concentrations of thiol groups (-SH) were significantly reduced ($P < 0.05$) in infected horses compared to control horses (290.7±15.02 vs. 233.80±12.08; Fig. 5b).

TCS evaluation demonstrated that horses infected with *T. equi* had significantly more DNA damage than control horses ($P < 0.001$; Fig. 5c). The amount of DNA damage in the cell population was significantly more variable in infected horses (Fig. 5d).

Distribution of relative activity of LDH isoenzymes (Fig. 6a) and their electrophoretic profiles (Fig. 6b) showed that only LDH_5 had significantly increased activity ($P < 0.05$) in infected horses. There were no significant changes in the activity of other isoenzymes (LDH_1-LDH_4) when infected and uninfected horses were compared.

Discussion

In the present study, horses naturally infected with *T. equi* showed important changes in blood markers of oxidative stress. These findings fit with potential host strategies to develop complex mechanisms such as the production of reactive oxygen and/or nitrogen species (RNS/ROS) in response to parasite infection. There was a significant increase in PHb concentrations in horses infected with *T. equi* compared to control horses. Similary, Saleh (2009) reported increased PHb in crossbred cattle infected with *B. bigemina.* It is noteworthy that increased concentration of PHb reflects cell lysis and haemolysis (Fairbanks and Klee 1994).

Analysis of EH in this study indicated decreased resistance to hemolysis in infected horses. This finding is in accord with increased PHb concentrations reported here and suggests that *T. equi* may affect the integrity of red blood cells. This parallels the results of Esmaeilnejad et al. (2014), who reported increased osmotic fragility in *B. ovis*-infected sheep.

In this study, spectrophotometric analyses showed that horses naturally infected with *T. equi* had significantly increased SOD1 activity, which was confirmed by densitometry. This suggests that *T. equi* could have modified isoenzyme activity via free radical production. Similarly, Chaudhuri et al. (2008) reported a significant rise in SOD activities in dogs infected with *B. gibsoni*. By contrast, significant decreases in SOD activity have been observed in sheep with babesiosis and theileriosis (Nazifi et al., 2011; Esmaeilnejad et al., 2014). The discrepancy could be explained by possible inactivation of SOD by its product, hydrogen peroxide (H_2O_2; (Nikolić-Kokić et al., 2010), which can arise due to dismutation of superoxide anion.

Hydrogen peroxide, generated by SOD, is potentially dangerous for cells and is a substrate for another antioxidant enzyme, CAT. Our study demonstrated significantly increased SOD activity and significant reduction in CAT activity in horses infected with *T. equi*, which may indicate the weakening of CAT antioxidant capacity. One possible reason for reduced CAT activity is its inactivation by nitric oxide (Brunelli et al., 2001). In goats with babesiosis, CAT activity significantly decreased with increased parasitemia (Esmaeilnejad et al. 2014). However, contrary to our results, Baghishani et al. (2011) reported increased CAT activity and decreased SOD activity in sheep naturally infected with *Theileria*. ROS homeostasis depends on balanced activity of antioxidant enzymes (Nikolić-Kokić et al., 2010) and this balance results from interdependent changes in the intracellular activity of antioxidant enzymes. It is possible that increased CAT activity detected by Baghishani et al. (2011) could have resulted from decreased activity of SOD and consequent decreased production of H_2O_2.

Besides CAT and SOD, the activity of PON1 was also evaluated in this study. Several studies have investigated PON1 activity in pathological conditions in animals (Rossi et al., 2014) but none has been conducted in horses with theileriosis. We observed that infected horses had reduced plasma PON1 activity, which agrees with the findings of Azimzadeh et al. (2013) who reported decreased activity of PON1 in sheep infected with *B. ovis.* Reduced PON1 activities in *Theileria*-infected horses in our study may indicate excess free radicals that could trigger cell membrane damage caused by lipid peroxidation.

In our study, significant increases in NO_2^- and NOx were demonstrated in *T. equi*-infected horses. Deger et al. (2009) also reported increased NO production in horses with *T. equi* and *B. caballi*. Increased NO production has also been associated with other parasitic infections (Dimitrijevic et al., 2012).

The results of this study indicate that *T. equi* enchances lipid peroxidation, as reflected in increased MDA concentrations and cell membrane damage associated with increased production of free radicals. These findings are in accordance with a previous study which demonstrated significantly increased MDA in horses infected with *T. equi* and *B. cabballi* (Deger et al., 2009). Similar findings have been reported by Cingi et al. (2012) and Salem and El-Sherif (2015), indicating that theileriosis is clearly associated with increased lipid peroxidation. Ovine theileriosis and babesiosis have also been associated with increased concentrations of MDA (Baghishani et al., 2011; Esmaeilnejad et al., 2014), indicating that increased lipid peroxidation may be the major mechanism of cell membrane destruction caused by piroplasmosis.

Peroxidation of polyunsaturated fatty acids induced by ROS can cause increased membrane rigidity, permeability and serious consequences, such as membrane disruption and release of haemoglobin. Therefore, the haemolysis demonstrated in our study may be related to increased lipid peroxidation caused by free radicals. Previous studies have reported positive correlations between lipid peroxidation and the progression of anemia (Asri and Dalir-Naghaded, 2006; Gopalakrishnan et al., 2015). Thus, oxidative damage might be an important factor in pathogenesis of anaemia in animals infected with *T. equi*.

Highly reactive secondary products of lipid peroxidation can react with thiol groups in plasma proteins, which are susceptible to the impact of free radicals. The present study revealed decreased concentrations of thiol groups in horses with theileriosis, confirming our suspicions that the intensity of free radical processes were enhanced. Ducrocq et al. (1999) reported that thiol radicals can react with oxygen and promote oxidative stress by propagating free radical reactions.

Although numerous studies have reported increased DNA damage in animals infected with parasites (De Oliveira et al., 2011; El Sayed and Aly, 2014), to our knowledge, no such investigation has been conducted in horses with theileriosis. In this study, horses infected with *T. equi* had higher amounts of lymphocyte DNA damage compared to non-infected horses. Similar findings have also been reported in goats infected with *B. ovis* (Kucukkurt et al., 2014). The

DNA damage we observed in equine lymphocytes can be attributed to the immune response against *Theileria*. Superoxide radicals and hydrogen peroxide produced in parasitic infections can form potent hydroxyl radicals (OH•; Jackson and Loeb, 2001), which are capable of reacting with DNA nitrogen bases and forming critical biomarkers of oxidative stress, such as 8-hydroxyguanine (8-OHG). Significantly increased 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations have been demonstrated in dogs with *B. vogeli* infection (Ciftci et al., 2014), suggesting the involvement of free radical-mediated oxidative DNA damage in the pathogenesis of babesiosis.

The appearance of a cellular enzyme such as LDH in the extracellular space is a useful marker of disrupted cellular integrity. There are five forms of LDH (isoenzymes LDH_1-LDH_5) and the distribution of each isoenzyme varies between tissues (Jović et al., 2013). We observed that only LDH_5, related to liver damage, was significantly increased in *Theileria* infected horses. It is known that *B. divergens* infection in gerbils causes an increase in LDH activity and that babesiosis induces oxidative stress in hepatic tissue (Dkhil et al., 2013). Additionally, increased serum AST and ALT, markers of hepatic pathology, has been reported in cattle infected with *B. bigemina* and *T. annulata* (Lotfollahzadeh et al., 2011).

Conclusions

In this study, equine theileriosis was associated with increased markers of oxidative stress. Increased superoxide and NO associated with *Theileria* infection resulted in the disruption of host defense mechanisms, demonstrated by significant changes in the activity of antioxidant enzymes (SOD, CAT, PON1) and increased LDH_5 isoenzyme activity. Increased production of ROS/RNS in *Theileria*-infected horses resulted in changes in osmotic fragility, oxidative damage of lipids, proteins and DNA, haemolysis, and hepatocellular damage. These markers of oxidative stress could be involved in the pathogenesis of theileriosis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with people or organizations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig. 1. Duplex PCR detection of *Theileria equi* and *Babesia caballi*. M, 100 bp ladder DNA marker; Lane 1, Positive control for *T. equi*; Lanes 2, positive control for *B. caballi*; Lane 3, positive control for mixed infection; Lanes 4-8, specimens infected with *T. equi.* The sizes of the positive bands are indicated on the left.

Fig. 2. Results of haemolysis in *T. equi* infected and control horses. (a) Extent of hemolysis curve. (b) Bar chart of plasma free haemoglobin (mean ± standard deviation). $^{**}P < 0.01$ vs. control group.

Fig. 3. The activity of enzymes (mean ± standard deviation) in *T. equi* infected and control horses. (a) Bar chart of Cu, Zn superoxide dismutase (SOD) SOD1 activity. (b) SOD1 electropherogram on PAGE (Lines 1-5, control horses; Lines 6-10, infected animals). (c) Bar chart of relative activity of SOD1a and SOD1b isoenzymes. (d) Bar chart of catalase (CAT) activity. (e) Bar chart of paraoxonase (PON1) activity. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control group.

Fig. 4. Nitrite (NO_2^{-}) and total nitrate and nitrite concentrations (NOx; mean ± standard deviation) in *T. equi* infected and control horses. (a) bar chart of NO_2^{-} concentration. (b) bar chart of NO_x concentrations. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control group.

Fig. 5. Oxidative stress parameters (mean ± standard deviation) in *T. equi* infected and control horses. (a) Bar chart of malondialdehyde (MDA) concentration. (b) Bar chart of free thiol (-SH) groups concentration. (c) DNA damage expressed as total comet score (TCS). (d) Distribution of classes of DNA damage. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. control group.

Fig. 6. Relative distribution of lactate dehydrogenase (LDH) LDH_1- LDH_5 isoenzymes (mean ± standard deviation) in *T. equi* infected and control horses. (a) Bar chart of isoenzymes expressed as percentages; (b) Activity staining of LDH isoenzymes on PAGE (Lines 1-4, control horses; Lines 5-8, infected animals). $^{*}P < 0.05$ vs. control group.