Kinetics of plasma procalcitonin, soluble CD14, CCL2 and IL-10 after a sublethal infusion of lipopolysaccharide in horses

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Abstract

Endotoxemia is still an important clinical and economical problem in the equine industry. The aim of the present study was to assess the kinetics of soluble CD14 (sCD14), chemokine (C-C motif) ligand 2 (CCL2), interleukin 10 (IL-10) and plasma procalcitonin (PCT) in healthy horses after intravenous infusion of lipopolysaccharide (LPS) in order to support basic understanding of the species-specific kinetic of horses to LPS exposure for these molecules. Investigating the kinetics of these molecules in an equine experimental model could support further findings in clinical studies and could identify valuable inflammatory biomarkers for equine practice. Eleven healthy horses were involved in this experimental *in vivo* study. Horses were classified as healthy before LPS infusion. After the pre-infusion blood collection (T0), all horses received an infusion of *E. coli* endotoxin (30 ng/kg over 30 minutes). Data and samples were collected at 1 hour (T1), 2 (T2), 3 (T3) and at 24 hours (T24) after infusion. Plasma sCD14, CCL2 and IL-10 were evaluated with a fluorescent bead-based assay, while PCT was evaluated with an equine PCT ELISA assay. One-way ANOVA test was performed on results between each blood-sampling time for PCT, sCD14 and IL-10, while Friedman test was performed for CCL2. Plasma PCT, IL-10 and CCL2 concentrations were statistically increased at T1, T2 and T3 compared to T0. No statistically significant differences were found between plasma IL-10 and CCL2 concentrations between T0 vs T24, although plasma PCT values remained elevated 24 hours after LPS infusion. Plasma sCD14 concentration showed no statistically significant differences for all sampling times. Our results demonstrate that LPS injection into healthy horses results in PCT, CCL2 and IL-10 increases in plasma in the absence of an increase of sCD14. The increase of PCT, CCL2 and IL-10 are related to the inflammatory response inducted by circulating lipopolysaccharide.

Key words

Equine induced endotoxemia, Procalcitonin (PCT), Soluble CD14 (sCD14), Chemokine ligand 2 (CCL2), Interleukin-10 (IL-10).

List of abbreviations

CARS	compensatory anti-inflammatory response syndrome
CCL2	chemokine (C-C motif) ligand 2
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
IL-10	interleukin 10
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
PCT	procalcitonin
RCF	relative centrifugal force
sCD14	soluble cluster of differentiation antigen 14
SIRS	systemic inflammatory response syndrome
TLR4	toll-like receptor 4

69 Endotoxemia is an important clinical and economical problem in equine industry (Taylor,
70 2015). Lipopolysaccharide (LPS) is a component of gram negative bacterial cell wall and one
71 of the most potent bacterial signal molecules known (Moore and Barton, 1998). LPS is
72 present in large quantities in the site of infection, but clinical problems occur when
73 gastrointestinal translocation or absorption of bacteria from an infected site (for example
74 uterus or lung) took place (Moore and Vandenplas, 2014; Taylor, 2015). Sensitive detection
75 of LPS by the innate immune system provides animals with an early immune response.
76 However, in many subjects an exaggerated response to LPS can be harmful, leading to
77 endotoxemia and sepsis, organ failure (MODS), and death (Moore and Vandenplas, 2014;
78 Taylor 2015). Endotoxemia leads to activation of the inflammatory cascade, including release
79 of pro-inflammatory cytokines, release of acute phase proteins and production of eicosanoids
80 (Moore and Vandenplas, 2014; Taylor, 2015).
81 Intravenous infusion of LPS in horses mimics the inflammatory cascade and many of the
82 clinical signs observed in naturally occurring endotoxemia (Werners et al., 2005). Several
83 studies have investigated the pathways of LPS transduction in laboratory animals; however,
84 well documented species and individual differences in responses to LPS exposure suggests
85 that species specific research is required (Werners et al., 2005).
86 Once LPS has reached the bloodstream, it is recognized by the innate immune system (Fang
87 et al. 2004; Moore and Vandenplas, 2014; Taylor, 2015), LPS is bound to LPS-binding
88 protein (LBP) (Moore and Vandenplas, 2014) and transported to the cellular receptor cluster
89 of differentiation antigen 14 (CD14). CD14 is a 53-kd serum/cell-surface glycoprotein that is
90 expressed by activated monocytes and tissue macrophages in response to LPS-induced
91 signaling through TLR4 (Endo et al., 1994; Werners et al., 2005; Riedel, 2012; Moore and
92 Vandenplas, 2014). CD14 is also found in a soluble form (sCD14), bound to cell types
93 lacking CD14, such as endothelial cells (Duriex et al., 1994). Circulating concentrations of

sCD14 greatly increases during inflammation, which makes it a useful marker of acute and chronic inflammation (Stelter, 2000). Thus, during endotoxemia the LPS-CD14 bond leads to the activation of some molecular pathways resulting in production and release of inflammatory mediators and enzymes such as chemokines, cytokines and acute phase proteins (Endo et al., 1994; Werners et al., 2005; Riedel, 2012, Moore and Vandenplas, 2014).

Chemokines target many cell types similar to cytokines. Chemokines are produced by a wide variety of cell types in the body and any nucleated cell is capable of producing at least some chemokines constitutively or upon activation (Gangur et al., 2002). One of the most studied chemokine in veterinary species is chemokine (C-C motif) ligand 2 (CCL2) (old name MCP-1), a 76– amino acid member of the C-C subfamily of chemokine with chemoattractant activity for monocytes, T cells, mast cells, and basophils (Chensue et al., 1996). CCL-2 is produced in response to various stimuli, including TNF, IL-1, IL-4, viruses, and endotoxin (Bossink et al., 1995; Chensue et al., 1996). Chemokines and adhesion molecules, such as selectins and integrins, work together in order to facilitate leukocyte rolling and adhesion to the vascular endothelium during diapedesis, a step of chemotaxis mechanism (Mackay, 2001).

Cytokines are synthesized and released by mononuclear phagocytes and then have effects on other cells or other mediators. The most well known of the cytokines are tumor necrosis factor (TNF) and the interleukins. Interleukins are cytokines that regulate inflammatory and immunological responses in animals (Gangur et al., 2002). Interleukin 10 (IL-10) is involved in the type-2 cytokines response to LPS-induced inflammation (Compensatory Anti-inflammatory Response Syndrome, CARS). IL-10 partially mediates the immune changes during CARS, including functional deactivation of monocytes, decreasing production of inflammatory cytokines (Gullo et al., 2005).

In response to activation of the inflammatory pathways during endotoxemia, several acute phase proteins (APPs) are synthetized and released in the bloodstream. In recent years, researchers studied the possible role of these APPs as biological laboratory markers (biomarkers) in diagnosing endotoxemia, guiding antimicrobial therapy and assessing response to therapy in human medicine (Taylor, 2015). Procalcitonin (PCT) is the 116-amino acid peptide precursor of the hormone Calcitonin, encoded by the CALC-I gene. In response to circulating LPS the expression of the CALC-I gene is up regulated and PCT is released from many body tissues and cell types in hamsters, dogs and horses, as well as in humans (Riedel, 2012). PCT appears to be an early marker of endotoxemia and SIRS caused by bacterial infections and LPS release in human medicine (Riedel, 2012; Afsar and Sener, 2015).

The aim of the present study was to assess the kinetics of plasma sCD14, CCL2, IL-10 and PCT concentrations after 30 minutes sub-lethal (iv) administration of LPS in order to support basic understanding of the species-specific kinetic of horses to LPS exposure for these molecules. Investigating the kinetics of these molecules in an equine experimental model could support further findings in clinical studies and could identify valuable inflammatory biomarkers for equine practice.

Materials and Methods

Horses

The study involved 11 clinically healthy adult mares (4 Thoroughbreds, 4 Warmbloods, 2 Quarter Horses, 1 Oldenburg) from an academic teaching facility[1]. The average age was 13.1±5.7 years and the average weight was 565.4±39.6 kg with an average Body Condition Score (BCS) of 3.6± 0.2. All the animals were kept in individual 10 x 10 foot stalls, fed free choice hay and 1 kg of a commercial concentrate twice daily. All horses were treated against gastrointestinal parasites and vaccinated according to the guidelines of the American Association of Equine Practitioners Infectious Disease Committee (http://www.aaep.org/info/vaccination-guidelines-265).

Before starting the experimental protocol a complete physical examination, along with a standard blood hematological and biochemical analyses was performed. Only horses that showed no remarkable clinical and laboratory findings were included in the study (Mackay, 2009).

An Institutional Laboratory Animal Care and Use Committee approved all study procedures and certificated that all experiments were conducted conforming to the relevant regulatory standards.

Experimental design

Endotoxin preparation and infusion

All horses received an infusion of *E. coli* 055:B5 endotoxin[2] (30 ng/kg over 30 minutes). The lyophilized powder was sterilely reconstituted in phosphate buffered saline solution at a concentration of 10 μg/ml and stored frozen in individual aliquots in siliconized glass containers until the day of infusion. One hour prior to infusion, an LPS aliquot was thawed at room temperature for one hour and then vortexed. After vortexing, 30 ng/kg LPS was added

to 500 ml 0.9% saline and infused as a constant rate infusion at a rate of 1000 ml/hour for 30 minutes (Watts et al., 2014).

Specimen collection

A 10 ml blood sample was collected from each horse via jugular venipuncture using a 17G needle attached to a plastic catheter with stopper-piercing needle[3] immediately before LPS infusion (0 h), than 1 h, 2 h, 3 h and 24 h after LPS infusion. Blood samples were collected in 2 aliquots: a 1 ml aliquot was placed in an EDTA tube[4] for WBC evaluation[5]; a second 9 ml aliquot was collected in heparin anticoagulant tubes[6], immediately centrifuged at 3000 RPM for 10 minutes, plasma was frozen at -18°C for one month. Plasma samples were analyzed in a single batch for PCT, sCD14, CCL2 and IL-10 concentrations evaluation.

Clinical data

The horses were also monitored for alterations in clinical signs specific for endotoxemia, throughout all the study period. Data evaluated at pre-infusion time (0h) and at 1h, 2h, 3h and 4h were: body temperature (BT), heart rate (HR), respiratory rate (RR) and WBC concentrations.

At time 2h after LPS infusion, the presence of two or more of the following symptoms was used in order to confirm SIRS: BT < 36.7 or >38.6 °C, HR>50 beats/min, RR>25 breaths/min, and WBC < 5.000 or > 14.500 cells/mm (Mackay, 2009).

Soluble CD14, CCL2 and IL-10 evaluation

Cytokines were quantified using species-specific singleplex ELISA (CCL2) or multiplex ELISA (IL-10, sCD14), as described previously (Wagner et al., 2009; Wagner et al., 2013). In brief, monoclonal antibodies against recombinant equine cytokines were produced either in a mammalian expression system using the equine IgG1 heavy chain (Wagner et al., 2005) (anti-equine IL-10 clone 492-228 coupled to bead 34; anti-equine CD14 clone 105 coupled to bead 38) or anti-CCL2 (coupled to bead 37), were coupled to fluorescent beads. Mixed

coupled antibodies (final concentration of 105 beads/mL each) were added to each well of the microtiter plate before adding samples or standard curves. The latter were prepared using serial 3-fold dilutions of supernatants containing recombinant cytokine/IgG-fusion proteins. Biotinylated antibodies specific for the cytokines were then added antiequine IL-10 clone 165-228; anti-CCL2 (Wagner et al., unpublished data); anti-CD14 mAb 59 (Wagner et al., 2013) followed by streptavidin–phycoerythrin.[7] The assay was analyzed in a Luminex IS 100 instrument[8] and the data were reported as median fluorescence intensities. Calculation of the cytokine concentrations in samples was performed according to the logistic 5p formula (y = a + b/(1 + (x/c)^d)^f).[9] The multiplex intra- and inter-assay variability has been described elsewhere (Wagner et al., 2009; Wagner et al., 2013). The detection threshold was >15 pg/mL IL-10, 1 pg/mL for CCL2, and 1 ng/ml for sCD14.

PCT evaluation

Plasma PCT concentrations were determined with a commercial equine PCT ELISA assay.[10] The intra-assay coefficient of variation was determined from 10 replicates of equine plasma samples containing low and high PCT concentrations. These samples were obtained by addition of standard PCT in equine blank samples. The inter-assay coefficient of variation was determined from values obtained by repeating the analysis of duplicate samples with low and high PCT concentrations in 5 different assays. To establish the detection limit for equine PCT, we performed repeated PCT measurements (inter-assay and intra-assay) using equine samples with low PCT concentrations (<10.0 pg/ml). Samples were measured in 10 replicates in a single assay and in 5 different assays. The intra-assay and the inter-assay coefficients of variation were less than 15%. The detection limit for equine PCT was 10 pg/ml (Bonelli et al., 2015a).

Data analysis

Data are reported as mean±standard deviation and Kolmogorov-Smirnov test was applied to verify data distribution. One-way ANOVA test for paired data and Tukey's multiple comparisons test as post hoc were carried out on values of BT, HR, RR and WBC count in order to evaluate changes related to time. One-way ANOVA test for paired data and Tukey's multiple comparisons test as post hoc were also carried out on values of PCT, sCD14 and IL-10 concentrations, while Friedman test for paired data and Dunn's test were performed for CCL2 to verify temporal changes. Significance level was set at $P < 0.05$. A commercial statistical software was used.[11]

Results

Values of clinical signs, WBC evaluation, PCT, sCD14 and IL-10 data were normally distributed, while CCL2 data were not.

Clinical and WBC data

Data concerning WBC concentration, HR, BT and RR are reported in table 1. Prior to LPS infusion all the horses remained clinically healthy without abnormalities in vital signs and WBC counts. Following LPS infusion, all horses demonstrated similar temporal patterns of statistically increased HR ($p<0.05$) and BT starting ($p<0.05$) from 60 minutes after LPS infusion, while RR statistically increased at 180 minutes ($p<0.05$). Values returned within normal limits 180 minutes after LPS infusion for HR and 24 hours for BT and RR. All horses showed a statistically significant decrease in WBC ($p<0.05$) 60 minutes after LPS infusion. This decrease in value was no longer detected by 24 hours after LPS infusion. All horses returned to be clinically normal within 24 hours after LPS infusion and no horse required medical treatment.

Soluble CD14 kinetic

Results of sCD14 kinetic are reported in table 2 and figure 1. No statistically significant differences were found between concentrations *vs* time.

CCL2 kinetic

Results of CCL2 kinetic are reported in table 2 and figure 2. Plasma concentrations were statistically higher at T1, T2 and T3 respect to T0. Values peaked at T2 with average values 278 times fold higher than T0, than drop down to pre-infusion level at T24. No statistically differences were found between T0 and T24.

IL-10 kinetic

Results of IL-10 kinetic are reported in table 2 and figure 3. Plasma concentrations were statistically higher at T1, T2 and T3 respect to T0. Values peaked at T1 with average values 3 times fold higher than T0, than drop down to pre-infusion level at T24. No statistically significant differences were found between T0 and T24.

PCT kinetic

Results of PCT values are reported in table 2 and figure 4. Plasma concentrations were statistically higher at T1, T2, T3 and T24 respect to T0.

250 **Discussion**

251 Due to the species-specific response of LPS exposure (Moore and Vandenplas, 2014) our aim
252 was to assess changes in plasma sCD14, CCL2, IL-10 and PCT concentrations in healthy
253 horses after a single sub-lethal LPS dose by i.v. injection.
254 Infusion of LPS as performed in this study is a well-established method of experimental
255 induction of endotoxemia in horses (Tadros et al., 2012). Prior to LPS infusion, all the horses
256 were healthy on the basis of physical examination and blood work values were within normal
257 ranges. Starting from 60 minutes after LPS infusion all horses had similar temporal pattern of
258 increased HR, RR, BT, colic signs and markedly leukopenia and neutropenia. These
259 alterations were probably due to the increasing of the inflammatory cytokine concentrations
260 after LPS infusion that leads to SIRS becoming apparent (Tadros et al., 2012). Some studies
261 have been conducted in humans and veterinary species concerning sCD14 after LPS
262 challenge (Leturcq et al., 1996; Verbon et al., 2001; Lee et al., 2003). To the authors
263 knowledge there are no studies about plasmatic sCD14 concentrations following LPS
264 infusion in horses. Pre infusion sCD14 values obtained in this study were higher than data
265 reported by others for healthy horses (Silva et al., 2013; Fogle et al., 2016). This could be due
266 for differences in studied horses population and potential underlying inflammatory status not
267 detected at pre-infusion sample time in our population. Moreover, it could be due to different
268 type of assay performed, gender and time of the year in which the experiment was done.
269 Previous papers reported an increase in sCD14 concentrations in horses with acute
270 gastrointestinal diseases or illnesses causing endotoxemia, and in humans with gram negative
271 sepsis (Landmann et al., 1995; Silva et al., 2013). Our results showed no differences related
272 to sampling time for sCD14 levels. However, in previous studies LPS concentrations were
273 not measured, thus duration of suspected endotoxemia were unproven. Two forms of CD14
274 are reported: membrane bound (mCD14), constitutively expressed on the surface of

monocytes, macrophages, and neutrophils and (sCD14), derived from both secretion and enzymatic cleavage of mCD14 (Jack et al., 1995). Lack of increase in sCD14 levels in our population does not rule out that an increase in mCD14 occurred. Furthermore, the stimulation of mCD14 could be insufficient to cause a measureable increase in sCD14.

Studies conducted in mice, dogs and healthy human volunteers showed that CCL-2 increases after LPS infusion (Sylvester et al., 1993; Sonnier et al., 2012; Floras et al., 2014). To the authors' knowledge there are no studies about kinetics of plasma CCL-2 in healthy horses or after LPS infusion. CCL-2 concentrations at T0 are higher than data reported for humans and dogs (Haukeland et al., 2006; Floras et al., 2014), but lower than murine (Zisman et al., 1997). This could be due to differences in species.

The kinetic of plasma CCL-2 obtained in this study is similar to what reported by others (Sonnier, 2012; Floras et al., 2014) in dog and mice after LPS infusion, suggesting the increment of CCL-2 during endotoxemia in horses.

Circulating IL-10 concentrations are increased in humans and laboratory animals during endotoxemia, sepsis and septic shock (Marchant et al., 1994; Flohe et al., 1999). Moreover, mRNA IL-10 expression increased quickly during LPS-induced inflammation in horses (Sykes et al., 2005; Pusterla et al., 2006; Lopes et al., 2010a; Lopes et al., 2010b; Wagner et al., 2010; Frellstedt et al., 2012; Tadros et al., 2012).

IL-10 concentrations obtained in the present study were too low to be calculated in pg/ml and the program could not extrapolate the exact concentrations. Thus, we cannot compare our results with literature.

Our results on kinetic of IL-10 in horses show a faster rise respect to what reported for men (1 h vs 2.5 h after LPS infusion) (Kox et al., 2011). This difference could be due to the sensitivity of the horse to endotoxin (Taylor, 2015) and to the higher LPS dose used for endotoxemia induction in this study (30 ng/kg IV over 30 minutes) *vs* in the humans one (2

300 ng/kg by IV bolus) (Kox et al., 2011). Despite that, the kinetic of equine plasma IL-10
301 concentrations is similar to what reported by others in in men after LPS infusion (Kox et al.,
302 2011).
303 Previous studies on PCT reported differences between healthy and septic SIRS foals (Bonelli
304 et al., 2015a), and adult horses (Bonelli et al., 2015b). Also, gene expression of calcitonin and
305 procalcitonin has been studied in adult horses (Toribio et al., 2003) and foals (Pusterla et al.,
306 2006) and recently, some authors evaluate PCT in plasma of healthy and septic horses
307 (Rieger et al., 2014) and in foals affected by *Rhodococcus equi* (Barton et al., 2016).
308 To the authors' knowledge, no data have been reported on plasma PCT kinetic after LPS
309 infusion in horses. In this study, detectable amounts of PCT have been found in pre-infusion
310 samples, while no PCT is detectable in healthy pre-endotoxin infusion humans (Dandona et
311 al., 1994; Brunkhorst et al., 1998). This difference is likely due to the different species
312 studied. The equine normal intestinal flora is composed by large quantities of gram-negative
313 bacteria, so a large amounts of LPS normally exists in the equine intestinal lumen, especially
314 in the large hindgut (Dicks et al., 2014; Costa et al., 2015). Even in healthy horses a small
315 amounts of LPS might cross the intact mucosal barrier and reach the portal circulation and
316 the liver (Dicks et al., 2014). This small amount of LPS could lead to slightly higher basal
317 PCT concentrations in horses.
318 The PCT kinetic obtained in the present paper is similar to what reported in human after LPS
319 infusion or iatrogenic sepsis. Furthermore, plasma PCT concentration was still elevated at 24
320 h after LPS infusion in horses, as reported also in humans (Dandona et al., 1994; Brunkhorst
321 et al., 1998).
322
323 In conclusion, LPS infusion in healthy horses results in CCL2, IL-10 and PCT increase in the
324 absence of an increase in sCD14. PCT concentrations were still elevated at 24 hours after

infusion, whereas plasmatic CCL-2 and IL-10 concentrations at 24 hours returned to the pre-infusion concentrations. The kinetics of these molecules followed the changing of clinical finding. All PCT, CCL-2 and IL-10 concentrations increased within 60 minutes after LPS infusion, as happened to the clinical signs typically associated with endotoxemia such as BT and HR. Both clinical values and molecules concentrations, except for PCT, returned to the pre-infusion levels at 24 hours after LPS infusion. These results might suggest that the increase of PCT, CCL2 and IL-10 are related to the inflammatory response inducted by circulating concentrations of LPS even if the plasma kinetics are different. Evaluation of PCT, CCL-2 and IL-10 might represent diagnostic tools for inflammatory status, especially those caused by LPS. In particular, CCL-2 and IL-10 seem not only to rise early, but also to decrease fast after the end of the insult. The limits of this study were the low number of animals included and the enrollment of only mares. Further investigation would be focused in improving study population and improving variability of gender and age.

Footnotes

[1] Cornell University Equine Park, Ithaca, NY

[2] LPS, Sigma-Aldrich, St. Louis, MO 63103

[3] Blood Collection Set, Hospira Inc, Lake Forest, IL

[4] BD Vacutainer K2 EDTA and Vacutainer Heparin, BD, Franklin Lakes, NJ

[5] PFA-100\ System, Siemens Diagnostics, Tarrytown, NY

[6] Heparin-tubes BD Vacutainer, Sodium Heparin BD Franklin Lakes N.J.

[7] Invitrogen, Carlsbad, CA

[8] Luminex Corp, http://www.luminexcorp.com

[9] Luminex 100 Integrated System 2.3, Austin, TX

[10] MyBiosource.com, USA.

[11] Graph Pad Prism 6, USA.

Authorship

Francesca Bonelli: conception and design of the study, acquisition, analysis and interpretation of data, drafting the article, final approval.

Valentina Meucci: conception and design of the study, analysis and interpretation of data, drafting the article, final approval.

Luigi Intorre: revising the article critically for important intellectual content, final approval.

Bettina Wagner: analysis and interpretation of data, final approval.

Thomas J Divers: conception and design of the study, acquisition, revising the article critically for important intellectual content, final approval.

Micaela Sgorbini: conception and design of the study, analysis and interpretation of data, drafting the article and revising critically, final approval.

References

1. Afsar, I., Sener, A.G., 2015. Is Procalcitonin a diagnostic and/or prognostic marker in sepsis? Infect. Dis. Clin. Pract. 23, 3-6.
2. Barton, A.K., Rieger, M., Teschner, D., Gehlen, H., 2016. Procalcitonin - a useful biomarker for pneumonia associated with Rhodococcus equi? Modern Research in Inflammation. 5, 13-9.
3. Bonelli, F., Meucci, V., Divers, T.J., Jose-Cunilleras, E., Corazza, M., Guidi, G., Tognetti, R., Intorre, L., Sgorbini, M., 2015b. Plasma procalcitonin concentration in healthy horses and horses affected by Systemic Inflammatory Response Syndrome. J. Vet. Intern. Med. 29,1689-1691.

4. Bonelli, F., Meucci, V., Divers, T.J., Radcliffe, R., Jose-Cunilleras, E., Corazza, M., Guidi, G., Tognetti, R., Castagnetti, C., Intorre, L., Sgorbini, M., 2015a. Evaluation of plasma procalcitonin concentrations in healthy foals and foals affected by septic Systemic Inflammatory Response Syndrome. J. Equine Vet. Sci. 35(8), 645-649.
5. Bossink, A.W.J., Paemen, L., Jansen, P.M., Hack, C.E., Thijs, L.G., Van Damme, J., 1995. Plasma levels of the chemokines monocyte chemotactic proteins-1 and -2 are elevated in human sepsis. Blood 86, 3841–3847.
6. Brunkhorst, F.M., Heinza, U., Forycki, Z.F., 1998. Kinetics of procalcitonin in iatrogenic sepsis. Intensive Care Med. 24, 888-892.
7. Chensue, S.W., Warmington, K.S., Ruth, J.H., Sanghi, P.S., Lincoln, P., Kunkel, S.L., 1996. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (Mycobacterial) and Th2 (Schistosomal) antigen-induced granuloma formation. J. Immunol. 157, 4602-4608.
8. Costa, M.C., Silva, G., Ramos, L.V., Staempfli, H.R., Arroyo, L.G., Kim, P., Weese, J.S., 2015. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. Vet. J. 205, 74-80.
9. Dandona, P., Nix, D., Wilson, M.F., Aljada, A., Love, J., Assicot, M., Bohuon, C., 1994. Procalcitonin increase after endotoxin injection in normal subjects. J. Clin. Endocrinol. Metab. 79(5),1605-1608.
10. Dicks, L.M.T., Botha, M., Dicks, E., Botes, M., 2014. The equine gastro-intestinal tract: an overview of the microbiota, disease and treatment. Livest. Sci.160, 69-81.
11. Durieux, J.J., Vita, N., Popescu, O., Guette, F., Calzada-Wack, J., Munker, R., Schmidt, R.E., Lupker, J., Ferrara, P., Ziegler-Heitbrock, H.W., Labeta, M.O., 1994. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. Eur. J. Immunol. 24(9), 2006-2012.

12. Endo, S., Inada, K., Kasai, T., Takakuwa, T., Nakae, H., Kikuchi, M., Yamashita, H., Yoshida, M., 1994. Soluble CD14 (sCD14) levels in patients with multiple organ failure (MOF). Res. Commun. Chem. Pathol. Pharmacol. 84, 17-25.

13. Fang, H., Pengal, R.A., Cao, X., Ganesan, L.P., Wewers, M.D., Marsh, C.B., Tridandapani, S., 2004. Lipopolysaccharide-induced macrophage inflammatory response is regulated by SHIP. J. Immunol. 173, 360-366.

14. Flohe, S., Dominguez Fernandez, E., Ackermann, M., Hirsch, T., Borgermann, J., Schade, F.U., 1999. Endotoxin tolerance in rats: expression of TNF-alpha, IL-6, IL-10, VCAM-1 and HSP 70 in lung and liver during endotoxin shock. Cytokine 11, 796-804.

15. Floras, A.N.K., Holowaychuk, M.K., Bienzle, D., Bersenas, A.M., Sharif, S., Harvey, T., Nordone, S,K., Wood, G.A., 2014. N-Terminal Pro-C- natriuretic peptide and cytokine kinetics in dogs with endotoxemia. J. Vet. Intern. Med. 28, 1447-1453.

16. Fogle, J., Jacob, M., Blikslager, A., Edwards, A., Wagner, B., Dean, K., Fogle, C., 2016. Comparison of lipopolysaccharides and soluble CD14 measurement between clinically endotoxaemic and nonendotoxaemic horses. Equine Vet. J. 2016; DOI: 10.1111/evj.12582

17. Frellstedt, L., McKenzie, H.C., Barret, J.G., Furr, M.O., 2012. Induction and characterization of endotoxin tolerance in equine peripheral blood mononuclear cells in vitro. Vet. Immunol. Immunopathol. 149, 97-102.

18. Gangur, V., Birmingham, N.P., Thanesvorakul, S., 2002. Chemokines in health and disease. Vet. Immunol. Immunopathol. 86, 127-136.

19. Gullo, A., Iscra, F., Di Capua, G., Berlot, G., Lucangelo, U., Chierego, M.L., Ristagno, G., Peratoner, A., Fasiolo, S., Consales, C., De Martino, G., Tufano, R.,

2005. Sepsis and organ dysfunction: an ongoing challenge. Minerva Anestesiol. 71, 671-699.

20. Haukeland, J.W., Damas, J.K., Konopski, Z., Loberg, E.M., Haaland, T., Goverud, I., Torjesen, P.A., Birkeland, K., Bjoro, K., Aukrust, P., 2006. Systemic inflammation in nonalcoholic fatty liver disease characterized by elevated levels of CCL2. J. Hepatol. 44(6), 1167-1174.

21. http://www.aaep.org/info/vaccination-guidelines-265. Accessed on June 2016.

22. Jack, R.S., Grunwald, U., Stelter, F., Workalemahu, G., Schutt, C., 1995. Both membrane-bound and soluble forms of CD14 bind to Gram –negative bacteria. Eur. J. Immunol. 25, 1436-1441.

23. Kox, M., de Kleijn, S., Pompe, J., Ramakers, B.P., Netea, M.G., van der Hoeven, J.G., Hoedemaekers, C.W., Pickkers, P., 2011. Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia. Crit. Care Med. 39(8), 1866-1870.

24. Landmann, R., Zimmerli, W., Sansano, S., Link, S., Hahn, A., Glauser, M.P., Calandra, T., 1995. Increased circulating soluble CD14 is associted with high mortality in Gram-negative septic shock. J. Infect. Dis. 171, 639-644.

25. Lee, J.W., Paape, M.J., Elsasser, T.H., Zaho, X., 2003. Elevated Milk Soluble CD14 in Bovine Mammary Glands Challenged with *Escherichia coli* Lipopolysaccharide. J. Dairy Sci. 86, 2382-2389.

26. Leturcq, D.J., Moriarty, A.M., Talbott, G., Winn, R.K., Martin, T.R., Ulevitch, J., 1996. Antibodies against CD14 Protect Primates from Endotoxin-induced Shock. J. Clin. Invest. 98(7), 1533-1538.

447 27. Lopes, M.A., Salter, C.E., Vandenplas, M.L., Berghaus, R., Hurley, D.J., Moore, J.N.,
448 2010a. Expression of inflammation-associated genes circulating leukocytes collected
449 from horses with gastrointestinal tract disease. Am. J. Vet. Res. 71, 915-924.
450 28. Lopes, M.A., Salter, C.E., Vandenplas, M.L., Berghaus, R., Hurley, D.J., Moore, J.D.,
451 2010b. Expression of genes associated with inflammation induced by ex vivo
452 exposure to lipopolysaccharide in peripheral blood leukocytes from horses with
453 gastrointestinal disease. Am. J. Vet. Res. 71, 1162-1169.
454 29. Mackay, C.R., 2001. Chemokines: immunology's high impact factors. Nat. Immunol.
455 2 (2), 95-101.
456 30. Mackay, R.J., 2009. Endotoxemia. In: Smith, B. (Ed), Large Animal Internal
457 Medicine. 4. edn., Mosby Elsevier, Missouri, pp. 711–723.
458 31. Marchant, A., 1998. Interleukin-10 production during septicaemia. Lancet 343, 707-
459 708.
460 32. Moore, J.N., Barton, M.H., 1998. An update on endotoxaemia. Part 1: mechanism and
461 patways. Equine Vet. Educ. 10(6), 300-306.
462 33. Moore, J.N., Vandenplas, M.L., 2014. Is it the systemic inflammatory response
463 syndrome or endotoxemia in horses with colic? Vet. Clin. North Am. Equine Pract.
464 30, 337-351.
465 34. Pusterla, N., Magdesian, G., Mapes, S., Leutenegger, C.M., 2006. Expression of
466 molecular markers in blood of neonatal foals with sepsis. Am. J. Vet. Res. 67(6),
467 1045-1049.
468 35. Riedel, S., 2012. Procalcitonin and the role of biomarkers in the diagnosis and
469 management of sepsis. Diagn. Microbiol. Infect. Dis. 73, 221–227.
470 36. Rieger, M., Kochleus, C., Teschner, D., Rascher, D., Barton, A.K., Geerlof, A.,
471 Kremmer, E., Schmid, M., Hartmann, A., Gehlen, H., 2014. A new ELISA for the

quantification of equine procalcitonin in plasma as potential inflammation biomarker in horses. Anal Bioanal Chem 2014;406:5507–5512.

37. Silva, A., Wagner, B., McKenzie, H.C., Desrochers, A.M., Furr, M.O., 2013. An investigation of the role of soluble CD14 in hospitalized, sick horses. Vet. Immunol. Immunopathol. 155, 264-269.

38. Sonnier, D.I., Bailey, S.R., Schuster, R.M., Gangidine, M.M., Lentsch, A.B., Pritts, T.A., 2012. Proinflammatory chemokines in the intestinal lumen contribute to intestinal dysfunction during endotoxemia. Shock 37(1), 63-69.

39. Stelter, F., 2000. Structure/function relationships of CD14. Chem. Immunol. 74, 25-41.

40. Sykes, B.W., Furr, M., Giguere, S., 2005. In vivo pretreatment with PGG- glucan fails to alter cytokine mRNA expression of equine peripheral blood mononuclear cells exposed to endotoxin ex vivo. Vet. Ther. 6, 67-76.

41. Sylvester, I., Suffredini, A.F., Boujoukos, A.J., Martich, G.D., Danner, R.L., Yoshimura, T., Leonard, E.J., 1993. Neutrophil attractant protein-1 and monocyte chemoattractant protein-1 in human serum. Effects of intravenous lipopolysaccharide on free attractants, specific IgG autoantibodies and immune complexes. J. Immunol. 151, 3292-3298.

42. Tadros, E.M., Frank, N., 2012. Effects of continuous or intermittent lipopolysaccharide administration for 48 hours on the systemic inflammatory response in horses. Am. J. Vet. Res. 73(9), 1394-1402.

43. Taylor, S., 2015. A review of equine sepsis. Equine Vet. Educ. 27(2), 99-109.

44. Toribio, R.E., Kohn, C.W., Leone, G.W., Capen, C.C., Rosol, T.J., 2003. Molecular cloning and expression of equine calcitonin, calcitonin gene-related peptide-I and calcitonin gene-related peptide II. Mol. Cell. Endocrinol. 199, 119-128.

497 45. Verbon, A., Dekkers, P.E., ten Hove, T., Hack, C.E., Pribble, J.P., Turner, T., Souza,
498 S., Axtelle, T., Hoek, F.J., van Deventer, S.J., van der Poll, T., 2001. IC14, an anti-
499 CD14 antibody, inhibits endotoxin-mediated symptoms and inflammatory responses
500 in humans. J. Immunol. 166(5), 3599-3605.
501 46. Wagner, B., Ainsworth, D.M., Freer, H., 2013. Analysis of soluble CD14 and its use
502 as a biomarker in neonatal foals with septicemia and horses with recurrent airway
503 obstruction. Vet. Immunol. Immunopathol. 155, 124-128.
504 47. Wagner, B., Burton, A., Ainsworth, D., 2010. Interferon-gamma, interleukin-4 and
505 interleukin-10 production by T helper cells reveals intact Th1 and regulatory T_R1 cell
506 activation and delay of the Th2 cell response in equine neonates and foals. Vet. Res.
507 41(4), 47.
508 48. Wagner, B., Freer, H., 2009. Development of a bead-based multiple assay for
509 simultaneous quantification of cytokines in horses. Vet. Immunol. Immunopathol.
510 127(3-4), 242-248.
511 49. Wagner, B., Robeson, J., McCracken, M., Wattrang, E., Antczak, D.F., 2005. Horse
512 cytokine/IgG fusion proteins–mammalian expression of biologically active cytokines
513 and a system to verify antibody specificity to equine cytokines. Vet. Immunol.
514 Immunopathol. 105, 1-14.
515 50. Watts, A.E., Ness, S.L., Divers, T.J., Fubini, S.L., Frye, A.H., Stokol, T., Cummings,
516 K.J., Brooks, M.B., 2014. Effects of clopidogrel on horses with experimentally
517 induced endotoxemia. Am. J. Vet. Res. 75(8), 760-769.
518 51. Werners, A.H., Bull, S., Fink-Gremmels, J., 2005. Endotoxemia: a review with
519 implications for the horse. Equine Vet. J. 37(4), 371-383.

52. Zisman, D.A., Kunkel, S.L., Strieter, R.M., Tsai, W.C., Bucknell, K., Wilkowski, J., Standiford, T.J., 1997. MCP-1 protects mice in lethal endotoxemia. J. Clin. Invest. 99(12), 2832-2836.

Figure Legends

Figure 1

Curve of plasma sCD14 (ng/ml) concentrations for each blood-sampling time (T0, T1, T2, T3, T24) in a group of healthy horses (n=11) pre- and after LPS infusion (*E. coli* 055:B5 endotoxin at 30 ng/kg dose over 30 minutes). Plasma sCD14 concentrations for each horse was graphically represented using different color, pattern line and symbols. See the legend in the figure for more details.

Figure 2

Curve of plasma CCL-2 (pg/ml) concentrations for each blood-sampling time (T0, T1, T2, T3, T24) in a group of healthy horses (n=11) pre- and after LPS infusion (*E. coli* 055:B5 endotoxin at 30 ng/kg dose over 30 minutes). Plasma CCL-2 concentrations for each horse was graphically represented using different color, pattern line and symbols. See the legend in the figure for more details.

Figure 3

Curve of plasma IL-10 (MFI) concentrations for each blood-sampling time (T0, T1, T2, T3, T24) in a group of healthy horses (n=11) pre- and after LPS infusion (*E. coli* 055:B5 endotoxin at 30 ng/kg dose over 30 minutes). Plasma IL-10 concentrations for each horse was

graphically represented using different color, pattern line and symbols. See the legend in the figure for more details.

Figure 4

Curve of plasma PCT (pg/ml) concentrations for each blood-sampling time (T0, T1, T2, T3, T24) in a group of healthy horses (n=11) pre- and after LPS infusion (*E. coli* 055:B5 endotoxin at 30 ng/kg dose over 30 minutes). Plasma PCT concentrations for each horse was graphically represented using different color, pattern line and symbols. See the legend in the figure for more details.

554 **Table 1.** Average and standard deviation of white blood cell count (WBC), heart rate (HR),
555 respiratory rate (RR) and body temperature (BT) in each blood-sampling time (pre-infusion
556 and T1, T2, T3, T24 hours post infusion blood-sampling time). Legend: within rows different
557 superscripts denote a significant difference (a≠b: $p<0.05$).
558

	Blood-sampling time					
	T0	**T1**	**T2**	**T3**	**T24**	
WBC (10^9 cells/L)	8.2 ± 0.7^{a}	2.2 ± 0.5^{b}	3.2 ± 0.9^{b}	4.3 ± 0.8^{ab}	10.3 ± 1.5^{a}	P<0.05
HR (bpm)	37.3 ± 4.4^{a}	56.4 ± 10.3^{b}	51.5 ± 7.5^{a}	43.6 ± 4.5^{a}	41.6 ± 5.1^{a}	P<0.05
RR (bpm)	16.2 ± 4.8^{a}	23.6 ± 11.1^{a}	19.4 ± 7.8^{b}	27.4 ± 19.6^{b}	15.3 ± 4.0^{a}	P<0.05
BT (°C)	37.3 ± 0.3^{a}	37.9 ± 0.4^{b}	38.5 ± 0.6^{b}	38.8 ± 0.4^{b}	37.3 ± 0.4^{a}	P<0.05

559
560 **Table 2.** Average and standard deviation of plasma PCT, sCD14 and IL-10 concentrations in
561 each blood-sampling time (pre-infusion and T1, T2, T3, T24 hours post infusion blood-
562 sampling time). Legend: within rows different superscripts denote a significant difference
563 (a≠b: $p<0.05$).
564

	Blood-sampling time					
	T0	**T1**	**T2**	**T3**	**T24**	
sCD14 (ng/ml)	2011±405.2	2144±369.6	2191±431.6	2089±361.2	2134±377.4	NS
CCL2 (pg/ml)	223.7 ± 281.7^{a}	7059 ± 3536^{ab}	62168 ± 80738^{b}	42026 ± 20964^{b}	445.2 ± 392.1^{a}	p<0.05
IL-10 (MFI)	31.2 ± 15.6^{a}	97.4 ± 67.8^{b}	97.3 ± 54.4^{b}	99.2 ± 57.0^{b}	33.2 ± 19.7^{a}	p<0.05
PCT (pg/ml)	28.5 ± 23.3^{a}	193.0 ± 144.5^{b}	219.1 ± 143.9^{b}	239.7 ± 138.6^{b}	271.0 ± 131.9^{b}	p<0.05

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