Expression of interleukin-1β, interleukin-8, and interferon-γ in blood samples obtained from healthy and sick neonatal foals

Carolina Castagnetti, DVM, PhD; Jole Mariella, DVM, PhD; Alessandro Pirrone, DVM, PhD; Stefano Cinotti, DVM; Gaetano Mari, DVM; Angelo Peli, DVM, PhD

Objective—To evaluate and compare the gene expression of interleukin(IL)-1β, IL-8, and interferon-γ during the first 72 hours after birth in healthy foals and during the first 72 hours after hospitalization in sick neonatal foals and investigate correlations of clinicopathologic variables with cytokine expressions in healthy and sick neonatal foals.

Animals—33 foals < 7 days old (10 healthy foals, 7 foals with sepsis, 6 foals with peripartum asphyxia syndrome, and 12 foals with other diseases [2 with failure of passive transfer of immunity only were not further evaluated]).

Procedures—A blood sample (15 mL) was collected from each foal immediately after birth or hospital admission (0 hours) and at 24 and 72 hours later. Clinicopathologic variables were evaluated, and cytokine gene expression in WBCs was measured with an absolute quantitative real-time reverse transcriptase PCR assay.

Results—At all time points, gene expression of interferon-γ was low in all groups. No time-dependent changes in cytokine expressions were detected in healthy or sick foals. Foals with sepsis had significantly higher IL-1β gene expression than did healthy foals, foals with peripartum asphyxia syndrome, or foals with other diseases. At 0 hours, IL-1β expression was correlated with plasma fibrinogen concentration in healthy foals and with the neutrophil-to-lymphocyte ratio in foals with sepsis; IL-8 expression was correlated with monocyte count in foals with sepsis and with arterial pH, plasma fibrinogen concentration, and plasma lactate concentration in foals with peripartum asphyxia syndrome.

Conclusions and Clinical Relevance—Data have suggested that evaluation of IL-1β expression in sick neonatal foals could help identify those with sepsis. (*Am J Vet Res* 2012;73:1418–1427)

It is well-known that perinatal infections and PAS are the major causes of morbidity among equine neonates. An in-depth understanding of the pathophysiology of these diseases could help generate new diagnostic and prognostic tools that would have a major impact on equine medicine.

Cytokines are endogenous inflammatory mediators and integral components of the adaptive and innate immune responses. They may be classified as proinflammatory (eg, IFN-γ, TNF, IL-1, IL-2, IL-6, and IL-8) or anti-inflammatory (eg, IL-4, IL-5, IL-10, IL-11, IL-13, and TGF-β). The balance between pro- and anti-inflammatory cytokines is critical to allow the reestablishment of homeostasis after an inflammatory insult. Proinflammatory cytokines mainly have host-protective actions, but accentuation of their normal protective functions may lead to damaging effects on the integrity of the host organism. Ultimately, the deleterious events resulting from the derangement of the normal function of the cytokine network may lead to shock, multiorgan failure, and death.[1,2]

In adult horses, as in other species, TNF-α is involved in early stages of inflammation and is responsible for the induction of IL-1.[3] A marked increase in

Abbreviations

cDNA	Complementary DNA
FPT	Failure of passive transfer of immunity
IFN	Interferon
IL	Interleukin
MAP	Mean arterial blood pressure
N:L	Neutrophil-to-lymphocyte
PAS	Peripartum asphyxia syndrome
PBMC	Peripheral blood mononuclear cell
TGF	Transforming growth factor
TNF	Tumor necrosis factor

Received November 15, 2010.
Accepted July 22, 2011.
From the Department of Veterinary Medical Sciences, Faculty of Veterinary Medicine, University of Bologna, 40064 Ozzano Emilia, Bologna, Italy.
Supported by funds of the University of Bologna.
Presented in abstract form at the 54th American Association of Equine Practitioners Annual Convention, San Diego, December 2008.
Address correspondence to Professor Castagnetti (carolina.castagnetti@unibo.it).

serum TNF-α activity seems to be associated with higher mortality rate in some clinical colic cases.[4] Serum activity of IL-6 increases in horses after experimental infusion of lipopolysaccharide[5,6]; increases in the blood or peritoneal fluid during acute abdominal disease have also been detected.[7] In the latter study,[7] high IL-6 activity in serum or peritoneal fluid was correlated with mortality rate, whereas high serum TNF activity was not correlated.

Previous clinical studies of cytokines in neonatal foals have revealed that serum TNF activity is correlated with clinical criteria of sepsis and disease severity[8] and that IL-6 and TNF-α concentrations increase in foals that receive an infusion of lipopolysaccharide.[9,10] Boyd et al[11] studied the temporal changes in PBMC cytokine expression in 72 foals and found that the expression of IFN-γ, IL-1α, and TGF-1 increased significantly during the first month after birth. Particularly, IFN-γ expression was low at birth but more than doubled from the day of birth to day 28 after birth, suggesting a weaker helper T-cell 1 response during the perinatal period or a subsequent exposure to environmental antigens. Transforming growth factor-β1 is a chemoattractant for neutrophils and monocytes, but it also limits inflammatory responses and promotes wound healing.[12] Interestingly, in the study of Boyd et al,[11] expression of TGF-β1 was less in foals that developed clinical signs of infectious disease, compared with findings in foals that did not. In the same study,[11] IL-2, IL-4, IL-6, IL-10, or TNF-α expression did not significantly differ with age, but IL-1β and IL-8 expression appeared to increase with age. Interleukin-1 plays an important role in nonspecific immunologic defense, and IL-8 is an important proinflammatory chemokine for neutrophil migration.

It has been shown that lymphocytes from newborn foals have a profound deficiency in both IFN-γ gene expression and IFN-γ protein production in vitro.[13] The production of IFN-γ approached a normal adult level when foals reached approximately 3 months of age. The inability to express the IFN-γ gene and produce the IFN-γ protein may explain the susceptibility of newborn foals to intracellular pathogens.

In a study of molecular markers in blood samples obtained from sick and healthy neonatal foals, Pusterla et al[14] found that IL-10 expression was significantly greater in nonsurviving neonatal foals, compared with findings in sick foals that did survive. Expression of TNF-α or TGF-β was lower and expression of IL-8 was significantly greater in sick foals with or without sepsis, compared with findings in healthy foals.[14] Expression of IL-1β, IL-6, or procalcitonin did not differ between sick and healthy foals.[14] These results suggest that foals with sepsis have an immunosuppressive status.

Gold et al[15] investigated the gene expression of IFN-γ, IL-1β, IL-6, IL-4, and IL-8 as well as the gene expression of toll-like receptor 4 in PBMCs isolated from 21 foals with sepsis during the first 72 hours after hospital admission and from 20 healthy neonatal foals at comparable ages. The results of that study indicated that at admission, toll-like receptor 4 gene expression was upregulated in foals with sepsis; such upregulation may enhance proinflammatory cytokine production. Compared with findings for healthy neonatal foals, there was a 6-fold decrease in IL-4 gene expression in foals with sepsis at the time of hospital admission; however, IFN-γ, IL-1β, IL-6, and IL-8 gene expression did not differ between the 2 groups of foals. In 3 foals with sepsis that died, there was a 15-fold increase in IL-6 expression at admission, compared with findings in foals with sepsis that survived.[15]

To our knowledge, reports of studies regarding cytokine expression in neonatal foals with PAS are lacking. The objective of the study reported here was to determine the temporal gene expression of IL-1β, IL-8, and IFN-γ in healthy foals during the first 72 hours after birth and in sick neonatal foals during the first 72 hours of hospitalization and compare gene expression of the 3 cytokines in the 2 groups of foals at all sample collection times. In addition, differences in cytokine gene expression among healthy foals, foals with sepsis, foals with PAS, and foals with other diseases were evaluated, and the correlation of gene expression of any of the 3 cytokines with clinicopathologic variables in healthy and in sick neonatal foals was investigated. We hypothesized that gene expression of the cytokines would be higher in sick neonatal foals than in healthy neonatal foals and that foals with PAS but no sepsis would have different patterns of gene expression of these cytokines, compared with findings in neonatal foals with sepsis. Another hypothesis was that gene expression of one or more of the cytokines would correlate with one or more of the recorded clinicopathologic variables.

Materials and Methods

Animals—All procedures performed on the foals were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, in accordance with DL 116/92, and approved by the Ministry of Health. Oral informed consent for foal participation was given by the owners. Foals included in the study were classified as healthy or sick. The healthy foals were the offspring of mares referred to the Equine Perinatology Unit Stefano Belluzzi of the University of Bologna for assisted delivery. Healthy foals had an Apgar score ≥ 9,[16] and results of clinical evaluation were considered normal; at 24 hours after birth, serum IgG concentration was > 800 mg/dL. Sick foals were either born at the Equine Perinatology Unit or were referred after birth (< 7 days old). All sick foals born at the Equine Perinatology Unit were also assigned an Apgar score to evaluate the neonates' vitality.[16]

Data collection immediately after birth or at hospital admission—For healthy and sick foals, the following data were recorded at 0 hours (ie, immediately after birth or at the time of hospital admission): mare's age and parity; foal's gestational age, breed, and sex; age at time of evaluation; rectal temperature; WBC count; lymphocyte, monocyte, and neutrophil counts; N:L ratio (calculated); plasma lactate, serum magnesium, and plasma fibrinogen concentrations; and results of arterial blood gas analysis (pH, $Paco_2$, and Pao_2). All sick foals received a complete and standardized clinicopathologic evaluation at 0 hours, and the following additional data were recorded: duration of clinical signs before hospitalization, heart and respiratory rates, MAP,

results of microbial culture of a blood sample, blood glucose concentration, and serum IgG concentration. Medications administered prior to or during hospitalization were also recorded. The sepsis score of each sick foal was calculated from the data obtained at 0 hours, as described by Brewer and Koterba.[17]

The mare's age and parity, foal's gestational age, foal's age, and duration of clinical signs before hospitalization were recorded from information provided by the owner. The MAP was measured indirectly[a] via a cuff over the coccygeal artery.

For each sick foal, microbial culture of a blood sample was performed regardless of prereferral antimicrobial treatment. Ten milliliters of blood was withdrawn from a jugular vein after clipping of hair and aseptic preparation of the skin. The sampling needle was then discarded, and a new needle was used to inoculate the blood into the commercially available culture bottle.[b]

Serum IgG concentration[c] was measured at the time of hospital admission if the sick foals were at least 18 hours of age[18]; otherwise, testing was performed when the foals attained that age. A 12.5-mL blood sample was placed into plastic vials[d] containing serum separation gel (for serum biochemical analysis), EDTA (for hematologic and immunoglobulin evaluation), or sodium citrate (for plasma fibrinogen concentration assessment). Blood (1.2 mL) was also collected into a tube containing sodium fluoride and potassium oxalate for evaluation of lactate concentration. The samples were delivered to the laboratory within 30 minutes after collection. After centrifugation, samples of serum and plasma were processed or were stored at –20°C until analyses. All analyses were performed within a 2-month period after collection.

Hematologic assessments were performed with a commercial semiautomated multichannel blood cell-counting system,[e] and differential counts were performed by manual cytologic examination of blood smears. Biochemical variables were evaluated with a commercial automated analyzer.[f] Blood glucose concentration was determined with a rapid method.[g] For blood gas analysis, an arterial blood sample (1 mL) was collected anaerobically from a dorsal metatarsal artery into a 1-mL plastic syringe, the dead space of which was filled with heparin sodium (1,000 U/mL). Blood gases, electrolytes, base excess, and anion gap were evaluated with an automated blood gas analyzer[h] within 10 minutes after sampling. Blood gas and pH were corrected for divergence of the temperature of the analyzer (37°C) from the rectal temperature of the foal at the time of sample collection.

All data were collected by 1 of the 2 admitting clinicians (CC and JM). A foal was classified as having sepsis on the basis of positive results of microbial culture of the blood sample, positive results of culture of pathogens from local sites of suspected infection, or findings of a postmortem examination. A foal was defined as unclassifiable if it had diarrhea of unknown etiology, a clinical response to antimicrobials without documented infection, or clinical evidence of inflammation without documented infection. A foal was classified as having PAS on the basis of anamnesis and clinical signs, especially neurologic signs,[19] after other causes of neurologic disease such as meningitis had been ruled out. A foal was classified as premature when born prior to 320 days of gestation and as dysmature when born after 320 days of gestation but with immature physical characteristics (eg, low birth weight and inability to maintain body homeostasis).[19] During the hospitalization, all sick foals were treated on the basis of the clinical signs and additional clinical data were collected to address the therapeutic choices. All nonsurviving foals underwent postmortem examination to confirm the diagnosis.

Cytokine expression evaluation—For each foal, a blood sample (2.6 mL) was obtained from a jugular vein for cytokine expression evaluation in the immediate postpartum period or at admission to the hospital (0 hours) and at 24 and 72 hours thereafter. Each jugular blood sample was collected into a tube containing EDTA and was overlaid on sterile gradient density medium[i] for isolation of PBMCs.

Total cellular RNA from PBMCs was extracted by use of a total RNA isolation reagent[j] according to the manufacturer's instructions, and the concentrations of IL-1β, IFN-γ, and IL-8 gene products were determined by a real-time PCR assay. On the basis of previous studies[15,20,21] to characterize cytokine profiles in PBMCs of healthy and sick foals, the β-actin gene was used as an internal reference gene to normalize the variation of cell numbers in the samples. The β-actin primers were chosen on the basis of the partial equine mRNA sequence as used by Giguère and Prescott.[22] The primer pair for each cytokine was designed by use of software[k] on the basis of published sequences (**Appendix**). The RNA extracted from PBMCs of individual foals underwent reverse transcription into first-strand cDNA with random hexamers and purified avian myeloblastosis virus reverse transcriptase[l] in 20 µL of reaction after a DNAse treatment and stored at –80°C until use.

The PCR reactions were performed with the following cycle conditions: a denaturation step for 15 minutes at 95°C and 40 cycles of 30-second denaturation at 94°C, 30 seconds of annealing (IL-1β at 55°C, IL-8 at 55°C, IFN-γ at 55°C, and β-actin at 56°C), 15 seconds at 72°C, and 7 minutes of final extension at 72°C. The PCR products were visualized by electrophoresis on agarose gel 2% stained by ethidium bromide. The purified PCR products were cloned into a plasmid vector, transformed into an *Escherichia coli* kit, and purified with a kit[m] containing reagents needed to isolate plasmid DNA.

The recombinant plasmid was linearized upstream of the target sequence by use of the restriction endonuclease *Pme*I.[n] Ten-fold dilutions of recombinant plasmid from 10^6 copies to 10^1 copies were used as standards.

The real-time PCR assay was developed and evaluated on a real-time DNA detection system.[o] The reaction was performed in a final volume of 25 µL containing 1X premix reagent,[p] 200nM each of forward and reverse primers, 1X reference dye,[q] and 2 µL of cDNA. Cycling conditions were as follows: 10 minutes at 95°C for polymerase activation, followed by 40 cycles of 15 seconds at 95°C, 15 seconds of annealing (IL-1β at 55°C, IL-8 at 55°C, IFN-γ at 55°C, and β-actin at 56°C), and 20 seconds at 72°C.

The signal was acquired on the 6-carboxyfluorescein (FAM) channel (multichannel machine; source, 470 nm; detector, 510 nm; gain set to 5), with the fluorescence reading obtained at the end of each 72°C step. All of the data were then expressed as the ratio between copies of the target gene and copies of the reference gene β-actin.[23]

A melt step was added after a cycling run performed with the same conditions of the SYBR Green assay.[p] During the melt cycle, the temperature was increased by increments of 1°C from 72° to 95°C, and the signal was acquired on the FAM channel (source, 470 nm; detector, 510 nm; gain set to 5).

To distinguish specific from nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 72°C for 12 seconds, followed by a gradual increase in temperature to 95°C at a rate of 0.1°C/s, with the fluorescence signal acquisition mode set to step. In fact, melting curve analysis is considered essential in designing an efficient and specific quantitative PCR assay.

Statistical analysis—All data were evaluated for normal distribution with a Shapiro-Wilk normality test before statistical analyses were performed. Time-dependent changes in cytokine gene expressions were analyzed by the nonparametric Friedman test in healthy foals, all sick foals, and foals with various diseases (sepsis, PAS, and other).

The Kruskal-Wallis test was used to compare the gene expression among the 3 cytokines at all sample collection times in healthy foals and foals with various diseases (sepsis, PAS, and other). The Kruskal-Wallis test was used also to evaluate the differences among healthy foals and foals with various diseases (sepsis, PAS, and other) at all sample collection times.

On the basis of data distribution, the Pearson or the Spearman rank correlation test was used to evaluate the correlation of gene expression of the 3 cytokines immediately after birth or at admission to the hospital and the recorded data (Apgar score; mare's age and parity; gestational age; foal's age; duration of clinical signs; rectal temperature; heart and respiratory rates; MAP; WBC count; neutrophil, lymphocyte, and monocyte counts; N:L ratio; plasma fibrinogen, serum magnesium, and plasma lactate concentrations; arterial pH; Pa_{O_2}; Pa_{CO_2}; and sepsis score). All analyses were performed with commercial software,[r] and values of $P < 0.05$ were considered significant.

Results

Foals—Thirty-three foals (10 healthy foals and 25 sick foals) were initially included in the study. The breed distribution of foals reflected that of the local equine population: Standardbred (n = 23), Italian Saddlebred (4), Arabian (4), Appaloosa (2), Quarter Horse (1), and Paint (1). It was determined that 2 Arabian foals had FPT only, and were not further evaluated; data from these foals were not included in the descriptive statistics or any analyses.

The mean age of the 10 healthy foals was 0 hours. The mean age of the 23 sick foals at time of admission to the hospital was 41 hours. Two sick foals were born at the Equine Perinatology Unit, and 21 foals were referred after birth. Clinical diagnoses for the sick foals included sepsis (n = 7), PAS (6), meconium impaction (2), dysmaturity (2), congenital malformation (2), and umbilical hematoma (1). The remaining 3 foals had weakness of undetermined etiology.

Table 1—Clinical and laboratory findings (including mare's age and parity [median ± SD]) for healthy and sick foals at the time of birth or admission to the hospital (0 hours).

Variable	Healthy foals (n = 10)	Foals with sepsis (n = 7)	Foals with PAS (n = 6)	Foals with other diseases (n = 10*)
Mare's age (y)	14 ± 4	13 ± 5	15 ± 6	12 ± 8
Mare's parity	4 ± 3	5 ± 4	6 ± 4	5 ± 2
No. of males/No. of females	5/5	3/4	3/3	6/4
Gestational age (d)	349 ± 8	332 ± 4	338 ± 7	339 ± 23
Rectal temperature (°C)	38 ± 0.5	38.5 ± 0.2	37.9 ± 0.4	38.4 ± 0.5
Heart rate (beats/min)	—	116 ± 28	115 ± 15	113 ± 20
Respiratory rate (breaths/min)	—	30 ± 7	33 ± 17	44 ± 24
MAP (mm Hg)	—	67 ± 14	64 ± 7	75 ± 8
Duration of signs before hospitalization (h)	—	13 ± 8	15.5 ± 16.5	8.5 ± 8.5
Sepsis score	—	11.3 ± 4.3	4.2 ± 2.8	6.6 ± 4.6
WBCs (cells ×10^3)	8,460 ± 1,960	5,700 ± 4,400	8,510 ± 2,140	8,290 ± 4,300
Lymphocytes (cells ×10^3)	1,720 ± 580	1,380 ± 850	1,390 ± 470	1,680 ± 1,170
Monocytes (cells ×10^3)	95 ± 70	460 ± 620	1,170 ± 1,980	550 ± 460
Neutrophils (cells ×10^3)	6,580 ± 2,150	3,870 ± 3,830	5,960 ± 3,440	6,780 ± 3,340
N:L ratio	5.6 ± 6.8	2.5 ± 1 .5	5.5 ± 2.6	5.4 ± 4.0
Plasma lactate (mmol/L)	3.4 ± 1.1	0.9 ± 1.4	0.7 ± 0.5	1.1 ± 2.3
Serum magnesium (mmol/L)	0.65 ± 0.08	0.82 ± 0.49	0.90 ± 0.16	0.41 ± 0.20
Plasma fibrinogen (g/L)	3.2 ± 0.6	5.0 ± 2.7	3.2 ± 0.9	4.0 ± 1.7
Blood glucose (mmol/L)	—	5.2 ± 2.9	6.9 ± 3.0	7.1 ± 3.0
Arterial blood pH	7.37 ± 0.0	7.35 ± 0.04	7.35 ± 0.03	7.38 ± 0.03
Pa_{CO_2} (mm Hg)	53.8 ± 5.8	46.6 ± 4.5	51.3 ± 9.0	49.9 ± 4.9
Pa_{O_2} (mm Hg)	40.7 ± 12.8	78.5 ± 15.2	77.5 ± 11.2	70.6 ± 12.7

*Two other foals initially included in the study had FPT only and were not further evaluated; no data from those animals were used in any analyses

— = Not applicable.

Clinical and laboratory findings for healthy and sick foals—For purposes of data analysis, sick foals were classified as foals with sepsis (n = 7), foals with PAS (6), and foals with other diseases (10, including the 3 foals for which a diagnosis was not made). Total FPT (serum IgG concentration < 400 mg/dL) was detected in 5 of the sick foals and partial FPT (serum IgG concentration > 400 mg/dL but < 800 mg/dL) in 2 foals. In 6 foals, serum IgG concentration was not determined at time of admission to the hospital because the foals were < 18 hours old. When they reached 18 hours of age, 2 foals had serum IgG concentration < 400 mg/dL and 4 foals had serum IgG concentration > 800 mg/dL. For each of the healthy foals, serum IgG concentration at 24 hours after birth was > 800 mg/dL. Clinical and laboratory findings for healthy and sick foals were summarized (**Table 1**).

Microbial culture of blood samples obtained at the time of admission to the hospital from 7 foals with sepsis yielded positive results (growth of gram-negative organisms, n = 4; growth of gram-positive organisms, 3); for 1 foal, a synovial fluid sample was also collected, which yielded positive results following microbial culture. Among the 7 foals with sepsis, only 3 had a sepsis score ≥ 11 (scores of 14, 16, and 17).

One foal included in the other diseases group had received glucocorticoids prior to hospitalization but not during hospitalization. Two foals (1 foal with sepsis and 1 foal with other diseases) had been treated with flunixin meglumine (1 mg/kg, IM) prior to admission; 14 foals (7 foals with sepsis and 7 foals with other diseases) were treated with flunixin meglumine during the first 72 hours of hospitalization with the same dosage (0.25 mg/kg, IV, q 8 h). Three foals had received a plasma transfusion before admission to the hospital; 10 foals received a plasma transfusion during the first 72 hours after admission.

Five foals had been treated with antimicrobials before admission, and all were treated after admission with the same protocol (ampicillin [50 mg/kg, IV, q 6 h] and amikacin sulfate [30 mg/kg, IV, q 24 h]). Twenty-four foals survived, and only 1 sick foal died of septic shock.

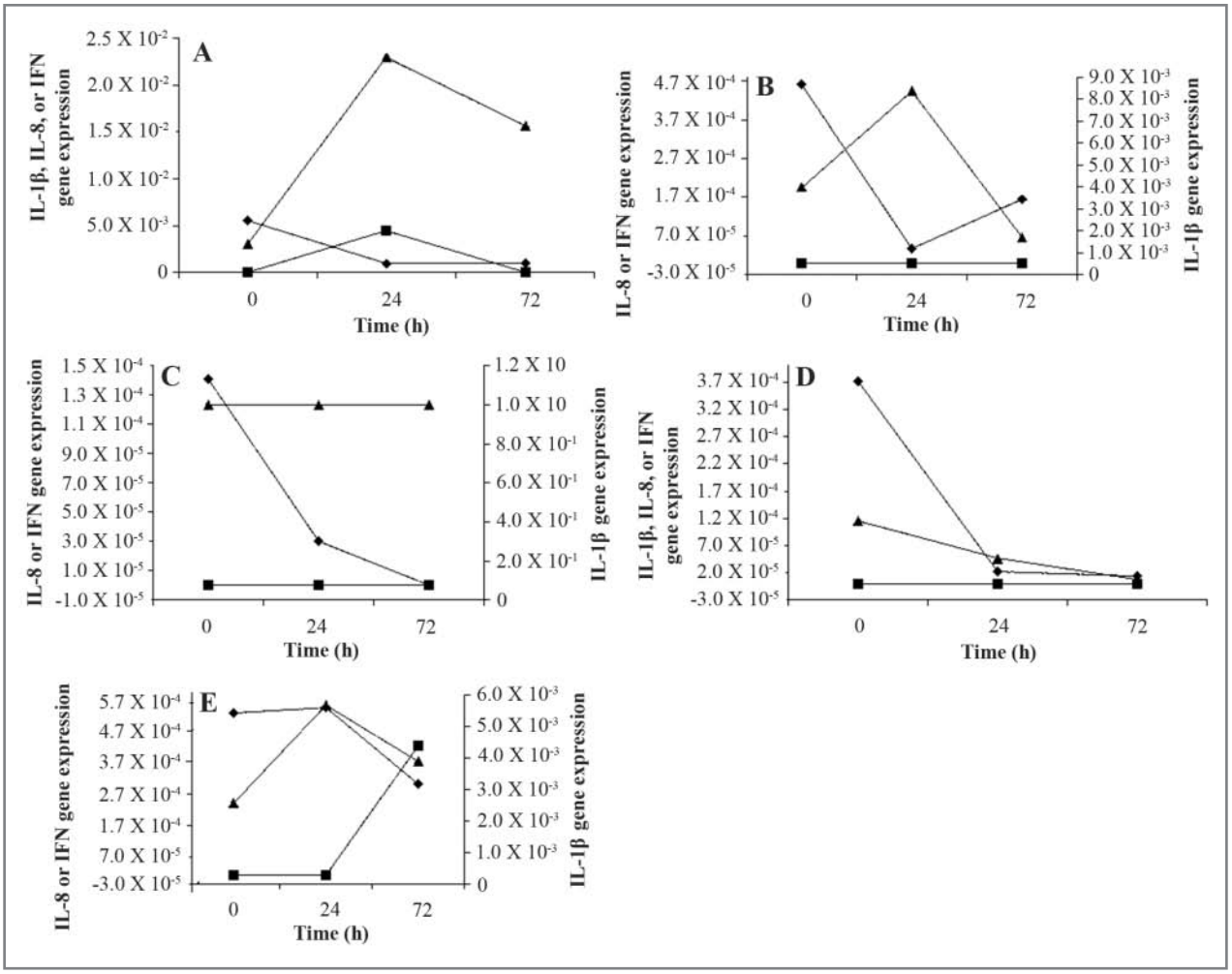

Figure 1—Gene expression of IL-1β (triangles), IL-8 (diamonds), and IFN (squares) in blood samples collected from 10 healthy foals (A) at birth and from 23 sick foals (B) at the time of birth or admission to the hospital (0 hours) and at 24 and 72 hours later. Sick foals (all < 7 days old) were classified into subgroups (foals with sepsis [C; n = 7], foals with PAS [D; 6], and foals with other diseases [E; 10 {2 other foals initially included in the study had FPT only and were not further evaluated; no data from those animals were used in any analyses}]). Gene expression is reported as the number of cDNA copies of cytokine/β-actin (housekeeping gene); IL-1β gene expression is marked on the right y-axis when present (B, C, and E).

Table 2—Data regarding gene expression of IL-1β, IL-8, and IFN-γ (median, minimum, and maximum values and SD of the number of cDNA copies of cytokine/β-actin [housekeeping gene]) in blood samples collected from healthy foals at birth and from sick foals (all < 7 days old) at the time of birth or admission to the hospital (0 hours) and at 24 and 72 hours later.

	IL-1β			IL-8			IFN-γ		
Foal group	**0 hours**	**24 hours**	**72 hours**	**0 hours**	**24 hours**	**72 hours**	**0 hours**	**24 hours**	**72 hours**
Healthy foals (n = 10)									
Median	1.8×10^{-4}	1.0×10^{-2}	7.2×10^{-3}	3.8×10^{-3}	5.2×10^{-4}	4.2×10^{-4}	0.0	1.8×10^{-3}	0.0
Minimum	0.0	0.0	8.8×10^{-4}	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	4.7×10^{-1}	6.3×10^{-2}	4.3×10^{-2}	1.3	1.3×10^{-2}	2.7×10^{-3}	4.2×10^{-5}	2.4×10^{-1}	2.3
SD	1.9×10^{-1}	2.8×10^{-2}	1.8×10^{-2}	5.1×10^{-1}	5.0×10^{-3}	1.3×10^{-3}	1.9×10^{-5}	1.1×10^{-1}	1.1
Sick foals (n = 23*)									
Median	4.0×10^{-3}	8.4×10^{-3}	1.7×10^{-3}	4.6×10^{-4}	3.8×10^{-5}	1.7×10^{-4}	0.0	0.0	0.0
Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	4.6×10^{3}	5.9×10^{3}	1.7×10^{3}	7.4×10	6.0	1.3×10	6.5	7.7×10^{-2}	2.7×10
SD	1.1×10^{3}	1.3×10^{3}	3.8×10^{2}	1.6×10	1.6	2.7	1.4	1.9×10^{-2}	5.6
Foals with sepsis (n = 7)									
Median	1.0	1.0	1.0	1.4×10^{-4}	3.0×10^{-5}	0.0	0.0	0.0	0.0
Minimum	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	4.6×10^{3}	5.9×10^{3}	1.7×10^{3}	7.4×10	6.0	1.3×10	6.5	7.7×10^{-2}	9.9×10^{-5}
SD	1.7×10^{3}	2.2×10^{3}	6.5×10^{2}	2.8×10	2.3	4.9	2.5	2.9×10^{-2}	3.7×10^{-5}
Foals with PAS (n = 6)									
Median	1.2×10^{-4}	4.6×10^{-5}	8.8×10^{-6}	3.7×10^{-4}	2.3×10^{-5}	1.5×10^{-5}	0.0	0.0	0.0
Minimum	3.5×10^{-6}	2.3×10^{-6}	0.0	5.5×10^{-6}	8.3×10^{-6}	0.0	0.0	0.0	0.0
Maximum	2.1×10^{-2}	2.2×10^{-3}	7.3×10^{-3}	3.6×10^{-2}	6.8×10^{-3}	9.6×10^{-4}	0.0	1.4×10^{-4}	0.0
SD	8.3×10^{-3}	8.6×10^{-4}	2.9×10^{-3}	1.5×10^{-2}	2.7×10^{-3}	3.9×10^{-4}	0.0	5.9×10^{-5}	0.0
Foals with other diseases (n = 10*)									
Median	2.6×10^{-3}	5.7×10^{-3}	3.9×10^{-3}	5.4×10^{-4}	5.6×10^{-4}	3.0×10^{-4}	0.0	0.0	4.3×10^{-4}
Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Maximum	2.6×10^{3}	2.1×10^{3}	6.7×10^{2}	5.5	4.9	8.7×10^{-2}	1.0	4.7×10^{-2}	2.7×10
SD	8.2×10^{2}	6.6×10^{2}	2.1×10^{2}	1.7	1.5	2.8×10^{-2}	3.2×10^{-1}	1.5×10^{-2}	8.5

For analysis, sick foals were classified into subgroups (foals with sepsis, foals with PAS, and foals with other diseases).
See Table 1 for key.

Cytokine expression evaluation—Blood samples were collected from all 33 foals at 0 hours (at birth or at the time of hospital admission), 24 hours, and 72 hours. With regard to β-actin, used as an internal reference gene, the ratio between the SD and the mean expression within each foal group was constant and did not differ by more than 2 SDs from the mean (1.7 in healthy foals, 1.8 in foals with sepsis, and 1.8 in foals with PAS), except for foals with other diseases (2.9). Time-dependent changes in IL-1β, IL-8, and IFN-γ gene expression were evident (**Figure 1**), and the median, minimum values, maximum values, and SD of cDNA copies of cytokine/β actin were summarized (**Table 2**).

Interferon-γ was expressed in much lower copy numbers than were the other 2 cytokines. Interleukin-1β gene expression in healthy foals at birth was evaluated in only 6 of 10 foals. Among all foals, the highest IL-1β and IL-8 copy numbers were detected in the nonsurviving foal. The highest IL-8 copy numbers in foals with PAS were detected in the foal with the most severe neurologic signs.

Data analysis—Among healthy foals, data that were normally distributed included Apgar score; mare's age; foal's gestational age; rectal temperature; WBC count; lymphocyte, monocyte, and neutrophil counts; serum magnesium concentration; arterial pH; $Paco_2$; and plasma lactate concentration. Among healthy foals, data that were not normally distributed included the mare's parity, N:L ratio, plasma fibrinogen concentration, Pao_2, and cytokine gene expression at all sampling times. Among sick foals, data that were normally distributed included the mare's age, rectal temperature, respiratory rate, WBC count, neutrophil and lymphocyte

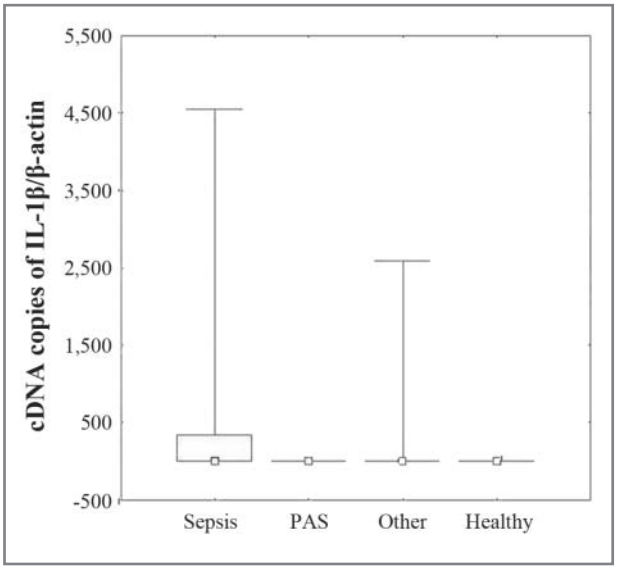

Figure 2—Box-and-whisker plots of IL-1β gene expression in blood samples collected from 10 healthy foals, 7 foals with sepsis, 6 foals with PAS, and 10 foals with other diseases. For each box, the square represents the median and the upper and lower boundaries represent the 75th and 25th percentiles, respectively. Whiskers represent the minimum and maximum values. Gene expression is reported as the number of cDNA copies of cytokine/β-actin (housekeeping gene).

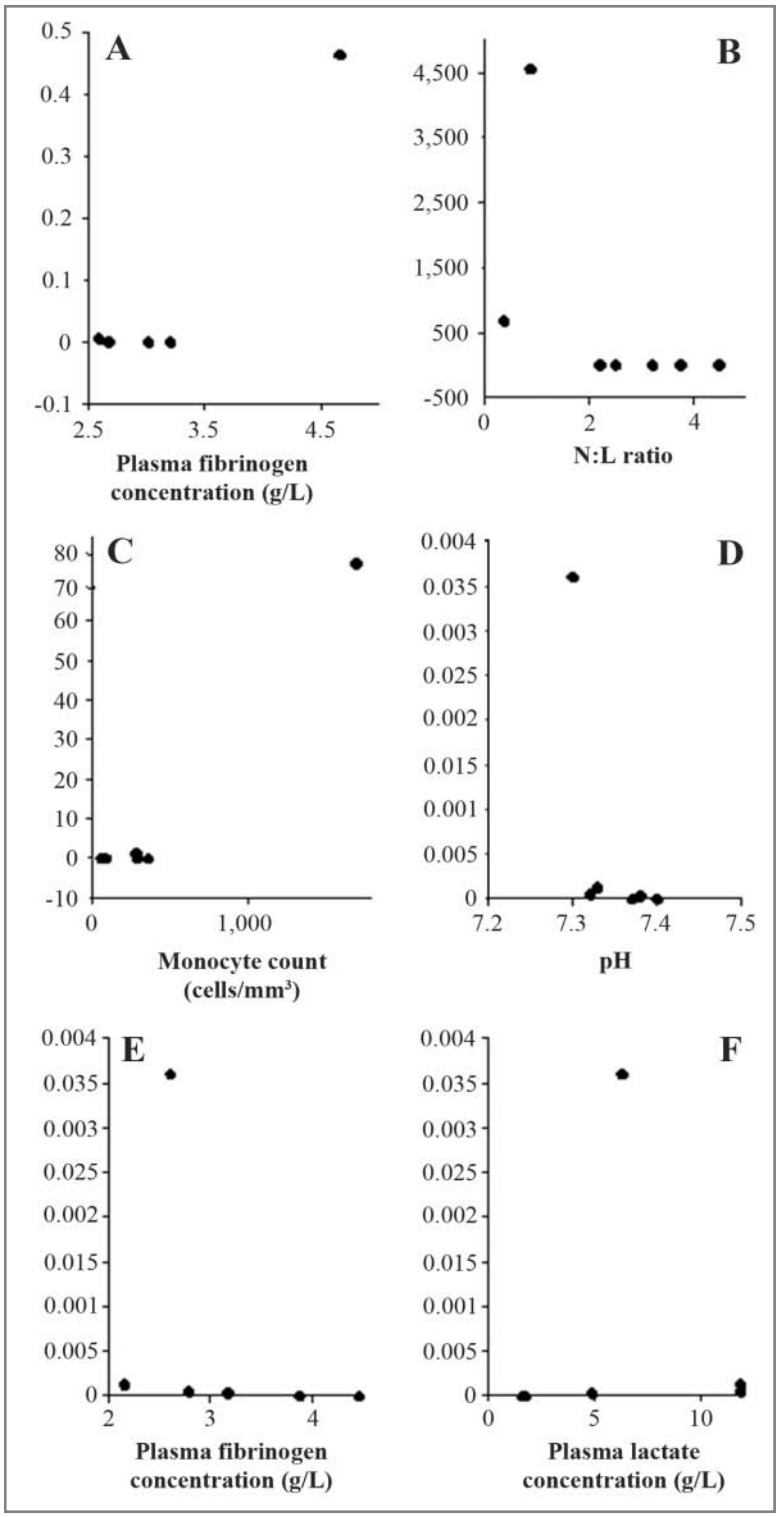

Figure 3—Plots of IL-1β gene expression against plasma fibrinogen concentration in 6 healthy foals (A [2 values are superimposed]); N:L ratio (B) and monocyte count (C [2 values are superimposed]) in 7 foals with sepsis; and arterial blood pH (D), plasma fibrinogen concentration (E), and plasma lactate concentration (F) in 6 foals with PAS. Data were obtained from healthy foals at birth and from sick foals at birth or at the time of admission to the hospital; all sick foals were < 7 days old. In healthy foals, IL-1β gene expression was correlated with plasma fibrinogen concentration ($r = 0.93$; $P < 0.05$). In foals with sepsis, IL-1β gene expression was correlated with N:L ratio ($r = –0.77$; $P < 0.05$) and with monocyte count ($r = 0.98$; $P < 0.01$). In foals with PAS, IL-1β gene expression was correlated with arterial blood pH ($r = –0.89$; $P < 0.05$), plasma fibrinogen concentration ($r = –0.89$; $P < 0.01$), and plasma lactate concentration ($r = 0.83$; $P < 0.05$).

counts, N:L ratio, Pa_{O_2}, and sepsis score. Among sick foals, data that were not normally distributed included mare's parity, foal's gestational age, foal's age, duration of clinical signs before hospitalization, heart rate, MAP, monocyte count, plasma fibrinogen and serum magnesium concentrations, arterial pH, Pa_{CO_2}, lactate concentration, blood glucose concentration, and cytokine gene expression at all sample collection times. Breed, sex, and serum IgG concentration were not evaluated statistically.

No time-dependent changes in IL-1β and IL-8 gene expressions were found in healthy foals during the first 72 hours after birth or in sick neonatal foals during the first 72 hours of hospitalization. In healthy foals, there were no significant differences in IL-1β or IL-8 gene expression at all sample collection times. Similarly, there were no significant differences in IL-1β or IL-8 gene expression at all sample collection times in foals with sepsis, PAS, or other diseases. In foals with sepsis, IFN-γ gene expression was significantly lower, compared with that of IL-1β, at 0 hours ($P < 0.05$), 24 hours, ($P < 0.01$), and 72 hours ($P < 0.05$). In foals with PAS, IFN-γ gene expression was significantly lower, compared with expressions of IL-1β and IL-8, at 0 hours (each $P < 0.01$), 24 hours, (each $P < 0.05$), and 72 hours (each $P < 0.01$). Because no time-dependent changes in IL-1β and IL-8 gene expressions were found, all results were considered together for the comparison between groups. Interleukin-1β gene expression in foals with sepsis was significantly ($P < 0.01$) higher than findings in healthy foals, foals with PAS, and foals with other diseases (**Figure 2**).

At birth, IL-1β gene expression was correlated with plasma fibrinogen concentration ($r = 0.93$; $P < 0.05$) in healthy foals (n = 6; **Figure 3**). In foals with sepsis (n = 7), IL-1β gene expression was correlated with the N:L ratio ($r = –0.77$; $P < 0.05$) and IL-8 gene expression was correlated with monocyte count ($r = 0.98$; $P < 0.01$) at time of admission or at birth. In foals with PAS (n = 6), IL-8 gene expression was correlated with arterial blood pH ($r = –0.89$; $P < 0.05$), plasma fibrinogen concentration ($r = –0.89$; $P < 0.01$), and plasma lactate concentration ($r = 0.83$; $P < 0.05$) at time of admission or at birth.

Discussion

As in previous investigations in foals,[11,13,15] IFN-γ was expressed at low levels at birth or in a short period thereafter in foals (healthy or sick) of the present study; moreover, IFN-γ gene expression did not differ between healthy foals and foals with

sepsis. This finding is in disagreement with results of a study[24] of preterm human infants with sepsis, in whom serum concentrations of IFN-γ and other cytokines increase and remain increased for at least 48 hours after sepsis develops.

In the present study involving foals < 7 days old, no time-dependent changes in expression of any cytokine were detected, which is similar to other reported findings.[15] Considering that blood samples were collected from healthy foals at birth and before colostrum consumption, it appears that colostrum intake does not influence cytokine gene expression. However, because of the low number of foals with total FPT (n = 5), we did not compare their cytokine gene expressions with those of foals with adequate serum IgG concentration. Breathnach et al[13] investigated whether colostrum itself was responsible for the observed IFN-γ suppression in young foals by comparing data from blood samples collected within 30 minutes after birth (prior to nursing) and again 36 hours later in 5 healthy foals. Unlike the results of the present study, an extremely low level of IFN-γ gene expression prior to colostrum ingestion was detected within 30 minutes after birth in that investigation[13]; 36 hours later, that gene expression had increased. On the basis of data from other species,[25–29] the effects of colostrum and milk ingestion on immune maturation in foals should be studied in greater depth, in our opinion. Goto et al[25] found that IL-1β could be transferred to newborn calves via ingestion of colostrum. In women, most of the cytokines that are known to be deficient in neonates, particularly in preterm infants, have been found in considerable amounts in human milk and are thought to provide immunologic benefit and to promote the development of the digestive system.[26–29]

The absence of time-dependent changes in cytokine expression in sick foals suggested that treatments administered during the first 72 hours of hospitalization did not influence cytokine expression. Also, it has been reported that plasma administration in foals with FPT does not seem to influence cytokine expression.[15] With regard to treatments administered before admission to the hospital in the present study, only the foal that received a corticosteroid dose had high expressions of IL1-β and IL-8 (2,594.24 cDNA copies/β-actin and 5.47 cDNA copies/β-actin, respectively) at 0 hours.

The results of the present study disagree somewhat with those of Pusterla et al[14] and Gold et al[15] in that our findings suggested that foals upregulate IL-1β gene expression in response to sepsis and that IL-1β gene expression is positively correlated with plasma fibrinogen concentration in healthy foals and negatively correlated with the N:L ratio in foals with sepsis. Because fibrinogen is an acute-phase protein, its production is stimulated by proinflammatory cytokines such as IL-1β.[1] A low N:L ratio in full-term sick foals is generally attributable to neutropenia; overzealous production of cytokines in overwhelming or uncontrolled sepsis induces margination of neutrophils, extravasation into affected tissues, or apoptosis, which results in neutropenia with or without a left shift.[30]

The differences between the results of the present study and those of the studies of Pusterla et al[14] and Gold et al[15] could reflect the different numbers of foals or a slightly different classification of foals with sepsis in each study. Also, different methods were used; in the present study, an absolute quantification of the amount of a target gene was performed, whereas in the other 2 studies, a comparative threshold cycle method was used.

In human medicine, serum IL-1β, IL-6, IL-8, and TNF-α concentrations are significantly higher in infants with sepsis, compared with findings in healthy infants,[31] although results of different studies are contradictory.[32,33] In our opinion, the difference in results could also reflect different phases of the disease; as stated by Lam and Ng,[34] IL-6 and IL-8 concentrations have a good diagnostic value as early-phase markers in infants with sepsis, although acute-phase reactants (eg, C-reactive protein and procalcitonin) are more useful during the later phases.

The large SDs of the values of IL-1β and IL-8 gene expression, mainly in foals with sepsis, could be related to the degree of sepsis in those foals, as suggested by Pusterla et al.[14] In the present study, the highest gene expression was measured in a nonsurviving foal with septic shock.

Serum concentrations of IL-8 in human infants with sepsis, necrotizing enterocolitis, other inflammatory states, and perinatal asphyxia are greater than those in healthy infants, and it has been suggested that large quantities of IL-8 have a trophic function in the developing human intestine.[27] In 1 study,[35] a high gene expression of IL-8 in umbilical cord blood was a sensitive marker of neonatal infection.

Unlike results of studies[36,37] of asphyxiated neonatal humans, foals with PAS in the present study did not express any cytokine at levels higher than those in healthy foals, but IL-8 gene expression was correlated with some variables that are typically altered in association with PAS (arterial blood pH and lactate concentration). It is worth noting that the foal with the more severe neurologic signs had the highest IL-8 gene expression among foals with PAS in the present study. Further studies with a larger number of foals with PAS are necessary. Fotopoulos et al[36] found that serum IL-8 concentration was significantly elevated in perinatally asphyxiated and perinatally infected human neonates during the first day after birth, compared with healthy controls, probably because of its passage from the ischemic brain of the affected neonates. It was suggested that inhibition of inflammatory chemokines could possibly contribute to the prevention of brain damage in neonates.[36]

The correlations between clinicopathologic data and cytokine gene expression identified in the present study could be influenced by data from the 2 foals with the highest IL-1β and IL-8 gene expressions. In accordance with previous studies that revealed an overall reduced variability of β-actin gene expression in PBMCs of adult horses[38] and neonatal foals,[15,20,21] results of the present study suggested that this housekeeping gene is expressed at more or less constant levels.

In the present study, we did not perform an evaluation of the prognostic value of cytokine expression because there was only 1 foal with sepsis that died. How-

ever, it is interesting to note that this foal had much higher values of IL-1β gene expression than did the other foals with sepsis.

Because of the rapid progression of diseases in neonates, it is well-known that the key to a successful outcome is early diagnosis and treatment. In the authors' opinion, once suitable reagents and detection systems have been developed, cytokine evaluation will be useful as a diagnostic and prognostic tool, as it is for human infants. After a complete clinical evaluation, while waiting for results of microbial culture of a blood sample, detection of a high level of IL-1β gene expression, together with other clinicopathologic data (eg, high plasma fibrinogen concentration and neutropenia), could help clinicians to more promptly diagnose disease in and initiate treatment of foals with sepsis.

a. Dynamap Pro Series 300, Critikon Co LLC, Tampa, Fla.
b. OXOID signal blood-culture system, Oxoid Ltd, Basingstoke, Hampshire, England.
c. SNAP Foal IDEXX, IDEXX Laboratories Inc, Milan, Italy.
d. S-Monovette, Sarstedt Inc, Verona, Italy.
e. CELL-DYN 3500R, Abbott Laboratories Inc, Santa Clara, Calif.
f. Chemistry Analyzer AU400, Olympus Diagnostica GmbH, Lismeehan, Ireland.
g. Medisense Optium, Abbott Laboratories Inc, Bedford, Mass.
h. Roche Opti CCA, Roche Diagnostic Corp, Indianapolis, Ind.
i. Ficoll-Paque, Amersham Bioscience Corp, Uppsala, Sweden.
j. Tri-reagent, Sigma-Aldrich, Deisenhofen, Germany.
k. Oligo, Molecular Biology Insights, Cascade, Colo.
l. Takara Bio Inc, Otsu, Japan.
m. Turbo Kit, QBIOgene Inc, Irvine, Calif.
n. MBI Fermentas Inc, Burlington, ON, Canada.
o. Rotor-Gene 3000 system, Corbett Research, Sydney, NSW, Australia.
p. SYBR PREMIX Ex Taq, Takara Bio Inc, Otsu, Japan.
q. ROX Reference Dye, Roche Applied Science, Indianapolis, Ind.
r. Analyse-it, version 2.03, Analyse-it Software Ltd, Leeds, West Yorkshire, England.

References

1. Dinarello CA. Proinflammatory cytokines. *Chest* 2000;118:503–508.
2. Tsiotou AG, Sakorafas GH, Anagnostopoulos G, et al. Septick shock; current pathogenetic concepts from a clinical perspective. *Med Sci Monit* 2005;11:RA76–RA85.
3. Morris DD, Crowe N, Moore JN. Correlation of clinical and laboratory data with serum tumor necrosis factor activity in horses with experimentally induced endotoxemia. *Am J Vet Res* 1990;51:1935–1940.
4. Morris DD, Moore JN, Crowe N. Serum tumor necrosis factor activity in horses with colic attributable to gastrointestinal tract disease. *Am J Vet Res* 1991;52:1565–1569.
5. Morris DD, Moore JN, Crowe N, et al. Effect of experimentally induced endotoxemia on serum interleukin-6 activity in horses. *Am J Vet Res* 1992;53:753–756.
6. MacKay RJ, Lester GD. Induction of the acute-phase cytokine, hepatocyte-stimulating factor/interleukin 6, in the circulation of horses treated with endotoxin. *Am J Vet Res* 1992;53:1285–1289.
7. Barton MH, Collatos C. Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J Vet Intern Med* 1999;13:457–464.
8. Morris DD, Moore JN. Tumor necrosis factor activity in serum from neonatal foals with presumed septicemia. *J Am Vet Med Assoc* 1991;199:1584–1589.
9. Robinson JA, Allen GK, Green EM, et al. Serum interleukin-6 concentrations in endotoxin-infused neonatal foals. *Am J Vet Res* 1993;54:1411–1414.
10. Allen GK, Green EM, Robinson JA, et al. Serum tumor necrosis factor alpha concentrations and clinical abnormalities in colostrum-fed and colostrum-deprived neonatal foals given endotoxin. *Am J Vet Res* 1993;54:1404–1410.
11. Boyd NK, Cohen ND, Lim W-S, et al. Temporal changes in cytokine expression of foals during the first month of life. *Vet Immunol Immunopathol* 2003;92:75–85.
12. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-β. *Annu Rev Immunol* 1998;16:137–161.
13. Breathnach CC, Sturgill-Wright T, Stiltner JL, et al. Foals are interferon gamma-deficient at birth. *Vet Immunol Immunopathol* 2006;112:199–209.
14. Pusterla N, Magdesian KG, Mapes S, et al. Expression of molecular markers in blood of neonatal foals with sepsis. *Am J Vet Res* 2006;67:1045–1049.
15. Gold JR, Perkins GA, Erb HN, et al. Cytokine profiles of peripheral blood mononuclear cells isolated from septic and healthy neonatal foals. *J Vet Intern Med* 2007;21:482–488.
16. Vaala WE, House JK, Madigan JE. Initial management and physical examination of the neonate. In: Smith BP, ed. *Large animal internal medicine*. 3rd ed. St Louis: Mosby, 2002;277–293.
17. Brewer BD, Koterba AM. Development of a scoring system for the early diagnosis of equine neonatal sepsis. *Equine Vet J* 1988;20:18–22.
18. LeBlanc MM. Immunologic considerations. In: Koterba AM, Drummond WH, Kosch PC, eds. *Equine clinical neonatology*. Philadelphia: Lea & Febiger, 1990;275–294.
19. Knottenbelt DC, Holdstock N, Madigan JE. Neonatal syndromes. In: Knottenbelt DC, Holdstock N, Madigan JE, eds. *Equine neonatology medicine and surgery*. Philadelphia: Saunders, 2004;155–363.
20. Svansson V, Roelse M, Olafsdòttir G, et al. Immune response against equine gammaherpesvirus in Icelandic horses. *Vet Microbiol* 2009;137:363–368.
21. Flaminio MJBF, Nydam DV, Marquis H, et al. Foal monocyte-derived dendritic cells become activated upon *Rhodococcus equi* infection. *Clin Vaccine Immunol* 2009;16:176–183.
22. Giguère S, Prescott JF. Quantitation of equine cytokine mRNA expression by reverse transcription-competitive polymerase chain reaction. *Vet Immunol Immunopathol* 1999;67:1–15.
23. Bowels KS, Beadle RE, Mouch S, et al. A novel model for equine recurrent airway obstruction. *Vet Immunol Immunopathol* 2002;87:385–389.
24. Ng PC, Li K, Wong RPO, et al. Pro-inflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. *Arch Dis Child Fetal Neonatal Ed* 2003;88:F209–F213.
25. Goto M, Maruyama M, Kitadate K, et al. Detection of interleukin-1β in sera and colostrum of dairy cattle and in sera of neonates. *J Vet Med Sci* 1997;59:437–441.
26. Srivastava MD, Srivastava A, Brouhard B, et al. Cytokines in human milk. *Res Commun Mol Pathol Pharmacol* 1996;93:263–287.
27. Maheshwari A, Lu W, Lacson A, et al. Effects of interleukin-8 on the developing human intestine. *Cytokine* 2002;20:256–267.
28. Takahata Y, Takada H, Nomura A, et al. Interleukin-18 in human milk. *Pediatr Res* 2001;50:268–272.
29. Chirico G, Marzollo R, Cortinovis S, et al. Antiinfective properties of human milk. *J Nutr* 2008;138:1801S–1806S.
30. MacKay R. Inflammation in horses. *Vet Clin North Am Equine Pract* 2000;16:15–27.
31. Kurt AN, Aygun AD, Godekmerdan A, et al. Serum IL-β, IL-6, IL-8, and TNF-α levels in early diagnosis and management of neonatal sepsis. *Mediators Inflamm* 2007;2007:31397.
32. Ng PC, Cheng SH, Chui KM, et al. Diagnosis of late onset neonatal sepsis with cytokines, adhesion molecule, and C-reactive protein in preterm very low birthweight infants. *Arch Dis Child Fetal Neonatal Ed* 1997;77:F221–F227.
33. Santana Reyes C, García-Muñoz F, Reyes D, et al. Role of cytokines (interleukin-1β, 6, 8, tumour necrosis factor-α, and soluble receptor of interleukin-2) and C-reactive protein in the diagnosis of neonatal sepsis. *Acta Paediatr* 2003;92:221–227.
34. Lam HS, Ng PC. Biochemical markers of neonatal sepsis. *Pathology* 2008;40:141–148.
35. Berner R, Tuxen B, Clad A, et al. Elevated gene expression of

interleukin-8 in cord blood is a sensitive marker for neonatal infection. *Eur J Pediatr* 2000;159:205–210.
36. Fotopoulos S, Mouchtouri A, Xanthou G, et al. Inflammatory chemokine expression in the peripheral blood of neonates with perinatal asphyxia and perinatal or nosocomial infections. *Acta Pediatr* 2005;94:800–806.
37. Okazaki K, Nishida A, Kato M, et al. Elevation of cytokine concentrations in asphyxiated neonates. *Biol Neonate* 2006;89:183–189.
38. Cappelli K, Felicetti M, Capomaccio S, et al. Exercise induced stress in horses: selection of the most stable reference genes for quantitative RT-PCR normalization. *BMC Mol Biol* 2008;9:49.

Appendix

Real-time PCR primers designed with software according to published gene sequences and used to assess gene expressions of IL-8, IFN-γ, and IL-1β in healthy and sick foals.

Target gene	Primer forward (5′-3′)	Primer reverse (5′-3′)	Frame (bp)	Reference sequence Genbank code
β-actin	CTGGCACCACACCTTCTACAACGAG	TCACCGGAGTCCATCACGA	214	AF035774.1
IL-8	TCTCTTGGCCGTCTTCCTG	CCGTTGACGAGCTTTACAA	195	AY184956.1
IFN-γ	GTGTGCGATTTTGGGTTCTTCTA	TTGAATGACCTGGTTATCT	235	D28520.1
IL-1β	GAGGCAGCCATGGCAGCAGTA	TGTGAGCAGGGAACGGGTATCTT	257	D42165.1