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Monitoring of inflammatory blood biomarkers in foals with *Rhodococcus Equi pneumonia* during antimicrobial treatment

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ABSTRACT

Rhodococcus equi (R. equi), a gram-positive facultative intracellular pathogen, is a common cause of pneumonia in foals and represents a major cause of disease and death. The aim of the present study was to investigate the time-depended changes in White Blood Cells (WBC), basophils (Baso), neutrophils (Neu), lymphocytes (Lymf), monocytes (Mon), eosinophils (Eos), platelet (PLT) counts, fibrinogen (Fbg) concentration, interferon (IFN-α, IFN-γ) and interleukins (IL-2 and IL-10) in foals with clinical *R. equi* pneumonia. The main treatment was with azithromycin-rifampicin for 14 days. Blood was sampled prior to, 7 and 14 days after starting therapy. Treatment was associated with significantly decreased counts of WBC, (25.6 ± 6.7 and 14.2 ± 2,7 × 10^3/ml), Neu (18.6 ±6.2 and 10.7 ± 3.1 × 10^3/ml), Mon (1.5 ± 0.5 and 0.9 ± 0.2 × 10^3/ml) and Fbg (539 ± 124 and 287 ± 26 g/dl) between day 0 and day 14. IL-2 and IL-10 concentrations were significantly increased ($P = 0.028$, $P = 0.013$, respectively) after treatment, whereas IFN-α and IFN-γ concentrations were not. The diagnostic potentials of INF-α, INF-γ, IL-2 and IL-10 *per se* seems not very high, however, the study suggests that the activity change of selected interleukins in the course of the disease may be associated with amelioration. We concluded that patterns of serum concentration changes of INF-α, INF-γ, IL-2 and IL-10 may help in the study of the innate immune response in foals during infection and treatment of *R. equi* pneumonia.

1. Introduction

For at least six decades, pyogranulomatous *Rhodococcus equi* (*R. equi*) infection has been a major problem in large breeding studs all over the world. Effective antimicrobial treatment options are under the pressure of developing macrolide resistance [1]. Prevention of infection by vaccination is not an option, since efforts with classical vaccines were not successful. This is due to the immaturity of the immune response of suckling foals [2]. Studies suggest that equine neonates and young foals have an impaired T helper 2 (Th2) cell response. The immune response of suckling foals, however, is T helper (Th1) cells biased. A generally suitable anti-pathogen response appears possible in foal as is suggested by the Interferon (IFN-γ) production of Th and cytotoxic T cells, which work similar to adult horses [3]. Interleukin-10 (IL-10) production by Treg cells reaches to maturity in foals during the first three months of life [4,5].

Taxonomically *Rhodococcus* and *Mycobacterium spp* are members of the actinomycetes group. There are similarities in pathology and intracellular replication between *R. equi* and *Mycobacterium tuberculosis* (Mtb) infections. The major host mechanism for elimination of Mtb is the Th1 response, which depends on the induction of Th1 cytokines (e.g., IFN-γ) in the host. Interestingly, using the cytokine-based biomarkers patterns in the peripheral blood of man, pulmonary tuberculosis, pulmonary aspergillosis or mixed infection could be distinguished [6]. With this result in mind, we hypothesized that INF-γ, INF-α, IL-2, and IL-10 would be useful biomarkers for innate immune response to *R. equi* infection in young foals and to predict if infection will resolve subclinically or develop into pyogranulomatous pneumonia. In this respect, we

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considered IL-2 as indicator of Th1 cell activity and IL-10 as indicator of Th2 cell activity, while INF-γ was considered to represent macrophage activity. At first, the choice of IL-10 as a marker seems illogical due to its anti-inflammatory reputation, but IL-10 is a pleiotropic cytokine with a strong role in limiting the scope and extent of immune activation [7]. Regarding INF-γ, Wagner et al. (2010) showed that the INF-γ levels in suckling foals indicate a Th1 phenotype immune signature. This occurs against a background of an emerging Th2 response, which is at 3 months of age still below that of adults [4].

Laboratory tests such as SAA have been investigated for diagnosis and monitoring of inflammatory disease in equines [8-12]. Currently, a point-of-care test kit for SAA is commercially available (Stable lab, EQ-1 Handheld Reader. Zoëtis). However, results are not univocal in case of *R. equi* infections. Furthermore, increase in serum fibrinogen concentration still is the better marker of inflammation [11].

To substantiate our hypothesis, we started with a description and analysis of the time-depended changes in the IFN-α, IFN-γ, IL-2 and IL-10 concentrations in relation to established clinical parameters such as WBC, basophils (Baso), neutrophils (Neu), lymphocytes (Lymf); monocytes (Mon), eosinophils (Eos) as well as concentrations of fibrinogen (Fbg), platelet (PLT) counts in foals suffering *R. equi* pneumonia. Blood sampling was prior to, at midway and at the end of treatment with azithromycin-rifampicin. Administrative obstacles prevented the formation of a control group. Furthermore, we tried to find out whether the cytokine patterns also provided information about involvement of innate immunity.

2. Materials and methods

2.1. Ethics committee

This study was approved by Erciyes University Animal Experiments Local Ethics Committee and the approval certificate (Decision no: 22/217) was obtained.

2.2. Animals and diagnosis

A total of 185 foals were born in the Stud of the Turkish Jockey Club during the breeding season of 2022. Foals were daily observed by experienced farm staff. Those showing pneumonia were examined at least once daily by professional stable veterinarians with at least 10 years of clinical experience. During the breeding season, 65 foals (35 %) developed signs of *R. equi* pneumonia. Foals with fever (T >39.5 °C), respiratory rate > 80/min, cough or abnormal lung sounds were examined by thoracic ultrasonography (USG). USG thorax examination was by using a multi-frequency 5.0–7.5 MHz linear transducer. Ethanol (70 %) was used contact medium. The thorax was scanned bilaterally from the 16th to 3rd intercostal space in a dorsal to ventral plane. Pulmonary lesions (thick reflections, areas of consolidation and pulmonary abscess) were recorded. Transtracheal fluid samples were used for confirmative bacteriology. From the 35 diseased foals, only 15 could be used for this study. This loss of cases was due to owners' refusal of transtracheal sampling. The ages of the remaining 15 foals ranged between 32–48 days. At the start of treatment all were in good bodily condition with weights ranging from 112 to 168 kg. Diagnoses of pulmonary *R. equi* infection was based on the ACVIM guidelines for diagnosing *R. equi* infection [2].

2.3. Sample collection and handling

Tracheal mucus samples were obtained by using the technique described by Sponseller (2018) [13]. Briefly, after sedition (xylazine 0.6 mg/kg and 1butorphanol 0.02 mg/kg iv), a small area of the ventral neck just proximal to the chest was clipped, cleaned and anesthetized with 2–4 ml 2 % lidocaine cum adrenaline. A 14G introducer was inserted between two tracheal rings and progressed ventrally into the tracheal lumen. Subsequently, a 16G × 60–70 cm flushing catheter was advanced through the access devise and 30 ml of sterile 0.9 % saline solution was administered. Tracheal mucus was obtained by aspiration. Samples were stored at 4 °C till analyses within the next 3 h. Samples were inoculated onto agar plates containing 5 % defibrinated sheep blood, mannitol salt, and MacConkey agar. The plates were aerobically incubated at 37 °C for 48 h. Identification of *R. equi* was by colony shape and gram-positivity. Subsequently, doubtful samples were analyzed using automated microbiology testing equipment (BD Phoenix 100, Biosciences, USA) for accurate identification. From all 15 foals *R. equi* monocultures were cultured. Virulence of the isolates was determined by PCR according to Haites et al. [14].

DNA from single colonies was obtained using the GeneJET Genomic DNA Purification Kit (Thermo Fischer Scientific, USA) following manufacturer's instructions. PCR was performed in a thermal cycler (T100, BioRad, USA) programmed for 40 cycles of 94 °C for 1.5 min, 57 °C for 1 min, 72 °C for 2 min. The reaction mix included 25 μL Taq DNA polymerase master mix (Ampliqon, Denmark), 0.1 μM forward and reverse primers of vapA gene sequences (5′ GACTCTTCACAAGACGGT and 3′TAGGCGTTGTGCCAGCTA), and isolated DNA of strains. Each reaction was analyzed to determine if it produced a DNA fragment of around 563 base pairs by using electrophoresis through a 1 % agarose gel stained with ethidium bromide.

On days 0, 7 and 14 at 7:00 a.m. jugular blood samples were collected in Plain Vacutainer (Terumo Co, Tokyo, Japan), Na-EDTA and Na-citrate tubes. Blood samples were kept at 4 °C until analysis within 3 h for routine hematology and fibrinogen concentration. Na-citrate samples were centrifuged at 3000 r.p.m (Hettich ROTOFIX 32A) for 15 min to obtain plasma. Plain tubes with clot activator were centrifuged at a speed of 3000 r.p.m. for 10 min. After centrifugation from each foal at least 3 batches of 2–5 serum samples were collected into Eppendorf tubes. The serum samples were coded for foal and day of sampling and kept at –80 °C until least 3 analyses. Fibrinogen analysis was performed by using a coagulation analyzer (Rayto RT-2204C).

2.3.1. Analysis of IFN-α, IFN-γ, IL-2 and IL-10

Due to technical reasons cytokines could only be determined in the serum samples of day 0 and 14. The following tests were performed: IFN-α ELISA (kit, 201-03-0635, Sunredbio), IFN-γ (ELISA kit 201-03-0117, Sunredbio), IL-2 (ELISA kit 201-03-1045, Sunredbio) and IL-10 (ELISA kit 201-03-0054, Sunredbio). All tests were performed according to manufacturer's protocol. Testing kits reached room temperature. Blank first well. Next, 50 μl of each standard (S1-S5) was applied to 10 wells, with duplicates. Next, 40 μl serum samples were applied to the remaining wells. Sample wells were treated with cytokine-specific anti-antibodies (10 μl). To all wells except those with no material, 50 μl of Streptavidin-HRP was added. The plate was sealed and incubated for 60 min at 37 °C. The plate was cleaned five times after incubation. 50 μl of Chromogen Solution A and B were added to each well. The mixture was carefully stirred and incubated at 37 °C without light for 10 min. To stop the reaction, 50 μl of stop solution was added to each well. An ELISA reader (BioTek ELX800) measured blank, standard, and sample absorbance at 450 nm after 15 min. As a component of our internal validation procedure, we performed redundant measurements on six samples. In this specific situation, we have incorporated the mean value of the coefficient of variation for intraassay.

2.4. Treatment

Treatment included azithromycin (Azomax, Kocak Farma) 10 mg/kg, orally, once daily for 15 days and rifampicin (RIFCAP, Kocak Farma) 5 mg/kg orally, twice daily for 15 days. Supportive therapy was by 0.8 μg/kg clenbuterol (Ventolin syrup, Glaxosmithkline) orally, twice daily for 15 days. This was to enhance the function of the mucociliary escalator as has been shown to occur in tracheal cell cultures [15]. The decision to stop antimicrobial treatment was based on return to normal

values of WBC counts and Fbg concentrations [16,17]. In fact, this coincided always with 15 days of treatment in the current trial.

Nonspecific supportive therapy was by 5 ml vitamin B complex (Berovit B12, Ceva) intramuscularly (IM), once daily for 5 days, as well as a 5 ml vitamin C (Vital C, Santa Vet) IM, once daily for 5 days. In cases of high body temperature (40°–41 °C) and dehydration (poor suckling, increased hematocrit and poor skin turgor) 40 mg/kg of metamizole (Dolarjin, Topkim) was administered IM once daily for 5 days. Dehydration was treated IV by 0.9 % NaCl (Polifleks, Polifarma) and/or 5 % dextrose (Medifleks, Eczacıbaşı) and vitamin-mineral-amino acid solution (Duphalyt, Zoetis). Fluids were given at a flowrate of 3–5 ml.kg/h, Foals suffering hyperpnea received 5 % oxygen intranasally. All foals tolerated treatments without complications and returned to a good health at the end of therapy.

2.5. Statistical analysis

Descriptive statists were performed to present the data using the SPSS 25.0 package (SPSS Inc, Chicago, IL, USA). Furthermore, analysis of variance (ANOVA) was used to analyze changes in concentrations of blood cells and biomarkers associated with antimicrobial treatment. Since currently, F-tests have been identified as robust enough for testing smaller sample sizes and data that violate against normality [18]. A P value <0.05 was considered significant. Graphical images were made with Graph Pad Prism 9.0 software (Graph Pad Software Inc., San Diego, CA, USA). Data were round if appropriate

3. Results

Hematology data given in Table 1. WBC, Neu, Mon counts and Fbg levels were significantly lower after the 14 days in which treatment took place than before ($P < 0.001$). Counts of Lymph, Eos, Bas and Plt were not significantly changed over time. The average coefficient of variation (CV) values for IFN-α, IFN-γ, IL-2, and IL-10 were 3.92, 8.88, 7.71, and 4.88, respectively. Cytokine changes per timepoint are given in Fig. 1. Mean (± sd) cytokine concentrations in pg/ml before and after 14 days were for IFN-α: 25.2 ± 12.5 and 27.4 ± 6.4; IFN-γ: 58.9 ± 21.1 and 66.1 ± 13.2; IL-2: 12.2 ± 3.5 and 15.1 ± 3.7; IL-10: 57.5 ± 23.1 and 101.7 ± 29.4. Fig. 1. suggests that the 4 cytokines are lower at the start of clinical overt pneumonia. Only IL-2 and IL-10 concentrations were significantly increased after treatment as shown in Fig. 1.

Table 1
Comparison of hematological parameters and fibrinogen concentration at 0, 7th, and 14th days. WBC; White Blood Cell, Baso; basophil, Neu; neutrophil, Lymph; lymphocyte, Mono; monocyte, Eos; eosinophil, PLT; platelet and fibrinogen concentration, Fbg in mg/dl;. Data were expressed as mean ± standard deviation (SD). *$P < 0.001$. Different letters in the same row indicate differences between groups.

Parameters	0 ($n = 15$)	7th ($n = 15$)	14th ($n = 15$)	P Value
WBC (10^3/μL)	25.6 ± 6.7 a 24.5 (21.6–27.8)	21.3 ± 4.2 b 15.7 (13.4–29.1)	14.2 ± 2.7 c 14.4 (11.2–16.2)	<0.001*
Neut (10^3/μL)	18.6 ± 6.2 a 18.0 (14.8–19.4)	14.7 ± 4.0 b 13.1 (8.1–21.1)	10.7 ± 3.1 c 10.1 (9.2–12.5)	<0.001*
Lymph (10^3/μL)	5.5 ± 0.9 5.3 (5.0–6.0)	5.8 ± 1.1 5.7 (3.9–9.6)	5.65 ± 1.0 5.7 (5.1–6.6)	0.748
Mono (10^3/μL)	1.5 ± 0.5 a 1.5 (1.2–1.9)	1.1 ± 0.3 b 1.0 (0.7–1.7)	0.9 ± 0.2 c 0.9 (0.8–1.0)	<0.001*
Eos (10^3/μL)	0.03 ± 0.02 0.01 (0.00–0.05)	0.04 ± 0.03 0.13 (0.00–0.13)	0.05 ± 0.03 0.05 (0.01–0.08)	0.112
Baso (10^3/μL)	0.03 ± 0.01 0.04 (0.03–0.04)	0.03 ± 0.01 0.03 (0.02–0.05)	0.04 ± 0.01 0.03 (0.03–0.04)	0.596
Plt (10^3/μL)	362 ± 181 351 (196–429)	385 ± 100 333 (248–581)	371 ± 100 351.00 (318–446)	0.863
Fbg (mg/dl)	539 ± 124 a 487 (441–628)	410 ± 65 b 345 (227–572)	287 ± 26 c 286 (269–305)	<0.001*

4. Discussion

Many young foals recover spontaneously from subclinical *R. equi* infections, while 18–50 % develop pyogranulomatous pneumonia but recover with treatment, whereas 2–5 % perish [5]. When respiratory disease does become clinically apparent, disease is initially often insidious and becomes chronic and progressive [3]. Given the prevalence of subclinical infection and high false positive rate in current screening methods, there is a critical need to identify factors contributing to the hosts susceptibility [5]. As first step in this search we studied blood concentrations of INF-α, INF- γ, IL-2 and IL-10 during clinical infection and its resolution by antimicrobial treatment. We could not find appropriate literature on the baseline and elevated cytokine concentrations in foal or resting adult horse serum. Conflicting results are reported for in man. A set of studied interleukins were not detectable in the blood of healthy blood donors [20], whereas in another study with healthy people, mean (± sd) base-line serum concentrations for Il-2, IL-10, and INF-γ were 0.7 ± 0.3, 4.5 ± 112 and 1.3 ± 1.9, respectively [21]. A recent study on normal values in plasma reported lower baseline concentrations values for Il-2, IL-10 and higher for INF-γ [22]. Remarkably, for these cytokine samples most standard deviations were larger than their means, which suggested a high variation in values.

Due to the lack of a control group in our study, the usefulness of the studied cytokines as specific diagnostic tests could not be determined, but we tried to link the observed presence and changes in concentrations of the cytokines with putative running processes of innate immunity, especially that of pathogen phagocytosis.

The alveolar macrophages play an ambivalent role in the pathogenesis of intracellular *R. equi* infection. They provide a niche for survival and replication of the rhodococci, while on the other hand they are essential in the innate immune mechanisms and possibly the start-up of the adaptive immunity. How this is precisely regulated cannot be answered; the nature of the innate immune milieu generated by *R. equi* infection is still ill-defined anyhow [5]. In both their roles macrophages likely cross-talk with other actors in innate immunity. We believe to have noticed a small fragment of this cross-talk, but too little to understand the message and thus still there is much room for speculations.

Regarding the WBC dynamics, the foals had increased neutrophil production as was shown by hyperleukocytosis at the start of therapy, which was mainly caused by the neutrophil fraction. The initial leukocytosis, granulocytosis, monocytosis and hyperfibrinogenemia on average were returned to normal at the end of treatment, which is consistent with another study [23]. This indicates successful killing of rhodococci by neutrophils and in debris cleaning likely by monocyte/macrophages. Moreover, neutrophils killing of *R. Equi* appears to have been stimulated continuously as the measurable serum levels of IFN-γ suggests. Tissue damage and a disturbed homeostasis is the trade-off of the inflammatory killing process and is likely limited by increasing serum levels of IL-10 as we observed [7,24]. So far it is clear that IFN-γ and INF-α expression by the host are involved in the pathogenesis of *R. equi* [19]. Darah et al [25] even showed that pro-inflammatory cytokine production is required for clearing *R. equi.* In summary, IFN-γ activates certain macrophages to produce reactive oxygen species that limit intracellular replication and kill *R. equi* [21]. Vail et al. [5] found evidence that *R. equi* activates the cytosolic DNA sensing pathway during macrophage infection and suggest that type I IFN (INF-α/β) signaling likely plays a critical role in the pathogenesis of *R. equi*. In general, intracellular bacterial infections induce low levels of type I IFNs (INFα/β) as is also suggested by Fig. 1. IFN-α is a multi-facetted cytokine and one its effect is on T and B cell differentiation, as well as on cytotoxic T cell activities, and enhances the microbicidal function of macrophages [26]. The significantly increased IL-10 concentration after treatment suggests a developing Th2 response,

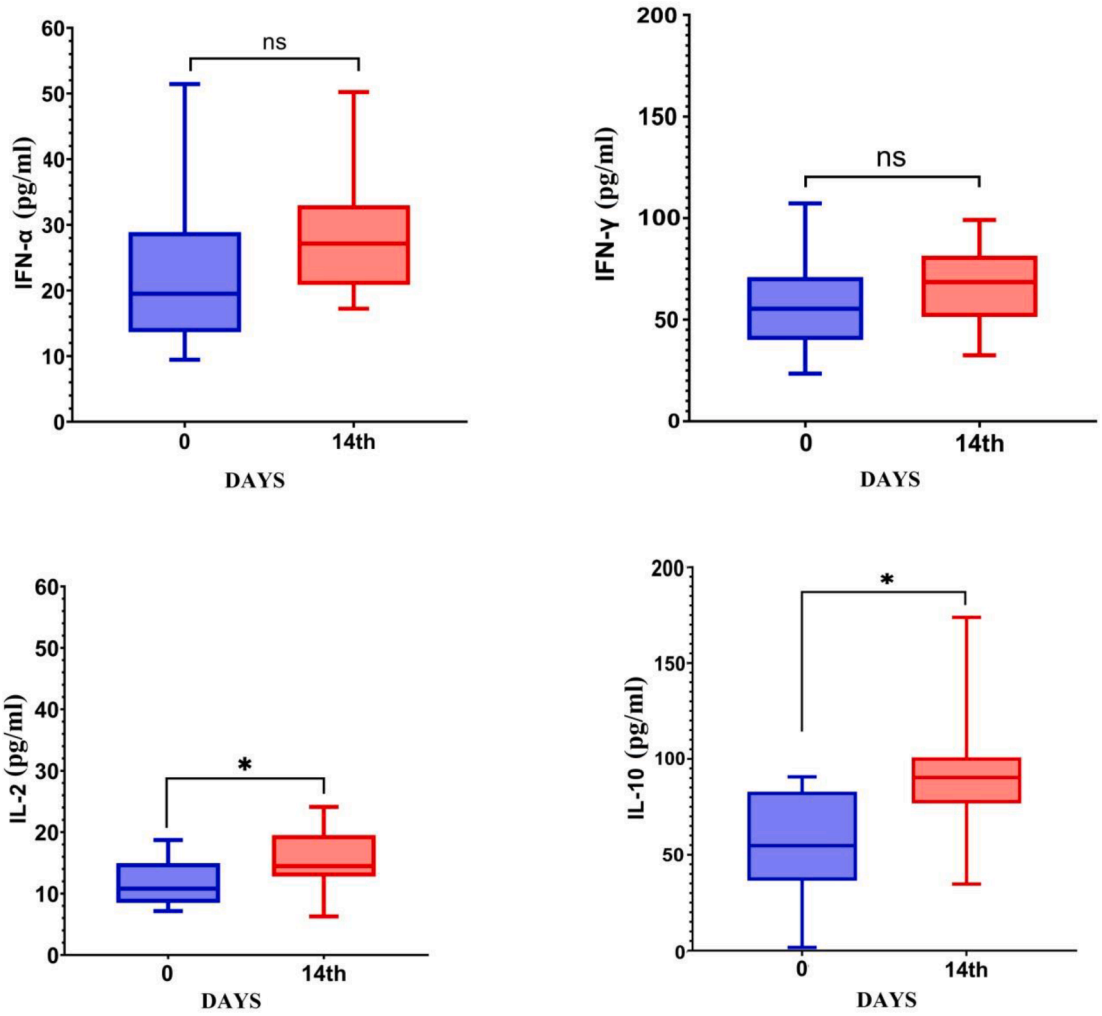

Fig. 1. Box and whisker plots of INF-α, INF-γ, IL-2 and IL-10 (pg/ml). showing medians, Q1, Q3, minima and maxima. Only medians for IL-2 and IL-10 were significantly different before and after treatment ($P = 0.04$, $P = 0.013$, respectively). Symbols *: $P < 0.001$.

which is likely related to the onset of production of immunoglobulins heling to clear the rhodococci [3].

We associated a weak T helper cell type 1 (Th1) response with an average 19 % increase of IL-2 serum concentration. The IL-10 serum concentration was increased by 77 % over 14 days. The interpretation of this is manyfold. Possibly one effect of IL-10 was limiting tissue damage, another may be the execution of its critical function in innate and adaptive cell-mediated immunity against intracellular bacterial infections.

In foals, absence of a noticeable Th1 response is likely due to this very low level of IFN-γ and may contribute to their susceptibility to *R. equi* [3,5]. In the present study, INF-γ concentrations had not significantly changed over time, which may indicate that a Th1 response was uninterrupted. A possibly other cause may be the macrophage cleaning activities of the pyogranulomatous cell debris.

The general picture of our study suggests that foals had already had an active Th1 response at the moment that clinical signs of pyogranulomatous pneumonia were observed and that a Th2 response developed over time.

A weakness of this explorative self-controlled study is that the effects of antimicrobial treatment are not known. There is a general consensus to treat *R. equi* pneumonia with azithromycin in combination with rifampicin [2]. Macrolides, in particular azithromycin, which is a first-line drug for nontuberculous mycobacterial infections too, have a well-established immune-modulatory role and regulate the secretion of several proinflammatory mediators from phagocytes and epithelial cells [27,28]. Rifampicin may have anti-inflammatory effect and could inhibit the production of cytokines [29]. Such effect cannot be excluded on the post-treatment serum concentrations of IL-2. Furthermore, an effect of clenbuterol on immune cells cannot be excluded either, since clenbuterol increases the expression of IL-10 gene in equine peripheral leukocytes after an intrabronchial challenge with lipopolysaccharides [30].

In practice, accurate quantification of cytokines is challenging, because if they are present in body fluids, it is in very low amounts in the range of picograms (pg/ml). Moreover, their secretion dynamics and short half-lives provide both sampling and analytic [31,32] and interpretation problems. Other unquantified effects are the rate and the timing of release into the blood compartment from the inflammatory spots into the extravascular compartment. Furthermore, cytokines maybe released in the blood itself due to traveling lymphocytes and monocytes and may increase concentrations of cytokines which cannot straightly be associated with presence and volume damaged tissue.

5. Conclusions

Next to the classical pattern of decreased leukocyte counts, our study putatively shows that serum cytokines were associated with innate immunity activities in foals recovering from *R. equi* pneumonia. Observed changes coincided with oral treatment with azithromycin-rifampicin.

Our findings suggest activation, if not a transition of the foals' innate immunity towards specific immunity, possibly supported by the antimicrobial treatment.

Ethical

This study was approved by Erciyes University Animal Experiments Local Ethics Committee and the approval certificate (Decision no: 22/217) was obtained.

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Data statement

Data are available on request or on a public repository.

CRediT authorship contribution statement

Ömer Deniz: Writing – original draft, Conceptualization, Project administration, Investigation. **Gencay Ekinci:** Methodology, Formal analysis. **Ali Cesur Onmaz:** Conceptualization, Supervision. **Fatih Mehmet Derelli:** Resources, Investigation. **Francesco Fazio:** Validation, Methodology, Supervision. **Francesca Aragona:** Investigation, Resources. **René van den Hoven:** Data curation, Writing – review & editing.

Declaration of competing interest

None.

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