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Original article

Could Serum Collectin-11, surfactant protein-A and D be suitable biomarkers for assessing pulmonary damage caused by *Rhodococcus equi* infection in foals on large farms?

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Abstract

Rhodococcus equi (*R. equi*) is a primary cause of pyogranulomatous pneumonia of foals between three weeks and five months of age. Early diagnosis of rhodococcal pneumonia has always been considered a preferable approach as it can lead to more successful treatment and better outcomes. Horse stud farms where the disease is common, neonatal foals are subjected to tests such as complete blood count and fibrinogen analysis at certain intervals. However, new biomarkers are needed in addition to blood count and fibrinogen measurement in this field for early diagnosis of diseases. Based on this need, in this study, the diagnostic importance of CL-11, SP-A, SP-D was investigated for the early diagnosis of pneumonia in foals naturally infected with *R. equi*. In a case-control design, fourteen 1-5 month-old foals with proven *R. equi* pyogranulomatous pneumonia and 10 healthy 1-5 month-old control foals were enrolled in this study. The median white blood cell count (WBC) and fibrinogen concentration in the case group were significantly higher than in the control group. The median CL-11, SP-A, and SP-D concentrations in the case group were also significantly higher than in the control group. However, there were overlaps in concentrations between groups for SP-A and SP-D. There was less overlap between the groups for the CL-11 concentration. The CL-11 assay was sufficiently accurate but over-diagnosed *R. equi* infection. The correlation plot between fibrinogen and CL-11 concentrations shows that this problem may be solved if CL-11 is used as the first biomarker and fibrinogen as the second check for those foals with CL-11 serum levels >0.8 and ≤1.5 ng/mL. As a result, in this study, it is recommended to use CL-11 together with fibrinogen to obtain more accurate results in diagnosing pyogranulomatous pneumonia caused by *R. equi* in foals on large horse stud farms.

Keywords: Collectin-11, foal, pneumonia, *Rhodococcus equi*, surfactant protein A, surfactant protein D.

Introduction

Rhodococcus equi (*R equi*) is a facultative intracellular, non-motile, non-endospore-forming rod-shaped gram-positive bacterium (Hoffman et al. 1993, Giguère et al. 2011a). *R. equi* was reclassified as *Rhodococcus hoagii* (*R. hoagii*) in accordance with the Bacteriological Code after its taxonomy was recently altered due to sequencing techniques (Kämpfer et al. 2014). However, some still argue that it is best to use the name *R. equi* in articles (Giguère et al. 2017, Vázquez-Boland et al. 2020, Kahn et al. 2023).

R. equi and *Streptococcus equi* subspecies *zooepidemicus* are the two most common causes of pneumonia in foals between one and six months of age (Hoffman et al. 1993, Bordin et al. 2022, Arnold-Lehna, 2020). Especially on horse farms, *R. equi* can be a principal cause of pyogranulomatous pneumonia in this age group (Prescott and Hoffman 1993, Giguère et al. 2011b). Apart from passive immune protection by hyperimmune serum (Giguère et al. 2002, Giguère et al. 2011b, Kahn et al. 2023), no vaccine against *R. equi* is available. For more than a decade, affected foals have received antimicrobial treatment (Giguère et al. 2011a), which increases the risk of development of antimicrobial resistance (Giguère et al. 2010, Giguère et al. 2017, Arnold-Lehna et al. 2020), especially large stud farms.

Recent studies have shown that antimicrobial treatment of foals with small pulmonary abscesses is unnecessary and costly and increases the risk of antimicrobial resistance on farms (Arnold-Lehna et al. 2020). Foals may have lung lesions (pulmonary consolidations or abscess formation) identified by ultrasonography but do not always show clinical signs of pneumonia. Spontaneous regressions are uncommon and were shown in a challenge model (Perkins et al. 2002).

The suitable immune mechanisms must become activated in foals. Genetic factors may be involved; McQueen et al. (2014) showed that one region on chromosome 26 was moderately associated with susceptibility to *R. equi* pneumonia. This region is involved in neutrophil function. Other significant players in innate immunity are the collagenous lectins. This group of pattern-recognition proteins has essential roles in the innate immune system. Collagenous lectin deficiency is associated with increased susceptibility to infectious diseases in various species (Fraser et al. 2018). In a genetic study, these authors identified 4559 sequence variants in the equine collagenous lectin and related mannose-associated serine protease (MASP) genes, 113 of which may have relevance in horses with infectious or autoinflammatory diseases. Of particular interest are the genes for the transcription of collectin-10 (CP-11), surfactant protein A (SP-A), and surfactant protein D (SP-D). COLEC11, SFTPA1, and SFTPD were ranked 3rd, 4th, and 5th of 12 equine collagenous lectin genes concerning variants identified per kB of DNA. Although most of these variants occurred in non-coding DNA and had no predicted biological relevance, 280 mutations located within predicted transcription factor binding sites and 95 mutations located within predicted microRNA binding elements still may be related to genetic roles of the quantitative release of different collagenous lectins and subsequent enhancement of innate immunity.

SP-A and SP-D were identified as surfactant-associated proteins involved in pulmonary surfactant homeostasis. It is widely accepted that the underlying lung disease mechanism increases the alveolar-capillary permeability and leads to the leakage of the surfactant proteins into the circulation, increasing the overall serum concentrations (Nayak et al. 2012). CL-11 is a member of the lectin family of pattern recognition molecules, with known antimicrobial functions and the ability to trigger complement activation via the lectin pathway. CL-11 expression was first noticed in the kidney, but adrenal glands and the liver are also primary expression sites. Its presence in the circulation plays an essential role in the innate immune defense by activating complement, opsonized microbes, or apoptotic cells (Hansen et al. 2010). Since collectin 11 (CL-11) is considered a significant sentinel at epithelial surfaces and a key pattern recognition molecule in complement-mediated ischaemic injury (Nauser et al. 2018, Nauser and Sacks 2023), it may also be involved in pulmonary damage of foals. A pilot study showed that CL-11 SP-A and SP-D serum concentrations were elevated in a developing *R. equi*-associated pyogranulomatous pneumonia. We think the presence and extent of the lung damage can be predicted by a response pattern of serum CL-11, SP-A, and SP-D.

Materials and Methods

Study design

Prospective, case-control study.

Ethics statement

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (Decision no: 22/044).

Foals

During one breeding season, 190 foals at a thoroughbred stud were observed daily by experienced

farm personnel. Foals with suspected disease were referred to the stud veterinarians for a diagnostic workup. In total, 40 less than six months old foals were diagnosed with broncho-pneumonia.

Case group

Inclusion criteria for the case group were: age between 3 and 6 months, clinical signs of pyogranulomatous pneumonia, and confirmation by a positive *R. equi* monoculture of tracheal secretions (Giguère et al. 2011a, ACVIM Consensus Statement).

Criteria for the diagnosis of granulomatous pneumonia were as given by Giguère et al. (2011a). Briefly, clinal signs included fever (>38.2°C), decreased appetite and lethargy, cough, dyspnea, and murmurs and crackles on lung auscultation. The diagnosis was further supported by thoracic ultrasonographic detection of lesions.

Of 40 foals, 19 with chronic pyogranulomatous pneumonia had an established bacterial infection and were allowed by the owners to be included in the study. However, five foals were excluded due to mixed bacterial populations in the tracheal aspirates.

Control group

Inclusion criteria for the control group were that the foals were healthy before and at the time of sampling. Owners included ten clinically healthy foals in the control group. The foals were randomly selected from healthy foals at inspection and whose fibrinogen levels were within the reference range (< 400 mg/dL).

Tracheal sample collection and culture of secretum

Percutaneous tracheobronchial aspirates (TBAs) were performed in sedated (xylazine 0.6 mg/kg and butorphanol 0.02 mg/kg iv.) and locally anesthetized foals according to established methods (Sponseller and Sponseller 2017).

Sterile-obtained aspirates were inoculated onto 5% defibrinated sheep blood agar, mannitol salt agar, and MacConkey agar. Plates were incubated at 37°C for 48 h under aerobic conditions. After incubation, *R. equi* was identified by colony morphology and gram staining. Suspicious isolates were determined by an automated microbiology testing system (BD Phoenix 100, Biosciences, USA) for definitive identification. In total, 14 foal monocultures were identified.

Blood sample collection

Blood from the jugular vein was collected in plain tubes with gel, K_2 EDTA, and Na citrate tubes (BD Vacutainer®) for serology, hematology, and fibrinogen concentration. Serum and plasma were obtained within 2 hours by centrifugation of the samples for 15 minutes at 3000 rpm (Hettich ROTOFIX 32A).

Hematology and fibrinogen

Hematological parameters were determined using an automated hematological analyzer (Sysmex XT-1800i, Sysmex Corporation, Japan). Fibrinogen concentrations were also analyzed using an automated Start St Art coagulation analyzer (Diagnostica Stago Inc., Asnieres, France). The most suitable reference values were chosen as given by Santos et al. (2014) and Faramarzi and Rich (2019) for foals between 30-90 days old.

Analysis of CL-11, SP-A, and SP-D

Serum samples were assayed with experimental purpose ELISAs for the quantification of equine CL-11, SP-A, and SP-D (Horse ELISA Kit MBS9361413, MBS033347, and MBS040510; MyBioSource), according to the manufacturer's protocols. Absorbance at 450 m was determined with a microplate reader (BioTek ELX800, Absorbance Microplate Reader, BioTek Instruments, Colmar, France).

Statistical analysis

Statistical analyses were performed using SPSS for Windows Release 25.0 Program (SPSS Inc, Chicago, IL, USA). Descriptive statistics were performed, and data were graphically presented. The normal distribution of data sets was tested with the Shapiro-Wilk test. The Mann-Whitney U-test tested group differences. Association between the CL-11, SP-A, SP-D, and fibrinogen concentrations were calculated with Spearman's rho correlation test. For all tests, significancy was set at $p<0.05$.

To estimate the potential diagnostic suitability of the biomarkers, receiver operating characteristics (ROC) curves, sensitivity, specificity, and best cut-off values for CL-11, SP-D, and SP-A values were calculated using MedCalc version 20.014 statistical software (MedCalc Software, Ostend, Belgium). Graphs were prepared with Graph Pad Prism 9.0 software (Graph Pad Software Inc., San Diego, CA, USA).

Results

Groups were roughly balanced for sex (case group 57% males, 43% females; control group 50% males and 50% females). The ages of the case group ranged from

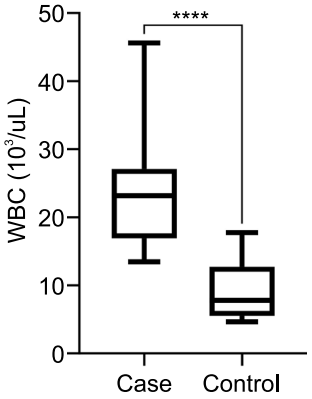

Fig. 1. Boxplot of WBC x10^3/µL in foal cases and controls.

32-48 days, and for the control group, 36-59 days. Mean body weights were 110±18.2 kg (range 112-168 kg) and 102±16.4 kg (range 126-149 kg) for the case and control group, respectively.

Monocultures of *R. equi* were isolated in the case group in 14 out of 19 aspirated samples. Mixed cultures in five cases apart from *R. equi* included *Klebsiella aerogenes, Pantoea agglomerans, Staphylococcus xylosus, Staphylococcus gallinarum,* and *Staphylococcus aureus*. These foals were excluded from this study.

At presentation, the foals in the case group all had fever (temp. ≥38.3°C), rectal temperatures ranged from 38.3-40.2°C, respiratory rates varied from 62 to 88 /min; heart rate varied from 90 to 140 /min, respiratory signs varied from mild to severe. Foals in the control group had normal rectal temperatures ranging from 37.2-38.2°C. The respiratory rate ranged from 40 to 60/min, which was no indication of respiratory discomfort. Heart rate varied from 60 to 100/min. Decreased appetite, anorexia, lethargy, dyspnea, lung auscultation murmurs, and crackles were noticed only in the foals of the case group.

Boxplots of WBC per group are shown in Fig. 1. The reference ranges for WBC, neutrophils, and lymphocytes were 8.4-9.8 x10^6/mL, 5.1-5.9 x10^6/mL, and 2.5-3.3 x10^6/mL, respectively. In all case group foals (14 foals) WBC was >9.8 x10^6/mL (lowest 13.5 and highest 45.6 x10^6/mL) and this was mainly due to increased neutrophil counts, which ranged from 8.3 to 38.0 x10^6/mL. On the other hand, very mild lymphocytosis in 12 foals (out of 14 foals), was observed which ranged between 2.9 and 6.1 x10^6/mL. Therefore, we concluded that case foals had no evidence of marked insufficient lymphopoiesis. WBC in the control group ranged from 4.7 to 17.7, suggesting moderate leukocytosis in seven foals. Twelve foals had mild leukocytosis, and all foals had counts ranging from 3.9 to 7.5 x10^6/mL.

In the case group, the ultrasonographic findings included vertical hyperechoic lines that are reverberation artifacts (n=5), consolidation areas (n=3), and pulmonary abscesses (n=6). The reverberation artifact is due to gas shadowing from the interface of the aerated lung deep into the abscess. Pleural abnormalities are also referred to as reverberation artifacts and manifest as vertical hyperechoic lines (Ramirez et al. 2004).

Sonograms were obtained from the foals in the case group with *R. equi*; some of these are displayed in Fig. 2.

The mean fibrinogen concentration in the case group was significantly higher ($p<0.001$) than in the control group, 660 ± 54.05 mg/dl and 330 ± 55.18 mg/dl, respectively.

Plasma fibrinogen and serum CL-11, SP-A, and SP-D concentrations

The violin plots of CL-11, SP-A, and SP-D are shown in Fig. 3. These show that fibrinogen and CL-11 had no or little overlap between the groups. Data for fibrinogen, CL-11, and SP-A were not normally distributed. Therefore, non-parametric tests were performed, whereas it was deemed prudent to analyze SP-D data non-parametrically. Data are given in Table 1.

ROC curves and sensitivity analysis determined the potential diagnostic value of the biomarkers. Cut-off levels were calculated using the maximal Youden's Index. Data are shown in Table 2. Next, to calculate the diagnosed properties at an optimal cut-off value, slightly larger or commonly used cut-off values were calculated for CL-11 and fibrinogen, respectively.

The associations between the potential marker variables and the fibrinogen as the gold standard were analyzed by Spearman's Rho correlation test. All correlations were positive and statistically significant but varied moderately between 0.48 and 0.60 (Table 3).

Discussion

The selected *R. equi*-infected foals in our study showed pathognomonic signs of pyogranulomatous

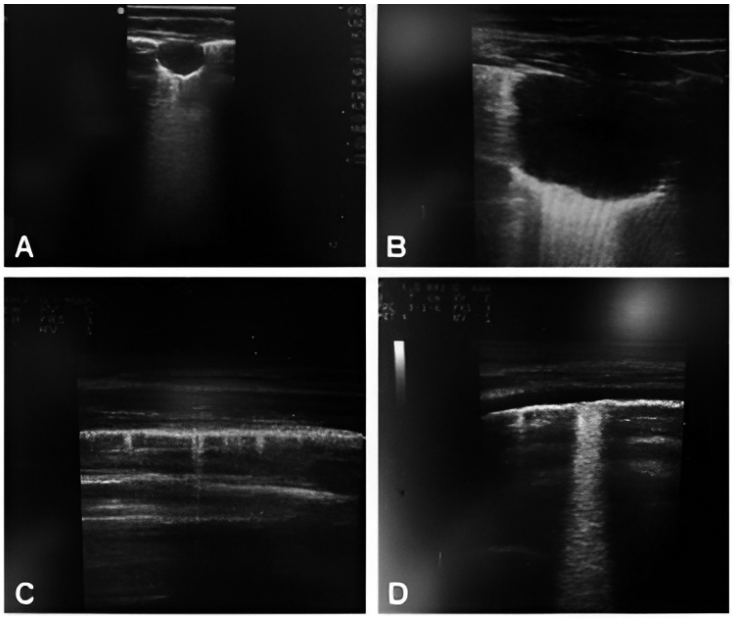

Fig. 2. (A, B): Sonograms of the thorax from foals with pulmonary abscessation (a well-defined, circular hypoechoic region in the periphery of the lung) secondary to *R. equi* pneumonia. (C, D): Sonograms of the thorax from foals with thin (C) and thick (D) reflections (Note the vertical hyperechoic lines that are reverberation artifacts) secondary to *R. equi* pneumonia.

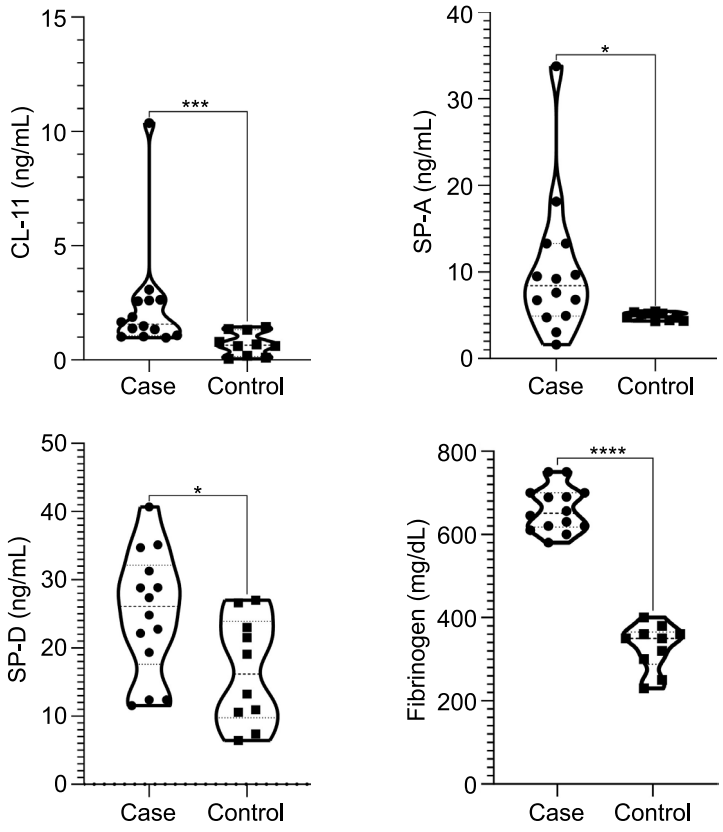

Fig. 3. Violin plots show that fibrinogen discriminates between foal cases and controls.

lung lesions, identified as *R. equi* after isolation of the pathogen. The WBC and neutrophil counts in the case group were statistically higher than in the controls, which agrees with the presence of infection (Leclere et al. 2011). However, there was too much overlap in the respective distributions to make firm conclusions on each case. This confirms what others have already shown (Giguère et al. 2011a). Lymphocyte counts could give some room for speculation. Twelve of 14 foals showed lymphocytosis, as did all control foals. This meant that the lymphocyte production rate of infected foals seemed to be similar to that of healthy foals. How

Table 1. Median (and range) of 3 collectins and fibrinogen in foals.

	Case	Control	p values
CL-11 (ng/mL)	1.7 (1.2-2.6)	0.7 (0.1-1.3)	<0.001
SP-A (ng/mL)	8.6 (6.3-13.3)	5.0 (4.4-5.2)	0.021
SP-D (ng/mL)	26.2 (11.5-40.7)	16.2 (6.4-27.0)	0.018
Fibrinogen (mg/dL)	650 (580-750)	350 (230-400)	<0.001

CL-11 – Collectin-11, SP-A – Surfactant protein A, SP-D – Surfactant protein D

Table 2. ROC data by free software analyse-it for Excel (foal case n=14; control n=10).

	AUC	95% CI	SE	P=
Marker		2-sided		
CL-11 (ng/mL)	0.89	0.76-1.02	0.07	<0.001
SP-A (ng/mL)	0.78	0.57-0.99	0.11	0.008
SP-D (ng/mL)	0.79	0.60-0.97	0.09	0.0025
Fibrinogen (mg/dL)	1.00	NA	NA	NA

Sensitivity analysis

Marker	Cut-off value	Sensitivity (TP)	Specificity (TN)	FP	FN	Max. Youden's index
CL-11	0.8	1.00	0.70	0.30	0	0.70
CL-11	1.2	0.71	0.70	0.30	0.30	0.41
SP-A	5.4	0.71	1.0	0	0.30	0.71
SP-D	27.0	0.50	1.0	0	0.50	0.50
Fibrinogen	400	1.00	1.0	0	0	1.00
Fibrinogen*	700	0.14	1.0	0	0.86	0.14

* cut-off value according to literature (Leclere et al. 2011), CL-11 – Collectin-11, SP-A – Surfactant protein A, SP-D – Surfactant protein D, TP – True positive, TN – True negative, FP – False positive, FN – False negative

Table 3. Spearman's rho correlation results in foals.

Variables	CL-11	SP-A	SP-D	Fibrinogen
CL-11	1.000			
SP-A	0.511*	1.000		
SP-D	0.392	0.568**	1.000	
Fibrinogen	0.595**	0.480*	0.489	1.000

Correlation is significant at the 0.05 level (2-tailed)
Correlation is significant at the 0.01 level (2-tailed)

adequately these lymphocytes responded in immune defense cannot be concluded from our hematology counts.

The context of our study was the prevention of overtreatment with antimicrobials of foals with self--healing *R. equi* infection. Therefore, we hypothesized that biomarkers of pulmonary tissue would refine the diagnosis of pyogranulomatous pneumonia if CL-11, SP-A, and SP-D were used as biomarkers. Our hypothesis was not confirmed since CL-11 overdiagnosed *R. equi* pyogranulomatous by a false positive rate of 30%. Based on the AUCs, fibrinogen's maximal discriminative capacity was 100%, whereas CL-11 AUC was 89%. Shifting the cut-off value from 0.8 to 1.2 ng/ml decreased CL-11 sensitivity from 100% to 71%, while specificity remained at 70%. Thus, the false positive rate could not be lowered by a slight increase in cut-off value. In principle, false positives are less severe than false negatives, but a further test is needed to predict the chance of spontaneous recovery. Maybe a testing algorithm based on combinations of variables could deliver a decision to treat or to wait.

Due to overlap, the biomarkers SP-A and SP-D were less discriminative, with AUCs of 78% and 79%,

respectively. These tests classified none of the healthy foals as having pyogranuloma but missed 30-50% of the affected foals.

Currently, clinical suspicion of pneumonia in foals is mostly based on blood test results, such as plasma fibrinogen concentrations and white blood cell counts (WBC), as well as clinical symptoms (Giguère et al. 2003, Passamonti et al. 2015, McCracken et al. 2019). Fibrinogen at our calculated 400 mg/dl cut-off level performed best, with no false positives or negatives. Increasing the cut-off level to the commonly used 700 mg/dl dramatically decreased sensitivity and resulted in 86% false negatives. This confirms the statement of Giguère et al. (2011a) that improving either the sensitivity or specificity of ELISA assays by changing the cut-off value of the tests could only be done to the detriment of the other.

The authors' rationale for selecting diagnostic tests in foals linked to immunology was based on the following facts. In contrast to foal macrophages, neutrophils from foals can kill R. equi entirely (Giguère et al. 2011a, Giguère et al. 2011b). The exact process of neutrophil-assisted intra- and extracellular killing of *R. equi* bacteria, including the role of the complement system, acute phase proteins, interleukins, interferons, and lectins, is unclear in immune naïve foals. Complement may be activated only or sequentially through the classical, the alternative, or the lectin pathway. In this respect, a biomarker of the latter may be advantageous in clinical decisions on antimicrobial treatment. The common biomarker of inflammation in horses is the acute phase protein serum amyloid A (SAA) (Cohen et al. 2005). SAA takes part in the innate immune response via the recruitment of leucocytes and has antimicrobial activity (Abouelasrar Salama et al. 2021). However, SAA as a possible marker in *R. equi*-induced pyogranulomatous pneumonia in foals could not be associated with the presence of sonographic evidence of lung abscesses (Long and Nolen-Walston 2020). Independent studies on the diagnostic performance of serological tests for *R. equi* infection made it clear that such tests either have low sensitivity, low specificity, or both (Martens et al. 2002, Giguère et al. 2003a, Giguère et al. 2003b, Phumoonna et al. 2006); new biomarkers should be searched for this reason, the authors hypothesized that selected collections could improve diagnosis.

A significant weakness of this study is the unbalanced case and control group and the small number of foals that could be included. The reason is that many foals at the stud were privately owned, and the owners did not permit trans tracheal washing. Therefore, potential cases and controls were lost.

Conclusion

In summary, we showed that fibrinogen in our stud population of foals at a cut-off level of 400 mg/dL was the best test to identify foals with existent pulmonary pyogranuloma, but still did not bring an indication for or against antimicrobial treatment. The CL-11 assay was sufficiently accurate but over-diagnosed *R. equi* infection. A correlation plot between fibrinogen and CL-11 concentrations shows that this problem may be solved if CL-11 is used as the first marker and fibrinogen as the second check for those foals with CL-11 serum levels >0.8 and ≤1.5 ng/mL. The caseload and data in this study are too low to investigate this option.

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