Cytological findings in bronchoalveolar lavage fluid of foals with pneumonia caused by *Rhodococcus equi* and other bacteria

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Abstract

The distinction between lower respiratory tract infections caused by *Rhodococcus equi* and those caused by other pathogens is difficult.

The aim of this retrospective study was to describe cytological findings in bronchoalveolar lavage fluid (BALF) of foals with pneumonia caused by *R. equi* or other bacteria.

Nineteen foals aged from 3 weeks to 6 months with evidence of respiratory disease between 2015 and 2016 were selected from the database of the Veterinary Teaching Hospital “Mario Modenato” of the University of Pisa.

Eight foals out of 19 (42.1%) had *R. equi* pneumonia while eleven out of 19 (57.9%) had another bacterial pneumonia. *R. equi* positive foals had statistically significant higher TNCC (P=0.02) and neutrophils percentage (P=0.002) than *R. equi* negative ones. Macrophages proportion (P=0.01) was lower in *R. equi* positive than in *R. equi* negative foals.

BAL is a quite easy procedure that can be performed in the field with minimal equipment required. Here we reported significant differences in the cellular composition of BALF that can be used to differentiate foals with *R. equi* bronchopneumonia from those with other bacterial pneumonias, while waiting for culture results.

Keywords: BAL, *Rhodococcus equi*, bronchopneumonia, foal, cytology

1. Introduction

Respiratory disease is a major cause of disease and death in foals and weanlings [1,2]. *Rhodococcus equi* (*Rhodococcus hoagii/Prescotella equi*), a Gram-positive facultative intracellular microorganism [3], is one of the most important causes of pneumonia in foals between 1 and 6 months of age [1,4,5]. Clinically distinguishing *R. equi* pneumonia and that caused by other bacterial agents is problematic because of their similar presentation [1,2]. The early and accurate diagnosis of *R. equi* infection is important because foals are poorly responsive to the common antibiotics used to treat other types of bacterial pneumonia [2,6]. Success in treating *R. equi* infection is greatly enhanced by the use of appropriate antimicrobials [4,7]. Recently, much effort has been directed toward identification of biomarkers that are useful in the differential diagnosis of infectious conditions in foals [8-11].

Bronchoalveolar lavage (BAL) is frequently performed as part of a diagnostic workup for equine respiratory disease [12,13]. It is most commonly performed in mature horses for the diagnosis of non-infective inflammatory diseases, such as equine asthma and exercise-induced pulmonary hemorrhage [14,15]. However, BAL may be used also in foals with clinical pneumonia that is refractory to treatment [13,16].

The aim of this retrospective study was to describe cytological findings in bronchoalveolar lavage fluid (BALF) of foals with pneumonia caused by *R. equi* or other bacteria, and to detect possible differences that could help clinicians in reaching an early diagnosis.

2. Material and methods

2.1 Case inclusion

Foals aged from 3 weeks to 6 months with evidence of respiratory disease between 2015 and 2016 were selected from the database of the Veterinary Teaching Hospital “Mario Modenato” of the University of Pisa. Selection criteria included: evidence of pulmonary disease on physical examination (abnormal thoracic auscultation) and thoracic ultrasonography; BAL performed at their respective breeding farm, no requirement for hospitalization due to the respiratory disease; results of cytology, bacterial culture and polymerase chain reaction (PCR) of the BALF sampled. Thoracic auscultation was considered abnormal when crackles, wheezes, rubs or abnormally increased broncho-vesicular sounds were reported [17]. Thoracic ultrasonography was considered abnormal when ring-down artifact, consolidation areas or abscessation were present [17]. Other data collected were: signalment, duration of clinical signs prior to the BAL, previous treatment administrated at the breeding farm and outcome. Thoracic radiography was not available.

2.2 BAL procedure

The procedure of BAL was standardized and performed with a modified protocol: a 240 cm length and 10x2.5 mm diameter silicone cuffed tube (large animal broncho-alveolar lavage catheter, Mila International, USA) was used [18,19]. Foals were not sedated but only manually restrained, according with the owner’s wishes. The nostrils were cleaned to reduce contamination prior to pass the cuffed tube via the ventral nasal meatus into the lower airways without touching the external nostrils until it could not be further advanced.

To avoid coughing during the passage of the tube through the larynx, trachea and carina, 60 ml of diluted 0.4% lidocaine without epinephrine in 30 ml aliquots was infused. Once the tube

was wedged in a bronchus, the distal cuff was inflated with 5 ml of air [18] to form a seal with the bronchus and 180 ml pre-warmed sterile saline were infused in 60 ml aliquots and immediately aspirated. The volume of fluid retrieved was not recorded. Successively the cuff was deflated and the tube removed. During the whole procedure sterile gloves were used for the manipulation of the tube and the samples to reduce contamination. Samples were collected in EDTA tubes for cytology and PCR analysis, and in sterile tubes with no anticoagulant for microbiology. Samples were stored at 4°C and processed within 2h for cytology. Samples for bacteriology and PCR were stored at 4°C and processed within 24h [20].

2.3 BALF processing

Total nucleated cell count (TNCC) of the BALF was performed on EDTA samples with automated cell counter machine (Idexx, USA), while differential TNCC counts were performed manually by counting 400 cells/smear at 100X after cytocentrifugation of EDTA sample, air drying and Romanowski staining using an automatic colorimeter machine (Aerospray 7152, Delcon, Italy).

BALF samples collected in sterile tubes were cultured on Tryptic Soy Agar containing 5% sheep blood, with and without *Streptococcus* supplement, Mannitol Salt Agar, Mac Conkey Agar, Cetrimide Pseudomonas Agar and Hektoen Agar. Plates were incubated aerobically at 37°C for 72 hours. Within 48 hours, bacterial mucoid, light, large colonies suggestive of *R. equi* were sub-cultured and phenotypically identified using standard procedures that included colony morphology, pink color under natural or artificial light, Gram staining, CAMP testing, urease and catalase positive rods [21].

DNA was obtained from bacterial colonies by the QIAamp DNA Mini Kit (Qiagen, Germany). *R. equi* suspected colonies were tested by PCR as previously described [22]. Beta-haemolytic streptococci were identified by multiplex PCR as previously described [23]. Samples were classified as monoinfection whether only single bacteria (i.e. *R. equi*) have been cultured (pure growth), while samples were classified as mixed infection whether more than one bacterium type (i.e. *R. equi plus Pseudomonas* spp.) were cultured. Foals were considered *R. equi* positive and *R. equi* negative for purposes of comparison.

2.4 Statistical analysis

Data were compared for normality using the method of Kolmogorov-Smirnov. Since not all the data were normally distributed, data were expressed as median±standard error with minimum and maximum value. Statistical analysis was carried out using a Mann-Whitney comparison test. $P < 0.05$ was considered significant. A commercial statistical software package was used (Graph Pad Prism 6, USA).

3. Results

Within the database, 19 foals met the inclusion criteria, 11 colts and 8 fillies, all standardbreds, with a median age of 2.5 months (1-4.5 months). The subjects included came from the same breeding farm located in Tuscany district (Italy). All of them presented unspecific respiratory signs since 4 days and received a treatment with ampicillin (20 mg/kg, every 8 hours, IM) and gentamicin (6.6 mg/kg, every 24 hours, IM), to which they responded poorly, according to the owner. In all of them BAL was performed on the 5th day of clinical signs.

Eight foals out of 19 (42.1%) had *R. equi* pneumonia and in all of them the culture was characterized by a pure growth of *R. equi*. Of the remaining 11/19 (57.9%) foals, 6/19 (31.6%) of them had a mixed bacterial infection and 5/19 (26.3%) a pure growth of either *Staphylococcus* spp. (3/5) or *Streptococcus equi subsp. zooepidemicus* (2/5). Other bacteria cultured included: *Proteus* spp., *Corynebacterium* spp., *Pseudomonas* spp., *E. coli*, *Serratia marcescens* and alpha-hemolytic *Streptococcus* spp.

Based on microbiology and PCR results, foals were divided in *R. equi* negative (n=8) and *R. equi* positive (n=11) foals. The median age of *R. equi* negative foals was 2.5 months, while the median age of *R. equi* positive ones was 2.7 months. No statistically significant difference was found relating to age.

Results of the cytological evaluation of BALF within the two groups are presented in table 1. *R. equi* positive foals had statistically significant higher TNCC (P=0.02) and neutrophils percentage (P=0.002) than *R. equi* negative ones. Macrophages proportion (P=0.01) was lower in *R. equi* positive than in *R. equi* negative foals, while no differences were recorded for the percentage of eosinophils and lymphocytes. In both groups mast cells were not recorded.

Foals were treated according to the results of culture and antimicrobial sensitivity and all of them recovered without the need of further diagnostic tests. Antibiotics used for *R. equi* positive foals included different association of erythromycin (25 mg/kg, every 8 hours, PO), azithromycin (10 mg/kg, every 24 hours, PO for 7 days, then 10 mg/kg every other days), clarithromycin (7.5 mg/kg, every 12 hours, PO) and rifampin (5 mg/kg, every 12 hours, PO). Antimicrobials used for *R. equi* negative foals included: ceftiofur (10 mg/kg, every 12 hours, IM) alone or associated with amikacin (25 mg/kg every 24 hours, IM).

4. Discussion

The purpose of this study was to describe cytological findings in BALF of foals with *Rhodococcus equi* or other bacterial causes of pneumonia, and to identify characteristics that could be used by clinicians to differentiate pneumonic foals with *R. equi* from those infected with other bacteria in order to facilitate appropriate early antimicrobial therapy while awaiting results of culture. The use of macrolides and rifampin without the definitive diagnosis *of R. equi* is contraindicated for several reasons: they may be not effective against other bacteria [1,2]; they are not free of adverse effects, such as diarrhea, fatal colitis in the mares of foals treated and severe hyperthermia due to drug-induced anhidrosis [4]; they are considered critically important antimicrobials for human medicine [24]; there is increasing development of antimicrobial resistance [4,24].

As stated elsewhere, the distinction between lower respiratory tract infections caused by *R. equi* and those caused by other pathogens is difficult [1]. Detection of abscesses by thoracic ultrasonography or radiography may raise the degree of suspicion that pneumonia might be caused by *R. equi* rather than by another microorganism [1,25]. However, bacteriologic culture or amplification of the Vap A gene by PCR from a tracheobronchial aspirate (TBA) are the only acceptable ways of establishing a diagnosis of *R. equi* pneumonia [1,26]. Nevertheless, TBA is frequently eschewed by many practicing veterinarians at breeding farms because of the invasiveness of the technique, lack of consent by the owner, risks and costs related to the procedure [1,26]. Furthermore, bacterial culture can take up to 7 h before an accurate identification can be made. While waiting microbiological results, clinicians often have to

choose to either commence specific antimicrobial therapy targeting *R. equi*, or to use broad-spectrum antimicrobials affective against other bacteria [2,4]. Whenever possible, positive culture should be supported by cytological evidence of septic inflammation and pleomorphic, Gram-positive rods found intracellularly in TBA fluid. The supportive cytological results have the purpose of reducing the likelihood of false-positive results that can occur because of the ubiquity of *R. equi* in the environment of foals [26].

Compared with TBA, BAL is less invasive; frequently more accepted by the owners and can be performed also without the guidance of an endoscope with a blinded technique [12,13]. Furthermore, the analysis of cellular composition of BALF may be immediate and does not necessitate a specific laboratory [12]. Bacteriological culture of BALF does not reliably reflect the microflora of the lower respiratory tract because of the possible contamination during the passage through the upper airways [27]. However, it has been suggested that BAL may be a diagnostic tool useful also in foals with clinical pneumonia that is refractory to treatment [13]. Furthermore, contamination of the lower respiratory tract by materials from the upper airways is often reflected by the recovery of large number of anaerobic bacteria and mixed growth [27] that we did not identify in our samples.

The *R. equi* negative foals were slightly older than *R. equi* positive ones, as already reported [2]. In the current study, in all the *R. equi* positive cases, *R. equi* was present as a pure growth that is in agreement with the study of Giguère et al. [28], but in contrast with what reported by Leclere et al. [2]. All the foals included in the present study came from the same stud farm, thus they all shared the same environment. A less variability of pathogens involved it might be

possible in the present study, compared to the population of Leclere and colleagues [2] that might have been came from different environments.

Age-related changes in the cellular composition of BALF in foals from 1 week to 1 year of age have been reported by Hostetter and colleagues [13]. If we compare the values reported in healthy foals with those found in our population, *R. equi* positive foals showed extremely high TNCC and neutrophils percentage, while foals with other types of pneumonia had more moderate increases. Considering the age-related changes in cellular composition of BALF, *R. equi* negative foals presented a normal proportion of macrophages, in contrast this values found in *R. equi* positive foals. The higher TNCC and neutrophils percentage in foals with *R. equi* pneumonia could be related with a more chronic or severe disease compared with other bacterial infections. Indeed, bronchopneumonia caused by *R. equi* has been characterized by an insidious course, as pulmonary lesions may be quite extensive before the onset of clinical signs [25,26].

This study has some limitations related to its retrospective nature. Firstly, the number of patients is quite limited and further studies are needed to confirm our findings. Also, contamination of samples during the passage of the tube through the upper airways cannot be excluded, nevertheless all the foals responded favorably to the treatment based on the culture results. Finally, it is possible that some of the foals could have recovered spontaneously but, regardless of the treatment and outcome, we were interested in the description of the cytological findings according to the diagnosis.

5. Conclusions

BAL is a quite easy procedure that can be performed in the field with minimal equipment required [12]. Here we reported significant differences in the cellular composition of BALF that can be used to differentiate foals with *R. equi* bronchopneumonia from those with other bacterial pneumonias, while waiting for culture results. It could be interesting to know if also the TBA cytology reflects the same differences in these two populations of foals.

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Authorship Declaration

VV, MS, BF - conception and design of the study, acquisition, analysis and interpretation of data; VC, SP, ARA – analysis and interpretation of data VV - drafted the article; MS and BF revised the manuscript critically for important intellectual content. All the authors approved the final version to be submitted.

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Table 1

Results of total nucleated cell count (TNCC) and differential cell count of bronchoalveolar lavage fluid (BALF) in *R. equi* positive and *R. equi* negative foals. Data are presented as median±standard error, minimum and maximum values.

Variable	*R. equi* positive	*R. equi* negative	*P* value
TNCC (cells/µL)	1300±322* 500-2800	500±25* 400-650	0.0291
Macrophages (%)	52±4* 20-58	67±3* 40-80	0.0140
Lymphocytes (%)	5±0 3-7	17±2 4-30	0.0554
Neutrophils (%)	40±4* 39-75	21±3* 1-40	0.0020
Eosinophils (%)	0±0 0-1	0±0 0-5	0.8571

* Indicates statistically significant differences between groups. P values are reported on the right column.

Declarations of interest: none.

Ethical Statement

All the procedures were carried out in order to diagnose a pathological problem in sick animals; thus an owner's written consent was obtained before carrying out all the clinical procedures described.

Due to the retrospective nature of the study, an owner written consent has been obtained in order to use clinical data of foals for research purpose.