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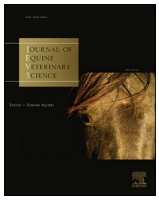

Short Communication

Evaluation of bronchoalveolar lavage fluid from donkeys using four different cytological stains: A pilot study

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ABSTRACT

Bronchoalveolar lavage fluid (BALF) cytology is used for the diagnosis of non-infectious lower airway inflammation in equids. Discrepancies have been reported in the differential cell count when different staining methods were used both in humans and horses. The objective of this study was to compare the results of BALF cytology in donkeys using four different staining methods: modified May-Grunwald Giemsa (mMGG), Diff-Quick (DQ), Toluidine blue (TB) and Perls Prussian blue (PPB). Nine healthy Amiata female donkeys were enrolled. The BAL procedure was performed as previously described and pairs of cytocentrifuged BALF slides were stained with each method. No differences between mMGG and DQ were found for macrophages, neutrophils, and eosinophils, while differences were found in mast cell count between DQ *vs.*TB, but not between mMGG *vs.* DQ or mMGG *vs.* TB. Finally, no differences were obtained in the differential count for hemosiderophages comparing mMGG, DQ and PPB. The mMGG appears to be an excellent stain for the identification of all possible cell types, including mast cells in the BALF of donkeys. DQ, if used alone, may lead to inappropriate identification of mast cells. These results are consistent with the literature on BALF staining methods in horses.

1. Introduction

Bronchoalveolar lavage fluid (BALF) cytology is a diagnostic tool used for the diagnosis of non-infectious lower airway inflammation in horses [1,2]. Different staining methods for BALF cytology evaluation have been reported, however, some discrepancies exist in the differential count of nucleated cells between them, especially for mast cells and hemosiderophages [3,4]. In horses, Fast Romanowsky (FAST-R) does not stain metachromatic granules, making mast cell identification hard to perform [3]. Toluidine blue was reported as the stain of choice for mast cells [5]; however, it does not allow the identification of other cell types making impossible to perform a complete differential cell count [1, 3]. Staining with an automated Romanowsky stain (AUTO-R) and May-Grunwald Giemsa gave the most accurate estimation of mast cells, without altering the identification of other cells in the BALF of horses [3, 6]. Concerning hemosiderophages, the Perls Prussian blue is considered the staining method of choice in humans and horses [7–9]. Due to the importance of a correct identification of all cell types in the BALF cytology for diagnostic purposes, it is important to choose an optimal staining method [1].

Literature about BALF cytological findings and interpretation in healthy and sick donkeys is limited and often is extrapolated from literature from horses [10–13]. However, a slightly different percentage of macrophages and eosinophils between horses and donkeys have been reported; [11,12]. This study aimed to compare results of BALF cytology obtained from donkeys, performed according to four different staining methods.

2. Material and methods

2.1. Ethical approval

The study took place between May and October 2022 after approval by the Research Ethical Committee of the University of Pisa (Approval number, 3/21; Approval date, 22/01/21).

2.2. Animals

Nine female donkeys used for reproduction purposes, aged between 8 and 17 years and weighed 357–425 kg with an average body condition

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score of 5/9, were enrolled in the study. The herd was housed at the Veterinary Teaching Hospital, University of Pisa (Italy) in a large sand paddock (20 × 30 m) with two shelters. They were fed a diet consisting of *ad libitum* grass hay and were vaccinated against tetanus, influenza, and herpesvirus-1 and -4 once per year [14]. They were routinely dewormed, based on faecal egg count, with ivermectin (0.2 mg/kg orally), at least twice annually. Inclusion criteria were that donkeys were normal on physical examination, including thoracic and tracheal auscultation, for 3 months prior inclusion and until the end of the study. For this reason, all the animals underwent to weekly physical examinations performed by two trained clinicians.

The day before the scheduled BAL, donkeys were housed around 7 p.m. in 4 × 4 m boxes with a cement floor and straw bedding. Animals were housed in pairs to avoid unnecessary stress. Food was withheld for 12 h, while access to water remained *ad libitum*.

2.3. Procedure

All the donkeys were sedated with an intramuscular injection of romifidine (Sedivet, Boeheringer, dose 0.1 mg/kg) and butorphanol (Nargesic, ACME, dose 0.05 mg/kg). Drugs were administered to donkeys staying in their own boxes; then, animals were immediately transferred to the examination room where they were restrained in stocks. BAL procedures were performed as previously reported [12]. Briefly, a large animal BAL catheter (Mila International Inc, USA) measuring 240 cm in length, 2.5 mm of inner diameter, and 10 mm of external diameter, and provided with an inflatable cuff was passed through the nasal passage and moved into the trachea. For each donkey, five syringes were prefilled with 20 mL of a solution of lidocaine (Lidocaina 2 %, Ecuphar Italia srl) and 0.9 % saline solution (dilution 0.66 %). The solution was used to desensitize the tracheal lumen while advancing the tube for antitussive purposes. Once the BAL tube reached the third- to fourth-generation bronchi, the cuff was inflated with air, and 300 mL of warm, sterile 0.9 % saline solution was infused followed by 60 mL of air to remove all the fluid from the tube. BALF samples were collected by gentle and constant manual aspiration using a 60 mL syringes. When at least 40 % of the infused volume was recovered, the cuff was deflated, and the tube was removed. The BALF obtained was immediately assessed by visual inspection to ensure the presence of foam, indicative of a well performed procedure. Subsequently, the syringes were pooled to gather a representative aliquot of BALF that was collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 1 h from the collection. No fixatives were used to avoid altering cell morphology [15].

When sedation effects ended, donkeys were moved back to the barn and feed access was restricted until the normalizing of intestinal motility. No side effects of both sedation and BAL procedures were recorded and all the animals included went back to the main sand paddock around 6 PM. of the same day.

2.4. Slide preparation and staining methods

Slides were prepared by cytocentrifugation (Cytofuge 2, Statspin, USA; 400 µL *per* slide and 300 g rpm for 10 min) and air-dried. For each donkey, slides were stained in pairs using four different colorations, thus eight slides were prepared and assessed for each animal making a total of 72 slides. Staining methods were the modified May-Grunwald Giemsa (mMGG) stain, the Diff-Quick (DQ) stain, Toluidine Blue (TB), and the Perls Prussian blue (PPB) stain. The mMGG stain was performed with an automated slide stainer (Aerospray Hematology Slide Stainer mod. 7150, Delcon, Italy). The staining time was 12 min. The DQ stain (Bio-Optica Milano S.p.a., Italy) was based on the Romanowsky modified metachromatic staining which is made by one alcoholic fixative (methanol) and two stains (eosin and methylene blue). The staining was performed according to the manufacturer's recommendations. Briefly, each slide was immersed five times for 1 to 2 s in methanol, eosin, and methylene blue solutions following this order. Before passing from a solution to another, the stain was held above a clean disposable paper towel for few seconds to eliminate the excess of solution. At the end of these passages, the slides were washed with water and subsequently air-dried. For the TB (Bio-Optica Milano S.p.a., Italy) staining, slides were prepared by removing background material using a sulphate solution, immersed into the stain for 1-5 min and then fixation with absolute ethanol. The PPB stain was performed by a first step of fixation in methanol for 3 min, followed by the air-dry of the slides. Subsequently, 10 drops each of reagent A (potassium ferrocyanide) and reagent B (hydrochloric acid) were added to the slides and left to act for 20 min. Slides were then washed in distilled water and counterstained with 10 drops of reagent C (neutral red or carmallume Mayer) which was left to act for 5 min. After another wash with distilled water, the slides were air-dried again. The total staining time was 35 min.

No media was used to mount the slides. A single experienced blinded veterinarian carried out all the differential cell counts. Initially, the stained smears were scanned at low magnification (100x), then at higher magnification (400x) to identify an optimal monolayer area. Subsequently, the cells were counted with oil immersion (1,000x) and an automated counter (Leucoform 83, Crison Instruments S.A., Spain). The technique of scanning started from the upper edges and continued to the center, until arriving at the lower edges to avoid counting already seen areas. Four hundred cells were counted per each differential count, using bright-field light microscopy.

2.5. Statistical analysis

Data was analysed for distribution using the D'Agostino and Pearson test. Data with Gaussian distribution was presented as average and standard deviation, while the one with non-Gaussian distribution was expressed as median, 1st, and 3rd quartile. No power analysis was performed since the animals were included on an opportunistic manner.

Due to the limited sample size, non-parametric tests were used. The Wilcoxon matched pairs signed rank test was applied to verify differences in the differential cell count between the mMGG vs. DQ stains for macrophages, lymphocytes, neutrophils, eosinophils, and mast cells. The Friedmann test for paired data and Dunn's multiple comparison test as post-hoc were applied to detect differences in the differential cell count for mast cells among mMGG vs. DQ vs. TB and for hemosiderophages among mMGG vs. DQ vs. PPB.

The statistical analysis was performed using a commercial software (GraphPad Prism 9, USA) and the cut off for significance was set at $P < 0.05$.

3. Results

The percentage of macrophages, lymphocytes, eosinophils stained with mMGG and DQ staining, and the percentage of mast cells stained with mMGG and TB showed a Gaussian distribution. The percentage of neutrophils stained with mMGG and DQ, the percentage of mast cells stained with DQ staining, and the percentage of hemosiderophages stained with mMGG, DQ and PPB showed non-Gaussian distribution. In Table 1, the results of the differential cell counts for macrophages, lymphocytes, neutrophils, mast cells obtained with mMGG and DQ, and the percentages of mast cells and hemosiderophages revealed by the TB and PPB staining were reported.

Comparing the mMGG and DQ staining, no significant differences were observed for the differential counts of macrophages ($p = 0.09$), neutrophils ($p = 0.6$), eosinophils ($p > 0.99$), while significant differences were found for mast cell count ($p = 0.03$) and lymphocytes ($p < 0.01$).

Significant differences were found for mast cells ($p < 0.01$) differential cell count between DQ vs. TB, but not between mMGG vs. DQ or mMGG vs. TB. No differences ($p = 0.11$) were obtained in the differential count for hemosiderophages comparing mMGG, DQ and PPB.

Table 1
Differential cells counts (%) of bronchoalveolar lavage fluid obtained from nine donkeys and stained using four different staining methods Results are presented as average±standard deviation, or median, 1st, and 3rd quartile, depending on data distribution.

	mMGG						DQ						TB	PPB
	M (%)	L (%)	N (%)	E (%)	MC (%)	HS (%)	M (%)	L (%)	N (%)	E (%)	MC (%)	HS (%)	MC (%)	HS (%)
1	52	42	2	1	3	0	39	56	2	3	0	0	4	0
2	30	61	7	2	0	0	25	68	6	1	0	0	1	1
3	19	77	3	0	1	0	16	80	3	1	0	0	1	0
4	24	58	9	4	5	0	29	63	5	3	0	0	1	0
5	49	29	18	2	2	0	35	37	27	1	0	0	1	0
6	45	44	7	1	3	0	45	48	4	3	0	0	4	0
7	32	56	5	7	0	0	36	56	5	3	0	0	0	0
8	36	59	1	3	1	0	28	67	2	3	0	0	1	1
9	16	81	3	0	0	0	10	89	1	0	0	0	1	1
X±SD - Med (1st-2nd IQ)	33.7 ±13.0	57.4 ±14.6	5 (2.5-8.0)	2.2 ±2.2	1.7 ±1.7	0 (0-0)	29.2 ±11.1	62.7 ±14.6	4 (2.0-5.5)	2.0 ±1.2	0 (0-0)	0 (0-0)	1.6 ±1.4	0 (0-1)

Legend – mMGG: modified May-Grunwald Giemsa; DQ: Diff-Quick; TB: Toluidine blue; PPB: Perls Prussian blue; M: macrophages; L: lymphocytes; N: neutrophils; E: eosinophils; MC: mast cells; HS: hemosiderophages; X±SD: average and Standard Deviation; Med (1st-2nd IQ): median value (1st and 2nd Interquartile).

4. Discussion

This study described the cytological findings with four different staining methods of the BALF in nine healthy female Amiata donkeys. The mMGG is a metachromatic stain widely used in equine clinical pathology that is particularly indicated to recognize macrophage morphology and differentiation stage [3]. The DQ is commonly used in equine practice for staining cytological preparations of respiratory tract samples; however, several authors believe that detection of mast cells is hindered by the utilization of this stain as it may stain their granules poorly and lead to an underestimation of their numbers [15,16]. The TB is a basic thiazine metachromatic stain with an affinity for acid tissue components, such as mast cell granules [17]. The PPB stain is a commonly used method to detect the presence of iron in tissue or cell samples. The method does not involve the application of a dye, but rather causes the pigment Prussian blue to form directly within the tissue. This method stains mostly iron in the ferric state which includes ferritin and hemosiderin [18]. Results from the present study supported previous research with slightly decreased macrophages and slightly increased eosinophils' percentage in donkeys compared to horses [1, 10–12].

No differences between mMGG and the DQ staining procedures for the differential cell count of macrophages, neutrophils, and eosinophils were found, in line with previous findings in horses [15,16], while differences were observed in lymphocyte percentage. This discrepancy might be related to the low number of donkeys included.

Significant differences in the mast cells count between DQ and TB confirmed that DQ might be considered an inappropriate staining method for the identification of mast cells, in line with research using horses [15,16] and small animals [19]. The lack of identification of mast cell by the DQ has been related to its non-metachromatic characteristics which fail to stain mast cell granules. This may result into an incorrect classification of mast cells as macrophages or epithelial cells and to an underestimation of the number of mast cells [15]. Compared to horses, donkeys affected by respiratory diseases may not show a significant increase in mast cells count [11,12], thus, the limitation of DQ may not represent an issue for donkeys. No differences were found in detecting mast cell percentages between TB and mMGG in the current study, confirming that mMGG provided appropriate straining. Unfortunately, although TB is considered as the stain of choice for mast cells [5], it does not allow the identification of other cell types [3]. Moreover, it can induce metachromasia in the intracellular granules of macrophages, leading to an incorrect classification between macrophages and mast cells [3,16].

Although no differences were found among mMGG, DQ and PPB for hemosiderophages percentage, only the PPB stain was able to identify a small percentage of hemosiderophages, as previously described in humans and horses [7,9,15]. The limited percentage of hemosiderophages found in the BALF of our donkey population composed of resting animals supports the speculation that a small number of macrophages containing hemosiderin can be identified also in subjects with no history of pulmonary haemorrhage [20]. Moreover, a recent study [21] reported the association between severe asthma and the presence of haemosiderin in BALF of lightly active or sedentary horses; thus, the use of PBB staining could be considered useful for the assessment of BALF not only in athlete horses with suspected EIPH, but also in sedentary horses and donkeys with different respiratory diseases, such as asthma.

The limited numbers of animals included in this study constitutes the main limitation of this research. It cannot be excluded that no statistically significant differences have been detected for some of the cell percentages obtained with the different staining methods due to a type II error. Furthermore, due to the same breed, age, sex, and environmental management of the donkeys included in this study, it is not possible to extrapolate our results to a larger donkey population housed in different conditions. However, our findings are in line with the previous research on this species [11,12] and our aim was limited to compare the cytological results obtained with the four staining methods.

5. Conclusions

Based on the results of this study, mMGG seems to be an excellent stain for the identification of all possible cell types, including mast cells in the BALF of donkeys. DQ, if used alone, may lead to an inappropriate identification of mast cells. Further studies are needed to verify the impact on the diagnostic and prognostic approach, including a higher caseload and sick and/or athletic donkeys.

Ethical statement

The study has been approved by the Research Ethical Committee (OPBA) of the University of Pisa (n. 3/21) on the 22/01/21.

CRediT authorship contribution statement

V. Vitale: Writing – original draft. **F. Bindi:** Investigation. **A. Briganti:** Supervision. **F. Bonelli:** Formal analysis. **C. Parietti:** Supervision. **M. Sgorbini:** Conceptualization, Writing – review & editing.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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