

Neutrophils function in parasite clearance via phagocytosis, production of reactive oxygen species (ROS), production of antimicrobial products or by the generation of neutrophils extracellular traps (NETs)²¹⁵. They also produce cytokines and chemokines and may be associated with antigen presentation, thereby playing a crucial role in activating and regulating the immune response^{216,217}. In human malaria, various changes are described within the neutrophil population in the peripheral circulation²¹⁸. Stable neutrophil numbers have been reported during the asymptomatic liver stage of non-immune persons with controlled human malaria infection²¹⁹. Olliaro et al (2011) demonstrated a 43% rise in circulatory neutrophil numbers during the acute stage in persons with uncomplicated malarial infections. This neutrophil rise was directly related to the level of parasitaemia²²⁰. Neutrophils have different subsets with different functional properties²²¹. Differing behaviour amongst the neutrophil sub-populations are detected amongst persons infected with falciparum malaria compared to uninfected persons²¹⁸, where a subgroup of neutrophils in persons with falciparum malaria is associated with decreased respiratory oxidative burst function suggesting changes within neutrophil subgroups as the disease progresses²²². During falciparum malaria, increased levels of neutrophil chemoattractants are present, but malarial pathology is rarely associated with notable neutrophil infiltration at parasitized red blood cell sequestration sites²¹⁸. In brain microvasculature, as well as in placentas and pulmonary tissue during falciparum malaria, the number of neutrophils were not significant^{223–225}. Similarly, in canine babesiosis neutrophils were found to be scant in the splenic and pulmonary tissues from infected animals^{187,188}. In these studies, it was concluded that neutrophils are not a major contributor to the injury observed during canine babesiosis^{187,188}. Data suggests the recruitment of neutrophils to infection sites during falciparum malaria and canine babesiosis is not always present and Aitken et al (2018) postulated that neutrophil chemotaxis may be inhibited by infection²¹⁸. Decreased neutrophil chemotaxis is observed in human falciparum malaria, with parasite antigens likely involved as is evident by the restoration of chemotaxis after treatment²²⁶. Neutrophil chemotaxis is suppressed by *P. falciparum* protein MSP1-19 blocking the neutrophil's response to S100P, a proinflammatory protein²²⁷. A similar mechanism may be involved with canine babesiosis. It is possible that neutrophil nets play a role in the acute inflammation, coagulopathy and platelet activation that are present in babesiosis.

A study conducted by Atkinson et al (2021), demonstrated an inverse relationship between the number of segmented neutrophils and the cytokine keratinocyte chemotactic-like (KC-like)¹⁹⁰. This cytokine plays an important role in the migration and activation of neutrophils²²⁸. Similarly, an inverse relationship was also demonstrated with IL-8 and the number of segmented neutrophils¹⁹⁰. This mirrors what Goddard et al (2016) found in their study where the concentration of IL-8 was notably decreased in *Babesia* infected dogs as compared to control dogs and this low level of IL-8 serum concentration correlated with a notable decreased circulating neutrophil count¹²². IL-8 plays an important role in neutrophil recruitment and activation²²⁹. A decrease in the segmented neutrophil number has also been reported in acute human falciparum malaria and various mechanisms for this have been proposed, namely a shift from the circulating pool to the marginated pool and early release of premature granulocytes from the bone marrow²³⁰. Increased levels of myeloperoxidase index (MPXI) in neutrophils were noted in dogs infected with *B. rossi*, ascribed to possible hastened maturation and release of immature neutrophils from the bone marrow²³¹.

4.2.3. Lymphocytes

There was no appreciable difference in the lymphocyte counts between the infected and control groups. The number of plasma cells were increased (as is evident by the number of MUM-1 positive labelling cells), but the increase was not statistically significant.

4.2.3.1. T-lymphocytes

This negligible difference in the T-cell population between the infected and control animal group mirrors what has been found in the canine spleen during babesiosis¹⁸⁷. Rautenbach et al (2017) in their study assessing the

peripheral immunophenotypes in dogs naturally infected with *Babesia rossi*, demonstrated a decline in CD3+ lymphocytes in dogs with complicated babesiosis, compared to uncomplicated babesiosis cases. The authors hypothesized about the possibility of a functional immunosuppression due to apoptosis or redistribution of the effector T-cells, or a combination of the above and other mechanisms⁵⁰. Martin (2019) in his study on the pathology associated with complicated *Babesia rossi* acute lung injury, demonstrated a significant increase in T-lymphocytes within alveolar walls of the lung, thereby suggesting that redistribution of these circulating T-cells may partially explain the relative decrease of T-cells observed within the Rautenbach et al (2017) study¹⁸⁸. Henning et al (2019) demonstrated in their study on the splenic pathology during canine babesiosis, that there is a negligible difference in the T-cell population between the infected and control animal group. This lack of significant T-cell increase may, apart from previously mentioned possibilities of rapid redistribution and accelerated apoptosis, be due to the influence of alternatively activated macrophages¹⁸⁷. Whilst classically activated macrophages are instrumental in protection of the host against a variety of microbial pathogens, alternatively activated macrophages play a major role in immune regulation and tissue repair²³². These macrophages are characterized by a distinct phenotype due to differing gene expression profiles²³³. Alternatively activated macrophages have been demonstrated to suppress T-cell function in a variety of infectious disease, including *Theileria* spp., *Trypanosoma* spp., and *Toxoplasma* spp.^{234–237}.

4.2.3.2. B-lymphocytes and plasma cells

There was a slight increase in B-lymphocytes within the infected animal group as compared to the control group in this study, but this increase was not statistically significant. The number of plasma cells were increased within the infected group, albeit not statistically significant. This plasma cell hyperplasia is consistent with inflammation and is deemed an appropriate bone marrow response. In human falciparum malaria, it has been demonstrated that the malarial parasite can negatively influence the generation and long-term maintenance of memory B-cells, ultimately with a negative effect on the humoral immune response²³⁸. Severe disruption of B-cell lymphopoiesis has also been demonstrated in mice infected with acute *Plasmodium chabaudi*²³⁹. Rautenbach et al (2017) demonstrated an increase in the B-lymphocyte population two to three days after presentation in dogs with complicated babesiosis⁵⁰. This increase in B-lymphocytes and plasma cells is time dependent with an increase noted in increasing chronicity. However, during the early stages of infection, several studies have demonstrated that resistance to babesiosis and malaria is antibody independent and an absence of B lymphocytes is not associated with impaired resistance^{3,207,240,241}. That said however, antibody responses are essential for parasite clearance after the acute infection is resolved²⁴². Although the time frame of disease within the infected animal group in this study is known, there is insufficient data to explore the possibility of parasite suppression of B-cell lymphopoiesis and memory B-cell production.

4.3. Megakaryocyte response

There was a left shift within the bone marrow megakaryocyte population of the infected group in this study. This is an appropriate response of the bone marrow considering the presence of a peripheral thrombocytopenia, which is common in canine babesiosis⁵⁴. The pathogenesis of thrombocytopenia during canine babesiosis is unknown, although from this study, bone marrow suppression with decreased platelet production appears unlikely. The finding of an adequate number of megakaryocytes in this study is similar as to what has been found in Kirtz, Leschnik et al.(2012) study. As concluded in their study, the thrombocytopenia during canine babesiosis is likely of peripheral origin via either immune-mediated destruction and increased consumption⁴⁴. An immune-mediated thrombocytopenia was also proposed by Scheepers et al (2011), where they found resolution of the thrombocytopenia in most *Babesia*-infected dogs after six days of infection, thereby suggesting a sufficient bone marrow response⁴⁵, which has now been confirmed with this study.

4.4. Bone marrow sequestration of parasites

Babesia parasites were present within vasculature, in contrast to what has been reported for *P. vivax* parasites, where the majority was located within the extravascular parenchyma of the bone marrow¹¹⁸. Sexual forms (i.e. gametocytes) of the malarial parasite are sequestered within the extravascular environment of the bone marrow¹¹⁸. Preliminary studies have also shown that this is the case in mouse models and has also suggested the occurrence of an asexual reservoir in the bone marrow¹²⁰. In order to complete its life cycle, it is essential for the malarial parasite to sequester within the bone marrow as the release of parasites into the peripheral blood circulation allows for parasite recognition by surveillance macrophages and subsequent splenic clearance¹²⁰. Asexually replicating parasites are sequestered via the attachment of the parasitized red blood cell to the vascular endothelium of various tissues, including the bone marrow¹²⁰. Sexual conjugation and sporogony of the *Babesia* parasite are completed within the transmitter tick and the asexual stage is present within the vertebrate host²⁴³. Whether the bone marrow serves as a reservoir of the asexual stages of the *Babesia* parasite still needs to be investigated in future studies, including the changes within the bone marrow endothelium upon infection.

In our study vascular endothelial cell hypertrophy was observed in the bone marrow of *Babesia* infected dogs. Endothelial cells play a crucial role in the regulation of haematopoiesis during inflammation. PPRs are expressed on endothelial cells and upon activation by TLR4, G-CSF is produced, thereby ensuring neutrophil production within the bone marrow with simultaneous neutrophil recruitment to the site of infection¹⁹². Upon stimulation by TNF- α and IL-1 β , bone marrow endothelial cells also produce GM-CSF leading to neutrophil recruitment and proliferation of haematopoietic progenitor cells in the bone marrow¹⁹². Future studies should evaluate the role of bone marrow vascular endothelium during canine babesiosis, including the interaction of the *Babesia* parasite with the host vasculature, the role of endothelium in the regulation of haematopoiesis and the role of endothelium as to potential parasite sequestration within the bone marrow.

No obvious differences were noted between the bone marrow smears and HE sections from the treated and non-treated infected cases. The degree of parasitaemia varied from low to high in the various infected cases, including the treated and non-treated cases. The highest parasitaemia was noted in the infected case that received Doxycycline treatment at home (case 6). This study is limited in the number of infected cases. As such, no conclusions can be drawn on the effect of treatment on the parasitic load within the bone marrow. This would have to be investigated in future studies.

4.5. Role of parasite factors on the bone marrow response

As discussed previously, an inappropriate erythroid bone marrow response was detected in half of the *Babesia* cases in this study. Included in the various hypotheses for this phenomenon (as discussed previously), is the influence of various parasite products, e.g. haemozoin produced by malarial parasites. Haemozoin is not produced by *Babesia* parasites¹⁰⁴, however, GPI molecules have been demonstrated to be present within *Babesia* parasites^{105,106}. Therefore, apart from haemozoin, other factors, e.g. GPI need to be considered as a cause of the inappropriate bone marrow response and reticulocyte suppression in cases of *Babesia*-induced anaemia.

4.6. Comparison between bone marrow cytology and bone marrow histopathology

The bone marrow cytological findings largely mirrored the histopathological findings. However, a discrepancy was noted in the number of macrophages within the infected group, where significantly raised values were not evident on bone marrow cytology as compared to the significantly increased CD204-reactive macrophages within histological specimens in the infected group. In *Babesia* case 4, the number of macrophages was decreased to such an extent that its value was comparable to the control dogs and this decreased value caused

for a statistically insignificant raise within the histiocytes. This discrepancy may be explained by the fact that the bone marrow smears were relatively thick in areas and evaluation of cellular niches were not always possible. Hence, evaluation focused on areas where the smears were thinner and as a result, the number of histiocytes may have been underestimated with cytological examination.

CONCLUSION

The bone marrow changes during canine babesiosis were characterized by a significant erythroid (rubriblast) and histiocytic response with sufficient iron stores. This macrophage response is characteristic of *Babesia rossi* infection, correlating with what other studies have found in the spleen and lungs of *Babesia* infected dogs and in natural and experimental malarial infections^{85,187,188}. The thrombocytopenia consistently observed during canine babesiosis is deemed to be of peripheral origin via either immune mediated destruction or increased consumption as megakaryocytes were adequate / increased in number in this study. The bone marrow response in infected dogs was inappropriate within the erythroid lineage in this study, mirroring what has been found in previous studies^{45,46,72}. This inappropriate response is likely due to a variety of causes, amongst which host factors (i.e. release of pro-inflammatory cytokines with subsequent reduced erythrocyte survival, impaired iron mobilization or utilization and impaired red blood cell production due to a nonresponsiveness to EPO) and dyserythropoiesis play a major role. Dyserythropoiesis was demonstrated in all *Babesia*-infected cases. Iron availability may play a role in this inappropriate response to the anaemia during canine babesiosis, although in this study there were sufficient iron stores within the bone marrow and reticulocyte indices did not support iron-restricted erythropoiesis. As demonstrated in previous studies¹²², it is likely that a cytokine dysregulation may be responsible for the inappropriate bone marrow response, but further investigation is necessary to explain this phenomenon. In contrast to what has been found in human and murine malaria in which parasitized erythrocytes are sequestered within the bone marrow, a similar finding is not supported by the findings of this study. The small sample size of *Babesia*-infected dogs is a limitation in this study and therefore future research should incorporate a larger infected animal group in an attempt to answer various research questions, including: 1). Is there sequestration of the *Babesia* parasite within the bone marrow? 2). What are the mechanisms behind the inappropriate bone marrow erythroid responses observed in the infected cases in this study? This should include an investigation of the role of various host factors (e.g. TNF, IL-1) and parasite factors (e.g. MIF, GPI) and 3.) What role does the vascular endothelium play in potential parasite sequestration within the bone marrow? From the results obtained in the present study, it is clear that evaluation of the bone marrow should be conducted in concordance with evaluation of the complete blood count / haemogram and bone marrow cytology to ensure accurate interpretation of bone marrow findings.

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APPENDICES

Appendix 1: Informed consent form

(To be completed by the patient's owner / authorised agent)

Encircle Yes or No where necessary

- | | | | |
|----|--|-----|----|
| 1. | Have you read the information sheet on canine babesiosis? | Yes | No |
| 2. | Have you had the opportunity to ask questions about the research project? | Yes | No |
| 3. | Have you received satisfactory answers to your questions? | Yes | No |
| 4. | Have you received enough information about this study | Yes | No |
| 5. | Supply the name of the person to whom you have spoken to:

..... | | |
| 6. | Do you grant consent that blood and urine samples can be drawn from your dog? | Yes | No |
| 7. | Do you grant consent that a post mortem examination can be performed in the case of death? | Yes | No |

I,, hereby give permission that my dog, a may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore I understand that the costs of the additional tests will be borne by the trial fund, and that I will only be liable for costs pertaining the treatment that would in any event be required by my dog, including any complications that may arise as a result of canine babesiosis.

Signed at Onderstepoort on the day of 20.....

Signature Owner/Agent

Home Tel:

Work Tel:

Cell No:

Appendix 2: Histopathology check-list

Observational matrix for histopathology of the bone marrow [modified from Raskin & Messick (2012)]

OVERALL CELLULARITY (%) (x10 objective)	Hypocellular	Mild	
		Moderate	
		Severe	
	Normocellular (25% - 75%)		
	Hypercellular	Mild	
		Moderate	
Severe			
MEGAKARYOCYTES (x10 objective)	Number	Decreased	
		Adequate	
		Increased	
	Distribution		
	General maturity	Mature	
Immature			
IRON CONTENT (PERLS' PRUSSIAN BLUE STAIN)	Absent / decreased		
	Within normal limits		
	Decreased		
Presence of erythrophagocytosis	Yes		
	No		
Parasite score			
Presence of vascular necrosis / vasculitis	Yes		
	No		
Thrombosis	Yes		
	No		
Haemorrhage	Yes		
	No		
Congestion / hyperaemia	Yes		
	No		
Endothelial cell hypertrophy	Yes		
	No		

Appendix 3: Histopathological findings of the bone marrow for each individual case

Babesia case #1 (Hospitalization number: N/A; Post mortem number: S847-19)

On low power magnification the sections show sparse fragments of bony trabeculae and few adipocytes. The bone marrow is hypercellular, approximately 90%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 4 to 11 per high power field, with a mean of 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident, approximately 6-7 per high power field (x40) and 62 per 10 high power fields (x400). Erythrophagocytosis is observed. Sparse haemosiderin pigment is evident.

Cellularity score: 4

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 4-11; mean 7.33

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: Yes, scattered mild

Thrombosis: No

Babesia case #2 (Hospitalization number: 2237315; Post mortem number: S2678-17)

On low power magnification the sections show few fragments of bony trabeculae and a low number of adipocytes. The bone marrow is hypercellular, approximately 85%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-7 per high power field, with a mean of 5.67. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines appear to predominate but few differentiated cells are seen. Multifocal mitoses are evident, approximately 59 per 10 high power fields (x400). Erythrophagocytosis is observed. A moderate number of haemosiderin pigment is evident.

Cellularity score: 4

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 5-7; mean 5.67

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: No

Thrombosis: No

Babesia case #3 (Hospitalization number: 5191717; Post mortem number: S1951-17)

On low power magnification the sections show scattered fragments of bony trabeculae and sparse adipocytes. The bone marrow is hypercellular, approximately 90 - 95%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 6-9 per high power field with the mean 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power

magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident - 54 per 10 high power fields (x400). Erythrophagocytosis is observed. Sparse haemosiderin pigment is evident.

Cellularity score: 4

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 6-9, mean 7.33

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: No

Thrombosis: No

Babesia case #4 (Hospitalization number: 4944217; Post mortem number: S879-17)

On low power magnification the sections show sparse fragments of bony trabeculae and moderate numbers of adipocytes. The bone marrow is hypercellular, approximately 90%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-6 per high power field, with the mean 5.33. Megakaryocytes of all maturational stages are evident and mature cells appear to be slightly predominating. On high power magnification (x40) immature cell lines appear to predominate and fewer differentiated cells are seen. Multifocal mitoses are evident - 43 per 10 high power fields (x400). Erythrophagocytosis is observed. A moderate to large amount of haemosiderin pigment is evident.

Cellularity score: 3

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 5-6, mean 5.33

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: No

Thrombosis: No

Babesia case #5 (Hospitalization number: Ozzy; Post mortem number: S776-19)

On low power magnification the sections show rare fragments of bony trabeculae and moderate numbers of adipocytes. The bone marrow is hypercellular, approximately 80%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 6-9 per high power field with a mean of 7.33. Megakaryocytes of all maturational stages are evident, but immatures appear to be predominating (left shift). On high power magnification (x40) immature cell lines predominate fewer differentiated cells are seen. Multifocal mitoses are evident - 42 per 10 high power fields (x400). Erythrophagocytosis is observed. Moderate number of haemosiderin pigment is evident.

Cellularity score: 4

Parasite score: 0

Endothelial cell reactivity score: 1

Megakaryocyte: Range 6-9, mean 7.33

Megakaryocyte score: 3

Congestion: Yes
Haemorrhage: Yes
Thrombosis: No

Babesia case #6 (Hospitalization number: 5376417; Post mortem number: S2710-17)

On low power magnification bony trabeculae are not clear. Few adipocytes are observed. The bone marrow is hypercellular, approximately 80-85%. There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 5-10 per high power field and a mean of 7.33. Megakaryocytes are mostly immature (i.e. left shifted megakaryocyte hyperplasia). On high power magnification (x40) immature cell lines predominate by far and only scattered few differentiated cells are seen. Multifocal mitoses are evident, approximately 59 per 10 high power fields (x400). Erythrophagocytosis is observed. Small amounts of haemosiderin pigment is evident.

Cellularity score: 4
Parasite score: 1
Endothelial cell reactivity score: 1
Megakaryocyte: Range 5-10, mean 7.33
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Control case #1 (Hospitalization number: N/A; Post mortem number: S4191-18)

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 4-9 per high power field and a mean of 6.67. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 21 per 10 high power fields (x400). Small to moderate amounts of haemosiderin pigment is evident.

Cellularity score: 3
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 4-9, mean 6.67
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Control case #2 [Hospitalization number: N/A; Post mortem number: S4191-18(2)]

On low power magnification few fragments of bony trabeculae are evident. Moderate to occasionally large numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –2-9 per high power field with a mean of 5. Megakaryocytes of various maturational stages are evident but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 13 per 10 high power fields (x400). Small amounts of haemosiderin pigment is evident.

Cellularity score: 2

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 2-9, mean 5

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: No

Thrombosis: No

Control case #3 [Hospitalization number: N/A; Post mortem number: S4191-18(3)]

On low power magnification few fragments of bony trabeculae are evident. Moderate to occasionally large numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –3-5 per high power field and a mean of 4. They are mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 23 per 10 high power fields (x400). Moderate amounts of haemosiderin pigment is evident.

Cellularity score: 3

Parasite score: 0

Endothelial cell reactivity score: 0

Megakaryocyte: Range 3-5, mean 4

Megakaryocyte score: 3

Congestion: Yes

Haemorrhage: Yes, scattered mild

Thrombosis: No

Control case #4 [Hospitalization number: N/A; Post mortem number: S4191-18(4)]

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from –4-9 per high power field and a mean of 6.67. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 18 per 10 high power fields (x400). Sparse haemosiderin pigment is evident.

Cellularity score: 3

Parasite score: 0

Endothelial cell reactivity score: 0
Megakaryocyte: Range 4-9, mean 6.67
Megakaryocyte score: 3
Congestion: Yes
Haemorrhage: No
Thrombosis: No

Control case #5 [Hospitalization number: N/A; Post mortem number: S4191-18(5)]

On low power magnification few fragments of bony trabeculae are evident. Moderate numbers of adipocytes are observed. The bone marrow appears normocellular mostly (slightly hypercellular in areas). There are randomly distributed megakaryocytes, and their number is variable per high power field (x40), but ranges from 1-4 per high power field with the mean of 2.33. Megakaryocytes of various maturational stages are evident, but appear mostly mature. On high power magnification (x40) differentiated cell lines predominate. Multifocal mitoses are evident, approximately 18 per 10 high power fields (x400). Moderate amounts of haemosiderin pigment is evident.

Cellularity score: 2
Parasite score: 0
Endothelial cell reactivity score: 0
Megakaryocyte: Range 1-4, mean 2.33
Megakaryocyte score: 2
Congestion: Yes
Haemorrhage: Yes, scattered mild
Thrombosis: No

Appendix 4: Cytological evaluation of the bone marrow for each infected and control case

Specimen	<i>Babesia</i> case #1	<i>Babesia</i> case #2	<i>Babesia</i> case #3	<i>Babesia</i> case #4	<i>Babesia</i> case #5	<i>Babesia</i> case #6	Control case #1	Control case #2	Control case #3	Control case #4	Control case #5
CBC: Erythron	Severe normocytic, normochromic anaemia	Moderate normocytic normochromic regenerative anaemia, moderate increase in nucleated RBCs	Severe macrocytic, hypochromic appropriately regenerative anaemia	Severe macrocytic hypochromic non-regenerative anaemia, RBC agglutination	Severe normocytic hypochromic non-regenerative anaemia	Severe normocytic hypochromic non-regenerative anaemia; moderate increase in nucleated RBCs	WNL. No retic count	WNL. No retic count	WNL. No retic count	WNL. No retic count	WNL. No retic count
CBC: Leukon	Mild left shift neutrophilia and eosinopaenia	Inflammatory leukogram: Moderate leukocytosis with left shift neutrophilia and monocytosis, moderate neutrophil toxicity	Leukocytosis with left shift neutrophilia and an eosinopaenia	Inflammatory leukogram: Moderate leukocytosis with left shift neutrophilia and monocytosis, moderate neutrophil toxicity	Leukopenia with neutropenia and a left shift	Inflammatory leukogram: Normal WBC with mildly left shifted neutrophils and many moderately toxic neutrophils	WNL.	WNL	WNL	Mild neutropenia and mild lymphocytosis	Mild mature neutrophilia and monocytosis - stress leukogram
CBC: Thrombon	Severe thrombocytopenia	Severe thrombocytopenia, although also severe platelet aggregation on smear so platelet count not accurate	Mild thrombocytopenia	Mild thrombocytopenia	WNL	Severe thrombocytopenia	WNL	WNL	WNL	Platelet count low with platelet aggregates - so probably WNL	WNL.
CBC: Other		<i>Babesia</i> noted		<i>Babesia</i> noted	<i>Babesia</i> noted.	<i>Babesia</i> noted. Plasma haemolytic					
Bone marrow evaluation:											
Adequate specimen	Very thick	Yes	Yes	Yes	Yes	Yes	Yes	Many degenerate cells	Yes	Yes	Yes
Cellularity %	>75%	>75%	>75%	50-75%	>75%	>75%	50-75%	50-75%	50%	50%	50-75%
Cellularity interpretation	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Hypercellular	Normal - hypercellular depending on age	Normal - hypercellular depending on age	Normocellular	Normocellular	Normal - hypercellular depending on age
Iron stores	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present	Present

Megakaryocytes cellularity	4/10 high power field (hpf)	6/ 10x hpf	10/ 10x low power field (lpf)	3/ 10x hpf	8/ 10x lpf	10/ 10x lpf	11/ 10x lpf	9/ 10 x lpf	6/10 hpf	9/10 hpf	4/10 hpf
Megakaryocytes maturity	Approximately 50% mature	Approximately 20% mature	Approximately 50% mature	Approximately 90% mature	Approximately 60% mature	Approximately 50% mature	Approximately 60% mature	Approximately 60% mature	Approximately 90% mature	Approximately 80% mature	Approximately 80% mature
Myeloid:Erythroid (M:E)	1: 1.2	1: 1.9	1: 1.9	1: 1.4	1:4	1: 1.9	1.92:1	1: 0.44	1:1.09	1: 1.3	1: 0.9
Myeloid cellularity	Increased	Increased	Increased	Hypercellular	Normal - increased	Increased	Normal	Normal	Normal	Normal	Normal
Myeloid morphology	Left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Marked left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Moderate left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Left shifted, normal morphology	Marked left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Marked increase in eosinophils.	Moderate left shift; maturation pool mostly metamyelocytes and bands, marked decrease in segmented forms. Neutrophilic metamyelocytes /bands/segmented neutrophils are often giant with some toxic change (basophilia, granulation). Mild increase in eosinophils.	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology
Erythroid cellularity	Increased	Increased	Increased	Hypercellular	Increased	Increased	Normal	Normal	Normal	Normal	Normal
Erythroid morphology	Moderate left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Orderly maturation, mild dysplastic changes in metarubricytes: pleomorphic nuclear shapes, binucleation	Moderately left shifted, mild dysplastic changes in metarubricytes: pleomorphic nuclear shapes	Marked left shift, moderate dysplastic changes in metarubricytes: nuclear pleomorphism, binucleation.	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology	Orderly maturation, unremarkable morphology
Differential 500-cell count Of All Nucleated Cells (%):											

Rubriblasts	3.6	5.2	6.6	1.4	4.2	6.6	2.0	0.8	1.0	1.0	0.8
Prorubricytes/ Rubricytes	46.0	31.4	38.2	39.0	49.4	38.2	21.0	22.6	31.4	47.8	39.8
Metarubricytes	4.4	2.8	7.6	14.2	6.0	7.6	8.6	5.0	15.4	4.0	5.6
Total Erythroid %	54.0	39.4	52.4	54.6	59.6	52.4	31.6	28.4	47.8	52.8	46.2
Of All Erythroid Cells:											
Rubriblasts	6.7	13.2	12.6	2.6	7.0	12.6	6.3	2.8	2.1	1.9	1.7
Prorubricytes/ Rubricytes	85.2	79.7	72.9	71.4	82.0	72.9	66.5	79.6	65.7	90.5	86.1
Metarubricytes	8.1	7.1	14.5	26.0	11.0	14.5	27.2	17.6	32.2	7.6	12.1
Of All Nucleated Cells (%):											
Myeloblasts	0.8	2.8	2.2	2.4	2.4	2.2	0.8	1.5	0.8	0.4	2.2
Promyelocytes/ myelocytes	19.0	7.2	10.4	19.8	5.8	10.4	8.6	6.0	6.8	7.8	5.0
Metamyelocytes/Bands/Segmented neutrophils	23.0	10.6	14.4	18.0	7.0	14.4	50.8	57.4	36.2	32.0	44.4
Total Myeloid %	42.8	20.6	27.0	40.2	15.2	27.0	60.2	64.9	43.8	40.2	51.6
Of All Myeloid Cells:											
Myeloblasts	1.9	13.6	8.1	6.0	16.0	8.1	1.3	2.3	1.8	1.0	4.3
Promyelocytes/ myelocytes	44.4	35.0	38.5	49.3	38.0	38.5	14.3	9.2	15.5	19.4	9.7
Metamyelocytes/Bands/Segmented neutrophils	53.7	51.5	53.3	44.8	46.0	53.3	84.4	88.4	82.6	79.6	86.0
Of All Nucleated Cells (%):											
Plasma cells	0.0	19.4	6.6	1.4	1.2	6.6	1.0	1.2	1.6	0.2	0.8
Plasma cell comment		Many immatures back to plasmablasts	Some immatures	Normal morphology	Some immatures	Some immatures	mature	mature	mature	mature	mature
Lymphocytes	1.2	4.8	2.2	3.2	3.8	2.2	5.8	1.6	5.4	5.0	0.8
Lymphocytes comment	small	Small, medium, large granular lymphocytes	Small	Mostly small, normal morphology	Increase in medium-sized	Small	small	small	Small and medium	small	small
Histiocytes/macrophages	2.2	15.6	11.8	0.6	20.2	11.8	1.4	4.0	1.4	1.8	0.6
Histiocytes / macrophages comment	Moderate activation, about 30% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Not active	Very active, some erythrophagocytosis and haemosiderin-laden	Moderate activation, about 50% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Mild activation	Moderate activation, about 30% show vacuolisation, phagocytosis of mature RBC, contain haemosiderin	Mild activation	Mild activation	Mild activation
Other cells comment										Occasional osteoclasts	
Stromal reaction	None	None	None	None	None	None	None	None	None	None	None
Infectious agents	<i>Babesia</i> +++	<i>Babesia</i> +++	<i>Babesia</i> +++	None seen	<i>Babesia</i> +++	<i>Babesia</i> +++	None	None	None	None	None

Megakaryocytes interpretation	Left shift. This would be an appropriate response if there was a peripheral thrombocytopenia	Marked left shift - this would be an appropriate response in the face of a peripheral thrombocytopenia	Left shift - this would be an appropriate response in the face of a peripheral thrombocytopenia	WNL - would expect some left shifting and hyperplasia given the CBC findings	WNL	Left shift - this is an appropriate response in the face of a peripheral thrombocytopenia	WNL	WNL	WNL	WNL	WNL
Erythroid interpretation	Hyperplasia and left shift. This would be an appropriate response if there was a peripheral anaemia	Marked hyperplasia with a left shift, appropriate in the face of haemolytic anaemia; mild dysplasia	Marked hyperplasia with a left shift, appropriate in the face of haemolytic anaemia; mild dysplasia	Some dysplastic changes. Lack of left shift unusual given the peripheral anaemia.	Marked hyperplasia with a left shift, appropriate given the haemolytic anaemia; mild dysplasia	Marked hyperplasia with a left shift, this is appropriate in the face of haemolytic anaemia; mild dysplasia	WNL	WNL	WNL	WNL	WNL
Myeloid interpretation	Hyperplasia and left shift. This would be an appropriate response if there was an inflammatory leukogram	Hyperplasia and left shift consistent with systemic inflammation	Hyperplasia and left shift consistent with systemic inflammation	Mild left shift - consistent with inflammatory leukogram	Marked left shift, increased eosinophilic granulocytes	Hyperplasia and left shift consistent with systemic inflammation	WNL	WNL	WNL	WNL	WNL
Plasma cells and lymphoid interpretation	WNL Unexpected given the <i>Babesia</i>	Marked plasma cell hyperplasia consistent with inflammation, reactive lymphoid hyperplasia	Marked plasma cell hyperplasia consistent with inflammation	Normal	WNL	Marked plasma cell hyperplasia consistent with inflammation	WNL	WNL	Mild lymphocytosis	WNL	WNL
Histiocyte/ macrophage interpretation	Moderate activation. Expected given the <i>Babesia</i>	Marked macrophagic/histiocytic inflammation	Marked macrophagic/histiocytic inflammation	Normal	Marked macrophagic/histiocytic inflammation	Marked macrophagic/histiocytic inflammation	WNL	Mild macrophagic activation	WNL	WNL	WNL
Other interpretation											
Summary	Apart from the lack of plasma cell hyperplasia this would be an appropriate bone marrow response to	Appropriate bone marrow response to haemolytic anaemia, thrombocytopenia and systemic	Appropriate bone marrow response to haemolytic anaemia and systemic inflammation	Myeloid left shift appropriate in the face of inflammatory leukogram. Lack of erythroid	Appropriate bone marrow response to severe haemolytic anaemia. The myeloid reaction may	Appropriate bone marrow response to severe thrombocytopenia and haemolytic anaemia. The	Normal bone marrow apart from a mild lymphocytosis	Apart from some macrophagic activity, this represents normal	Normal bone marrow apart from a mild lymphocytosis	Normal bone marrow, no evidence of a response or reason	Normal bone marrow

	haemolytic anaemia and systemic inflammation caused by <i>Babesia</i>	inflammation caused by <i>Babesia</i>	caused by <i>Babesia</i>	hyperplasia in the face of peripheral anaemia not appropriate; interesting that no parasites noted in BM smears.	not be sufficient, given the peripheral leukopenia. Histiocytic inflammation due to presence of parasite.	presence of increased nucleated RBCs in peripheral blood in the face of <u>no increase in reticulocytes</u> and a clear erythroid hyperplasia in bone marrow is an unusual finding. Either these samples have been taken just at the point where metarubricytes are about to mature into retics (i.e. 2-3 days after start of anaemia), or perhaps reticulocytes are being released but are being destroyed at high rate in the haemolytic process? The myeloid reaction may not be sufficient, given the left shift without neutrophilia and the toxic changes. Histiocytic inflammation due to presence of parasite.		bone marrow		for the mild neutropenia	
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Abbreviations: CBC: Complete blood count; RBC: Red blood cell; WNL: Within normal limits; HPF: High power field; LPF: Low power field

Appendix 5: The percentage of positive labelling per immunohistochemical marker/antibody

	B1	B2	B3	B4	B5	B6	C1	C2	C3	C4	C5
CD3 ***	9.78	8.3	11	7.12	9.92	8.47	7.06	13.23	9.15	10	7.61
CD20 ***	16.26	11.81	9.51	17.33	20.24	9.13	11.51	10.3	11.95	11.21	12.01
MUM1 ***	4	9.47	5.44	14.64	8.96	4.01	3.27	7.39	4.17	5.01	4.57
CD204 ***	21.41	24.06	22.94	22.34	27.19	21.17	8.82	14.32	15.39	15.76	15.61
MAC387 ***	66.6	45.82	44.63	52.62	39.1	41.01	38.17	42	38.74	37.4	46.38
Perls' Prussian blue stain***	74.43	20.44	63.19	28.22	29.15	13.12	9.01	17.17	8.23	10.58	26.78

* *Babesia* cases are denoted by the letter B and include case numbers 1 to 6

** Control cases are denoted by the letter C and include control case numbers 1 to 5

*** This represents the percentage of positive labelling per cell marker for each case

Appendix 6: Complete blood count values for each infected and control case (significantly decreased values are in bold and significantly increased values are underlined)

Case number	Case identifier	Haemoglobin (g/L)	Red cell count (x10 ¹² /L)	Haematocrit (L/L)	Mean corpuscular volume (f/L)	Mean corpuscular haemoglobin (pg)	Mean corpuscular haemoglobin concentration (g/dL)	Red cell distribution width (%)	White cell count (x10 ⁹ /L)	Segmented neutrophil (x10 ⁹ /L)	Band neutrophil (x10 ⁹ /L)	Lymphocyte (x10 ⁹ /L)	Monocyte (x10 ⁹ /L)	Eosinophil (x10 ⁹ /L)	Basophil (x10 ⁹ /L)	Platelet count (x10 ⁹ /L)	NRBC /100 WBC	Reticulocyte percentage	Absolute reticulocyte count (x10 ⁹ /L)
1	B1	22	1.03	0.06	63.1	20.1	31.8	15.4	14.18	8.65	<u>0.71</u>	3.83	0.99	0	0	20	3	-	-
2	B2	60	2.48	0.19	75.7	24.1	31.9	21.3	<u>24.83</u>	<u>14.9</u>	<u>3.97</u>	3.48	<u>2.48</u>	0	0	43	<u>16</u>	<u>5.9</u>	<u>147.1</u>
3	B3	30	1.16	0.12	<u>101.3</u>	25.8	25.5	17	<u>16.31</u>	<u>12.08</u>	<u>0.82</u>	2.12	1.31	0	0	154	<u>28</u>	<u>11.4</u>	<u>132.9</u>
4	B4	13	0.57	0.06	<u>96.7</u>	51.5	23.3	16.4	<u>36.64</u>	<u>22.35</u>	<u>5.5</u>	3.66	<u>5.13</u>	0	0	182	2	<u>11.7</u>	66.6
5	B5	70	1.67	0.12	70.8	42.2	<u>59.5</u>	18.2	4.01	1.72	<u>0.8</u>	1.12	0.36	0	0	500	3	<u>1.9</u>	31.6
6	B6	39	1.18	0.09	73.9	33.2	29.4	15.8	7.65	4.82	<u>0.54</u>	1.91	0.38	0	0	29	<u>15</u>	<u>1.8</u>	20.6
7	C1	153	6.29	0.47	75.4	24.3	32.2	15.1	11.6	6.26	0	3.83	0.35	1.16	0	231	0	-	-
8	C2	137	6.18	0.41	65.8	26.4	33.6	13.1	12.45	6.47	0	4.36	0.5	1	<u>0.12</u>	196	0	-	-
9	C3	177	7.57	0.53	70.1	23.3	33.3	12.8	11.03	5.63	0	3.86	0.66	0.77	<u>0.11</u>	321	0	-	-
10	C4	156	7.35	0.48	65.5	21.1	32.4	14.7	9.29	2.79	0	<u>5.85</u>	0.37	0.28	0	96	0	-	-
11	C5	178	7.66	0.53	69.2	23.2	33.5	12.8	<u>17.15</u>	<u>12.69</u>	0	2.92	<u>1.37</u>	0.17	0	276	0	-	-
Reference range		120-180	5.5-8.5	0.37-0.55	60-77	No reference range	32-36	No reference range	6-15	3-11.5	0-0.5	1-4.8	0.15-1.35	0.1-1.25	0-0.1	200-500	0-9	0-1 *	0-80*

* Reticulocyte percentage reference interval according to Stockham & Scott (2008) ¹⁴

** *Babesia* cases are denoted by the letter B and include case numbers 1 to 5.

*** Control cases are denoted by the letter C and include case numbers 1 to 5.