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ORIGINAL ARTICLE

Concurrent infections with vector-borne pathogens associated with fatal anaemia in cattle: haematology and blood chemistry

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Abstract An outbreak of a fatal haemolytic anaemia in a dairy herd of cattle in Switzerland was shown to be associated with infections with five vector-borne pathogens, namely *Anaplasma marginale*, *A. phagocytophilum*, *Babesia bigemina*, a *Theileria* spp belonging to the *buffeli/sergenti/orientalis* complex and haemotrophic *Mycoplasma* spp. The latter three had not been documented before this outbreak in Switzerland. To characterise the haematological and blood chemical changes in these unique cows, packed cell volume was determined in all 286 blood samples, blood smears, and complete haematology were performed from 285 and 173 blood samples, respectively, and biochemical parameters were assayed in 105 serum samples. Regenerative anaemia was the key sign of illness. Red blood cells of anaemic cattle were hypochromic and macrocytic. Anaemic animals had reduced platelet cell counts and increased total white cell counts. In addition, increased serum bilirubin, blood aspartate aminotransferase, gamma glutamyltransferase, glutamic dehydrogenase and blood urea nitrogen and decreased magnesium, calcium and albumin levels were found in anaemic cattle when compared to animals with normal packed cell volume. Most changes could not be attributed to a single infection. *A. marginale* seemed to be important in causing the outbreak, but co-infections may have aggravated the disease development and clinical signs. Thus, when encountering cattle with haemolytic anaemia, all of the mentioned pathogens should be included as differential diagnosis.

Keywords Vector-borne pathogens · Haemolytic anaemia · Haematology · Clinical chemistry · Cattle

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Introduction

A fatal disease outbreak of haemolytic anaemia in a large dairy herd in the Swiss Canton of Grisons located North of the Alps was reported of the summer of 2002 (Brulisauer et al. 2004; Hofmann-Lehmann et al. 2004). The cows suffered from fever, weakness, depression, anorexia, agalactia and rapid loss of condition. They had pale mucous membranes. Some animals were infested by lice, and others showed occasionally haemoglobinuria. After the death of

29 animals, the Swiss authorities decided to cull the entire herd to stop the spread of the outbreak to other herds (Brulisauer et al. 2004). Using microscopic and molecular detection methods, the presence of *Anaplasma marginale*, *A. phagocytophilum*, *Babesia*, *Theileria* and haemotropic *Mycoplasma* spp. was demonstrated (Brulisauer et al. 2004; Hofmann-Lehmann et al. 2004). Subsequently, the piroplasms were identified as *Babesia bigemina* and Theileria of the buffeli/sergenti/orientalis complex (Hilpertshauser et al. 2007). Fifty percent of the cows were anaemic, with some of them having packed cell volume (PCV) as low as 7% (Hofmann-Lehmann et al. 2004). Inclusion bodies of *A. marginale* were detected in blood samples of 47% of the animals examined. An association between the presence of *A. marginale* and anaemia was found. Anaemia was also significantly associated with the presence of *Babesia*, *Theileria* spp. and *Mycoplasma wenyonii*. The reported outbreak was unusual for two reasons: (1) observation of organisms not previously seen in the area (*B. bigemina*, *Theileria* spp. and *M. wenyonii*) and (2) a high percentage of ill animals apparently infected with several organisms (Hofmann-Lehmann et al. 2004).

The goal of the present study was to characterise the haematological and blood chemical changes in these unique cows. To our knowledge, no data had been available thus far on haematology and blood chemistry from cows concurrently infected with such a multitude of vector-borne pathogens.

Materials and methods

Blood and serum samples

The fatal outbreak affected a herd of more than 300 cattle, mostly dairy cows. During July and August 2002, 20 cows had died of unknown causes. Ethylenediaminetetraacetic acid anticoagulated blood samples and whole blood for serum preparation were collected from 286 surviving cows on August 25th before the entire herd was culled to reduce the potential spread of infection.

Polymerase chain reaction analyses and serological assays

Blood samples (n=58) were analysed by conventional polymerase chain reaction (PCR) for *A. marginale* (MSP5), *Babesia* and *Theileria* spp, *M. wenyonii* and by real-time TaqMan PCR for *A. phagocytophilum* (previously *Ehrlichia phagocytophila*) as described (Hofmann-Lehmann et al. 2004; Jensen et al. 2001; Persing et al. 1992; Pusterla et al. 1999; Torioni de Echaide et al. 1998).

Haematology and blood chemistry

Thin blood smears were prepared from all but one cow (n=285) within a few hours of blood collection and stained with Giemsa (AMES Hema Tek slide stainer, Bayer, Zurich, Switzerland). The PCV was determined in 113 samples by centrifugation of the blood in microhaematocrit capillary tubes. In addition, complete blood cell counting was performed in 173 samples using a haematology analyser (Cell-Dyn 3500, Abbott, Baar, Switzerland). These samples had been chosen randomly at the time of sample delivery to the laboratory. Anaemia (PCV<25%) was defined according to the reference values of our laboratory determined with identical methods using blood samples from 49 healthy lactating Swiss cows at the age of 3 to 11 years.

Because PCR results were available from only 58 cows, but within this selected group, the infection status correlated strongly with the key sign of anaemia, the haematological and blood chemistry values were grouped and analysed according to the PCV values of the cows. Animals were categorised into three groups: group I consisted of the severely anaemic cattle and had PCV<15%; group II was anaemic with intermediate PCV of 15 to <25%, and group III had normal PCV values of ≥25%.

Biochemical tests were carried out in 105 serum samples. These samples were chosen to represent approximately equal numbers of three PCV groups: 31 from group I, 37 from group II and 37 from group III. The following biochemical parameters were determined by automated analysis (Cobas-Integra 800, Roche Diagnostics, Rotkreuz, Switzerland) according to standard procedures recommended by the International Federation of Clinical Chemistry as compiled (Tieze 1995): calcium (Ca), sodium, potassium, chloride, phosphorus, magnesium (Mg), aspartate aminotransferase (ASAT), creatine kinase, gamma glutamyltransferase (GGT), glutamic dehydrogenase (GLDH), alkaline phosphates, total protein, albumin, total bilirubin and blood urea nitrogen (BUN). Reference values of our laboratory were determined with identical methods using blood samples from 50 healthy lactating Swiss cows at the age of 3 to 11 years.

Statistics

Data were compiled and analysed using Excel (Microsoft), Analyse-it Clinical Laboratory Version 1.65 (Analyse-it Software, Ltd., Leeds, UK) and GraphPad Prism Version 3.00 (GraphPad Software, San Diego, CA). To test for association between categorical variables, the chi-square statistic was used; for continuous variables, linear correlation and the non-parametric Spearman rank test were calculated (Siegel 1988); to test for statistical differences among several groups, values were tested using the non-

parametric Kruskal–Wallis test with Dunn's multiple comparison post test (Siegel 1988). Differences were considered significant when $P<0.05$.

Results

Sample characteristics

In a previous study, we showed evidence of infection with *A. marginale* in 135 of 286 cattle (47%; Hofmann-Lehmann et al. 2004). Of the 58 samples examined by PCR, 40 (69%) were positive for *A. marginale*, 37 (64%) for *Theileria* spp., 16 (28%) for *M. wenyonii* and 12 (21%) for *Babesia* spp. (Hofmann-Lehmann et al. 2004). For each of the four infectious agents, a high percentage of anaemic cows, but not of the non-anaemic animals, was found to be PCR-positive (Table 1). This observation was also made when only those samples that underwent complete haematological or biochemical analyses were included into the statistical analyses (Table 1). The majority of the cows in group I were infected with *A. marginale*, *Theileria* spp., *Mycoplasma* and *Babesia* spp (Table 1). Only five samples were found to be *A. phagocytophilum*-positive either by PCR or microscopically (Hofmann-Lehmann et al. 2004).

Haematological changes in diseased cattle

Anaemia (PCV<25%) was found in 143 of 286 (50%) cows, with 95 cows (33%) having PCV<15% (group I) and 148 cows (17%) having an intermediate PCV (group II). Fifty percent of the cattle had a PCV within the reference range (≥25%). In thin blood smears of anaemic cattle, polychromatic red blood cell (RBC), nucleated RBC, anisocytosis (variable size of RBC) and basophilic stippling within RBCs of varying size were present (Fig. 1).

Complete haematological tests were carried out in samples obtained from 173 cows (56 from group I, 31 from group II and 86 from group III). Fifty eight of them underwent also PCR analyses (Table 1). Figure 2a and b shows the degree of reduction in RBC numbers and haemoglobin concentration in anaemic cattle. RBCs of highly anaemic cattle (group I) were hypochromic (significantly decreased mean corpuscular haemoglobin concentration, MCHC) and macrocytic (significantly increased mean corpuscular volume, MCV, Fig. 2c and d). Unexpectedly, the highly anaemic cows showed increased MCH values (Fig. 2e). Linear regression analyses between MCH and MCV values demonstrated that this was probably due to the increased cellular volume of the RBC in anaemic cows ($y=0.268x+4.5$; $R^2=0.9716$; $P<0.0001$). In addition, anaemic cattle (groups I and II) had significantly decreased platelet

Table 1 Characterisation of the samples that underwent complete haematology and biochemical analyses according to PCR results and PCV values

Agent	No. of positive animals/no. of animals tested (% of positive animals)			
	Group I Severe anaemia (PCV <15)	Group II Mild anaemia (PCV 15 to <25)	Group III No anaemia (PCV ≥25)	*p* Chi-square
All samples investigated by PCR ($n=58$)[a]				
Anaplasma marginale	26/27 (96%)	14/16 (88%)	1/15 (7%)	<0.0001
Theileria spp.	23/25 (92%)	12/16 (75%)	1/15 (7%)	< 0.0001
Mycoplasma wenyonii	21/27 (78%)	1/16 (6%)	0/15 (0%)	< 0.0001
Babesia spp.	12/25 (48%)[2]	0/16 (0%)	0/15 (0%)	<0.0001
Samples that underwent PCR and complete haematology ($n=28$)				
Anaplasma marginale	11/11 (100%)	9/10 (90%)	1/7 (14%)	<0.0001
Theileria spp.	10/10 (100%)	8/10 (80%)	1/7 (14%)	0.0005
Mycoplasma wenyonii	10/11 (91%)	1/10 (10%)	0/7 (0%)	<0.0001
Babesia spp.	8/10 (80%)	0/10 (0%)	0/7 (0%)	<0.0001
Samples that underwent PCR and complete biochemical analyses ($n=38$)				
Anaplasma marginale	12/12 (100%)	12/14 (86%)	0/12 (0%)	<0.0001
Theileria spp.	11/11 (100%)	4/14 (29%)	1/12 (8%)	<0.0001
Mycoplasma wenyonii	9/12 (75%)	1/14 (7%)	0/12 (0%)	<0.0001
Babesia spp.	10/11 (90%)[b]	0/14 (0%)	0/12 (0%)	<0.0001

[a] Adapted from Hofmann-Lehmann et al. 2004

[b] Babesia PCR-positive samples could have been slightly overrepresented in group I in the samples selected for clinical chemistry compared to all samples that underwent PCR analyses ($P=0.0392$)

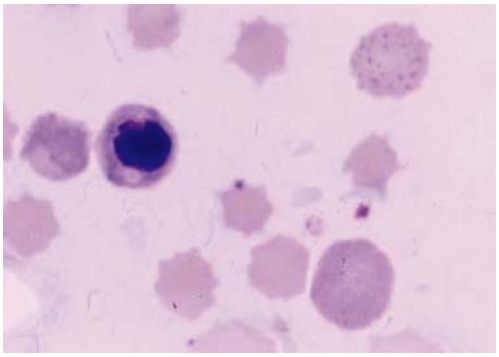

Fig. 1 A representative Wright-stained blood smear of an anemic cow demonstrating anisocytosis, polychromasia, basophilic stippling of RBC and normoblastemia indicative of regenerative response

counts, and highly anaemic cows (group I) had also increased total WBC counts when compared to animals with normal PCV (Fig. 2e and f).

Biochemical changes in diseased cattle

Anaemic cattle had significantly higher total serum bilirubin (>100 μmol/l; 95% quantile of reference values: 2.9 μmol/l) than animals with normal PCV (Fig. 3a). In addition, significantly increased ASAT, GGT GLDH and BUN as well as decreased Mg, Ca and albumin values were found in anaemic cows compared to animals with normal PCV (Fig. 3b–h).

Discussion

In the summer of 2002, a fatal disease outbreak was reported in a large cattle herd in Switzerland (Brulisauer et al. 2004; Hofmann-Lehmann et al. 2004). It was found that several of the infectious agents, i.e. *A. marginale*, *Theileria* and *Babesia* spp. and *M. wenyonii*, could have been causally linked to the disease, and it was speculated that *A. marginale* was the most important agent in causing anaemia, but co-infections with other agents may have aggravated disease development (Hofmann-Lehmann et al. 2004).

Haematological and biochemical analysis of blood samples revealed signs of severe haemolytic regenerative anaemia in affected cattle; PCV values, haemoglobin concentrations and RBC count values were below the respective reference ranges. Occurrence of polychromasia, anisocytosis, basophilic stippling and nucleated RBCs were indicative of regeneration. According to the erythrocyte indices, the anaemia was classified as hypochromic (low MCHC) and macrocytic (increased MCV). High MCV values are usually associated with regenerative anaemia

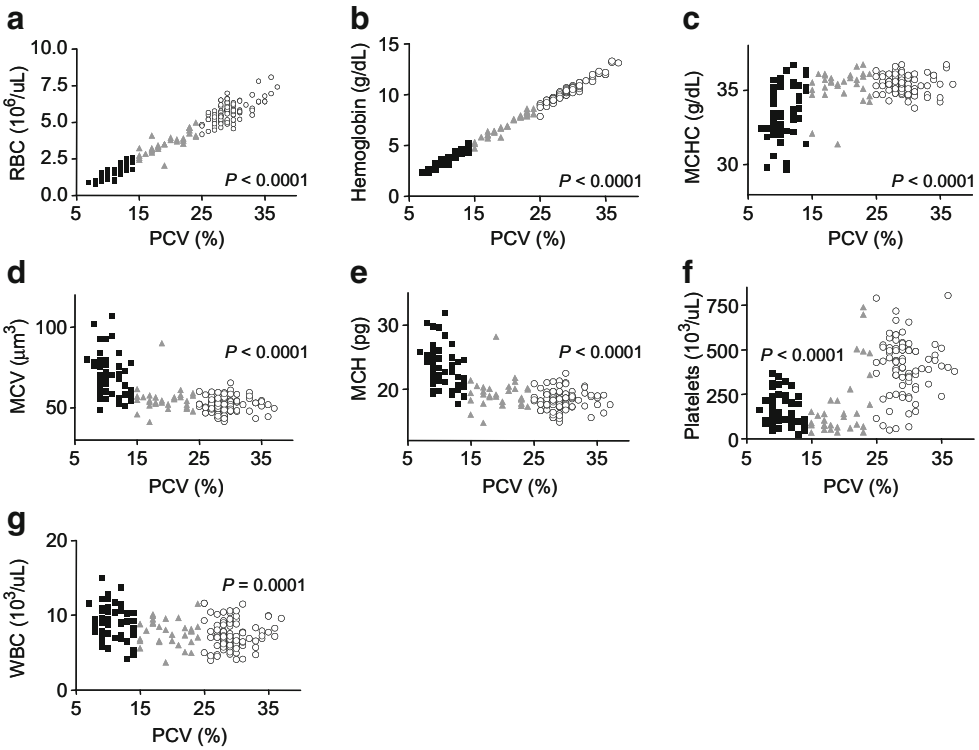

Fig. 2 Correlation of RBC (**a**), haemoglobin (**b**), MCHC (**c**), MCV (**d**), MCH (**e**), platelet (**f**) and WBC (**g**) with PCV. For easier analyses, samples with very low PCV <15% (group I) are plotted as *black squares*, samples with low to intermediate PCV (15 to <25%, group II) are given as *grey triangles*, and samples with normal PCV (≥25%, group III) are shown as *open circles*. Groups were compared using Kruskal–Wallis statistics (*P* values are given in the figure) with Dunn's post tests. The *P* values for the latter were as follows: in **a**, **b**, **d** and **e**, all groups compared were statistically different from each other ($P<0.001$); in **c** and **g**, group I was significantly different from groups II and III, respectively ($P<0.001$); and in **f**, groups I and II were significantly different from group III, respectively ($P<0.001$)

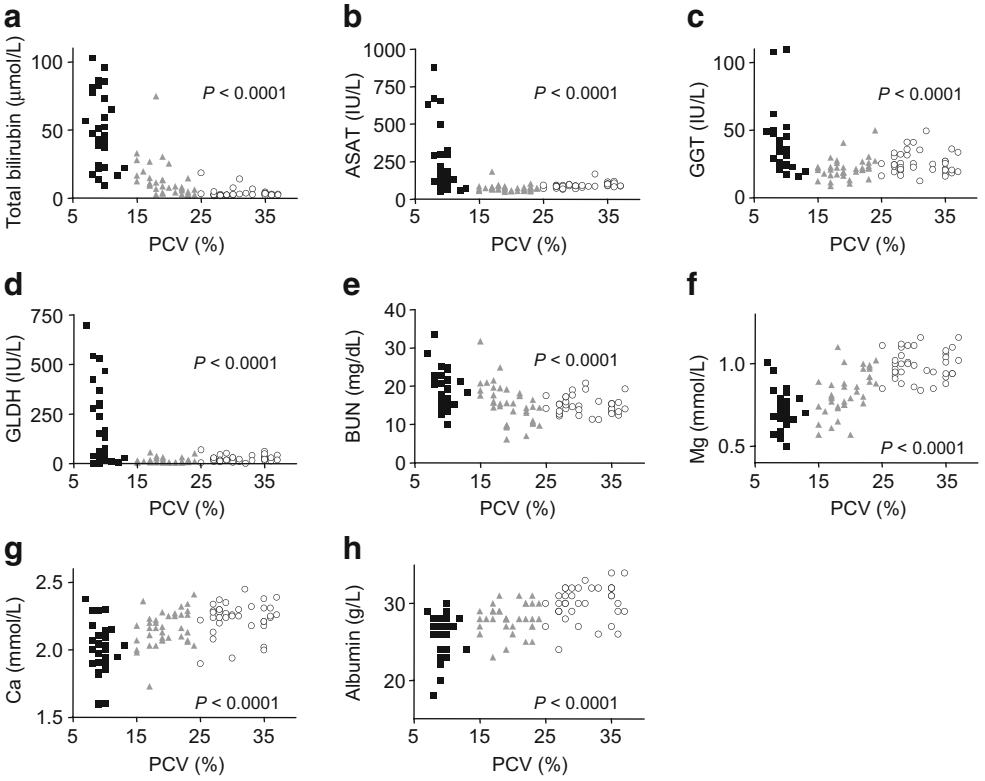

Fig. 3 Correlation of total bilirubin (**a**), ASAT (**b**), GGT (**c**), GLDH (**d**), BUN (**e**), Mg (**f**), Ca (**g**) and albumin (**h**) with PCV. For easier analyses, samples with very low PCV <15% (group I) are plotted as *black squares*, samples with low to intermediate PCV (15 to <25%, group II) are given as *grey triangles*, and samples with normal PCV (≥25%, group III) are shown as *open circles*. Groups were compared using Kruskal–Wallis statistics (*P* given in figure) with Dunn's post tests. The *P* values for the latter were as follows: all groups compared were statistically different ($P<0.001$) in all panels with the exception of **f** where only group I was significantly different from groups II and III, respectively ($P<0.001$). Only parameters are graphed for which significant differences were found

because immature RBCs, which are released from the bone marrow into the peripheral blood at times of increase demand, are bigger in size than mature red cells. This would also explain the decreased MCHC because immature RBCs may not yet have attained the cellular haemoglobin concentration of mature erythrocytes. Anaemic cattle from group I and II showed thrombocytopenia which is often associated with haemolytic anaemia caused by protozoa. The increased platelet consumption may be attributed to an immune-mediated process or result from intravascular disseminated coagulation. To rule out that the decreased platelet counts were due to platelet clumping and thus to incorrect readings of the electronic cell counter, platelet histograms of the Cell-Dyn as well as stained blood smears were evaluated. No platelet clumps were observed.

A. marginale is a haemoparasite that leads to extravascular haemolysis and anaemia in infected cattle (Ajayi et al. 1978; Kuttler 1984; Lotze 1947). In babesiosis and theileriosis, intravascular haemolysis is the predominant cause of erythrocyte destruction. Often, intravascular and extravascular haemolyses occur simultaneously. The changes in the biochemical parameters observed in infected animals of the present study are in accordance with those reported from experimental infection of mature cows with virulent *A. marginale* field isolates (Allen and Kuttler 1981). Initially, increased bilirubin, ASAT and BUN levels may be attributed to the rapid destruction of RBC via phagocytosis in the reticuloendothelial system (Ajayi et al. 1978; Allen and Kuttler 1981). An increased serum bilirubin concentration may be a hallmark of haemolytic anaemia; it results from haeme catabolites liberated from haemoglobin during RBC phagocytosis (Allen and Kuttler 1981). In general, haemolytic anemias are said to be associated with an increase in unconjugated bilirubin, whereas an increase in conjugated bilirubin is associated with cholestatic liver disease or bile duct obstruction (Feldmann et al. 2000). However, in ruminants, bilirubin concentrations are not consistently increased in animals with liver diseases. Cholestasis in ruminants can result in a moderate increase of serum bilirubin, but this is not a consistent finding. Significant hyperbilirubinemias most often result from haemolysis (Thrall 2004). In addition, anorexia can also cause hyperbilirubinemia. The historical determination of the concentrations of both conjugated and unconjugated bilirubin in ruminants seems not to be justifiable because they are unreliable indicators regarding the cause of hyperbilirubinemia (Thrall 2004).

Besides the increased serum bilirubin levels, increased enzyme activities of ASAT, GGT and GLDH were observed in these animals. Severe anaemia leads to hypoxic and toxic liver damage, supported by high ASAT und GLDH levels. This hepatocellular injury, and possibly also block-

age of small bile ducts due to excessive pigment load, may result in cholestasis and a reduced bilirubin metabolism. Cholestasis is in fact suggested by an increased GGT activity. Our results are in agreement with earlier findings that during the period of persistent infection with *A. marginale*, massive haemolysis may occur, which, in conjunction with hypoxia, may lead to hepatic cell degeneration and glomerular dysfunction (Allbritton and Seger 1962; Allen and Kuttler 1981). This in turn leads to increased bilirubin, ASAT, GGT and BUN. In the present study, BUN levels were only moderately elevated, which may rather indicate local hypoxia of the kidney than glomerular dysfunction (Allen and Kuttler 1981; Campbell and Watts 1970).

A reduction in serum albumin is associated with the acute phase of many infectious diseases (Allen and Kuttler 1981), and this in turn leads to decreased calcium levels (Meuten et al. 1982). In addition, albumin may be decreased due to decreased protein synthesis capacity of the affected liver or the prolonged insufficient caloric intake.

The aforementioned changes may not be entirely attributed to the *A. marginale* infection, as the cows were also shown to have been infected with *Babesia*, *Theileria* and *Mycoplasma* spp. Haemolytic anaemia has also been described in cows infected with those agents (Allen and Kuttler 1981; Cagnasso et al. 1983; Sandhu et al. 1998; Smith et al. 1980; Yadav and Sharma 1986). In addition, haemoglobinuria was documented in some of the diseased cows. Haemoglobinuria is an indicator of intravascular haemolysis, which can be expected in bovine babesiosis (De Vico et al. 1999; Donnelly et al. 1970) and recently has also been documented in a *T. buffeli*-infected cow (Cossio-Bayugar et al. 2002).

In conclusion, this report describes the haematological and blood biochemical changes in cows affected by concurrent infections with several vector-borne pathogens. Most of the haematological and blood chemical changes could not be attributed to a single infectious agent. The data from this study correspond with our earlier hypothesis in that *A. marginale* seemed to be the most important agent in causing anaemia and disease in the cows of this herd, but co-infections with the other agents may have aggravated the disease development. Practicing veterinarians encountering cattle with signs of haemolytic anaemia should be advised to include all of the aforementioned infectious pathogens in their list of differential diagnosis.

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