

The clinical pathology of heartwater. III. Changes in blood clotting, blood calcium, blood protein, haematocrit and white-cell counts in sheep with experimentally induced heartwater

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ABSTRACT

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Studies to evaluate changes in blood clotting, blood calcium and protein, the haematocrit and white-cell counts were undertaken in seven sheep with experimentally induced heartwater. A marked decline in thrombocyte count was recorded during the acute stage of the disease. This was associated with increases in both prothrombin time (PT) and activated partial thromboplastin time (APTT); fibrinogen increased while there was no detectable increase in fibrinogen degradation products (FDP). At the same time total serum protein (TSP), albumin and globulin dropped very sharply; total calcium showed a progressive drop but ionized calcium rose initially and was followed by a terminal decline. The total leucocyte count showed a terminal increase while the haematocrit dropped progressively.

INTRODUCTION

Laboratory evidence of disturbances in blood clotting has not been investigated in either natural or experimentally induced cases of heartwater. It may be possible that such abnormalities are present in heartwater, as some macropathological signs associated with a disseminated intravascular coagulopathy (DIC) have been described. These include oedema of the lungs and haemorrhages in various organs, including the conjunctiva, lymph nodes, heart, intestine and rumen, the central nervous system, vagina and urinary

bladder (Camus & Barré 1982; Prozesky 1987). The presence of microthrombi which can be indicative of the presence of DIC, has only once been reported on in experimentally induced heartwater in mice (Prozesky & Du Plessis 1985).

Van Amstel, Oberem, Didomenico, Kirkpatrick & Matthee (1988a) and Van Amstel, Myburgh, Mundell & Davidson (unpublished data 1993) showed the presence of endotoxin activity in the blood of sheep with experimentally induced heartwater. It has been shown that endotoxaemia in the horse may be associated with abnormalities in blood clotting which may indicate a laboratory diagnosis of DIC (Johnstone 1986).

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Laboratory changes associated with DIC include prolongations of prothrombin time (PT) and activated partial thromboplastin time (APTT) due to depletion of protein co-enzymes (V, VIII) and fibrinogen, a thrombocytopaenia and increased levels of fibrin-degradation products (FDP) (Slappendel 1989). Hereditary coagulopathies and other acquired causes besides a consumption coagulopathy, may also cause prolongation of PT and APTT. These include a severe hepatopathy and anticoagulant toxicosis which interfere with the synthesis of most of the serum-protease clotting factors (Jain 1986).

Van Amstel, Reyers, Guthrie, Oberem & Bertschinger (1988b) demonstrated a drop in the levels of total blood calcium in calves with experimentally induced heartwater, particularly in one case which ended fatally. The significance of this with regard to its contribution to the death of the animal was not clear, as the serum-albumin levels also decreased and ionized-calcium levels were not determined. The latter, being the pharmacologically active form, is very important for normal muscle contraction, including cardiac muscle (Ganong 1975).

The relationship between total and ionized calcium in sheep has been reported, and there appears to be some conflicting evidence in this regard (Belonje 1973; 1976a and 1976b). On the basis of the above-mentioned, it was decided to include the determination of ionized-calcium levels in this study.

In a review on the haematological findings in cases of heartwater, Van Amstel, Guthrie, Reyers, Bertschinger, Oberem, Killeen & Matthee (1987a) pointed out some conflicting findings with regard to the total white-cell count (WCC). Another intriguing aspect is the apparent development of an anaemia during the course of the disease. Consequently, changes in the WCC and haematocrit were also monitored in this study.

MATERIALS AND METHODS

Experimental animals

Seven healthy adult Merino sheep were used. The animals were given two weeks to adapt to their surroundings, during which period five blood samples were drawn. On the 14th day, heartwater was induced in the sheep by inoculating each of them intravenously with a single dose (5 ml of infected blood) of the "Welgevonden" stock of *Cowdria ruminantium* (Du Plessis 1985). The rectal temperature of each animal was recorded twice daily. Clinical disease was considered to have commenced when the morning temperature reached 40 °C. Rectal temperatures above 40 °C were recorded in all seven sheep from 8–15 days post-infection. Blood was collected for blood-clotting studies, blood-protein and -calcium de-

terminations, leucocyte count and haematocrit on days -13, -6, -5, and -1 from infection, on the day of infection and then daily from day 7–13 post-infection. On day 13 two samples were collected, several hours apart, from four of the sheep. One sheep (3428) died on day 12, five died, or were euthanased due to severe clinical signs, on day 13 and one (3559) was euthanased on day 15.

At autopsy, the presence of heartwater was confirmed by the demonstration of *C. ruminantium* organisms in smears prepared from the hippocampus and stained with a rapid Romanowski-type stain (Cams Diff Quick—CA Milsch, Krugersdorp, RSA)

Each of the seven sheep served as its own control, based on the serial laboratory determinations prior to infection (days -13, -6, -5, -1 and 0).

Experimental procedures

Haematology

Complete blood counts (CBC) were conducted, by means of a Baker System 9000 with Vet Pack (Serono-Baker Diagnostics, Inc., Allentown, Pennsylvania, USA) which measured the red-cell count (RCC), mean corpuscular volume (MCV), white-cell count (WCC) and thrombocyte count. Differential counts were not done.

Haematocrit (Ht) determinations were carried out with the use of microhaematocrit capillaries (75 mm/ μl) in a microhaematocrit centrifuge (Hawksley & Sons Ltd, England).

Protein determinations

Total serum protein (TSP) was measured by use of the biuret reaction on an automated analyser (RA 1000 Technicon Instruments Corp. Tarrytown, New York) (Reagent Cat. no. T01-1301-02).

For albumin, the Technicon method (Cat. no. T01-1377-02) was used, which makes use of bromocresyl green-dye binding.

Calcium (Ca)

Total calcium levels were determined by means of the Technicon method (Cat. no. T01-1476-01) based on the compleximetric method.

Ionized Ca determinations were performed with a Nova-8 ionized calcium-pH analyser (Nova Biomedical, Massachusetts, USA). Heparinized venous blood was used for this purpose.

Blood clotting studies

Prothrombin time (PT)

Venous blood collected in sodium citrate was centrifuged at 2 700 g for 10 min to obtain platelet-poor

plasma. The plasma was then separated into polystyrene test tubes. The test was then performed within 3 h on the separated plasma stored at room temperature.

0,2 ml of reconstituted coagulin-PT (Lennon Diagnostics (Pty) Ltd, 7 Fairclough Road, Port Elizabeth, 6001 South Africa) was pipetted into the bottom of a 13 x 100-mm glass tube and pre-incubated at 37 °C for 3 min. 0,1 ml of test plasma was then added to the reagent in the tube. Time is recorded on a stopwatch from this point until a gel clot forms recorded by a coagulation timer (MLA, Electra 750, Medical Laboratory Automation Inc., Pleasantvale, NY, USA). Determinations are done in duplicate and expressed as mean times.

Activated partial thromboplastin time (APTT)

Collection and handling of specimens and the test procedures are as described for PT, except that in this instance the reagent used is coagulin-APTT (Lennon Diagnostics, 10 Lindley Street, Bethlehem, 9700 South Africa). In addition, 3 min after mixing the reconstituted coagulin-APTT with the test plasma, 0,1 ml pre-warmed calcium chloride is added. The time until a gel clot forms is recorded by a coagulation timer (Lennon Diagnostics, 7 Fairclough Road, Port Elizabeth, 6001 South Africa).

Fibrinogen

The test plasmas were diluted 1:5 in prepared Coagulin-Fibrinogen Buffer (Lennon Diagnostics, 7 Fairclough Road, Port Elizabeth, 6001 South Africa) according to the manufacturers' directions. The test was then run as described for PT.

Fibrinogen degradation products (FDP)

Venous blood was collected by means of venepuncture of the jugular vein without prolonged venous occlusion, into specially prepared evacuated tubes supplied by the manufacturers of the Thrombo-Wellcotest (Wellcome Diagnostics, Dartford, England). Sample tubes were kept at room temperature for 30–60 min. Sera thus obtained were used for the Thrombo-Wellco rapid latex test which was carried out according to the manufacturers' directions.

Statistical analysis

Data for each measured variable were analysed by means of the computer-based statistical-analysis program "Statgraphics" (Statistical Graphics Corporation, USA, 1990). The data were subjected to analysis by the Least Significant Difference (LSD) multiple-range test (Snedecor & Cochran 1980) to compare sampling-day mean values. A difference was considered significant at the $P < 0,05$ level. Means from days -13, -6, -5, -1 and 0 were grouped as "pre-infection" levels.

RESULTS

The results of the blood-clotting studies are shown in Fig. 1–4.

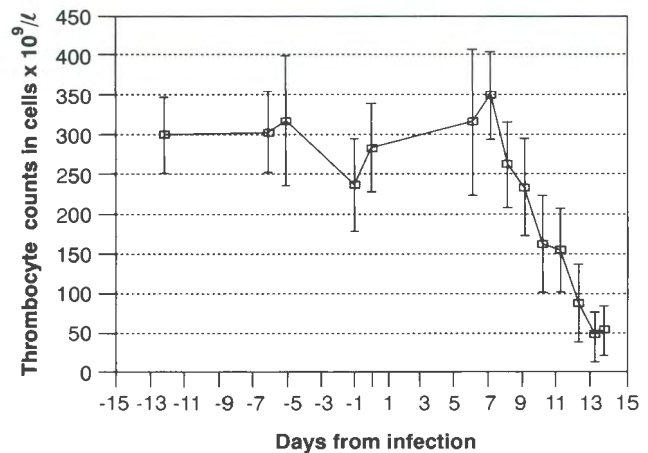


FIG. 1 Results of mean blood thrombocyte count in billion cells/ℓ ± 2 SE

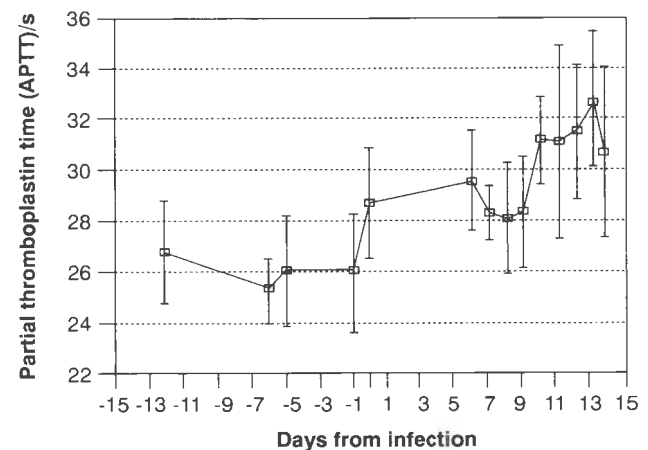


FIG. 2 Results of mean activated partial thromboplastin time ± 2 SE

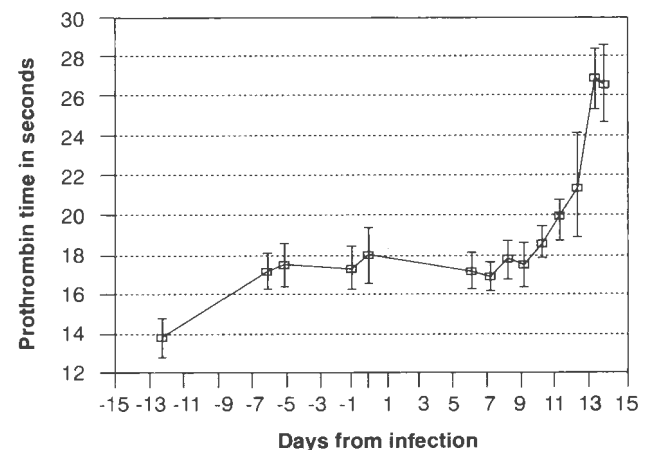


FIG. 3 Results of mean prothrombin time (PT) in seconds ± 2 SE

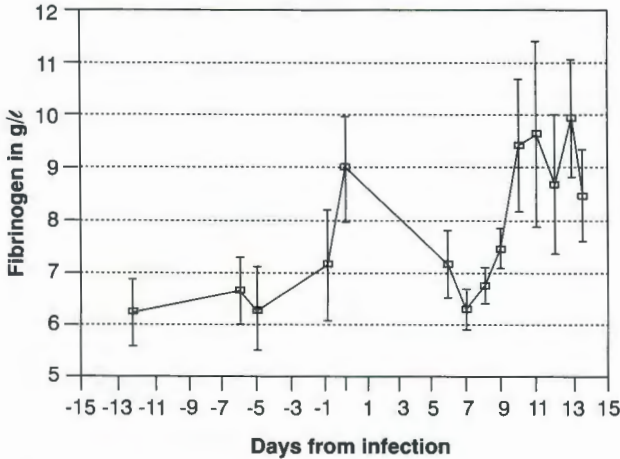


FIG. 4 Results of mean plasma fibrinogen in g/l \pm 2 SE

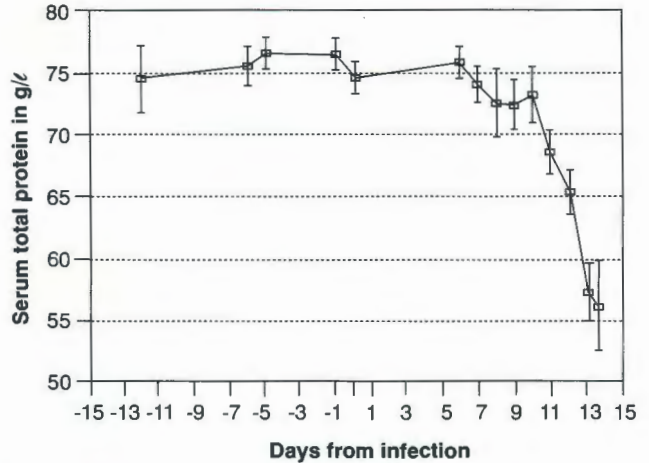


FIG. 5 Results of mean serum total protein in g/l \pm 2 SE

Fig. 1 shows the changes in the thrombocyte count. The data of one of the sheep (3459) was excluded as all its samples (from day -13) showed significant clotting with thrombocyte counts ranging from $2-13 \times 10^9/\ell$. From Fig. 1 it is clear that there is a marked drop in the thrombocytes during the acute stage of the disease. Thrombocyte counts obtained from days 10-13 differ significantly from all pre-infection means ($P < 0,05$). Thrombocyte counts obtained on days 11-13 differ significantly from days 6-8, which include the day of the temperature reaction above 40°C (day 7) ($P < 0,05$). The thrombocyte counts obtained from the two blood collections on day 13 differ significantly from days 10 and 11 ($P < 0,05$).

Fig. 2 and 3 show the changes associated with APTT and PT. Both APTT and PT rose steeply from day 10-13 post-infection, and became significantly ($P < 0,05$) prolonged, compared with pre-infection times excluding day 0. Fibrinogen increased during the acute stage of the disease and became significantly higher on days 10-13, compared with pre-infection levels, excluding day 0 (Fig. 4) on which there also seemed to have been a marked elevation in fibrinogen levels. The reason for this is not clear.

The Thrombo-Wellco rapid latex test showed no agglutination in any of the tests, indicating that concentrations above $2 \mu\text{g}$ per ml FDP were never reached in any of the sheep during the course of the disease.

It should be noted that PT, APTT and fibrinogen decreased terminally (second recording on day 13) as compared to the first reading on the same day. Similarly, there was a slight increase in the thrombocyte count in the second recording done on day 13.

The results of the blood-protein determinations are shown in Fig. 5-7.

Total serum protein (TSP), albumin and globulin showed a marked reduction during the course of the

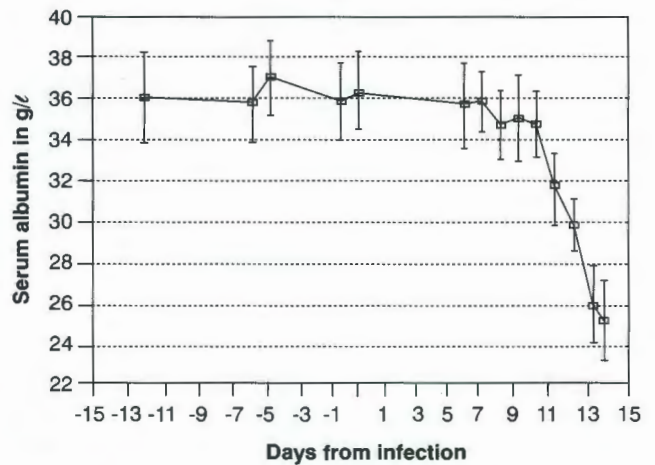


FIG. 6 Results of mean serum albumin in g/l \pm 2 SE

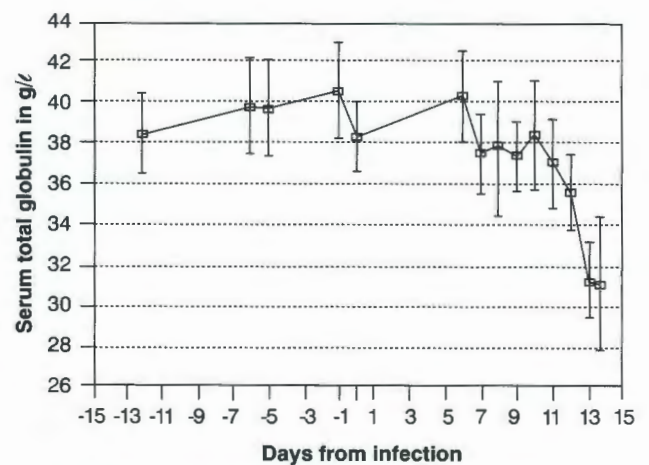
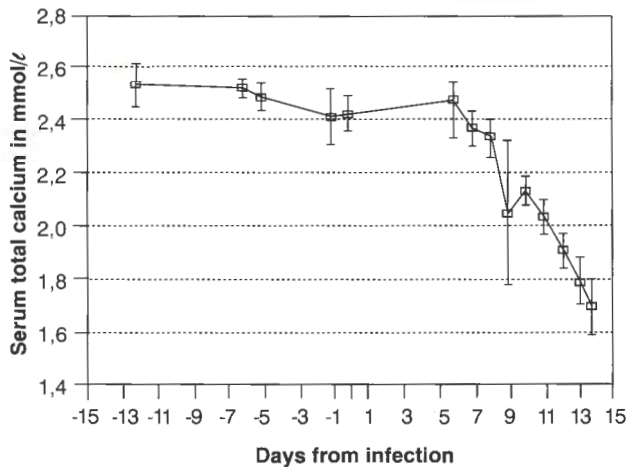
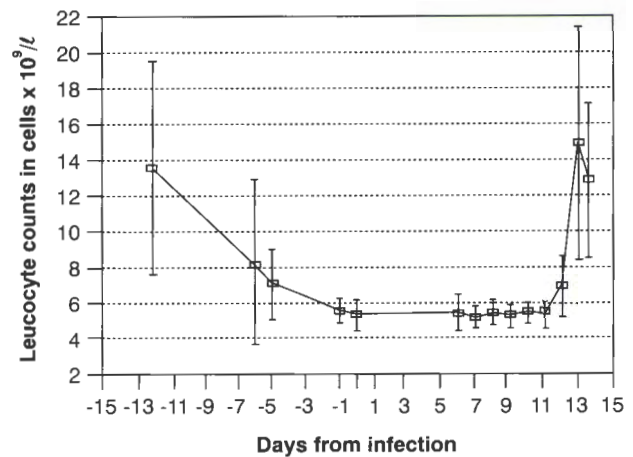
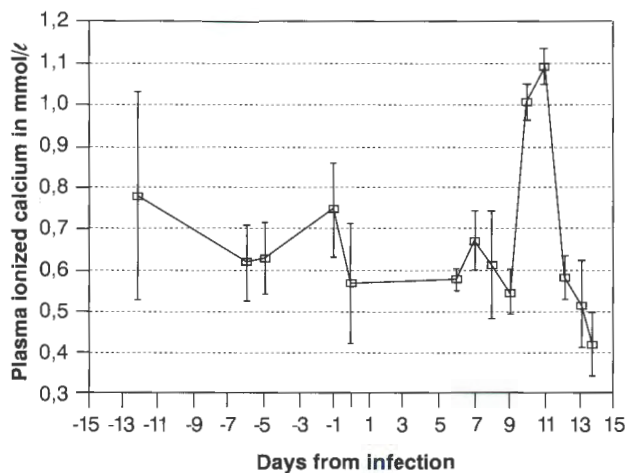
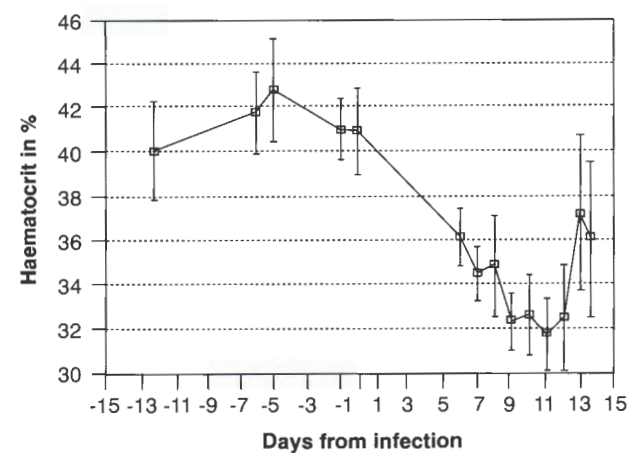


FIG. 7 Results of mean serum total globulin in g/l \pm 2 SE

disease. This started with the initial temperature reaction and became more severe towards the terminal stages. TSP levels and albumin recorded on

FIG. 8 Results of mean serum total calcium in mmol/l \pm 2 SEFIG. 10 Results of mean blood leucocyte counts in billion cells/l \pm 2 SEFIG. 9 Results of mean plasma ionized (pH corrected) calcium in mmol/l \pm 2 SEFIG. 11 Results of mean haematocrit (Ht) (%) \pm 2 SE

days 11–13 were significantly lower than pre-infection levels. Globulin, on the other hand, was significantly lower on day 13 only, compared with pre-infection levels. Albumin levels therefore are more severely affected as compared with globulin, probably since albumin is a smaller molecule and would therefore easily pass through semi-permeable membranes, compared with globulin (Ganong 1975).

Results of total and ionized calcium are shown in Fig. 8 and 9.

There was a marked drop in total calcium during the acute stage of the disease, starting with the temperature reaction. Levels of total calcium on days 9–13 were significantly lower, compared with those recorded on all pre-infection days. The decrease in total calcium was similar to that of total serum protein, which is not surprising, as much of the blood calcium is protein bound (Ganong 1975). In contradistinction to this, ionized calcium showed a marked rise during the acute stage of the disease, followed

by a terminal drop. Ionized calcium levels were significantly higher on days 10 and 11, compared with all the pre-infection days. However, on the second recording of day 13, the ionized calcium levels were significantly lower than pre-infection levels, excluding day 0.

The results of the white-cell count (WCC) and haematocrit (Ht) are shown in Fig. 10 and 11.

From Fig. 10 it is evident that there was an initial drop in the WCC during the pre-infection period. The initial leucocytosis was probably stress related (Jain 1986). The stress was probably caused by handling and the new stable environment. From Fig. 10 it is also evident that there was a terminal rise in WCC. If days -13 and -6 are excluded, then the WCC for both recordings on day 13 are elevated significantly over all other pre-infection values.

Fig. 11 shows that there was a marked decline in the haematocrit. From day 6 all Ht values, excluding the

first recording on day 13, were significantly lower than all the pre-infection levels.

DISCUSSION

One of the striking features of this study is the similarity in the course of the disease in the seven sheep. Rectal temperatures of 40°C and above were recorded in all the sheep on the morning of day 7. The height of the febrile reaction for all the sheep occurred on days 11 and 12. One sheep died on day 12, five died or were euthanased on day 13—due to severe clinical signs—and one was euthanased on day 15.

This aided in relating the clinical pathological changes observed to certain specific pathophysiological processes. One such process is the laboratory evidence of the development of a state of disseminated intravascular coagulopathy during the acute stage of the disease. For such a laboratory diagnosis, changes in at least three blood-clotting parameters are required (Van Amstel, Olivier & Reyers 1987b). In this study statistically significant changes in prothrombin time, activated partial thromboplastin time and thrombocyte counts were observed. The fact that the animals had not been exposed to anticoagulants and that there was no hepatopathy at autopsy, indicates that the prolonged PT and APTT are probably due to a consumption coagulopathy which is further supported by the thrombocytopenia.

The reason why fibrinogen-degradation products (FDP) were not elevated may possibly rest with the principle on which the test is based. Specific immunoglobulins against human fibrinogen fragments D and E are used in this test. It leaves, therefore, an open question as to whether this test is suitable for use in sheep. In addition, FDP fragments may complex with fibrinogen or fibrin monomers, which will result in the test being negative (Slappendel 1989).

It is well recognized that ruminants can produce large amounts of fibrinogen (Jain 1986). It is therefore quite feasible that the coagulation process present in these cases was not severe enough to have consumed sufficient fibrinogen to produce below-normal plasma levels. High fibrinogen levels can also be expected with chronic DIC or in cases where DIC is preceded by inflammatory conditions (Slappendel 1989).

Several compounds involved in normal blood clotting have been associated with an increase in capillary permeability, including alpha thrombin, fibrin and plasmin, through neutrophil and complement activation (Ferro & Malik 1989).

Both the involvement of endotoxin which can initiate DIC, and an increase in capillary permeability (Van Amstel *et al.* 1988a; Johnstone 1986) and comple-

ment (Du Plessis, Malan & Kowalski 1987) in cases of experimentally induced heartwater, have been demonstrated.

It therefore seems likely that the increase in capillary permeability observed in natural and experimentally induced heartwater is brought about by interrelated processes which include derangements in blood clotting, endotoxaemia, complement activation and other inflammatory products.

The presence of the abnormalities in bloodclotting as recorded in this study may be related to the pathogenicity of the infective agent as Du Plessis & Van Gas (1989) found the "Welgevonden" stock of *C. ruminantium* to be highly pathogenic. It is possible that the laboratory changes associated with the presence of DIC found in this study may not develop if other stocks of *C. ruminantium* are used in experimentally induced disease.

The presence and severity of the microvascular permeability defect was again demonstrated by the marked decline in protein, especially albumin, during the acute and terminal stages of the disease.

The fall in total calcium paralleled that of protein, as approximately 60% of the total calcium is in the bound form (Belonje 1976b). Changes in ionized calcium levels did not, however, follow those of total calcium. There was a significant increase over pre-infection levels during the acute stage of the disease and this was followed by a significant decline during the terminal stages. The reason for this increase in ionized calcium is not immediately apparent. (Belonje 1976b) demonstrated a correlation between blood-calcium levels and blood pH in sheep. He found a definite rise in total calcium which was paralleled by ionized calcium after infusion with lactic acid. In this study ionized calcium rose, despite the presence of a slight respiratory alkalosis during the acute stage of the disease (Van Amstel, Reyers, Sacks, Guthrie, Killeen, Myburgh & Pretorius, unpublished data 1993). The contribution of the terminal drop in ionized calcium to the death of these sheep is unclear. Further studies need to be carried out in this regard.

Although the total leucocyte count dropped during the course of the disease, a true leucopenia was not observed as previously reported (Ilemobade & Blotkamp 1978; Van Amstel *et al.* 1987 a). Figures for total white-cell count during the acute stage of the disease could be regarded as low normal (Jain 1986), whereas total leucocyte counts obtained during the terminal stage of the disease indicated a definite leucocytosis.

The reason for this terminal leucocytosis is not obvious, but it could be inflammatory or cortisol-induced. Van Amstel *et al.* (1988b) found elevated levels of cortisol in a calf with experimentally induced heartwater on the 2 d preceding death. Other factors as

yet undetermined, may also play a role as some of the eicosanoids will cause neutrophil aggregation and chemotaxis (Bottoms & Adams 1992).

A substantial drop in the haematocrits of the sheep was again demonstrated. In a previous study, Van Amstel *et al.* (1988b) also observed a progressive drop in both haemoglobin and the haematocrit, coinciding with the initial temperature reaction. In this and the afore-mentioned studies, there were no changes in either the red-cell mean corpuscular volume (MCV) or the mean corpuscular haemoglobin concentration (MCHC), indicating a normocytic normochromic anaemia (Jain 1986). These authors speculated that the cause of this anaemia might be a bone-marrow depression, on the basis that this type of anaemia was also accompanied by both a neutropenia and an eosinopaenia. The possibility of a transient bone-marrow depression in clinical cases of heartwater should still be investigated as other causes of a normocytic normochromic anaemia seem unlikely in this disease (Van Amstel *et al.* 1988b).

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