Different *Babesia canis* isolates, different diseases

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(*Received 22 January 1997; revised 27 May 1997; accepted 28 May 1997*)

SUMMARY

Using surface immunofluorescence isolate-specific antigens were detected on the membrane of erythrocytes infected with *Babesia* parasites. In addition, the strains reacted differently with Plasmagel in that the European isolate (*B.c. canis*) could be purified on Plasmagel effectively, whereas infected erythrocytes of the South-African isolate (*B.c. rossi*) could not. Experimental infection of dogs with *Babesia canis* isolates from geographically different areas revealed different pathology. The European isolate obtained from France exhibited transient parasitaemia, usually below 1 %, associated with low PCV values and congestion of internal organs. Clinical disease was correlated with an effect on the coagulation system, and not with peripheral parasitaemia. Infection of dogs with South-African-derived isolate induced high parasitaemia usually much higher than 1 %, which required chemotherapeutic treatment. In these animals clinical disease was correlated with peripheral parasitaemia and not with parameters of the coagulation system. The results show that the etiology of disease caused by these isolates of *B.c. canis* and *B.c. rossi* is different. This might have implications for the development of vaccines against these infections.

Key words: babesiosis, *Babesia canis rossi*, *Babesia canis canis*, dogs, pathology, antigenic diversity, coagulation.

INTRODUCTION

Babesiosis in dogs is caused by *Babesis canis* or *Babesia gibsoni* parasites. *B. canis* and *B. gibsoni* are easily distinguished as they differ considerably in size ($5 \times 2{\cdot}0$, $5 \times 3{\cdot}0$ and $1{\cdot}9 \times 1{\cdot}2$ μm respectively; Kuttler (1988)). There is also no doubt that these are different species. However, there is some controversy about the status of *B. canis* isolates derived from different geographical areas (Uilenberg *et al*. 1989). It has been noted that some isolates are restricted to specific tick-species with regard to transmission. Furthermore, scattered reports suggest that different isolates induce different types of pathology, ranging from fulminating disease with an exponentially increasing peripheral parasitaemia, to transient disease with few symptoms and low parasitaemia (Nuttall, 1904; Malherbe, 1956; Uilenberg *et al*. 1989). The fact that different isolates induce different types of pathology might have its implications for the development of an effective vaccine. Here we describe the course of infection in groups of animals that were infected with either a *B. canis canis* isolate derived from France, or a *B.c. rossi* isolate obtained from South Africa. As it was hypothesized that the coagulation system might be involved in the pathology of babesiosis (Wright & Goodger, 1988; Schetters *et al*. 1992) emphasis was put on the effects of parameters of the coagulation system after infection.

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MATERIALS AND METHODS

Animals

Beagle dogs (6–12 months old) of both sexes were used in the experiments. They received a standard amount of food daily. Drinking water was supplied *ad libitum*. Experimental groups of 5 dogs per group were formed assuring an even distribution of litter and sex in the groups.

Parasites

European isolate. Babesia canis canis was derived from a dog that had contracted babesiosis during a stay in France (Uilenberg *et al*. 1989).

South African isolate. Babesia canis rossi was derived from a dog that contracted babesiosis during a stay in Pietermaritzburg, South Africa (Uilenberg *et al*. 1989).

After isolation parasites were stored as a stabilate in liquid nitrogen, and passed through splenectomized dogs when required for infection.

Experimental infection

Experimental infection was carried out using heparinized blood of a dog that was infected with

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1 ml of stabilate. The blood was washed once with culture medium (modified RPMI-1640, Schetters *et al*. 1992). A total number of 2×10^6 parasitized erythrocytes was injected intravenously.

Parasitaemia

Parasitaemia was read from May-Grünwald/Giemsa-stained blood smears of peripheral blood. It was expressed as the log number of infected red blood cells/10^5 red blood cells (Jarra & Brown, 1985).

Clinical score

The clinical status of the animals was expressed as a clinical score value, as described before (Schetters *et al*. 1994). The clinical score was calculated from numerical values that expressed behaviour, body temperature, colour of mucosae, capillary refill time, size of lymph nodes and size of the spleen.

Chemotherapeutic treatment

Dogs were injected intramuscularly with 0·6 ml of imidocarbdipropionate solution (Imizol, Pittman-Moore) for 2 consecutive days. Within 3 days after this treatment no parasites were detectable in the peripheral blood.

Parameters of the coagulation system

Nine volumes of plasma were collected in 1 volume of sodium citrate solution (tri-sodium citrate . $2H_2O$; 38 g/l). The sample was centrifuged to pellet the blood cells (1500 ***g***, 10 min, 4 °C). The clear plasma was aspirated and kept at −20 °C until analysis. Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) and fibrinogen concentration were determined by Bergschot Centrum voor Onderzoek (BCO, Breda, The Netherlands) according to standard techniques. The amount of fibrinogen in the plasma is expressed as a value relative to the value of day 0; fibrinogen concentration at time x divided by fibrinogen concentration at day 0.

Normal dog serum

Normal dog serum was collected from healthy beagles. Serum samples were assayed with regard to sustaining *in vitro* growth of *B. canis*. A serum pool was prepared from selected dogs. The serum was sterilized by gamma-irradiation at 2·5 megarad, or filtration over a 0·2 μM membrane.

Immune dog serum

Immune serum against *B.c. canis* parasites was obtained from dogs that had recovered from infection. Immune serum against *B.c. rossi* parasites was obtained from dogs that were vaccinated with culture supernatant antigens followed by challenge infection (Schetters *et al*. 1994).

In vitro *culture of* B. canis

The parasites were cultured *in vitro* using dog erythrocytes essentially according to Trager and Jensen's method for the cultivation of *Plasmodium falciparum*, modified by Zolg (Trager & Jensen, 1976; Zolg *et al*. 1982). Briefly, infected blood was diluted with culture medium (RPMI 1640, Gibco cat. no. 21875, supplemented with 10 % (v/v) normal dog serum, 20–25 mM Hepes, and 0·6 g/l reduced glutathione, Sigma) to a concentration of 2 % packed cell volume. Cultures were gassed with a commercial gas mixture containing 5 % (v/v) CO_2 in air, and incubated at 37 °C in an incubator with 5 % (v/v) CO_2 in humidified air. Once a day the cultures were transferred to 50 ml tissue culture tubes (Falcon), and centrifuged (400 g, 5 min, 4 °C). The supernatant fraction was aspirated and replaced by fresh medium, pre-warmed in a 37 °C water bath. The cultures were gassed, and incubation resumed.

Immunofluorescence of live infected erythrocytes

Immunofluorescence analysis of parasitized erythrocyte surface antigens was performed by incubation of live *B.c. canis* or *B.c. rossi*-infected erythrocytes (parasitaemia 8–10 %) with different dog immune sera directed against *B.c. canis* or *B.c. rossi* according to the method of Allred (Allred, Hines & Ahrens, 1993). All dilutions were made in culture medium without serum supplements, and all incubations were carried out at 37 °C. Each incubation step was followed by washing with culture medium. Parasitized erythrocytes were collected from *in vitro* culture, washed with culture medium (500 ***g***, 5 min, room temperature) and resuspended in medium. Taking into account the differences of antibody titre of the immune sera (1 : 32000 for *B.c. canis* immune serum; 1 : 262000 for *B.c. rossi* immune serum), cells were incubated with undiluted anti-*B.c. canis* serum, or with 8-fold diluted anti-*B.c. rossi* serum. Bound antibodies were detected using a fluorescent antibody directed against dog immunoglobulins (FITC-conjugated rabbit anti-dog IgG, Sigma F7884) at a 1/100 dilution. Parasite nuclei were counter-stained using Hoechst 33258 (Sigma B2883) at 10 μg/ml. Finally, cells were washed by centrifugation at 500 ***g***, for 5 min and resuspended in culture medium with 10 % (v/v) normal dog serum. Fluorescence was detected using a fluorescence microscope (Axioscope, Zeiss), fitted with filters B2 (excitation 365 nm, emission 520 nm), for FITC, and UV (excitation 365 nm, emission 420 nm) for Hoechst.

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Separation of infected erythrocytes on Plasmagel

Erythrocytes infected with *Plasmodium falciparum* can be purified using Plasmagel (Roger Belon, France) (Pasvol *et al*. 1978; Reese, Langreth & Trager, 1979). The same method was used to separate *B.c. canis* and *B.c. rossi*-infected erythrocytes from *in vitro* cultures. One volume of cells suspended at 25% haematocrit in culture medium was mixed with 1 volume of Plasmagel in 10 ml tissue culture tubes filled to 5 ml. After 45 min of incubation at 37 °C, erythrocytes in the upper phase were collected, and extensively washed in culture medium supplemented with 10% (v/v) normal dog serum. Parasitaemia was read from blood smears stained with Diff Quick (Baxter, France).

RESULTS

Characterization of the different parasite strains in vitro

Detection of strain-specific parasite antigens on infected erythrocytes. *B.c. canis*-infected erythrocytes showed background fluorescence (20–30% of the infected cells) when reacted with FITC-conjugated anti-dog IgG serum (Table 1). This activity could not be blocked with unconjugated anti-dog IgG antiserum. No such reactivity was found when *B.c. rossi*-infected erythrocytes were used. All erythrocytes infected with *B.c. canis* or *B.c. rossi* exhibited specific surface fluorescence after incubation with their homologous antiserum only (Fig. 1). No specific fluorescence of the parasitized erythrocyte membrane (above background levels) was found when cells were incubated with heterologous antiserum. There was no evidence of staining of intracellular parasites with either immune serum, confirming the structural integrity of the infected erythrocyte membrane, and the surface localization of these isolate-specific antigens. Using phase-contrast and fluorescence microscopy it was found that uninfected erythrocytes did not react with immune serum. An additional difference between the two isolates was the occurrence of large agglutinates of erythrocytes infected with *B.c. rossi* parasites after incubation with homologous antiserum. In contrast, after incubation of *B.c. canis*-infected erythrocytes with either antiserum very small agglutinates were found.

Interaction of parasitized erythrocyte suspensions with Plasmagel. Erythrocytes infected with *B.c. canis* parasites could be separated effectively by Plasmagel. This method was less effective when using *B.c. rossi*-infected erythrocytes (Fig. 2). A compilation of results from 10 separate experiments is presented in Table 2. Using *B.c. canis*-infected erythrocytes, parasitaemia of the upper phase of the Plasmagel varied between 60 and 80%, whereas in the case of *B.c. rossi*-infected erythrocytes parasitaemia varied between 10 and 15% (Table 2). To show that the parasitized erythrocytes had retained their viability they were diluted with freshly prepared red blood cells to 1% parasitaemia, and put into culture. After 2 days of culture the number of infected erythrocytes rose to 8–10% parasitaemia.

Characterization of the different parasite strains in vivo

Peripheral parasitaemia after infection. Groups of 5 dogs were infected with either *B.c. canis*, or *B.c. rossi*. Parasitaemia was followed daily. Dogs infected with *B.c. canis* exhibited a transient parasitaemia (Fig. 3A). Parasites were first detected in the blood 3 days after infection. The average parasitaemia did not increase much, and declined from day 6 after infection onwards. Dogs that had been infected with *B.c. rossi* developed a progressive parasitaemia without any apparent restriction. Parasites were first detected in the peripheral blood at day 4 after infection. All *B.c. rossi*-infected dogs had to be treated chemotherapeutically at day 7 after infection. As the difference between the 2 types of infection was established, the experiment was stopped at day 8 after infection when the remaining dogs were chemotherapeutically treated.

Peripheral erythrocyte count after infection. From the first day after infection onwards, the packed cell volume (PCV) declined (Fig. 3B). Animals infected with *B.c. rossi* all had to be treated chemotherapeutically at day 7 after infection. At that point of time the mean PCV of the latter group of animals decreased at a faster rate than that of the dogs infected with *B.c. canis*. The dogs infected with *B.c. canis* parasites showed a gradual decrease which appeared to reach its lowest value at 8 days after challenge infection (Schetters *et al*. 1995). At that point of time the experiment was ended.

Coagulation parameters after infection. There was no statistically significant effect on the prothrombin time and thrombin time during the period of infection (data not shown). There was an increase of the activated partial thromboplastin time (APTT) from the moment the parasites were detected in the peripheral blood (Fig. 3C). The fibrinogen concentration in the plasma increased steadily from one day after infection onwards with either parasite (Fig. 3D). The relative increase of fibrinogen in the group of animals infected with *B.c. canis* reached higher values throughout the post-infection period as compared with the *B.c. rossi*-infected group.

Clinical score after infection. When infected with *B.c. canis* parasites, the clinical score increased from day

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Table 1. Detection of surface antigens on infected erythrocytes by immunofluorescence

(Data represent the percentage of infected erythrocytes that exhibited membrane fluorescence.)

	Babesia canis isolate	
Serum	*B.c. canis*	*B.c. rossi*
Anti-mouse	0	0
Anti-dog IgG	20–30	0
Normal dog serum	20–30	0
Anti-*B.c. canis*	100	0
Anti-*B.c. rossi*	20–30	100

3 onwards, peaked at day 7 after which it declined (Fig. 3E). In contrast, the clinical score of the group of animals infected with *B.c. rossi* parasites increased steadily from day 3 onwards. The highest values were reached at day 7 when it was decided to treat the animals.

Correlation between clinical score, parasitaemia and coagulation parameters. There was a positive correlation between the clinical score values and PCV of the animals infected with *B.c. rossi* (Fig. 4A). A similar correlation was found between the clinical score and parasitaemia (Fig. 4B). No correlation was found between APTT and parasitaemia, clinical score (Fig. 4C) or PCV (Fig. 4D) in this group of animals. In contrast, when infected with *B.c. canis*, there was no correlation between clinical score and peripheral parasitaemia (Fig. 5B). In this case, however, a positive correlation was found between the APTT and clinical score (Fig. 5C), and also between the PCV and APTT (Fig. 5D).

DISCUSSION

The clinical picture of dogs suffering from babesiosis due to infection with *Babesia canis* parasites is diverse, ranging from hyperacute, to acute, and finally chronic types of disease (Clarvoe, 1939; Malherbe, 1956; Shortt, 1973). This fact was recognized by early researchers who analysed the disease as it occurs in different geographical areas, and it was suggested that the parasite from South Africa was different from that causing the disease in France (Nuttall, 1904; Sanders, 1937). The first suggestion to classify the parasites from different geographical regions into subspecies of *B. canis* was made by Uilenberg (Uilenberg *et al.* 1989). The European parasites transmitted by *Dermacentor*

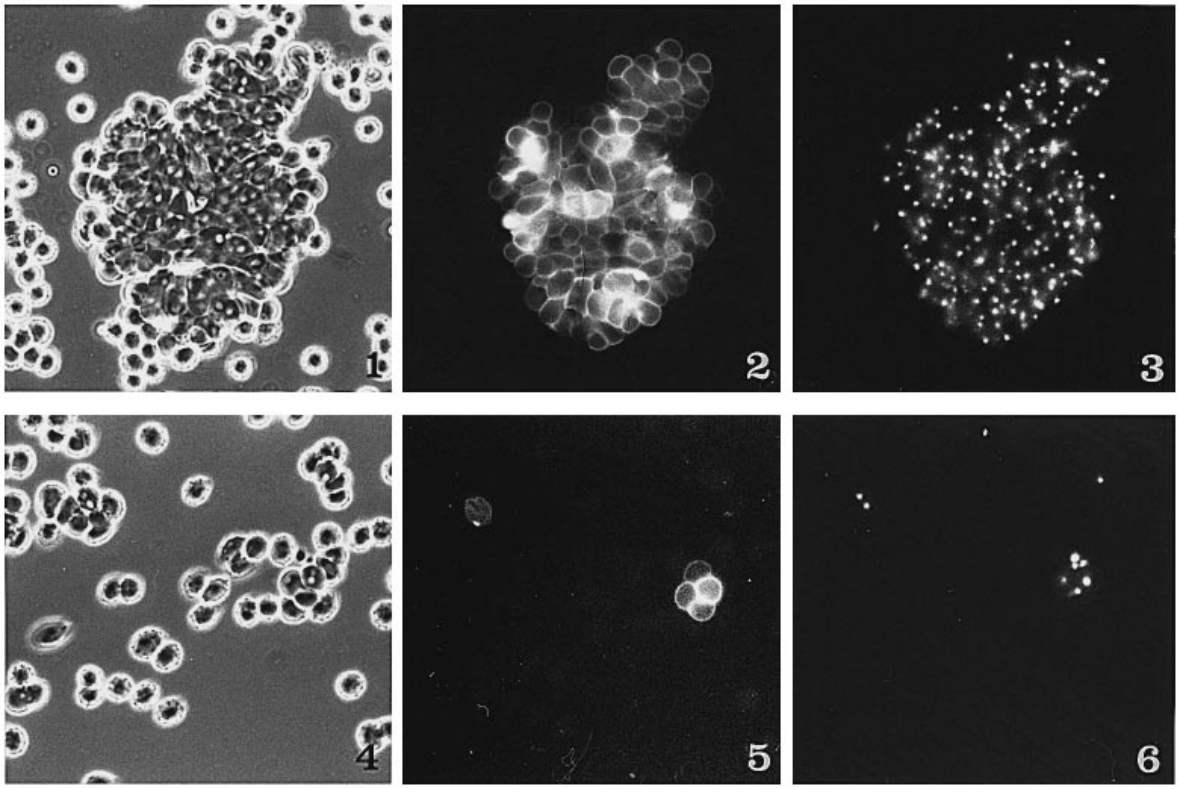

Fig. 1. Detection of antigens on the surface of *Babesia canis rossi* (1–3) or *B.c. canis* (4–6) infected erythrocytes by immunofluorescence. Live intact infected erythrocytes were incubated with homologous antisera, and bound antibodies were detected with FITC-conjugated anti-dog IgG antiserum. Surface fluorescence (2 and 5) was only observed on erythrocytes that contained *Babesia* parasites as confirmed by phase-contrast microscopy (1 and 4), and counterstaining using Hoechst 33258 (3 and 6).

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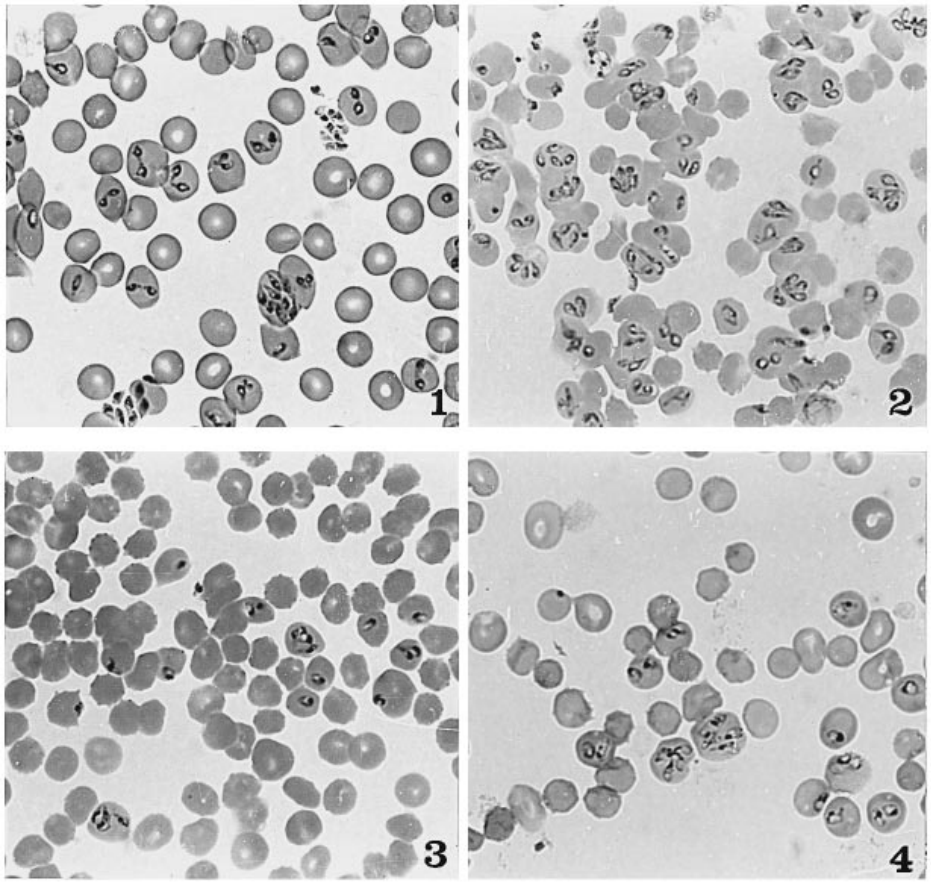

Fig. 2. Purification of *Babesia canis canis* (1, 2) and *B.c. rossi* (3, 4) infected red blood cells from *in vitro* cultures by sedimentation in Plasmagel. *B.c. canis*-infected erythrocytes could be effectively separated in the upper phase after incubation at 37 °C for 45 min with Plasmagel (2). On the contrary, with *B.c. rossi* this method was much less effective (4).

Table 2. Purification of infected erythrocytes from *in vitro* cultures of *B.c. canis* and *B.c. rossi* using Plasmagel

(Data represent the percentage of infected erythrocytes detected in the original cultures, and in the Plasmagel derived fraction (mean of 10 separate experiments).)

Infected erythrocyte suspension	*Babesia canis* isolates	
	B.c. canis	*B.c. rossi*
In vitro culture	8–10	8–10
Plasmagel fraction	60–80	10–15

reticulatus were classified as *B. canis canis*, the North African parasites transmitted by *Rhipicephalus sanguineus* as *B. canis vogeli*, and the South African parasites transmitted by *Haemaphysalis leachii* as *B. canis rossi*. Serological assays including immunofluorescence on fixed bloodsmears (Uilenberg *et al.* 1989), ELISA using soluble somatic parasite antigen (Hauschild, Shayan & Schein, 1995) and sandwich-ELISA using soluble parasite antigen from *in vitro* culture (Schetters *et al.* 1996*b*) all showed high isolate specificity. Cross-immunity studies confirmed the antigenic difference of the different isolates (Brumpt, 1938; Uilenberg *et al.* 1989), although this does not necessarily prove that the different isolates are subspecies, as such variations within isolates from the same subgroup have been shown, too (Schetters *et al.* 1995, 1996*a*).

In the experiments described here antigenic differences between live infected erythrocytes of *B.c. rossi* and *B.c. canis* could be detected at the erythrocyte surface, both by immunofluorescence using homologous antisera, and the reactivity with Plasmagel. As the infected erythrocyte surface is easily accessible to effector mechanisms of the immune system, this result provides a rationale for the specificity of immunity to *B. canis* infection (Uilenberg *et al.* 1989). Infection experiments in healthy non-splenectomized beagles confirmed that the 2 isolates tested were biologically different. The South African parasites continued to proliferate to such an extent that the animals had to be chemotherapeutically treated. In this group of animals the disease, read from the daily clinical score value, was positively correlated with the peripheral parasitaemia. High parasitaemias ($> 1\%$) were correlated with haemolysis, and in severe cases with haemoglobinurea (data not shown). Thus, using the

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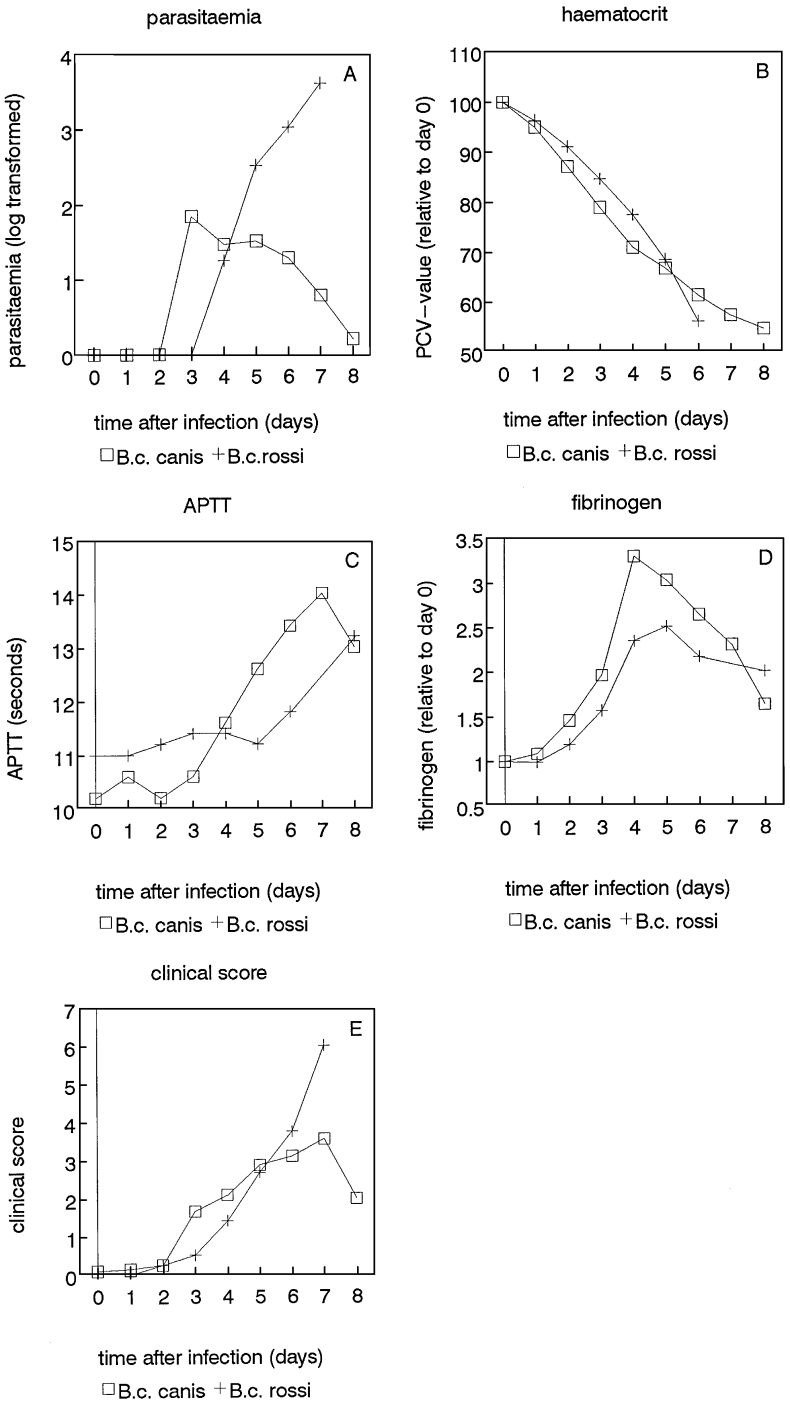

Fig. 3. Dynamics of parasitaemia (A), haematocrit (B), APTT of plasma (C), fibrinogen concentration in plasma (D), and clinical score (E) after infection of groups of dogs ($n = 5$) with *Babesia canis canis* or *B.c. rossi* parasites. Data represent the group mean values.

South African isolate, disease is caused by proliferation of parasites as such. One should be careful in making generalizations, however, as from a study on a number of dogs suffering from babesiosis in South Africa, it was found that the animals could be divided in groups of dogs that suffered from mild infections, and groups of dogs that suffered from severe infections (Moore & Williams, 1979). Thus, it is possible that different isolates from the group of *B.c. rossi* also differ with regard to virulence (Malherbe, 1956).

The European parasites did not proliferate to high peripheral parasitaemias, a feature also observed with an antigenically distinct European isolate (Schetters *et al*. 1995). *B.c. canis* parasites have been shown to proliferate in deep tissues, like *B. bovis* parasites, and appear to autoagglutinate (Schetters & Montenegro-James, 1995). This tendency to autoagglutinate has also been observed in *in vitro* cultures (Moubri, data not shown), and is associated with the fact that the infected erythrocytes can be purified on Plasmagel. At present it is uncertain as to

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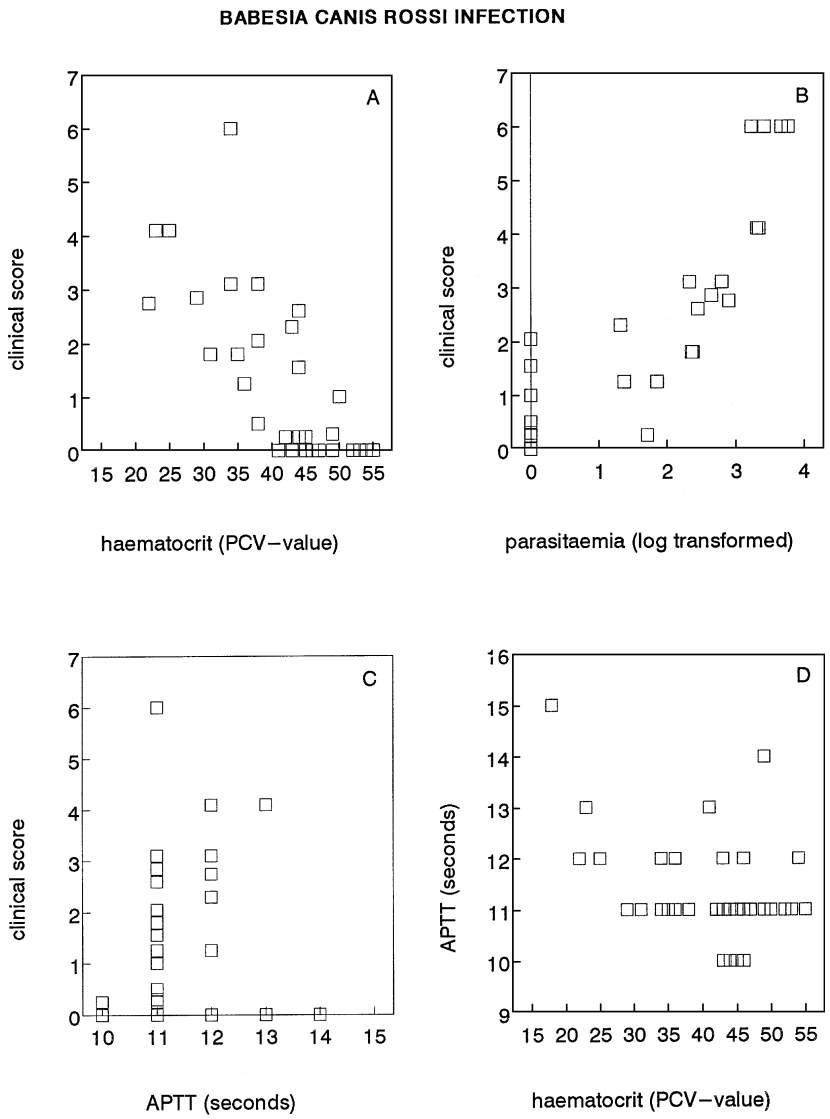

Fig. 4. Correlation between clinical score and haematocrit (A), parasitaemia (B) and APTT (C), and the correlation between the APTT and haematocrit after infection of dogs with *Babesia canis rossi* parasites. Data are sets of values of individual dogs during the post-infection period.

whether this relationship is causal, in that certain surface-exposed molecules on *B.c. canis*-infected erythrocytes, but absent on *B.c. rossi*-infected erythrocytes, cause the infected erythrocytes to stick in the microvasculature of internal organs. The fact that a proportion of the *B.c. canis*-infected erythrocytes reacted with anti-dog IgG antiserum corroborates the suggestion that due to parasitization the erythrocyte membrane is altered such that epitopes arise that are recognized by the particular antiserum used. It does not appear to be the result of interaction with dog immunoglobulins as the activity could not be blocked with another, unconjugated antiserum against dog IgG.

An alternative explanation could be that infected erythrocytes activate the coagulation system. Both parasite isolates affected the coagulation system, which is an important diagnostic criterium for babesiosis (Malherbe, 1956, Wright & Goodger, 1988). Activation of coagulation might lead to coating of infected erythrocytes with fibrinogen-like proteins, which render the cells sticky (Wright & Goodger, 1988). The European isolate appeared to affect the APTT 2 days earlier than the South African parasite. As the infection dose was comparable the results suggest that *B.c. canis* is more procoagulant than *B.c. rossi*. Moreover, if there were a 2-fold difference between the infection doses, then this difference would have been corrected in 8 h (the proliferation time of *B. canis*), which thus can not explain this differential effect on the APTT. In *B.c. canis* infection disease was positively correlated with the effect on the APTT, suggesting that the etiology of this type of disease is different from that in *B.c. rossi* infection. This is in line with the clinical picture observed by Nuttall (1904), who found that congestion of internal organs was more profound in dogs suffering from European babesiosis when compared to dogs suffering from South African babesiosis. From the study of Moore & Williams (1979) it also

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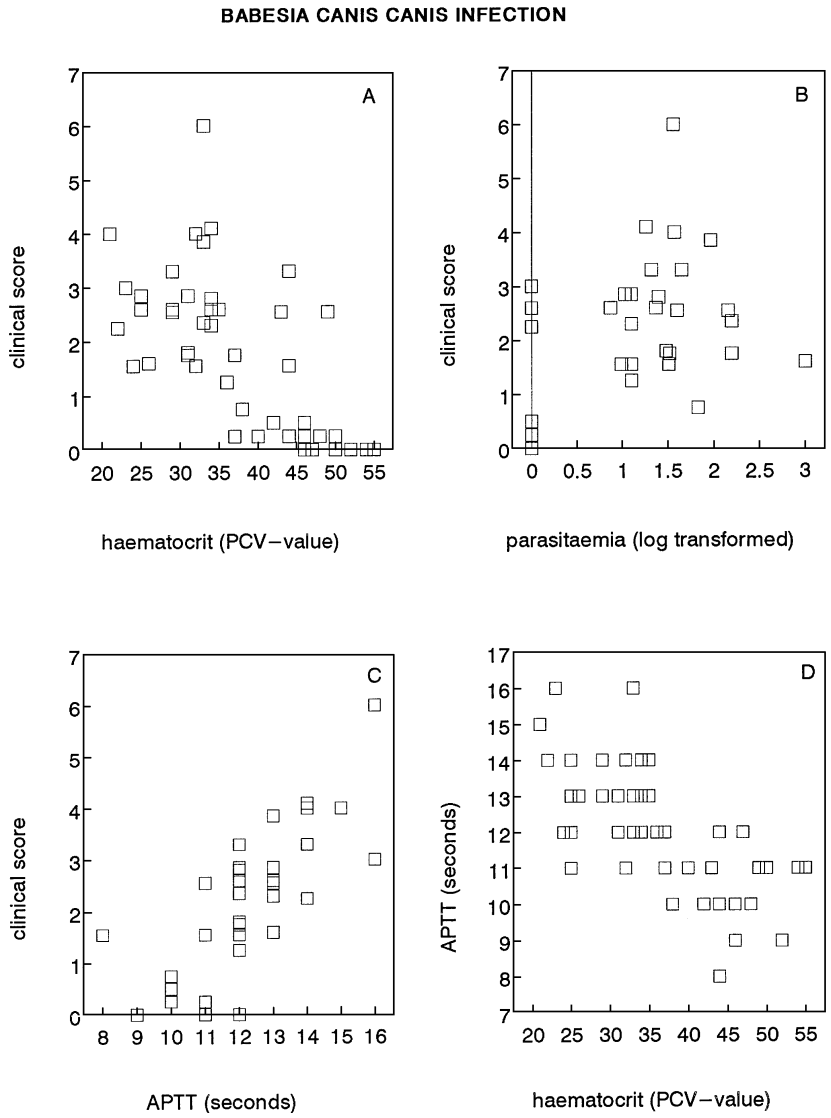

Fig. 5. Correlation between clinical score and haematocrit (A), parasitaemia (B) and APTT (C), and the correlation between the APTT and haematocrit after infection of dogs with *Babesia canis canis* parasites. Data are sets of values of individual dogs during the post-infection period.

was apparent (although not pointed out by these authors) that the animals that suffered from severe disease had significantly increased APTT values, in contrast to the group of animals with mild disease.

The increase of fibrinogen in the early days of experimental infection is most likely the result of the acute-phase response (Symons, Eastgate & Duff, 1992; Guelfi, Dubois & Boneu, 1984), when there is no other evidence for the coagulation system to be involved. As soon as the APTT value increases the fibrinogen concentration decreases rapidly, which is indicative for activation of the coagulation system (Feldman, Madewell & O'Neill, 1981). As yet it is unknown whether activation of the coagulation system results in increased stickiness and retention of infected erythrocytes in the microvasculature as we proposed earlier (Schetters & Montenegro-James, 1995), although in *B. bovis* infection it was shown that infected erythrocytes become coated with fibrinogen-like proteins (Wright & Goodger, 1988). Another explanation could be obstruction of capillary flow due to the deposition of fibrin clots in the microvasculature as a result of disseminated intravascular coagulation (Moore & Williams, 1979). That the coagulation system might be involved in retention of (infected) erythrocytes in the deep tissues is further substantiated by the fact that injection of heparin in a dog infected with *B.c. canis* was followed by a transient increase of peripheral parasitaemia and packed cell volume. A second consecutive injection showed the same effect (data not shown).

In conclusion, the results show that the etiology of disease due to these isolates of *B.c. canis* and *B.c. rossi* infection in dogs is different. Regarding the wide spectrum of canine babesiosis it appears that the 2 isolates tested are typical representatives of the groups of parasites that induced different types of pathology. It follows that the distribution of these strains is skewed towards *B.c. rossi*-type of parasites

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being more prevalent in South Africa, and *B.c. canis*-type parasites predominating in Europe. This might bear relevance to the development of vaccines against these parasites.

The authors wish to thank Evelien van Kempen-Hoogsteder for biotechnical assistance, Drs A. Vermeulen and J. Pasman for their helpful discussions, and Helma van Daal for preparing the manuscript.

REFERENCES

ALLRED, D. R., HINES, S. A. & AHRENS, K. P. (1993). Isolate specific parasite antigens of the *Babesia bovis*-infected erythrocyte surface. *Molecular and Biochemical Parasitology* **60**, 121–132.

BRUMPT, E. (1938). Identification des piroplasmes du chien du type *Piroplasma canis*. Transmission de la souche Française par la tique sud-africaine *Haemaphylsalis leachi*. Faible valeur des épreuves d'immunité croisée dans les piroplasmoses. *Annales de Parasitologie Humaine et Comparée* **2**, 97–116.

CLARVOE, H. M. (1939). Canine piroplasmosis. *The North American Veterinarian* **20**, 50–52.

FELDMAN, B. F., MADEWELL, B. R. & O'NEILL, S. (1981). Disseminated intravascular coagulation: antithrombin, plasminogen, and coagulation abnormalities in 41 dogs. *Journal of the American Veterinary Medical Association* **179**, 151–154.

GUELFI, J. F., DUBOIS, P. H. & BONEU, B. (1984). Exploration de l'hémostase chez des chiens atteints de babésiose. *Revue Médicinale Vétérinaire* **135**, 699–703.

HAUSCHILD, S., SHAYAN, P. & SCHEIN, E. (1995). Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. *Parasitology Research* **81**, 638–642.

JARRA, W. & BROWN, K. N. (1985). Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. Effectiveness and inter- and intraspecies specificity of immunity induced by infection. *Parasite Immunology* **7**, 595–606.

KUTTLER, K. L. (1988). World wide impact of babesiosis. In *Babesiosis of Domestic Animals and Man*, (ed. Ristic, M.), pp. 1–22. CRC Press, Inc., Boca, Raton, USA.

MALHERBE, W. D. (1956). The manifestations and diagnosis of *Babesia* infections. *Annals of the New York Academy of Sciences* **64**, 128–146.

MOORE, D. J. & WILLIAMS, M. C. (1979). Disseminated intravascular coagulation: a complication of *Babesia canis* infection in the dog. *Journal of the South African Veterinary Association* **50**, 265–275.

NUTTALL, G. H. F. (1904). Canine piroplasmosis. *Journal of Hygiene* **4**, 219–252.

PASVOL, G., WILSON, R. J., SMALLEY, M. E. & BROWN, J. (1978). Separation of viable schizont-infected red cells of *Plasmodium falciparum* from human blood. *Annals of Tropical and Medical Parasitology* **72**, 87–88.

REESE, R. T., LANGRETH, S. & TRAGER, W. (1979). Isolation of stages of the human parasite *Plasmodium falciparum* from culture and from animal blood. *Bulletin of the World Health Organization* **57** (Suppl. 1), 53–61.

SANDERS, D. A. (1973). Observations on canine babesiosis. *Journal of the American Veterinary Medical Association* **90**, 27–40.

SCHETTERS, T. P. M. & MONTENEGRO-JAMES, S. (1995). Vaccines against babesiosis using soluble parasite antigens. *Parasitology Today* **11**, 456–462.

SCHETTERS, TH. P. M., KLEUSKENS, J. A. G. M., SCHOLTES, N. C. & BOS, H. J. (1992). Vaccination of dogs against *Babesia canis* infection using parasite antigens from *in vitro* culture. *Parasite Immunology* **14**, 295–305.

SCHETTERS, TH., KLEUSKENS, J., SCHOLTES, N. & BOS, H. J. (1995). Strain variation limits protective activity of vaccines based on soluble *Babesia canis* antigens. *Parasite Immunology* **17**, 215–218.

SCHETTERS, TH. P. M., KLEUSKENS, J. A. G. M., SCHOLTES, N. C., PASMAN, J. W. & BOS, H. J. (1994). Vaccination of dogs against *Babesia canis* infection using antigens from culture supernatants with emphasis on clinical babesiosis. *Veterinary Parasitology* **52**, 219–233.

SCHETTERS, T., KLEUSKENS, J., SCHOLTES, N., PASMAN, J. & BOS, H. J. (1996*a*). Vaccination of dogs with soluble *Babesia canis* antigens derived from *in vitro* culture is strain specific. In *New Dimensions in Parasitology : Keynote Papers from the VIII International Congress of Parasitology ; Acta Parasitologica Turicca* **1996** (Suppl.) **1**, 1–8.

SCHETTERS, TH. P. M., SCHOLTES, N. C., KLEUSKENS, J. A. G. M. & BOS, H. J. (1996*b*). Not peripheral parasitaemia but the level of soluble antigen in plasma correlates with vaccine efficacy against *Babesia canis*. *Parasite Immunology* **18**, 1–6.

SHORT, H. E. (1973). *Babesia canis*: the life cycle and laboratory maintenance in its arthropod and mammalian hosts. *International Journal for Parasitology* **3**, 119–148.

SYMONS, J. A., EASTGATE, J. A. & DUFF, G. W. (1992). Interleukin 1 as an inflammatory mediator. In *Immunology and Medicine*, *Series Vol.* **18**, *Biochemistry of Inflammation*, (ed. Whicher, J. T. & Evans, S. W.), pp. 183–210. Kluwer Academic Publishers, Dordrecht.

TRAGER, W. & JENSEN, J. B. (1976). Human malaria parasites in continuous culture. *Science* **193**, 673–675.

UILENBERG, G., FRANSSEN, F. F. J., PERIÉ, N. & SPANJER, A. A. M. (1989). Three groups of *Babesia canis* distinguished and a proposal for nomenclature. *Veterinary Quarterly* **11**, 33–40.

WRIGHT, I. G. & GOODGER, B. V. (1988). Pathogenesis of babesiosis. In *Babesiosis of Domestic Animals and Man* (ed. Ristic, M.), pp. 99–118. CRC Press Inc., Boca Raton, USA.

ZOLG, W. J., MCLEOD, A. J., DICKSON, I. H. & SCAIFE, J. G. (1982). *Plasmodium falciparum*: modification of the *in vitro* culture conditions improving parasite yields. *Journal of Parasitology* **68**, 1072–1080.