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CONCURRENT BABESIOSIS AND EHRLICHIOSIS IN THE DOG: BLOOD SMEAR EXAMINATION SUPPLEMENTED BY THE INDIRECT FLUORESCENT ANTIBODY TEST, USING *COWDRIA RUMINANTIUM* AS ANTIGEN

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ABSTRACT

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Giemsa-stained, peripheral blood smears of 67 dogs, showing clinical signs typical of babesiosis or reminiscent of concurrent babesiosis and ehrlichiosis, were examined for the presence of *Babesia canis* and *Ehrlichia canis*. Since *Cowdria ruminantium* cross-reacts with *Ehrlichia*, the sera of these dogs were also subjected to the indirect fluorescent antibody (IFA) test in which *C. ruminantium* was used as antigen. Fifty-five per cent of these dogs had mixed infections of *B. canis* and *E. canis*, as judged by blood smear examination and serology. The serum of 32 % of these dogs with mixed infections reacted positively in the IFA test. Six out of 9 dogs, the blood smears of which were negative for both *B. canis* and *E. canis*, were serologically positive for *E. canis*. Furthermore, sero-conversion from a negative in the initial serum sample to titres of up to 1:160 in a subsequent sample was recorded in 9 out of 13 dogs with suspected mixed infection on blood smear.

INTRODUCTION

Concurrent babesiosis and ehrlichiosis is a well-documented phenomenon (Ewing & Buckner, 1965; Immelman & Button, 1973; Neitz & Thomas, 1938; Van Heerden, 1982; Van Heerden, Reyers & Stewart, 1983). While *Babesia canis* can usually be demonstrated quite readily in smears prepared from peripheral blood, *Ehrlichia canis* may often be difficult to detect in smears because of low parasite numbers. The clinical picture in these cases is usually dominated by the clinical signs attributable to babesiosis and ehrlichiosis is often overlooked. In many cases, however, the clinical signs may be vague and non-specific, and inappetence, a mild febrile reaction and loss in condition may be the only manifestations.

In the indirect fluorescent antibody (IFA) test currently used for ehrlichiosis (Ristic, Huxsoll, Weisiger, Hildebrandt & Nyindo, 1972), *E. canis* propagated in canine blood monocytes serves as antigen. Although the test has been found to be highly sensitive and specific for *E. canis* (Ristic *et al.*, 1972), difficulty in maintaining these cell cultures because of the decreasing virulence of the organism in successive passage has been experienced (Greene & Harvey, 1984). Problems with the preparation of the antigen were also encountered in the Republic of South Africa (C. G. Stewart, personal communication, 1989). Contrary to these findings, however, Davoust & Parzy (1989), using commercially available antigen, recently reported the successful application of the IFA test, developed by Ristic *et al.* (1972), in a serological survey involving 265 dogs in the south-east of France.

Since it is known that *Cowdria ruminantium* cross-reacts with *E. canis* and other *Ehrlichia* spp. (Logan, Holland, Mebus & Ristic, 1986; Du Plessis, Camus, Oberem & Malan, 1987), this investigation was undertaken to determine whether the IFA test, in which *C. ruminantium* is used as antigen, could supplement blood smear examinations and other laboratory procedures in the diagnosis of canine ehrlichiosis and perhaps throw light on the relationship between *B. canis* and *E. canis* in mixed infections.

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MATERIALS AND METHODS

To study the sensitivity of the IFA test as applied to canine ehrlichiosis, using the Kümm stock of *C. ruminantium* as antigen, serum was collected from 4 dogs immediately prior to their being infected intravenously with an isolate of *E. canis*. These control dogs were clinically normal German Shepherd dogs, housed in an experimental unit on a concrete floor and treated weekly with an acaricide. Pre-infection clinical and haematological examination findings were normal. Post-infection serum samples collected 29–32 days after infection were likewise subjected to the IFA test.

Paired peripheral blood smears were taken from 67 mixed-breed dogs of all ages and both sexes with clinical signs typical of babesiosis or showing vague and non-specific signs of inappetence, mild fever and loss in condition, which may be associated with concurrent babesiosis and ehrlichiosis. One of the smears was stained with Diff-Quik (Harleco) for a preliminary examination, and all the dogs with positive *B. canis* blood smears were treated with a babesiacidal drug and oxy-tetracycline. The other smear was stained with 5 % Giemsa for 50 min for a more thorough examination. Concentrating on the feather end of the smear, the red blood cells and monocytes, parasitized by *B. canis* and *E. canis* respectively, were counted, and the percentage of cells parasitized in both cases calculated. A smear was considered negative if after an examination of 5 min no parasites were observed.

Serum samples were also collected from the 67 dogs and subjected to the IFA test in which mouse peritoneal macrophages, infected with the Kümm stock of *C. ruminantium*, are currently used to detect antibodies to the heartwater agent (Du Plessis & Malan, 1987). Briefly, twofold serial dilutions of serum, commencing with a 1:10 dilution, were placed on 15-well antigen slides and incubated in a moist chamber for 30 min at 37 °C. The slides were washed in an IFA test buffer for 10 min and then flooded with a working dilution of fluorescein-isothiocyanate-conjugated anti-dog IgG (Bio-Yeda). After another incubation period of 30 min, the slides were again washed and mounted in buffered glycerine.

In the case of 13 dogs that were positive for both babesiosis and ehrlichiosis on blood smears, but serologically negative for the latter, blood smears and

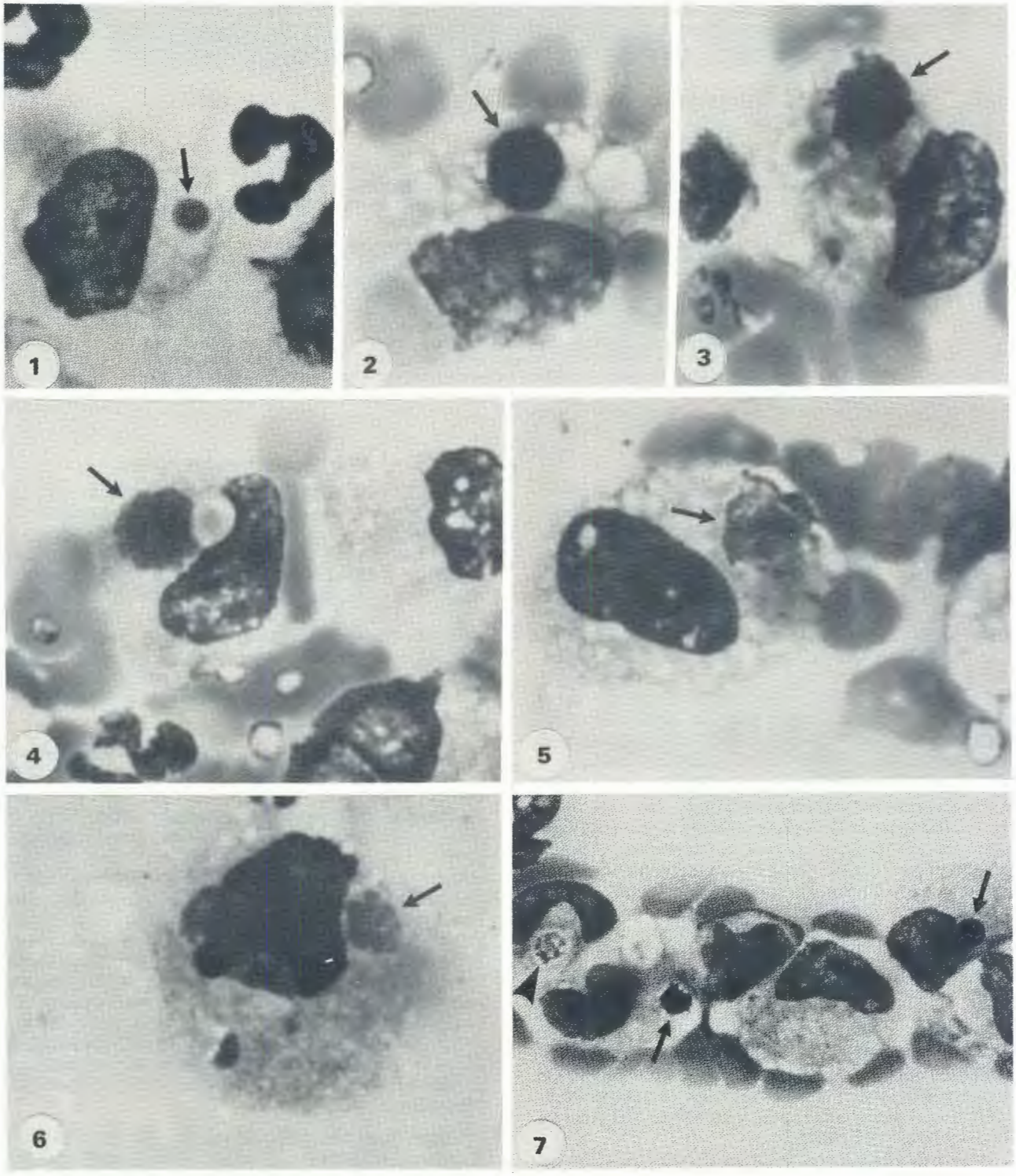

FIG. 1–7 *E. canis* (arrows) in the monocytes of dogs with concurrent ehrlichiosis and babesiosis

FIG. 1 & 2 Homogeneous, dense forms of *E. canis*

FIG. 3 & 4 Irregularly outlined forms of *E. canis* with internal structure becoming visible (Fig 4). Note the erythrocytes parasitized by *B. canis*

FIG. 5 & 6 Distinctly granular forms of *E. canis*

FIG. 7 Three monocytes with different developmental forms of *E. canis:* Homogeneous, dense bodies (arrows) and morula of *E. canis* (arrowhead) Giemsa, × 8000 (Fig. 1–6) & × 2000 (Fig. 7)

serum samples were again collected 6–43 days later and examined in a similar manner.

RESULTS

The sera of all 4 control dogs collected prior to infection were negative at a dilution of 1:10. The outlines of the colonies of *C. ruminantium* in the mouse peritoneal macrophages, used as antigen, could be vaguely distinguished, but these colonies showed no fluorescence. Distinct fluorescence, however, revealed titres of 1:320–1:2560 in the post-infection sera collected 29–32 days after infection. Sero-conversion from a total negative to high titres, corresponding with disease developing as a result of experimental infection, therefore proved that *C. ruminantium*-infected mouse peritoneal cells can be

used as antigen in the IFA test to detect antibodies to *E. canis*.

The combined results of smears and serology (Table 1) show that 37 out of 67 dogs (55 %) were infected with both *B. canis* and *E. canis*. In the case of 9 (13 %) and 10 (15 %) animals, a single infection with respectively *E. canis* and *B. canis*, was diagnosed, and 11 dogs (16 %) were free from infection with either of the parasites.

TABLE 1 Serology and Giemsa stained, peripheral blood smear findings on 67 dogs

	No. of dogs	*E. canis* +ive on		
		Blood smear only	Serology only	Blood smear and serology
B. canis +ive *E. canis* +ive	37	25	1	11
B. canis −ive *E. canis* +ive	9	0	6	3
B. canis +ive *E. canis* −ive	10	—	—	—
B. canis −ive *E. canis* −ive	11	—	—	—

In the case of the 37 dogs with mixed infections, the serum of only 12 (32 %) reacted positively to titres of 1:10 and 1:20 in the IFA test. The blood smears of only 3 out of 9 dogs, infected with *E. canis* alone, as determined with the IFA test, were positive. It is important to note, however, that they were all serologically positive to titres of 1:20 to 1:160.

The results of the subsequent examination of the 13 serum samples from dogs that were initially positive on smears for both *B. canis* and *E. canis*, but serologically negative for *E. canis*, are given in Table 2. It can be seen that 9 reacted positively to the IFA test to titres of 1:10–1:160. With the exception of 2 of these dogs in which small numbers of the morular stage of *E. canis* were detected in monocytes, the blood smears of these 13 dogs at the time when the 2nd serum sample was taken were negative.

TABLE 2 IFA test reactions of 13 dogs initially serologically negative for *E. canis* but positive for *B. canis* and *E. canis* on blood smears

Dog No.	Initial Giemsa smears*		IFA test titres of 2nd serum sample	Interval†
	B. canis	*E. canis*		
1	(2)	(5)	1:20	15
2	(1)	(5)	1:20	6
3	(2)	(6)	−ive	31
4	(1)	(6)	1:160	14
5	(2)	(6)	−ive	20
6	(2)	(4)	1:160	19
7	(1)	(6)	1:10	21
8	(3)	(6)	1:10	21
9	(1)	(5)	−ive	43
10	(2)	(4)	−ive	20
11	(1)	(6)	1:20	14
12	(1)	(6)	1:10	13
13	(1)	(6)	1:80	12

* (1) 5–30 % of red blood cells parasitized with *B. canis*
(2) 0,2–5 % of red blood cells parasitized with *B. canis*
(3) 0,2 % of red blood cells parasitized with *B. canis*
(4) 2–5 % of monocytes parasitized by *E. canis*
(5) 0,5–2 % of monocytes parasitized by *E. canis*
(6) 0,5 % of monocytes parasitized by *E. canis*
† Interval in days between initial and subsequent serum sampling

In the case of the 37 dogs with mixed infections, moderate to large numbers of red blood cells, parasitized with *B. canis*, were recorded in 32 of the dogs, and in only 5 of them were fewer than one per 500 cells infected (Table 3). Significantly fewer inclusions of *E. canis* were observed in 36 of these 37 dogs, only 1–4 out of 200 monocytes being parasitized in the great majority of the dogs.

TABLE 3 *B. canis* and *E. canis* infection rates of 37 dogs with mixed infections

	% red blood cells parasitized	No. of dogs
B. canis	5–30	20
	0,2–5	12
	≤0,2	5
	% monocytes parasitized	
E. canis	2–5	8
	0,5–2	24
	≤0,5	5

Different morphological forms, suspicious for *E. canis*, were demonstrable in monocytes. Some of these forms (Fig. 1, 2, 3 & 5), suspected of being different developmental stages of the parasite, differed from the typical morulae. Basophiliç, homogeneous, dense, round forms in the cytoplasm of monocytes (Fig. 1 & 2), which appeared to become less homogeneous, show some internal structure (Fig. 3 & 4), eventually loose their integrity and become granular (Fig. 5 & 6). Distinct morulae, consisting of clearly distinguishable individual organisms (Fig. 6 & 7, arrowhead), were rarely seen. If the diagnosis had been based solely on the observation of typical morulae, significantly fewer positive smears would have been recorded.

A characteristic feature, observed in a great number of the dogs with mixed infections, was the large numbers of monocytes at the feather end of the peripheral blood smear. These cells were irregularly shaped and large, with voluminous cytoplasm containing numerous vacuoles (Fig. 1–7). These were the cells that were not only parasitized with *E. canis*, but many of them contained phagocytosed *Babesia* parasites.

Discussion

The first important finding in this study was that antibodies to *E. canis* cross-react with the heartwater agent, harboured by peritoneal macrophages of mice infected with *C. ruminantium*. It confirms an earlier report on a study in which *C. ruminantium*-infected neutrophils were used as antigen in the IFA test (Logan *et al.*, 1986). Similar cross-reactions between the heartwater agent and *Ehrlichia equi* (Holland *et al.*, 1987), *Ehrlichia ovina, Cytoecetes phagocytophila* and *Ehrlichia bovis* (Du Plessis *et al.*, 1987) have been reported. This study therefore confirms an earlier observation that certain *Ehrlichia* spp. share common antigens with *C. ruminantium* (Du Plessis *et al.*, 1987; Holland *et al.*, 1987).

Although antibodies were detected in the sera of only 21 out of 46 dogs infected with *E. canis*, either as a single infection or concurrently with *B. canis*, based on suspicious blood smears combined with serology, the former was demonstrable in the monocytes of 14 of the 21 serologically positive dogs. This was further proof that the antibodies detected with the IFA test had developed in response to *E. canis*.

The sero-conversion from negative at a serum dilution as low as 1:10 to high titres in 4 experimentally infected control dogs was further confirmation of the sensitivity of the test.

The IFA titres recorded in the natural cases, particularly those of the dogs with a single *E. canis* infection and presumed to be sub-acute to chronic cases, were comparable with those recorded in an IFA test in which monocyte cultures infected with *E. canis* was used as antigen (Ristic *et al.*, 1972). The titres recorded in the experimentally infected control dogs, however, were significantly higher than in the natural cases, probably because antibody levels were at or near a peak when the post-infection serum sample was collected 29–32 days after infection.

The other 25 dogs with a mixed infection were initially serologically negative for ehrlichiosis, but a 2nd serum sample of 9 out of 13 of these dogs reacted positively in the IFA test. The low titres recorded and the absence of levels of antibody detectable with the IFA test in the other 4 can in all probability be ascribed to the fact that these animals had been treated. The seropositive cases nevertheless suggest that the *E. canis* component of the concurrent infection in these 25 dogs represented acute, primary infections and that the 1st serum sample had been collected before the development of antibody levels detectable with the IFA test.

The question arises whether these dogs with concurrent babesiosis and ehrlichiosis had been infected simultaneously with the 2 parasites or whether a primary infection of either of them was followed by a relapse of a sub-clinical latent infection of the other. The initial absence of antibodies to *E. canis* and the subsequent sero-conversion of 69 % of the dogs from which a follow-up serum sample was tested, in conjunction with outspoken monocytosis and *E. canis* inclusions suggestive of acute ehrlichiosis, indicates that these dogs had been sub-clinically infected with *B. canis* and were then newly infected with *E. canis*.

The assumption that the presence of *B. canis,* in many cases in large numbers, in the blood smears of these initially seronegative 25 dogs with mixed infections represented a precipitation of clinical babesiosis, is consistent with the finding that *B. canis* carrier dogs, experimentally infected with *E. canis,* developed clinical signs and haematological evidence of babesiosis, accompanied by severe parasitaemia with *B. canis* (Van Heerden *et al.,* 1983). The question arises whether it was necessary to treat cases like these with both a babesiacidal drug and a tetracycline, which is the usual procedure in mixed infections diagnosed for the 1st time. The danger of interference with the immunity against *B. canis* (Stewart, 1983) and the potentially harmful effects of drugs, such as phenamidine isethionate, if a babesiacidal drug is administered for a 2nd time (Naudé, Basson & Pienaar, 1970), have to be borne in mind. The *B. canis* infections were so severe in many of the cases in the present study that treatment with a tetracycline alone would have been dangerous.

The possibility that an acute *E. canis* infection may have an immunosuppresive effect on the immunity of dogs chronically infected with *B. canis* must be given serious consideration. This phenomenon has been reported (Nesbit, 1983; Nyindo, Huxsoll, Ristic, Kakoma, Brown, Carson & Stephenson, 1980) and is consistent with the possibility in the present study on mixed infections that the clinical manifestation of a chronic *B. canis* infection can be attributed to acute ehrlichiosis.

In the present study, the sero-conversion of some cases of suspected mixed infections, which were detected with the IFA test, suggested that monocytic inclusions other than typical morulae could possibly be developmental stages of *E. canis*. Developmental forms apart from morulae are by no means unprecedented in the literature. In the very first report on ehrlichiosis in the dog, Donatien & Lestoquard (1938) described monocytes containing dark-staining homogeneous bodies, 3–5 µm in diameter (initial bodies), others with smaller inclusions apparently resulting from fragmentation of the former, and still others with granular bodies (elementary bodies) in the form of morulae. Much later, similar inclusions were described in the monocytes of cattle infected with *E. bovis* (Rioche, 1966).

The test would also seem to be of value in the diagnosis of subacute and chronic cases of ehrlichiosis, where parasites are not demonstrable in a blood smear. The IFA test, using *C. ruminantium* as cross-reacting antigen for *E. canis*, may therefore prove to be a valuable addition to the criteria on which a diagnosis of canine ehrlichiosis should be based: anamnesis, clinical signs, haematological investigation, serum protein electrophoresis and a peripheral blood smear examination (Van Heerden *et al.*, 1983).

The sensitivity of the IFA test used in this investigation should be compared with that of the IFA test in which cultures of *E. canis* serve as antigen (Ristic *et al.*, 1972). If it compares favourably, the use of *C. ruminantium*-infected mouse peritoneal cells should be considered as an alternative source of antigen, particularly in view of the fact that the maintenance of the *E. canis* cultures is difficult.

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