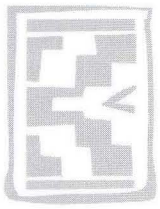

In vitro isolation of equine piroplasms derived from Cape Mountain zebra (*Equus zebra zebra*) in South Africa

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ABSTRACT

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Twenty blood samples of zebras (*Equus zebra zebra*) from the Karoo National Park and the Bontebok National Park in South Africa, all seropositive for *Theileria equi*, were subjected to *in vitro* culture to identify carrier animals and to isolate the parasites. Sixteen animals had a detectable parasitaemia in Giemsa-stained blood smears examined before culture initiation, the remaining four animals were identified as *T. equi* carriers by *in vitro* culture. Cultures were initiated either in an oxygen-reduced gas mixture or in a 5% CO_2-in-air atmosphere. Out of the 20 blood samples, 12 cultures of *T. equi* and two cultures of *T. equi* mixed with *Babesia caballi* were established. None of the four animals seropositive for *B. caballi* could be identified as carrier animals, whereas two seronegative samples became culture-positive for *B. caballi*.

Keywords: *Babesia caballi, Babesia equi,* Cape Mountain zebra, equine piroplasmosis, *Equus zebra zebra*, *in vitro* cultivation, *Theileria equi*

INTRODUCTION

Equine piroplasmosis is caused by two intraerythrocytic protozoa parasites, *Babesia caballi* and *Theileria equi*. The latter, previously known as *Babesia equi*, was recently reclassified (Mehlhorn & Schein 1998) due to the fact that it multiplies in lymphoid cells before invading erythrocytes (Schein, Rehbein, Voigt & Zweygarth 1981). Both parasites are tick-transmitted and infect horses, mules, donkeys as well as zebras.

Soon after *T. equi* was described in horses (Laveran 1901) it was also described in an east African zebra (species unnamed)(Kudicke cited by Koch 1905). Inoculation of zebra blood into a susceptible horse caused high fever and the appearance of "*Piroplasma equi*", a synonym of *T. equi,* in the recipient horse. These findings led to the conclusion that zebras are carriers of *T. equi*, as are horses, donkeys and their crosses (Government Veterinary Bacteriologist 1905–1906). Subsequently, small piroplasms, presumably *T. equi*, were found in the blood of zebras (*Equus quagga wahlbergi* Pocock) (Neitz 1931). Young, Zumpt, Boomker, Penzhorn & Erasmus (1973) described *T. equi* in another zebra subspecies, the Cape Mountain zebra (*Equus zebra zebra*). In contrast to *T. equi*, *B. caballi* was only described in zebras more than 50 years later and citations in the literature are very rare. To our knowledge, Van Niekerk (1962) may have been the first to be mentioned as having found *B. caballi* in

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zebras (cited by Neitz 1965). On the basis of morphological characteristics Salabarria, Godshaev, Ferrer, Jimenez, Villalba & Jorge (1981) reported having found *B. caballi* in zebras exported from South Africa into Cuba.

The aim of this study was to demonstrate and to isolate piroplasms from zebras using *in vitro* culture techniques.

MATERIALS AND METHODS

Blood samples

Blood and serum samples from 20 Cape Mountain zebras (*Equus zebra zebra)* were collected by venipuncture into sterile vacuum tubes (Vacutainer®, Becton Dickinson, Meylan, France) in the Karoo National Park (six samples in January 2000) and the Bontebok National Park (14 samples in March 2000). They were brought to the Onderstepoort Veterinary Institute (OVI) on ice in a polystyrene container. The blood samples from the Karoo National Park were severely haemolysed when processed. Upon arrival, all samples were processed as outlined below. Thin blood smears were prepared from all of them, and were stained with Giemsa stain and examined microscopically. Serum was prepared and examined by the indirect fluorescent antibody (IFA) test for anti-*T. equi* and anti-*B. caballi* antibodies. This test was performed as described for *B. caballi* by Madden & Holbrook (1968). A titre of 1:80 was considered to be positive.

Culture medium

A modified HL-1 medium (BioWhittaker, Walkersville, MD, USA) as described recently for the culture initiation of *T. equi* (*Babesia equi*) (Zweygarth, Just, De Waal & Lopez-Rebollar 1999), was used. It was supplemented with 20% horse serum, 2 mM L-glutamine, 0.2 mM hypoxanthine, 1 mM L-cysteine hydrochloride, 0.02 mM 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulphonic acid disodium salt (bathocuproine sulphonate, BCS; Serva Feinbiochemica, Heidelberg, Germany), 100 IU/mℓ penicillin and 100 mg/mℓ streptomycin. The medium was buffered with 15 mM HEPES and 2.2 gℓ $NaHCO_3$.

In vitro culture

To initiate cultures, the red blood cells (RBC) were washed four times by centrifugation (650 x *g* for 10 min at room temperature) and resuspension in a modified Vega y Martinez phosphate-buffered saline solution (Vega, Buening, Green & Carson 1985) omitting adenine, guanosine and the antibiotics (mVYM). After each wash, the white blood cell layer overlaying the RBC was removed. After the fourth and final wash, the RBC were resuspended in the culture medium at a concentration of 10 % (v/v). Aliquots of 1 mℓ were distributed into wells of 24-well culture plates (Corning, Bibby Sterilin, Staffordshire, England). Two different culture conditions were then applied: the plates were incubated at 37 °C, one in a humidified 5% CO_2-in-air atmosphere (Zweygarth *et al.* 1999), the other in a humidified gas mixture of 5 % CO_2, 2 % O_2 and 93 % N_2 (Zweygarth, Just & De Waal 1995). The culture medium was changed daily by replacing 700 µℓ medium overlaying the erythrocytes in each well. Every 4 days, 25 µℓ of uninfected horse RBC suspension was added to each well. For this purpose, blood from an uninfected donor horse, kept under tick-free conditions at the OVI, was collected and processed as described above. After the fourth wash, the horse RBC were suspended in mVYM solution and stored at 4 °C until used. Cultures were discontinued after 15 days for those samples in which no parasites were found in a Giemsa-stained thin blood smear, i.e. when no parasite was detected in approximately 20 000 RBC. Cultures were first examined on day 3 after culture initiation, and then at least every other day.

RESULTS

Before culture initiation, *T. equi* parasites were demonstrated in Giemsa-stained thin blood smears in 16 out of the 20 zebras. Of the cultures initiated with blood from the four zebras in which no parasites had been demonstrated before culture initiation, all became positive. In four zebras (4, 13, 18 and 19) parasites were not detected beyond the examination period of at least 15 days although parasites had been demonstrated before culture initiation. Two cultures became contaminated (2 and 5) and were terminated prematurely.

All of the zebra serum samples were serologically positive for *T. equi*, whereas antibodies against *B. caballi* were demonstrated only in zebras 1, 9, 15 and 20. No *B. caballi* parasites, however, were demonstrated in any of the samples serologically positive for *B. caballi.* In contrast, the blood of zebra 3, serologically negative for *B. caballi*, proved to be positive for both piroplasma species in culture. The results using an oxygen-reduced atmosphere for culture initiation are summarised in Table 1.

TABLE 1 Culture initiation of blood samples[1] obtained from zebras (*Equus zebra zebra*) from the Karoo National Park and the Bontebok National Park

Animal no.	Smear before culture initiation	IFA[2]		Culture results
		T. equi	*B. caballi*	
1	–	640	160	Te
2	Te	640	–	C(Te)
3	–	640	–	Te + Bc
4	Te	640	–	Te^{L14}
5	Te	640	–	C(Te)
6	Te	320	–	Te
7	–	640	–	Te
8	Te	320	–	Te
9	Te	640	80	Te
10	Te	320	–	Te
11	–	640	–	Te
12	Te	320	–	Te
13	Te	320	–	Te ^{L3}
14	Te	640	–	Te
15	Te	320	160	Te
16	Te	320	–	Te
17	Te	320	–	Te
18	Te	320	–	Te^{L3}
19	Te	160	–	–
20	Te	320	160	Te

[1] Samples 1–6 were derived from the Karoo National Park; samples 7–20 from the Bontebok National Park

[2] Reciprocal titres obtained by the indirect fluorescent antibody test

Te *T. equi* parasites were detected in Giemsa-stained smears prepared from culture material

Bc *B. caballi* parasites were detected in Giemsa-stained smears prepared from culture material

C Culture terminated due to bacterial contamination

L Parasites were positively identified in smears prepared from culture material but were no longer detectable on the day indicated

In cultures initiated using a 5 % CO_2-in-air atmosphere, *T. equi* grew beyond day 15 in only four samples (1, 6, 9 and 14). However, *B. caballi* was identified in sample 6, which was serologically negative.

DISCUSSION

Of the 20 blood samples drawn from Cape Mountain zebra from two different National Parks in South Africa, 16 tested positive for *T. equi* in Giemsa-stained blood smears. The remaining four samples were identified as positive by *in vitro* culture. In an investigation into diseases of zebras in East Africa (*Equus grevyi* and *Equus burchelli*), Dennig (1966) found that 108 of 121 blood smears were positive for *T. equi*. He assumed that 100 % of the zebra population may have been infected but not all could be identified due to the poor quality of some blood smears. De Waal (1995) demonstrated that 61 % of the blood smears examined from zebras were positive for *T. equi,* whereas in blood smears prepared from horses, parasites could only be detected in a few (~ 2 %). Similar low detection rates were found in 26 blood samples from carrier horses, only four being positive in thin Giemsa-stained blood smears (Zweygarth *et al.* 1999). On the other hand, Holman, Becu, Bakos, Polledo, Cruz & Wagner (1998) could not detect parasites in any of 23 horse blood samples, but *T. equi* was subsequently observed after culture in all of them.

In the case of the zebras serologically positive for both *B. caballi* and *T. equi*, all four samples gave positive culture results for *T. equi* only. *Babesia*

caballi was not detected in any of these samples. However, two *B. caballi*-seronegative zebras were identified by culture as carriers for this parasite. The reason for the low *in vitro* recovery rate of *B. caballi* remains speculative: false positive serological reactions, clearance of *B. caballi* parasites from the animals' circulation or other unknown factors related to the sampling, the transportation, the storage before cultivation or the culture system itself.

The comparison of the effects of an oxygen-reduced atmosphere and a 5 % CO_2-in-air atmosphere on culture initiation of *T. equi* from zebra blood showed that only the reduced oxygen system gave satisfactory results. This is in contrast with results of a recent survey of 43 horses of which 41 horses were identified as *T. equi* carriers using the technique applying a normal oxygen tension (Zweygarth, unpublished results 2001). Why these results could not be repeated with piroplasms derived from zebra blood is not clear. Possible reasons may be related to the host cells, the parasites or both. *Babesia caballi* was found in one culture sample each initiated in an oxygen-reduced atmosphere and in a 5 % CO_2-in-air atmosphere.

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