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In vitro cultivation of *Babesia equi*: detection of carrier animals and isolation of parasites

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ABSTRACT

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By means of an *in vitro* culture technique, 75 samples of horse blood were examined for *Babesia equi*, a causative agent of equine piroplasmosis. At the time of culture initiation, 15 samples were microscopically positive for *B. equi*, and this was subsequently confirmed by culture diagnosis. Sixty samples showed no parasites in Giemsa-stained thin blood smears. However, after the culturing process, parasites were found in blood smears of 36 of these samples. The sensitivity of the *in vitro* culture method was such that 2,5 µℓ (1/40 of the usual volume used for the above-mentioned samples) of packed erythrocytes obtained from a carrier horse still yielded positive results after cultivation Cultures were initiated from blood samples stored for up to 120 h at 8°C in vacuum tubes containing EDTA as anticoagulant. These results show that the *in vitro* culture method is highly sensitive. It can be used to identify *B. equi* carrier horses, to evaluate the effects of chemotherapeutic intervention, and to isolate field strains of *B. equi* for further characterization.

Keywords: *Babesia equi*, *in vitro* cultivation, carrier animals, parasite isolation

INTRODUCTION

Babesia equi is a tick-transmitted protozoan parasite and one of the causative organisms of equine piroplasmosis. This disease is characterized by signs of malaise, inappetence, fever, haemolytic anaemia and haemoglobinuria. After the animal has recovered from an acute attack of the disease, it becomes a carrier of the parasite (Hourrighan & Knowles 1979). Carrier status can be lifelong, and is responsible for much suffering in the carrier animal. In addition, *in utero* infection of the foetus may lead to abortion (Marlow & Bester 1994). In a recent survey of thoroughbred mares in South Africa, it was found that 11% of abortions were due to *B. equi* (J.A.W. Coetzer & D. T. De Waal, unpublished observation 1990).

Equine babesiosis caused by *B. equi* is widespread in South Africa (De Waal 1995), and interferes with the exportation of horses to disease-free countries such as the United States, Australia and Japan. Most serological tests used to detect *B. equi* antibodies are unsatisfactory as they often give false positive or false negative results (Tenter & Friedhoff 1986; Böse, Jorgensen, Dalgliesh, Friedhoff & De Vos 1995). There is, therefore, an urgent need to improve tests for the diagnosis of equine babesiosis caused by *B. equi*. *In vitro* techniques, such as those described recently by Holman, Chieves, Frerichs, Olsen & Wagner (1994) and Zweygarth, Just & De Waal (1995), could contribute to the identification of carrier animals and supplement other methods of parasite detection, such as microscopy, serology, or the use of PCR followed by DNA probing.

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MATERIAL AND METHODS

In vitro culture of parasites

The medium used, referred to as the complete medium, consisted of medium 199 (with Hanks' salts) with 40% horse serum, buffered with 10 mM TAPSO

(3-[N-tris-(hydroxymethyl) methyl-amino]-2-hydroxy-propanesulfonic acid, Sigma, St. Louis, Missouri) (Goff & Yunker 1988), and supplemented with 2 mM L-glutamine, 0,2 mM hypoxanthine, 100 IU/mℓ penicillin, and 100 µg/mℓ streptomycin (Zweygarth *et al.* 1995).

To initiate cultures, blood samples from the horses to be examined were collected by venipuncture into sterile vacuum tubes containing EDTA as anticoagulant (Vac-u-test^{R}, Radem Laboratory Supplies, Sandton, South Africa). The blood cells were washed four times by centrifugation (650 x *g*; 10 min; room temperature) and resuspended in a modified Vega y Martinez phosphate-buffered saline solution (Vega, Buening, Green & Carson 1985) without adenine, guanosine, and antibiotics (mVYM). After the first wash, the white-blood cells were removed from the interface of the plasma and horse red-blood cells (HRBC). After the fourth wash, the HRBC were suspended in complete medium at a concentration of 10% and aliquots of 1 mℓ were distributed into a 24-well culture plate (Corning, Bibby Sterlin, Staffordshire, England). Plates were incubated at 37°C in a humidified gas mixture of 5% carbon dioxide, 2% oxygen, and 93% nitrogen. The medium was changed daily by replacing 700 µℓ of the medium overlaying the erythrocytes in each well with fresh medium. Every 4 d, 25 µℓ of uninfected HRBC suspension were added to each well. The washing procedure for these HRBC, taken from an uninfected donor horse, was the same as that for parasitized erythrocytes. Cultures were discontinued after 15 d if no parasites were found in a thin blood smear, i.e., when no parasite was detected in 20 000 HRBC. When the percentage of parasitized erythrocytes (PPE) reached approximately 1%, the plates were transferred to a humidified atmosphere containing 5% carbon dioxide in air at 37°C.

For subcultures, erythrocytes in the culture wells were resuspended and 0,5 mℓ or 0,25 mℓ were transferred into two and four new wells, respectively. To each of these wells, a 10% suspension of uninfected HRBC in complete culture medium was added to make up a final volume of 1 mℓ.

Validation of the culture system was performed with blood obtained from a known carrier horse (212/5) infected with the OP stock of *B. equi* (De Waal, Van Heerden, Van den Berg, Stegmann & Potgieter 1988) as described below. This horse and the uninfected erythrocyte donor horse were kept under tick-free conditions at the Onderstepoort Veterinary Institute (OVI).

All blood samples used in these experiments were examined microscopically as a thin blood smear stained with Giemsa stain before the initiation of cultures.

Diagnostic sensitivity of the *in vitro* culture method

To evaluate the diagnostic sensitivity of the *in vitro* culture method, blood from a carrier horse (212/5) was mixed with various dilutions of uninfected HRBC before initiation of cultures. Small volumes of infected erythrocytes were used as follows: 0,5, 1,0, 2,5, 5,0, and 10,0 µℓ of packed infected HRBC were mixed with uninfected HRBC to give a final volume of 100 µℓ, which was then used to initiate fresh cultures as described above. As a positive control, 100 µℓ of infected blood was used. Each of these experiments was repeated twice.

Influence of storage conditions on the survival of parasites

To test the influence of storage conditions on unprocessed EDTA blood, samples were collected from horse 212/5 in eight vacuum tubes containing EDTA as anti-coagulant. One tube was used immediately to initiate cultures. Five tubes were stored at 8°C for 24, 48, 72, 96, and 120 h, respectively, and two tubes were left at room temperature (22°C) for 24 and 48 h, respectively. The stored samples were then processed as described above and the blood transferred in a 24-well culture plate. Each of these experiments was repeated twice.

Examination of field samples

A total of 75 blood samples were collected from horses at various localities from four different provinces of South Africa [Bon Accord, Kaalplaas and Onderstepoort (Gauteng); Krugersdorp and Vryburg (North-West Province); Tarkastad (Eastern Cape); Gillitts (Natal)]. Blood samples collected at Bon Accord and Kaalplaas were put on ice and brought immediately to the laboratory for culture examination. Blood samples collected at the remaining locations were sent to the OVI, overnight, in a polystyrene container with an ice block. Upon arrival, samples were processed without delay, as outlined above. In addition, blood from a carrier horse artificially infected with the USDA strain of *B. equi* (Frerichs, Holbrook & Johnson 1969) was included in these experiments.

Culture examination of blood from foals born to carrier mares

Blood was collected at various times from dams and their foals at the Kaalplaas farm, OVI. At the time of the first culture examination, the age of the foals ranged from 1–41 d. Samples were processed for culture examination and for serological tests. Three sets of blood samples, from three mares and their respective foals, were also received from the Veterinary Faculty, University of Pretoria, South Africa.

Detection of parasites after chemotherapy

In this part of the study, four horses were involved. Blood was collected from three horses previously treated (respectively 25, 43 and 50 d) by a private veterinarian for a patent *B. equi* infection. Each of these three animals received imidocarb dipropionate (Forray 65, Coopers Animal Health, Kempton Park, South Africa) as a single intramuscular injection at 2,4 mg/kg of body mass. Cultures were initiated as described above. Blood from horse 4 was successfully used to initiate a culture before treatment with imidocarb dipropionate. Then this animal was treated at 3-d intervals with two injections (2,4 mg/kg body mas) of imidocarb dipropionate, and re-examined after 8 weeks.

Estimation of parasite growth

Culture samples were taken, smeared on microscope slides, Giemsa-stained, and evaluated microscopically.

Preparation of stabilates

Parasite cultures were cryopreserved as previously described (Zweygarth *et al.* 1995), with minor modifications. Erythrocytes from cultures were centrifuged at room temperature instead of at 4°C, and the samples were finally stored at –196°C instead of at –74°C.

Indirect fluorescent antibody test (IFAT)

The IFAT was performed as described for *B. caballi* by Madden & Holbrook (1968).

RESULTS

Validation of culture initiation from a known *B. equi* carrier

Babesia equi parasites were successfully propagated on seven occasions, from blood taken from horse 212/5. In two samples, *B. equi* parasites were seen in blood smears before the cultures were initiated. In these cases, parasites were usually also found the following day, in smears made from the culture material. In the other experiments, in which parasites had not been seen in blood smears when the cultures were initiated, the parasites appeared in smears made from culture material 3–5 d later.

Sensitivity of the test

When suspensions of uninfected HRBC were mixed with infected HRBC, the smallest volume of packed infected cells giving a positive culture diagnosis, was 2,5 µℓ With lower volumes—0,5 and 1,0 µℓ—the cultures remained negative for 14 d. Growth responses observed in culture were dependent on the size of the inoculum: the larger the volume of infected HRBC was, the sooner parasites were seen. The results of a representative experiment are summarized in Table 1.

Influence of storage conditions on the survival of parasites

When unprocessed blood samples containing EDTA as anticoagulant were stored in a refrigerator (8°C), there was progressive haemolysis of the blood. Nevertheless, *B. equi* was successfully diagnosed in all cultures initiated from these samples, the parasites first being seen between days 3 and 10 after initiation. When samples were stored at room temperature (22°C) for 24 and 48 h, the detection periods were 6 and 10 d, respectively. The results of a representative experiment are summarized in Table 2.

Examination of field samples

Out of 75 blood samples examined, 15 were microscopically positive for *B. equi* at the time of culture initiation. Sixty samples initially showed no parasites in Giemsa-stained thin blood smears. After the culturing proces, parasites were found in smears of

TABLE 1 Influence of the inoculum volume of packed, infected erythrocyte suspension on the culture diagnosis of *B. equi*

Volume infected erythrocyte suspension (µℓ)	Volume uninfected erythrocyte suspension (µℓ)	Parasites first detected in culture (d)
2,5	97,5	12
5,0	95,0	7
10,0	90,0	6
100,0	–	4

TABLE 2 Influence of storage conditions of unprocessed EDTA blood samples on culture diagnosis of *B. equi*

Temperature (°C)	Period of storage (h)	Parasites first detected in culture (d)
–	0	3*
8	24	3
8	48	8
8	72	10
8	96	8
8	120	8
22	24	6
22	48	10

* Sample was processed immediately after blood collection

culture-derived erythrocytes initiated from 36 of the samples, whereas cultures initiated from the remaining 24 samples, remained negative. The time span for the culture diagnosis of *B. equi* varied In 32 cases (88,8%), the time span for a positive diagnosis was 6 d or less. One of the 36 microscopically negative samples became positive on day 12. The time span required to diagnose *B. equi* is shown in Table 3. Most cultures were expanded, and the parasite suspensions cryopreserved for further studies, before the experiment was terminated.

Culture examination of foals born to carrier dams

Blood samples were examined from ten dams and their foals (Table 4). All the dams were identified as *B. equi* carriers by *in vitro* diagnosis, but only three of the cultures initiated with foal blood became positive (157, 158 and 296). Among the foals which were negative when first examined, four (295, 297, 301 and 304) were observed for a total of 146 d, but all cultures remained negative. Anti-*B. equi* IFAT levels

TABLE 3 Time span for culture diagnosis of 36 positive *B. equi* blood samples from field infections

Days in culture	No. of positive samples	Cumulative % of positive samples
1	4	11,1
2	7	30,6
3	1	33,3
4	10	61,1
5	6	77,8
6	4	88,9
7	1	91,7
8	1	94,4
11	1	97,2
12	1	100,0

TABLE 4 Serological and culture results for *B. equi* in blood samples from dams and their foals

Animal numbers		IFAT*		Culture diagnosis		Age of foals on first examination (d)
Dam	Foal	Dam	Foal	Dam	Foal	
M	MF	nd	nd	+	–	1
V27	V27F	nd	nd	+	–	1
V81	V81F	nd	nd	+	–	1
9	304	1280	320	+	–	1
38	158	1280	640	+	+	41
168	301	1280	320	+	–	8
309	297	1280	160	+	–	28
379	296	1280	320	+	+	28
498	295	1280	640	+	–	33
500	157	640	320	+	+	40

* Reciprocal values of the titres determined with the immunofluorescent antibody test
nd Test was not done
\+ Parasites were detected in Giemsa-stained smears prepared from cultured erythrocytes
– No parasites were detected in Giemsa-stained smears prepared from cultured erythrocytes

TABLE 5 Course of antibody titres to *B. equi* in foals as determined by the immunofluorescent antibody test

Culture diagnosis	Animal number	Age of foals (d)	Reciprocal titre values				
			0	12	55	97	146 (d*)
Positive	157	40	320	nd	nd	1280	nd
	158	41	640	nd	nd	1280	nd
	296	28	320	nd	nd	1280	nd
Negative	295	33	640	nd	320	80	40
	297	28	160	nd	40	0	0
	301	8	320	nd	0	0	0
	304	1	320	640	320	80	0

* Data given here refer to the date of blood sampling, not to the age of the foals, i.e. when the first blood sample was taken, the age of the foals was as given under that column
nd Test was not done

in these foals are shown in Table 5. It can be seen that the titres dropped in three of the cases. In the case of foal 304, however, the antibody titre initially rose from 1:320 when it was 1 d old, to 1:640 12 d later. Subsequently, titres declined to 1:320, 1:80 and 1:40 on days 55, 97, and 146, respectively.

Detection of parasites after chemotherapy

Parasites were cultured from each of three animals treated only once with imidocarb dipropionate. However, blood obtained from horse 4, which received two injections, was negative 8 weeks after treatment.

DISCUSSION

A culture system recently developed by Zweygarth *et al.* (1995) was tested for its suitability for identifying *B. equi* carrier animals and for isolating strains of this parasite from various localities in South Africa. The present study shows that cultures from the blood of carrier horses can be reliably established if the samples are processed relatively soon after collection.

In order to evaluate the sensitivity of the culture system, the concentration of infected blood to initiate a culture, was reduced to 10, 5, and 2,5% of the total packed erythrocyte suspension. An approximate PPE could not be determined before culture initiation, as no parasites could be seen in blood smears. Nevertheless, after varying intervals, all the cultures became positive for *B. equi*. With one exception, these intervals correlated with the volume of infected blood used to initiate the culture. The smaller the volume of infected blood was, the longer the period before parasites could be detected in smears prepared from the culture material. If one judges by the range of volumes of infected blood used, it is evident that, under laboratory conditions, a volume 40 times smaller than that used in the routine *in vitro* diagnostic procedure, is sufficient to initiate cultures.

It is frequently impossible to process blood immediately after it has been collected, especially in the case of field samples. The influence of storage conditions on unprocessed blood samples for culture diagnosis, was therefore examined. It was shown, firstly, that it is better to store the blood samples at 8°C than at 22°C and, secondly, that the sooner the culture is initiated, the earlier parasites can be detected in the culture. It is therefore recommended that samples sent for culture diagnosis should be stored in a polystyrene container with an ice block, and should reach the laboratory within 24 h of collection.

The sensitivity of the test, and its suitability for isolating field strains of *B. equi*, were confirmed when a total of 75 blood samples were examined. All 15 samples that were positive in a thin blood smear, gave rise to a continuous culture from which stabilates were prepared. Of the 60 samples that were negative microscopically, 36 proved positive in the culture test. Furthermore, 88,8% of the positive cases were diagnosed within 6 d of the blood being cultured. Similar culture confirmation of the carrier status of *B. caballi*-infected horses was described by Holman, Frerichs, Chieves & Wagner (1993), who identified four *B. caballi* carrier horses among nine previously infected horses.

All ten dams examined were identified by the *in vitro* culture technique as being *B. equi* carriers, but only three infected foals were identified. The serological results in dams correlated well with those of the *in vitro* culture technique. All the dams examined showed high titres against *B. equi* antigen, and the cultures prepared from their blood also became positive with *B. equi*. In foals, serology would not be useful for diagnosis as it would not differentiate between colostral (maternal) antibodies and antibodies induced by a natural infection. However, when antibody titres were examined on a continuous basis, a titre increase was found only in the foals proved positive for *B. equi* infection by culture. Likewise, negative culture results correlated with a decrease in titre. Similar results were found by Donnelly, Phipps & Watkins (1982) when they detected complement-fixing antibodies in some English-born foals of Portuguese-born mares that were seropositive for *B. equi*. In their study, the titres in the mares remained constant over a 3-month observation period, whereas titres declined steadily in the foals. In our study, no cultures could be established from any of the four newborn foals, but cultures were established from three older foals which could have been in contact with infected ticks. Although the numbers are not statistically significant, this seems to support the hypothesis that foals of infected mothers are born uninfected, and become infected only later in life. It is speculated that if foals become infected *in utero*, they are either aborted or they die shortly after birth.

The culture technique demonstrated that parasites were again in circulation 25 d after treatment with a single dose of imidocarb dipropionate. Two injections of imidocarb dipropionate seemed to be more effective than a single administration. For example, horse 4 was still negative 8 weeks after its second dose, though ideally the animal should have been monitored for a longer period in order to reach a definitve conclusion. Control of clinical piroplasmosis caused by *B. equi* has been achieved with chemotherapy, whereas attempts at chemosterilization of infections have shown varying degrees of success (Frerichs, Allen & Holbrook 1973; Kuttler, Zaugg & Gipson 1987; Zaugg & Lane 1989). The reasons for the unsuccessful sterilization of *B. equi* infections have not been fully investigated.

In conclusion, it has been shown that culture diagnosis of *B. equi* is suitable both for the identification

of carrier horses and for the isolation of parasite strains. False positive results are unlikely, provided that the culture techniques are properly performed.

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REFERENCES

BÖSE, R., JORGENSEN, W.K., DALGLIESH, R.J., FRIEDHOFF, K.T. & DE VOS, A.J. 1995. Current state and future trends in the diagnosis of babesiosis. *Veterinary Parasitology,* 57:61–74.

DE WAAL, D.T., VAN HEERDEN, J., VAN DEN BERG, S.S., STEGMANN, G.F. & POTGIETER, F.T. 1988. Isolation of pure *Babesia equi* and *Babesia caballi* organisms in splenectomized horses from endemic areas in South Africa. *Onderstepoort Journal of Veterinary Research,* 55:33–35.

DE WAAL, D.T. 1995. Distribution, transmission, and serodiagnosis of *Babesia equi* and *Babesia caballi* in South Africa. Ph.D. thesis, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

DONNELLY, J., PHIPPS, L.P. & WATKINS, K.L. 1982. Evidence of maternal antibodies to *Babesia equi* and *B. caballi* in foals of seropositive mares. *Equine Veterinary Journal,* 14:126–128.

FRERICHS, W.M., HOLBROOK, A.A. & JOHNSON, A.J. 1969. Equine piroplasmosis: Production of antigens for the complement-fixation test. *American Journal of Veterinary Research,* 30:1337–1341.

FRERICHS, W.M., ALLEN, P.C. & HOLBROOK, A.A. 1973. Equine piroplasmosis (*Babesia equi*): Therapeutic trials of imidocarb dihydrochloride in horses and donkeys. *Veterinary Record,* 93:73–75.

GOFF, W.L. & YUNKER, C.E. 1988. Effects of pH, buffers and medium-storage on the growth of *Babesia bovis in vitro*. *International Journal of Parasitology,* 18:775–778.

HOLMAN, P.J., FRERICHS, W.M., CHIEVES, L. & WAGNER, G.G. 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. *Journal of Clinical Microbiology,* 31:698–701.

HOLMAN, P.J., CHIEVES, L., FRERICHS, W.M., OLSEN, D. & WAGNER, G.G. 1994. *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. *Journal of Parasitology,* 80:232–236.

HOURRIGHAN, J.L. & KNOWLES, R.C. 1979. Equine piroplasmosis. *American Association of Equine Practioners Newsletter,* 1:119–128.

KUTTLER, K.L., ZAUGG, J.L. & GIPSON, C.A. 1987. Imidocarb and parvaquone in the treatment of piroplasmosis (*Babesia equi*) in equids. *American Journal of Veterinary Research,* 48:1613–1616.

MADDEN, P.A. & HOLBROOK, A.A. 1968. Equine piroplasmosis: indirect fluorescent antibody test for *Babesia caballi*. *American Journal of Veterinary Research,* 29:117–123.

MARLOW, C.H.B. & BESTER, R.C. 1994. Infectious causes of equine reproductive failure, in *Infectious diseases of livestock with special reference to southern Africa*, edited by J.A.W. Coetzer, G.R. Thomson, & R.C. Tustin. Cape Town: Oxford University Press: 1554–1563.

TENTER, A.M. & FRIEDHOFF, K.T. 1986. Serodiagnosis of experimental and natural *B. equi* and *B. caballi* infections. *Veterinary Parasitology,* 20:49–61.

VEGA, C.A., BUENING, G.M., GREEN, T.J. & CARSON, C.A. 1985. *In vitro* cultivation of *Babesia bigemina*. *American Journal of Veterinary Research,* 46:416–420.

ZAUGG, J.L. & LANE, V.M. 1989. Evaluation of buparvaquone as a treatment for equine babesiosis (*Babesia equi*). *American Journal of Veterinary Research,* 50:782–785.

ZWEYGARTH, E., JUST, M.C. & DE WAAL, D.T. 1995. Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*. *Parasitology Research,* 81:355–358.