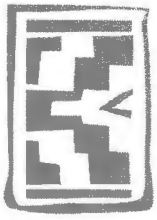

The occurrence of *Theileria* and *Cowdria* parasites in African buffalo *(Syncerus caffer)* and their associated *Amblyomma hebraeum* ticks

M.T.E.P. ALLSOPP[1], J. THERON[2], M.L. COETZEE[1], M.T. DUNSTERVILLE[1] and B.A. ALLSOPP[1]

ABSTRACT

ALLSOPP, M.T.E.P., THERON, J., COETZEE, M.L., DUNSTERVILLE, M.T. & ALLSOPP, B.A. 1999. The occurrence of *Theileria* and *Cowdria* parasites in African buffalo *(Syncerus caffer)* and their associated ticks. *Onderstepoort Journal of Veterinary Research*, 66:245–249

The polymerase chain reaction and oligonucleotide probing were used to detect *Theileria* and *Cowdria* species in DNA extracted from blood and ticks recovered from 24 African buffalo during a game-capture operation in the Kruger National Park, South Africa. Species-specific probing indicated that all but one of the buffalo were carrying at least one *Theileria* species. Indirect fluorescent antibody (IFA) serology indicated that all animals had been exposed to *Theileria parva* infection but only 33% were positive for *T. parva* by probing. Twelve (50%) of the animals but only six of the 214 adult *Amblyomma hebraeum* ticks examined (2,8%) were probe-positive for *Cowdria*. Only one *Cowdria* 16S genotype was detected in the animals and ticks.

Keywords: *Cowdria ruminantium*, oligonucleotide probes, polymerase chain reaction, *Theileria parva*

INTRODUCTION

The tick-borne diseases East Coast fever, corridor disease and heartwater are considered, along with tsetse-borne trypanosomosis, to be the most economically important vector-borne diseases of livestock in sub-Saharan Africa (Uilenberg 1981). East Coast fever (caused by the intra-cellular protozoan *Theileria parva parva*), corridor disease (caused by *T. parva lawrencei*) and heartwater (caused by the rickettsia *Cowdria ruminantium*) give rise to considerable economic losses to farmers in endemic areas. East Coast fever was eradicated from South Africa in 1954 (Anon.1 1981), but corridor disease remains a serious threat to cattle wherever domestic cattle come into contact with African buffalo (*Syncerus caffer*), the main natural reservoir of *T. p. lawrencei* in the presence of *Rhipicephalus appendiculatus* and *Rhipicephalus zambesiensis*. Parasites of the *T. parva* group are transmitted by *R. appendiculatus* and *R. zambesiensis* ticks in southern Africa and the areas in which these ticks occur are common to those in which *Amblyomma hebraeum*, a vector for both heartwater and *Theileria mutans*, is prevalent (Howell, Walker & Nevill 1978). Buffalo are known to be carriers of heartwater parasites and although they show no disease symptoms, they may represent a threat to domestic stock (Andrew & Norval 1989). It is therefore likely that, in areas where buffalo populations and both tick species occur, the buffalo may be carrying *Cowdria* and *Theileria* parasites.

Previous studies have shown that many buffalo carry a number of antigenically different *Theileria* parasites, piroplasms of which may be morphologically indistinguishable from the pathogenic *T. parva* group and which may be transmissible to cattle (Young, Brown, Burridge, Grootenhuis, Kanhai, Purnell & Stagg 1978; Conrad, Stagg, Grootenhuis, Irvin, Newson, Njamunggeh, Rossiter & Young 1987; Allsopp & Allsopp 1988; Conrad, Ole-moiyoi, Baldwin, Dolan,

[1] Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110 South Africa

[2] Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002 South Africa

Accepted for publication 23 July 1999—Editor

O'callghan, Njamunggeh, Grootenhuis, Stagg, Leitch & Young 1989; Allsopp, Baylis, Allsopp, Cavalier-Smith, Bishop, Carrington, Sohanpal & Spooner 1993). Since 1993 our laboratory has performed diagnostic testing, using the polymerase chain reaction (PCR; Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis & Erlich 1988) and oligonucleotide probing, on small numbers of buffalo which are being used in breeding programmes to generate disease-free animals. These tests have indicated that four *Theileria* species [*T. parva*, *T. mutans*, *T. buffeli* and *T.* sp. (buffalo)] are those most commonly encountered in South Africa (N.E. Collins & K. Mynhardt, unpublished data). A diagnostic survey using molecular tools and serology was carried out on 24 free-ranging buffalo and adult *A. hebraeum* ticks to investigate the prevalence of both *C. ruminantium* and *Theileria* species.

MATERIALS AND METHODS

Blood and serum samples from 24 randomly selected free-ranging adult buffalo were collected in Vac-u-test tubes during a game capture operation in the northern region of the Kruger National Park (KNP), South Africa in 1998. The blood and serum samples were stored frozen until processed. At the same time ticks were collected from each buffalo into separate tubes containing absolute ethanol. Tubes were stored at room temperature pending processing of the ticks. DNA was extracted from buffalo blood using the QIAamp tissue kit (Qiagen, Germany) as described (Allsopp, Visser, Du Plessis, Vogel & Allsopp 1997) except that samples were digested with RNAse A (final concentration 1 µg $\mu\ell^{-1}$, 1 h, room temperature) to eliminate any foot-and-mouth disease virus. All DNA samples were stored at –20 °C until required. DNA was extracted individually from ten ticks (where possible) from each buffalo using the QIAamp tissue kit. *A. hebraeum* adult ticks were chopped with a sterile scalpel blade before proteinase K digestion.

Aliquots containing 0,5–1,5 µg of purified blood DNA were subjected to polymerase chain reaction (PCR) amplification using primers 989 and 990 (Table 1). These primers target a part of the coding region (~1100 nucleotides) of *Theileria* parasite 18S rRNA genes (Allsopp *et al.* 1993). Aliquots of tick DNA containing 0,3–1,0 µg total nucleic acid were amplified similarly to check for the presence of *Theileria* DNA. Positive control samples included in each amplification experiment consisted of aliquots containing 10 µg of cloned *T. parva*, *T. buffeli*, *T. mutans* and *T.* sp (buffalo) full-length 18S rRNA genes in pGEM-T (K. Mynhardt, unpublished data). Buffalo and tick DNA samples were also amplified as previously described (Allsopp, Hattingh, Vogel & Allsopp 1998) using primers AB128 and AB129 (Table 1) which target a ~280 base pair region of the *Cowdria* pCS20 gene (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Barbet, Burridge & McGuire 1991; Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992). All experiments included a positive control comprising an aliquot of the complete coding region of the pCS20 gene cloned in pUC18. All PCR experiments included a negative (no DNA) control consisting of water. Amplicons were visualized by ethidium bromide staining and UV transillumination after electrophoresis on a 1 % agarose/TBE gel.

Amplicons were slot-blotted onto nylon membrane (Hybond N+, Amersham International) as described (Saiki, Bugawan, Horn, Mullis & Erlich 1986). *Theileria* 18S amplicons were probed with four different oligonucleotide probes (Table1) which were 3' end labelled using [α-^{32}P]-dATP (Anon.2 1991) and *Cowdria* pCS20 amplicons were probed with the full-length cloned pCS20 gene (Mahan *et al.* 1992) [α-^{32}P]-dATP labelled using the Megaprime kit (Amersham International) according to the manufacturer's instructions. Hybridization and stringency washing procedures were carried out using the conditions given in Table 1 and results were visualized by autoradiography.

DNA samples testing positive for *Cowdria* with the pCS20 probe were characterized further to ascertain the *Cowdria* 16S genotypes present (Allsopp *et al.* 1997; Allsopp, Hattingh, Vogel & Allsopp 1999).

T. parva-specific indirect fluorescence antibody (IFA) serology, using culture-derived *T. parva* schizont antigen (Burridge & Kimber 1972; Goddeeris, Katende, Irvin & Chumo 1982) was carried out on the serum samples.

RESULTS

All positive and negative controls in the PCR amplifications were positive and negative, respectively.

A visible band corresponding to a *Theileria* 18S amplicon of the expected size (approximately 1100 bp) was obtained from 11 of the 24 buffalo DNA samples and 52 of 214 tick DNA samples. Although only *A. hebraeum* ticks were collected from the buffalo, 46 of the 214 tick amplicons hybridized when probed with the *T. parva*-specific probe. Amplicons from 15 of the 214 ticks hybridized with the *T. mutans* probe but no hybridization signal was obtained with either the *T. buffeli* or the *T.* sp. (buffalo) probes (Table 2).

Oligonucleotide probing indicated that all but one of the animals were infected with at least one species of *Theileria*, as shown in Table 2. The most prevalent species detected was *T. buffeli*, found in 23 of the 24 buffalo. Eight of the animals were probe positive for *T. parva*, 14 for *T. mutans* and 13 for *T.* sp. (buffalo). All animals were positive when tested by *T. parva* specific IFA serology (Table 2).

TABLE 1 Primers and probes for amplification and detection of *Theileria* 18S genes, *Cowdria* pCS20 and 16S ribosomal RNA genes

Ident.	Length	Sequence 5'–3'	Amplifies/detects	T_m °C	Hyb. °C	Wash °C
989	17	ATG TTC TGA CCT ATC AG	*Theileria* 18S rRNA amplification	56,9	–	–
990	18	TTG CCT TAA ACT TCC TTG	*Theileria* 18S rRNA amplification	58,2	–	–
1348	18	CAA AGC GAA CTC CGT CCG	*Theileria parva* specific	67,3	45	55
IL313	20	GGA AAG AAA AAA TCA AAC CG	*Theileria buffeli* specific	60,3	40	50
1519	18	CAA AGT AAA CTC CGT CTG	*Theileria* sp. (buffalo) specific	60,4	40	50
IL268	18	ATA AGC CGC AAC GCT GGG	*Theileria mutans* (Intona) specific	67,3	45	55
AB128	24	ACT AGT AGA AAT TGC ACA ATC TAT	*Cowdria* pCS20 amplification	63,8	–	–
AB129	25	TGA TAA CTT GGT GCG GGA AAT CCT T	*Cowdria* pCS20 amplification	70,5	–	–
pCS20	~1300	*Cowdria* pCS20 coding region	*Cowdria* specific	–	45	65
930	18	GCT TAA CAC ATG CAA GTC	*Cowdria* 16S V1 loop amplification	60,4	–	–
BAA5	18	CCC ATT GTC CAA TAT TCC	*Cowdria* 16S V1 loop amplification	60,4	–	–
BAA13	18	TAC TCA TAG CCG AGG CTA	*Cowdria* Mara 87/7 16S genotype	62,7	45	57

T_m was calculated for 4xSSC as described by Davis *et al.* 1986

TABLE 2 Amplification of DNA and hybridisation of probes to buffalo blood and tick amplicons

Buffalo no.	*Theileria* species						*Cowdria ruminantium*			
	T. parva IFA	18S PCR	*T. parva* probe	*T. buffeli* probe	*T.* sp(buffalo) probe	*T. mutans* probe	pCS20 PCR	pCS20 probe	16S PCR	Mara *87/7* probe
1	+	–	–	+	–	+	–	–	nd	–
2	+	+	+	+	+	+	–	+	+	+
3	+	+	+	+	+	+	–	–	nd	–
4	+	+	+	+	+	+	–	+	+	+
5	+	+	–	+	+	+	–	–	nd	–
6	+	+	+	+	+	+	–	–	nd	–
7	+	–	–	+	–	–	–	–	nd	–
8	+	–	–	+	–	–	–	–	nd	–
9	+	+	+	+	+	+	–	–	nd	–
10	+	–	–	+	+	+	–	–	nd	–
11	+	–	–	+	–	–	–	+	+	+
12	+	–	–	+	–	–	–	–	nd	–
13	+	+	+	+	+	+	–	–	nd	–
14	+	+	+	+	+	+	–	+	+	–
15	+	–	–	+	–	–	–	–	nd	–
16	+	–	–	+	–	–	–	+	–	–
17	+	+	+	+	+	+	–	+	+	+
18	+	–	–	+	+	+	–	+	+	+
19	+	–	–	+	–	–	–	+	+	+
20	+	–	–	–	–	–	–	+	–	–
21	+	–	–	+	+	–	–	+	+	–
22	+	–	–	+	–	–	–	–	–	–
23	+	+	–	+	–	+	–	+	+	+
24	+	+	–	+	+	+	–	+	+	+
214 ticks		52	46	0	0	15	2	6	6	6

nd = not done

No visible *Cowdria* pCS20 amplicon was obtained from any buffalo DNA sample with primers AB128 and AB129, while of the 214 ticks examined, only two gave a visible amplicon of the correct size (~280 bp).

When amplicons were probed with the pCS20 probe 12 of 24 buffalo gave a positive signal, while only six of 214 tick DNA samples were positive. When these positive samples were re-tested to ascertain the *Cowdria* 16S genotypes present (Allsopp *et al.* 1999) only the Mara 87/7 genotype was detected. All six tick samples, and eight of the 12 buffalo samples, hybridized with the *Cowdria* Mara 87/7 probe and no other *Cowdria* 16S genotype could be detected.

DISCUSSION

Corridor disease outbreaks frequently occur when buffalo carrying *T. p. lawrencei* are introduced, either accidentally or in ignorance of their carrier status, into pastures grazed by cattle. However, no reports have

been received of heartwater outbreaks occurring at the same time, possibly because cattle in heartwater-endemic areas will have been exposed to *C. ruminantium* from an early age and will therefore be immune to local strains. However, *Cowdria* was found only in six of 214 of the *Amblyomma* ticks collected, which suggests that, while the buffalo are certainly carriers of *Cowdria*, transfer of the parasite to the adult ticks in a blood meal may be inefficient. The greatest threat therefore, from buffalo carrying *C. ruminantium*, lies in the possible introduction of antigenically different heartwater strains to which local cattle may have no immunity. No sex bias was observed in the six infected ticks, three being male and three female, but with such small numbers this cannot be considered significant. The failure of three of the buffalo 16S amplicons to hybridize with the *Cowdria* (Mara 87/7) probe could be due to very low levels of the organism, or it may indicate the presence of a new 16S genotype.

There was a notable contrast between the 100 % positive results for *T. parva* indirect fluorescence antibody (IFA) serology and the 33 % positive results after *T. parva* probing. This suggests that all the buffalo have been exposed to the parasite, as would be expected in an environment where neither dipping nor tick control is practised. However, while those animals which are *T. parva* probe-positive must be regarded as carriers, those which are probe-negative may be carriers with parasite levels below the level of detection at the time of sampling. Alternatively, they may have self-cured, although there is no information about whether this occurs in buffalo. If samples were examined from the same animals on different occasions this question might be resolved.

Ticks of the genus *Amblyomma* are the main vector of *T. mutans* (Uilenberg, Robson & Pedersen 1974), and 7 % of the ticks were *T. mutans* probe positive. Visible 18S amplicons were obtained from 24 % of the ticks and 21 % of the tick amplicons hybridized with the *T. parva*-specific probe. However, no hybridization signals were obtained between any of the amplicons and the *T. buffeli* and *T.* sp (buffalo) probes, an observation difficult to explain, since the tick blood meal would be expected to contain these parasites in addition to those of *T. parva* and *T. mutans*.

Free-ranging buffalo in the KNP are subjected to continual tick challenge and will therefore be carriers of a range of tick-borne parasites, including *Theileria* and *Cowdria*. If such animals come into contact with domestic cattle there are frequently serious immediate outbreaks of fatal disease, and there will also be a reservoir of newly infected ticks which perpetuate the problems. There is therefore an increasing demand in South Africa for "disease-free" buffalo for private game ranches. Disease-free means that the animals have been tested and are negative for foot-and-mouth disease, *Mycobacterium bovis* and *Theileria parva*. Such animals command prices 50–60 times greater than untested animals, so the temptation of quick financial reward is likely to lead to unwise or uninformed "disease-free" certification for which unsuspecting purchasers will pay heavily. There is an urgent need for a nationally agreed testing regimen for carrier state certification, and strict adherence to movement controls must be enforced. Failure to adhere to these procedures will have disastrous consequences for the cattle industry in the country.

ACKNOWLEDGEMENTS

We thank Dr D.G. Grobler, Mr H.J. Coetzee and Mr J. Malan for assistance with the collection of the blood samples, and Dr R.G. Bengis and Mr E.H. Dekker of the State Veterinary Services, for issuing of the relevant permits. We are grateful to Ms H. Bryden and the staff of Skukuza Scientific Services (KNP) for critical appraisal of the manuscript.

REFERENCES

ALLSOPP, B.A. & ALLSOPP, M.T.E.P. 1988. *Theileria parva*: genomic DNA studies reveal intra-specific sequence diversity. *Molecular and Biochemical Parasitology*, 28:77–84.

ALLSOPP, B.A., BAYLIS, H.A., ALLSOPP, M.T.E.P., CAVALIER-SMITH, T., BISHOP, R.P., CARRINGTON, D.M., SOHANPAL, B. & SPOONER, P. 1993. Discrimination between six species of *Theileria* using oligonucleotide probes which detect small subunit ribosomal RNA sequences. *Parasitology*, 107:157–165.

ALLSOPP, M.T.E.P., VISSER, E.S., DU PLESSIS, J.L., VOGEL, S.W. & ALLSOPP, B.A. 1997. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. *Veterinary Parasitology*, 71:283–300.

ALLSOPP, M.T.E.P., HATTINGH, C.M., VOGEL, S.W. & ALLSOPP, B.A. 1998. Comparative evaluation of 16S, *map*1 and pCS20 probes for the detection of *Cowdria* and *Ehrlichia* species in ticks. *Annals of the New York Academy of Sciences*, 849:78–84.

ALLSOPP, M.T.E.P., HATTINGH, C.M., VOGEL, S.W. & ALLSOPP, B.A. 1999. Evaluation of 16S, *map*1 and pCS20 probes for the detection of *Cowdria* and *Ehrlichia* species in ticks. *Epidemiology and Infection*, 122:323–328.

ANDREW, H.R. & NORVAL, R.I.A. 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. *Veterinary Parasitology*, 34:261–266.

ANON.1 1981. The eradication of East Coast fever in South Africa. *Journal of the South African Veterinary Association*, 52:71–73.

ANON.2 1991. 3'-End Labelling with Terminal Transferase. In: Promega Protocols and Applications Guide, 2nd edition. Madison: Promega Corporation.

BURRIDGE, M.J. & KIMBER, C.D. 1972. The indirect fluorescent antibody test for experimental East Coast fever (*Theileria parva* infection of cattle): evaluation of a cell-culture schizont antigen. *Research in Veterinary Science*, 13:4511–4515.

CONRAD, P.A., STAGG, D.A., GROOTENHUIS, J.G., IRVIN. A.D., NEWSON, J., NJAMUNGGEH, R.E.G., ROSSITER, P.B. & YOUNG, A.S. 1987. Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterization with anti-schizont monoclonal antibodies. *Parasitology*, 94:413–423.

CONRAD, P.A., OLE-MOIYOI, O.K., BALDWIN, C.L., DOLAN, T.T., O'CALLGHAN, C.J., NJAMUNGGEH, R.E.G., GROOTENHUIS, J.G., STAGG, D.A., LEITCH, B.L. & YOUNG, A.S. 1989. Characterization of buffalo-derived theilerial parasites with monoclonal antibodies and DNA probes. *Parasitology*, 98: 179–188.

DAVIS, L.G., DIBNER, M.D. & BATTEY, J.F. (Eds) 1986, in *Basic methods in Molecular Biology*. New York: Elsevier.

GODDEERIS, B.M., KATENDE J.M., IRVIN, A.D. & CHUMO, R.S.C. 1982. Indirect fluorescent antibody test for experimental and epizootiological studies on East Coast fever (*Theileria parva* infection of cattle). Evaluation of a cell-culture schizont antigen fixed and stored in suspension. *Research in Veterinary Science*, 33:360–365.

MAHAN, S.M., WAGHELA, S.D., MCGUIRE, T.C., RURANGIRWA, F.R., WASSINK, L.A. & BARBET, A.F. 1992. A cloned DNA probe for *Cowdria ruminantium* hybridises with eight heartwater strains and detects infected sheep. *Journal of Clinical Microbiology*, 30:981–986.

SAIKI, R.K., BUGAWAN, T.L., HORN, G.T., MULLIS, K.B. & ERLICH, H.A. 1986. Analysis of enzymatically amplified ß-globin and HLA-DQ DNA with allele-specific oligonucleotide probes. *Nature*, 324:163–166.

SAIKI, R.K., GELFAND, D.H., STOFFEL, S., SCHARF, S.J., HIGUCHI, R., HORN, G.T., MULLIS, K.B. & ERLICH, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487–491.

UILENBERG, G., ROBSON, J. & PEDERSEN, V. 1974. Some experiments on the transmission of *Theileria mutans* (Theiler, 1906) and *Theileria parva* (Theiler, 1904) by the ticks *Amblyomma variegatum* (Fabricius, 1794) and *Rhipicephalus appendiculatus* (Neumann, 1901) in Uganda. *Tropenmedizin und Parasitologie*, 25:207–216.

UILENBERG, G. 1981. Theilerial species of domestic livestock, in *Advances in the control of Theileriosis*, edited by A.D. Irvin, M.P. Cunningham & A.S. Young. The Hague: Martinus Nijhoff.

WAGHELA, S.D., RURANGIRWA, F.R., MAHAN, S.M., YUNKER, C.E., CRAWFORD,T.B., BARBET, A.F., BURRIDGE, M.J. & MCGUIRE, T.C. 1991. A cloned DNA probe identifies *Cowdria ruminantium* in *Amblyomma variegatum* ticks. *Journal of Clinical Microbiology*, 29:2571–2577.

YOUNG, A.S., BROWN, C.G.D., BURRIDGE, M.J., GROOTENHUIS, J.G., KANHAI, G.K., PURNELL, R.E. & STAGG, D.A. 1978. The incidence of theilerial parasites in East African buffalo (*Syncerus caffer*). *Zeitschrift für Tropenmedizin und Parasitologie*, 28:367–371.