# *Ehrlichia ruminantium* variants which do not cause heartwater found in South Africa

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## Abstract

In 1994 a batch of apparently healthy goats was selected for intended export to the USA from a heartwater-free and vector tick-free region of South Africa. The animals were tested serologically for heartwater, using either or both an IFA and an ELISA test, and 52% were found to be serologically positive. A PCR assay based on *Ehrlichia ruminantium* 16S gene sequences gave positive results for 54% of the animals, suggesting that apparently non-pathogenic *E. ruminantium* variants existed in this heartwater-free area. To identify and characterise the agents responsible for the positive serological and PCR results, ticks and animal blood samples were collected from two of the three farms involved in the original survey during two successive seasons of expected peak tick activity. Ticks were kept alive for a minimum of 3 weeks to allow digestion of any blood meal before being processed.

Over the two seasons, 28% of the livestock and 15% of the ticks sampled were found to be carrying *E. ruminantium*. *E. ruminantium* 16S and pCS20 sequences were detected in all of the four tick species collected from the livestock (*Rhipicephalus evertsi evertsi*, *Rhipicephalus evertsi mimeticus*, *Hyalomma truncatum*, *Hyalomma marginatum rufipes*), suggesting that some of the species may act as vectors. Animals generally carried multiple *E. ruminantium* 16S genotypes, whereas ticks rarely carried more than one. Infection levels in both animals and ticks were too low to generate a marked response

when a blood stabilate was sub-passaged in a clean sheep, preventing the subsequent establishment of any of the organisms in culture.

# Article Outline



# 1. Introduction

Heartwater is a disease of ruminants caused by the intracellular rickettsia *Ehrlichia ruminantium* (previously *Cowdria ruminantium*) in sub-Saharan Africa. The disease is only transmitted by ticks of the genus *Amblyomma* and its distribution coincides with that of its vector species (Walker and Olwage, 1987). Heartwater is a major obstacle to domestic livestock productivity in areas where it is endemic (Mukhebi et al., 1999), and is particularly serious when susceptible animals are moved from heartwater-free to heartwater-infected areas (Simpson et al., 1987). *Amblyomma variegatum*, the most widespread African vector of heartwater, was introduced, together with the disease, on to some Caribbean islands during the 18th or 19th centuries (Maillard and Maillard, 1998). It is possible that the disease could spread from the Antillean islands to the American mainland (Deem, 1998), where a competent tick vector is already present (Mahan et al., 2000), and this would have severe consequences for the cattle industry in the Americas.

In 1994, Boergoats from three farms in the Groblershoop district of the northern Cape Province of South Africa, a region free of heartwater and of the only South African heartwater vector tick, *Amblyomma hebraeum*, were held in quarantine in a facility near Cape Town prior to shipment to the USA. The goats were screened for heartwater using the ELISA and IFA tests available at the time (Du Plessis et al., 1993) and 52% of the animals tested positive for heartwater by either or both of the tests. These animals were not exported. A PCR assay which could detect five different *E. ruminantium* 16S genotypes gave positive results for 54% of the animals, suggesting that apparently non-pathogenic *E. ruminantium* variants existed in this heartwater-free area (Allsopp et al., 1997). One farm (Morgenswag) had contributed 47 animals, of which one was seropositive by ELISA, 19 were IFA positive, and 43 (91%) were probe positive for one or more of the *E. ruminantium* 16S genotypes.

We decided to try to further characterise, and if possible establish in culture, the organism(s) which gave rise to the confusing serological and PCR results in 1994 in order to improve the dependability of a "positive heartwater" diagnosis. We returned to the same area of the Northern Cape and collected blood samples and ticks from cattle, sheep and goats from two farms, Morgenswag and Doktershoek, for sequence investigation.

## 2. Materials and methods

Blood (10 ml aliquots in EDTA) and ticks were collected from cattle and sheep on the farm Morgenswag (28°32′S, 22°27′E) and from goats on the farm Doktershoek (28°34′S, 22°29′E). Blood stabilates were prepared *in situ* as follows: equal volumes of blood and SPG (sucrose: 74.62 g/l, $KH_2PO_4$: 0.517 g/l, $K_2HPO_4$: 1.643 g/l, K-glutamate: 0.907 g/l) were mixed and sealed in ~5 ml aliquots after separation of 200 µl for DNA extraction. Stabilates were frozen immediately in liquid nitrogen for attempted infection studies. The ticks were maintained alive at 27 ± 2 °C and 75–80% humidity for between 3 weeks and 3 months after identification. Individual ticks were then washed in ethanol and air dried before maceration in SPG (1 ml per tick). The resulting stabilates were stored in aliquots in liquid nitrogen, after separation of 200 µl for DNA extraction.

DNA was extracted from 200 µl aliquots of blood stabilates using the GENTRA 'Generation Capture Column' system (Gentra Corp. Minnesota, MN, USA) according to

the manufacturer's instructions and was eluted from the columns with 100 µl elution buffer. DNA was extracted from tick stabilates (200 µl) using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, except that proteinase K digestion was carried out at 56 °C overnight. DNA was eluted in 100 µl of the elution buffer supplied with the kit. DNA samples were stored at −20 °C pending analysis.

Five microlitre aliquots of DNA were subjected to a PCR using primers HH1-F and HH2-R for amplification of the pCS20 region (Van Heerden et al., 2004). A 'no-DNA' (water) negative control and an *E. ruminantium* (Welgevonden) (Du Plessis, 1985) tissue culture DNA positive control were amplified at the same time. Aliquots (5 µl) of each PCR product were run on 1% agarose/TBE gels with phiX174/*Hae*III markers to check for visible amplicons. Aliquots (10 µl) of each PCR product were slot blotted and probed as described (Allsopp et al., 1997). Samples giving strong hybridisation signals with the pCS20 probe were selected for pCS20 and 16S V1 loop amplification and sequencing. To obtain visible pCS20 products, a nested PCR using primer pair AB128/AB127 (Mahan et al., 1992) was carried out using 1 µl aliquots of the primary (HH1-F/HH2-R) amplicons. DNA samples positive for pCS20 were subjected to 16S V1 loop amplification and probing to determine the 16S genotypes present.

At least two PCR amplifications were carried out for each of the selected pCS20-positive samples and amplicons were pooled before cloning and sequencing to minimise the effects of polymerase misincorporation errors. Where multiple bands were observed PCR pools were gel-purified. Amplicons (pCS20, 903 bp; 16S, 328 bp) of the expected size were excised and extracted from gel slices using the MinElute PCR purification kit gel extraction protocol (Qiagen, Hilden, Germany). Some representative examples of bands of unexpected sizes were also purified. Purified amplicons were cloned into pGEM-T vector (Promega, Madison, USA) according to the manufacturer's protocol. Bacterial colonies positive for *E. ruminantium* were identified from colony lifts on nylon filters (Magna Lift, Osmonics Inc., Minnetonka, USA) by probing with a $^{32}$P-labelled oligonucleotide which hybridizes to all five *E. ruminantium* 16S V1 loop sequences (Allsopp et al., 1997) or with a $^{32}$P-labelled random primed fragment of the Welgevonden pCS20 region (Van Heerden et al., 2004). In the case of cloned amplicons of unexpected

sizes white colonies were picked at random. Picked colonies were grown overnight in 3 ml aliquots of LB medium with tetracycline and ampicillin as recommended in the Promega protocol and plasmid preparation was carried out using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany). Plasmid sequencing was carried out using 'big dye' chemistry on an ABI 3100 automated sequencer. 16S and pCS20 sequences were compared with existing *E. ruminantium* databases using the 'Seqlab' module of the Wisconsin Package version 10.1 (Genetics Computer Group, Madison, WI, USA). BLAST searches of GenBank were conducted with sequences from amplicons of unexpected sizes.

An aliquot (10 ml) of blood stabilate from one pCS20-positive sheep (sheep MS4) was injected intravenously into one pCS20-negative sheep (sheep S171) to check for pathogenicity. The temperature of the animal was monitored daily and blood was collected for attempted culture at peak temperature increase. DNA was extracted from a 200 µl blood aliquot as described and 16S amplicons were cloned and sequenced. Direct sequencing of the pCS20 amplicon was carried out.

All *E. ruminantium* sequences were submitted to GenBank and were assigned the following accession numbers:

16S: DQ640389–DQ640401; pCS20: DQ631917–DQ631931.

# 3. Results

### 3.1. Animal and tick samples

In the two seasons surveyed, the number of animals from which blood samples and ticks were collected are listed in Table 1, together with the numbers of animals testing pCS20 positive. A total of 106 ticks were collected from the animals in 2002, and 116 in 2003. The numbers and identities of the ticks, together with the numbers of ticks pCS20 positive, are given in Table 2. More ticks were collected from cattle in 2002 than in 2003, when only 16 were collected, probably because the animals had been dipped shortly before the survey. All 16 were pCS20 negative. The pCS20 probing results for animals and ticks sampled in 2003 are shown in Fig. 1.

Table 1.

Animals on two Northern Cape farms from which blood samples were collected in 2002 and 2003: total number of samples and numbers testing positive with the pCS20 probe

| **Host** | **2002** | | **2003** | |
|---|---|---|---|---|
| | **Total** | **pCS20 +ve** | **Total** | **pCS20 +ve** |
| Cattle | 7 | 3 | 10 | 2 |
| Sheep | 8 | 2 | 10 | 3 |
| Goats | 6 | 2 | 10 | 3 |

Table 2.

Tick species collected from animals on two Northern Cape farms during 2002 and 2003: total number of ticks collected and numbers testing positive with the pCS20 probe

| **Host and ticks testing pCS20 +ve** | **2002 tick species and number** | | | | **2003 tick species and number** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Ree** | **Rem** | **Ht** | **Hmr** | **Ree** | **Rem** | **Ht** | **Hmr** |
| Cattle | 1 | 35 | 0 | 3 | 0 | 10 | 0 | 6 |
| Sheep | 0 | 31 | 2 | 0 | 4 | 35 | 5 | 12 |
| Goats | 34 | 0 | 0 | 0 | 44 | 0 | 0 | 0 |
| Total ticks | 106 | | | | 116 | | | |
| | | | | | | | | |
| pCS20+ | 2 | 9 | 0 | 0 | 1 | 4 | 1 | 5 |

Key: Ree, *Rhipicephalus evertsi evertsi*; Rem, *Rhipicephalus evertsi mimeticus*; Ht, *Hyalomma truncatum*; Hmr, *Hyalomma marginatum rufipes*.

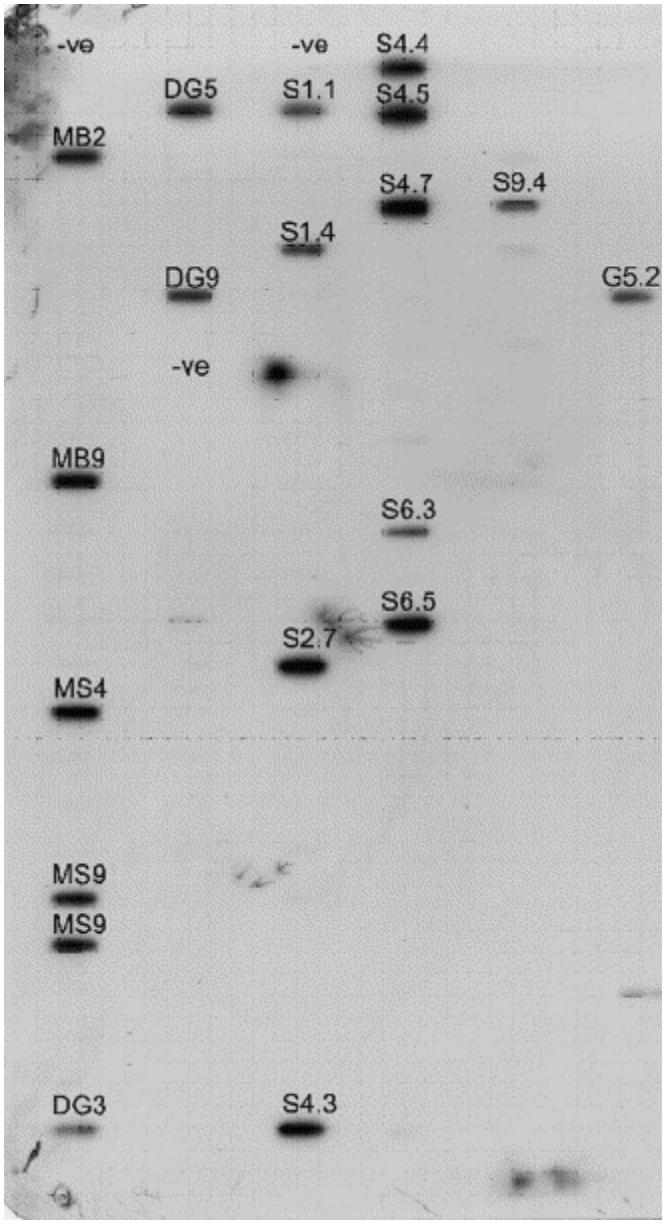


Fig. 1. Autoradiograph showing pCS20 probe results for animals and ticks collected in 2003.

Only *R. evertsi evertsi* ticks were collected from the goats on Doktershoek farm in both 2002 and 2003. The sheep and cattle on Morgenswag were infested predominantly with *R. evertsi mimeticus*, a sub-species closely related to *R. evertsi evertsi*, and some animals on this farm were also carrying *H. truncatum* and *H. marginatum rufipes* ticks. We found only these four tick species, and 9.8% of them tested positive for *E. ruminantium* using the pCS20 assay.

### 3.2. pCS20 and 16S probing

In 2002 the only ticks found to be pCS20 positive were of the two *Rhipicephalus* spp., whereas in 2003 all four species yielded pCS20-positive individuals (Table 2). We selected animals and ticks from 2003 for sequence analysis, in order to obtain better coverage of the *E. ruminantium* genotypes present, since it was possible that the different genotypes might be preferentially carried by different tick species.

In 2003 all the animals appeared to be carrying more than one *E. ruminantium* 16S genotype (Table 3), whereas the ticks rarely carried more than one. The Ball 3 genotype was detected in one animal and all the animals appeared to be carrying the Omatjenne 16S genotype. Only one tick (*H. marginatum rufipes*) gave a faint positive signal with the Omatjenne genotype probe.

Table 3.

Results obtained with five different *Ehrlichia ruminantium* 16S probes used to test all pCS20-positive blood and tick samples

| **Animal or tick ID** | **Species** | **16S V1 loop probe** | | | | |
|---|---|---|---|---|---|---|
| | | **Ball 3** | **Senegal** | **Omatjenne** | **Mara 87/7** | **Welgevonden** |
| MB1 | Bovine | – | + | + | + | + |
| MB9 | Bovine | + | ?+ | + | + | + |
| MS4 | Ovine | – | + | + | + | + |
| MS9 | Ovine | – | + | + | + | + |
| DG2 | Caprine | – | – | + | + | + |
| DG3 | Caprine | – | + | + | + | + |
| DG5 | Caprine | – | + | + | + | + |
| MS1.1 | Hmr | – | + | – | – | – |
| MS1.4 | Hmr | – | + | – | – | – |
| MS2.7 | Rem | – | + | – | – | – |

| Animal or tick ID | Species | 16S V1 loop probe | | | | |
|---|---|---|---|---|---|---|
| | | Ball 3 | Senegal | Omatjenne | Mara 87/7 | Welgevonden |
| MS4.3 | Hmr | – | + | – | + | + |
| MS4.4 | Hmr | – | – | – | + | – |
| MS4.5 | Hmr | – | – | ?+ | + | – |
| MS4.7 | Rem | – | + | – | + | – |
| MS6.5 | Rem | – | – | – | + | + |
| MS9.4 | Ht | – | – | – | + | – |
| DG5.2 | Ree | – | – | – | + | – |

Ree, *R. evertsi evertsi*; Rem, *R. evertsi mimeticus*; Ht, *H. truncatum*; Hmr, *H. marginatum rufipes*; (+) positive signal; (−) no signal observed; (?+) very faint signal, possibly negative.

### 3.3. pCS20 and 16S genotype sequence analysis

The sequence results are presented in Table 4. pCS20 sequences identical to those of *E. ruminantium* strains Welgevonden and Kiswani were detected in the bovine MB9, the same Kiswani pCS20 sequence was also detected in the *R. evertsi evertsi* tick DG5.2 recovered from goat DG5. The pCS20 sequence detected in goat DG5 was identical to that of *E. ruminantium* (Umbanein) and three new unique pCS20 sequences were also obtained, two from sheep MS9 and one from sheep MS4. In contrast, most of the 16S sequences were of the Senegal, Mara 87/7 and Welgevonden genotypes, although one previously unknown sequence was also obtained. In the case of the ticks, there was no correlation between their species and the *E. ruminantium* sequences which they carried. Although the probing results showed that all the animals appeared to be carrying the *E. ruminantium* (Omatjenne) genotype, no Omatjenne 16S sequences were obtained. We were also unable to obtain a Ball 3 16S sequence from the one animal in which that genotype had been detected by probing.

Table 4.

Genotype identities of different pCS20 sequences, and genotype identities of different 16S sequences, obtained from pCS20-positive blood and tick samples

| **Animal/tick ID** | **Species** | **pCS20 seqs** | **16S genotypes** |
|---|---|---|---|
| MB9 | Bovine | Welgevonden, Kiswani | Senegal, Welgevonden, Mara 87/7, 1 new |
| MS4 | Sheep | 1 unique | Welgevonden |
| MS4.3 | Hmr tick | No amplicon | Mara 87/7 |
| MS4.7 | Rem tick | No amplicon | Senegal |
| MS9 | Sheep | 2 unique | Senegal, Mara 87/7 |
| MS9.4 | Ht tick | No amplicon | Mara 87/7 |
| DG5 | Goat | Umbanein | Senegal |
| DG5.2 | Ree tick | Kiswani | Welgevonden, Mara 87/7 |

Ree, *R. evertsi evertsi*; Rem, *R. evertsi mimeticus*; Ht, *H. truncatum*; Hmr, *H. marginatum rufipes*.

### 3.4. Sequences from amplicons of unexpected sizes

Most of the amplicons of unexpected sizes were much less prominent than the 16S and pCS20 amplicons. All those from which sequences were obtained were found to match mammalian chromosomal sequences in GenBank.

### 3.5. Attempted transmission from blood stabilate to clean sheep

When blood stabilate from sheep MS4 was injected into sheep S171, the animal reacted 7 days post-inoculation with a temperature of 40.3 °C, but showed no signs of distress. The temperature fluctuated thereafter, reaching a maximum of 40.4 °C 30 days post-inoculation, after which no further records were made. No *Ehrlichia* organisms were observed in blood smears and the levels of infection in the animal were not sufficient for the establishment of cultures. Amplification of a blood sample taken at day 26 post-

inoculation (temperature 40.3 °C) and probing for pCS20 indicated that *E. ruminantium* was present. One 16S V1 loop sequence identical to that of *E. ruminantium* (Welgevonden) was obtained from this animal, but pCS20 sequencing indicated that a mixture of genotypes was present.

# 4. Discussion

The two farms, Doktershoek and Morgenswag, are situated on opposite sides of a road, close to a nature reserve. Four species of ticks were found on Morgenswag farm, *R. evertsi evertsi*, *R. evertsi mimeticus*, *H. truncatum* and *H. marginatum rufipes*, with the *Hyalomma* ticks being present in much smaller numbers than the *Rhipicephalus* species. *Hyalomma* ticks were not found at all on Doktershoek, possibly because fewer animals were sampled, and because these ticks are more restricted in their feeding preferences than *Rhipicephalus* species (I.G. Horak, personal communication). *R. evertsi mimeticus* ticks are not widespread in South Africa, but they are common in Angola and Namibia and are known to have been introduced into some sites in South Africa, presumably with imported livestock (Walker et al., 2003). The sub-species has obviously become well established on Morgenswag farm.

Diagnostic tests targeting the pCS20 region have long been considered to be specific for *E. ruminantium* (Allsopp et al., 1999), and the sequences of this region from 14 different virulent heartwater-producing isolates have been shown to be phylogenetically closely related, and much more divergent from the sequence of the homologous region of *Ehrlichia chaffeensis* (Van Heerden et al., 2004). Three of the pCS20 sequences obtained in this study were identical to those from known virulent heartwater-producing *E. ruminantium* isolates Welgevonden, Kiswani and Umbanein, and three were unique (Table 4). The new unique sequences were closely similar to the others, exhibiting only small numbers of SNPs, and they fell firmly into the *E. ruminantium* clade on phylogenetic analysis (data not shown). All except one of the 16S sequences we obtained were identical over the sequenced region to those of the well-characterised pathogenic *E. ruminantium* isolates Welgevonden, Senegal and Mara 87/7 found in heartwater-endemic areas. The new unique sequence was closely similar to the others, and it too fell firmly into the *E. ruminantium* clade on phylogenetic analysis (data not shown). No heartwater

disease is ever seen in this area of the Northern Cape, so we must assume that, despite sharing common pCS20 and 16S sequences, these *E. ruminantium* organisms differ markedly from pathogenic isolates in both infectivity and virulence. Infection levels in both animals and ticks also appear to be very low, as shown by our inability to obtain sequences from some samples which gave positive probe results.

It has been known for some years that at least one *E. ruminantium* genotype exists which does not cause heartwater in its area of origin, this is *E. ruminantium* (Omatjenne), obtained from a *H. truncatum* tick in a heartwater-free area of Namibia (Du Plessis, 1990). In the molecular probing survey carried out in 1994, and referred to in the introduction, 166 animals from the heartwater-free Northern Cape were examined. Seventy animals (42%) were probe positive for the Omatjenne 16S genotype (Allsopp et al., 1997) while 40 animals (24%) gave probe signals for 16S sequences from the pathogenic *E. ruminantium* genotypes Ball 3 and Welgevonden. In the present survey all seven *E. ruminantium*-positive animals gave a good signal with the Omatjenne 16S probe but only one *H. marginatum rufipes* tick gave a faint signal with this probe, suggestive of a very low infection rate.

Both *H. truncatum* and *H. marginatum rufipes* are two-host ticks, with the larvae and nymphs feeding exclusively on small animals and the adults feeding exclusively on large mammals. The immature stages of *H. truncatum* feed on scrub hares (*Lepus saxatilis*) and rodents, while the immature stages of *H. marginatum rufipes* feed on scrub hares and birds (I.G. Horak, personal communication). This means that ticks of the genus *Hyalomma* can only be vectors for *E. ruminantium* if larvae are infected transovarially or if adults transmit the infection intrastadially. Apart from a single unconfirmed report (Bezuidenhout and Jacobsz, 1986) it is not generally believed that *E. ruminantium* undergoes transovarial transmission, and intrastadial infection via *Hyalomma* adults would only occur in the event of accidental dislodgement of a partially fed tick, followed by re-attachment to a new host. *Hyalomma* ticks are therefore unlikely to be anything other than occasional and accidental vectors of *E. ruminantium*. *E. ruminantium* (Omatjenne) was originally obtained from an adult female *H. truncatum* tick taken from a bovine, triturated, and injected into a mouse (Du Plessis, 1990). This tick could well have

infected the mouse simply because it was carrying infected bovine blood, rather than because it was an active vector of *E. ruminantium*.

*R. evertsi* spp. are also two-host ticks but both immature and adult stages may infest large mammalian hosts, as well as scrub hares and small antelopes. The failure to detect *E. ruminantium* (Omatjenne) in any of the *Rhipicephalus* ticks suggests that this organism does not survive digestion of the blood meal in ticks of this genus. It is known that pathogenic strains of *E. ruminantium* can be transmitted by the tortoise tick, *Amblyomma marmoreum* (Peter et al., 2000), which is present in this area of the Northern Cape, so it is possible that this tick is a vector for less virulent *E. ruminantium* variants such as the Omatjenne strain. The cattle, sheep and goats in this study roam freely in the bush, hence we cannot discount the possibility that they may become infected, via these ticks, by any of the *E. ruminantium* variants we detected. Nevertheless, field collections of *A. marmoreum* from domestic animals are rare (Walker and Olwage, 1987) and none were recovered from the animals in our study. As for the *Rhipicephalus* species in which the *E. ruminantium* variants were detected, further work is needed to determine whether they are competent vectors for transmission of the organisms.

Positive serological results from heartwater-free areas cannot simply be ignored, but they also cannot be assumed to indicate the presence of potentially heartwater-causing organisms. Even the most sensitive and specific ELISA for *E. ruminantium* (MAP1B) gives positive cross reactions with *E. chaffeensis* and *Ehrlichia canis* sera (van Vliet et al., 1995), and while there is no molecular evidence that *E. chaffeensis* occurs in southern Africa (MTEPA, unpublished), and we have never detected *E. canis* 16S sequences in ruminants or in ticks recovered from ruminants, other uncharacterised *Ehrlichia* spp. could be a source of positive MAP1B reactions. In earlier work, for example, a 16S sequence (GenBank accession number U54805) closely similar to *E. ovina* (GenBank accession number AF318946.1) was obtained from a sheep on the farm Germishuys (Allsopp et al., 1997). It likely, however, that positive MAP1B results from heartwater-free areas, particularly if the animals are also pCS20 positive, indicates exposure to *E. ruminantium* of low pathogenicity. In the present work we were unable to obtain visible pCS20 amplicons from three of the ticks from which *E. ruminantium* 16S sequences were obtained. This is likely to be the result of polymorphisms in one or both of the pCS20

nested amplification primer target sites preventing effective hybridization and chain extension. It is also possible that serologically positive but pCS20-negative animals could be carrying other uncharacterised *Ehrlichia* spp. To summarize: positive "heartwater" serology in heartwater-free areas of southern Africa probably indicates exposure to *E. ruminantium* of low pathogenicity or to other uncharacterised *Ehrlichia* spp. The organisms may be unable to cause heartwater disease, but the animals involved should not be exported to heartwater-free regions, especially not to those where there are *Amblyomma* ticks capable of transmitting *E. ruminantium*. The danger that the virulence of the organisms could be affected by the tick host, as suggested by Du Plessis et al. (1993), has never been properly investigated, and such investigations must await establishment of the variant organisms in culture.

The data presented in Table 2 represent only adult ticks which survived for periods of 3 weeks to 3 months after collection. Detection of *E. ruminantium* in these ticks thus indicates that viable organisms were still present long after digestion of the blood meal. The detection of single *E. ruminantium* genotypes in ticks recovered from animals carrying multiple genotypes may be due to infection exclusion, an effect which has been shown to occur in *Dermacentor variabilis* ticks fed on animals infected with different strains of *Anaplasma marginale* (de la Fuente et al., 2003). The relatively high percentage of infection (10.5%) in the surviving *Rhipicephalus* spp. ticks suggests that these ticks may act as vectors for the transmission of apparently non-pathogenic *E. ruminantium* variants. This possibility was proposed as long ago as 1993 for both *H. truncatum* and *R. evertsi* (Du Plessis, 1993), but for the reasons stated in an earlier paragraph, *Hyalomma* spp. ticks are unlikely to be vectors. Du Plessis ascribed positive heartwater serological results in heartwater-free areas to uncharacterised *Ehrlichia* species, as opposed to what was then known as *C. ruminantium*, and the molecular sequence data obtained during this study represent a first step in the characterisation of the organisms. Infection levels in animals and ticks in the field are very low and we failed to induce a level of infection by blood stabilate passage into a clean sheep sufficient to initiate a culture.

# 5. Conclusion

It appears that there are many different *E. ruminantium* genotypes in circulation, with pathogenicities varying from high to low, and that pathogenicity does not correlate with either pCS20 or 16S genotype. Further molecular genetic characterisation, and transmission and pathogenicity studies, of the low pathogenicity genotypes must wait until the organisms are established in culture. As other workers have observed, a bacterial sample principally composed of disease-causing isolates is highly biased and represents a minor (and atypical) fraction of a much larger and more diverse population (Feil and Spratt, 2001).

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