

Evaluation of 16S, *map1* and pCS20 probes for detection of *Cowdria* and *Ehrlichia* species*

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SUMMARY

A panel of 16S ribosomal RNA gene probes has been developed for the study of the epidemiology of heartwater; five of these detect different *Cowdria* genotypes, one detects five distinct genotypes; one detects any Group III *Ehrlichia* species other than *Cowdria* and one detects any Group II *Ehrlichia* species. These probes have been used on PCR-amplified rickettsial 16S rRNA genes from over 200 *Amblyomma hebraeum* ticks. Control ticks were laboratory-reared and either uninfected or fed on sheep experimentally infected with different *Cowdria* isolates, field ticks were collected from animals in heartwater-endemic areas. All tick-derived DNA samples were also examined by PCR amplification and probing for two other *Cowdria* genes (*map1* and pCS20) which have previously been used for heartwater epidemiology. This paper describes the first direct comparison of all currently available DNA probes for heartwater-associated organisms.

INTRODUCTION

In South Africa, the obligate intracellular haemo-parasite *Cowdria ruminantium*, which causes heartwater in ruminants, is transmitted by the tick *Amblyomma hebraeum*. Clinical heartwater cases are observed within the distribution area of this tick. Current serological tests are of limited reliability, giving false positive and false negative reactions, and they cannot be used to detect the prevalence of heartwater-causative organisms in ticks. Different *Cowdria* genotypes [1] of differing immunogenicities [2] exist in the field and detailed information on the distribution of these geno- and immunotypes is essential if any large-scale vaccination programme is to be carried out. In addition, the detection and characterization of heartwater-associated pathogens

in local tick populations is an important guide to the threat posed to livestock in the areas concerned.

To improve our understanding of heartwater epidemiology we developed a panel of eight 16S ribosomal RNA (rRNA) probes for the detection of *Cowdria* and *Ehrlichia* species which may be associated with heartwater. We have six different *Cowdria* 16S probes. Five of these are specific for five different *Cowdria* genotypes (Ball 3, Senegal, Omatjenne, Crystal Springs, Mara 87/7) and one will detect all five of these genotypes. All 16S probes other than that for the *Cowdria* (Mara 87/7) genotype were characterized previously [1], that for Mara 87/7 was designed during this study. There are two further *Ehrlichia* 16S probes that will detect any Group III *Ehrlichia* species (the *E. canis*, *E. chafeensis*, *E. muris* group [3]) other than *Cowdria*, and any Group II *Ehrlichia* species (the *E. equi*, *E. platys*, *E. phagocytophila*, *Anaplasma marginale* group [3]).

We used these probes on polymerase chain reaction (PCR) amplified rickettsial 16S rRNA genes from

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Table 1. *Laboratory-reared control ticks*

No. ticks	Heartwater isolate	Region/country of origin of isolate	Reference
10	None	Uninfected negative controls	—
10	Nonile	Kwa Zulu/Natal, South Africa	7
10	Kwanyanga	Eastern Cape, South Africa	8
10	Mara 87/7	Northern Province, South Africa	2
10	Welgevonden	Northern Province, South Africa	9
10	Vosloo	North Western Province, South Africa	10
10	Kümm	Northern Province, South Africa	11
10	Omatjenne	Otjiwarongo District, Namibia	19
10	Senegal	Senegal, West Africa	12
9	Mali	Mali, West Africa	13

over 200 *A. hebraeum* ticks. We also used pCS20 [4] and *map1* [5] probes on PCR amplified gene fragments from the same DNA samples. This work compares all the currently available DNA probes for heartwater-associated organisms, a study which has not previously been performed.

MATERIALS AND METHODS

Laboratory-reared *A. hebraeum* ticks, fed as larvae on rabbits, were fed as nymphae on heartwater-susceptible sheep which were initially serologically negative by IFA [6] for this disease. Negative control ticks were fed on uninfected sheep. Positive control ticks were fed on sheep which had been individually experimentally infected with different cowdria isolates, as shown in Table 1. After moulting, adult ticks were washed with ethanol, air dried and frozen at -70°C . Other *A. hebraeum* ticks were collected from animals in the field in heartwater-endemic areas and these were ethanol-washed and air dried before processing. DNA was extracted from crushed frozen ticks using the QIAamp (Qiagen, Germany) tissue kit according to the manufacturer's instructions. DNA was extracted from an aliquot of the Kümm heartwater isolate blood stabilate, which was also used to infect the sheep upon which the Kümm control ticks were fed. This stabilate was prepared from blood of an experimentally infected sheep in 1994 and held in 5 ml aliquots in liquid nitrogen.

All DNA samples were subjected to PCR amplification using 16S [1], pCS20 [14] and degenerate *map1* gene primers (**f** *map1*: 5' ATG AAT TRC ARR RAA WTK TTT 3' and **r** *map1*: 5' AYA BRA AYC TTS CTC CAA 3') (UPAC symbols R = A+G, W =

A+T, K = G+T, Y = C+T, B = G+T+C, S = G+C). Amplicons were slot-blotted onto nylon membrane (Hybond N+ Amersham International) as described [15]. 16S amplicons were probed with eight different oligonucleotide probes as shown in Table 2. *map1* amplicons were probed with a cloned fragment of the cowdria (Welgevonden) *map1* gene coding region (base pairs 4-873) excluding the start codon [16] and pCS20 amplicons were probed with the full length cloned pCS20 gene [14]. Oligonucleotide probes were 3' end labelled [1] and pCS20 and *map1* probes were 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 2 and results were visualized by autoradiography.

A near full-length 16S rRNA gene amplicon from a tick experimentally infected with cowdria (Mara 87/7) [2] was cloned into pMOS-blue (Amersham International) according to the manufacturer's instructions. White colonies were picked from LB agar plates containing ampicillin ($200\ \mu\text{g ml}^{-1}$) and tetracycline ($15\ \mu\text{g ml}^{-1}$) with IPTG and X-gal and were grown overnight with gentle shaking in $100\ \mu\text{l}$ aliquots of LB with ampicillin and tetracycline in microtitre plate wells. Mara 87/7 16S positive recombinants were identified by probing a colony blot with a 3' end-labelled Mara 87/7 16S V1 loop oligonucleotide. The oligonucleotide was designed when it was found that V1 loop amplicons from this isolate differed in sequence from previously characterized cowdria 16S genes. Phagemids were prepared from 3 ml of overnight cultures [17] and were sequenced on both strands with T7, M13-20 and internal primers [1] using a standard protocol [18].

Table 2. Probes for the detection of heartwater-associated organisms

Probe	Length	Sequence 5'-3'	Detects	T_m °C	Hyb °C	Wash °C
BAA 10	20	ATACTTATAGCCGAAGCTAT	<i>Cowdria</i> Ball 3	60.3	45	57
BAA 11	20	ATACTTATAGCCAAGGCTAT	<i>Cowdria</i> Senegal	60.3	45	53
BAA 12	20	ATAACCGAGGCTATAAAACAA	<i>Cowdria</i> Omatjenne	60.3	45	62
BAA 13	18	TACTCATAGCCGAGGCTA	<i>Cowdria</i> Mara 87/7	62.7	45	58
BAA 14	18	TACTCATAGCCGAAGCTA	<i>Cowdria</i> Xtal Springs	60.4	45	57
BAA 15	20	ATTTCTAATAGCTATTCCAT	Any cowdria	56.2	45	53
BAA 16	18	ATTTCTAATGGCTATTCC	<i>Ehrlichia</i> spp. Group III	55.9	45	57
BAA 17	18	YTTCTAGTGGCTATCCYA	<i>Ehrlichia</i> spp. Group II	58.2–62.7	45	53
pCS20	~ 1300	ORF of unknown function	Most cowdria	—	45	65
map1	~ 870	<i>Cowdria</i> (Welgevonden) <i>map1</i> gene generated by primers <i>fmap1</i> and <i>rmap1</i>	<i>Cowdria</i> , (low stringency, Gp II <i>Ehrlichia</i> spp.).	—	45	60

RESULTS

The 16S probes

Amplified DNA from the 10 laboratory-reared control ticks which had been fed on uninfected sheep gave negative reactions with all 16S probes. Of the 89 laboratory-reared control ticks fed on cowdria-infected sheep, a total of 26% gave amplicons hybridizing with the 'all cowdrias' probe. No positive probe results were obtained from the ticks which had been fed on sheep infected with the Senegal, Mali, and Vosloo cowdria isolates.

The amplified DNA from ticks fed on sheep infected with Welgevonden (9/10), Kwanyanga (10/10) and Nonile (10/10) hybridized strongly with the cowdria (Crystal Springs) probe. The amplicons from ticks fed on sheep infected with Omatjenne (1/10), and Mara 87/7 (1/10) hybridized with their respective probes, and 5/10 Kümme-fed ticks hybridized strongly with the cowdria (Omatjenne) probe. Amplicons from the Omatjenne infected tick and the five Kümme infected ticks did not show any hybridization signal with the 'all cowdrias' probe under the stringency washing conditions originally used but hybridized strongly when the wash temperature was lowered from 57 to 53 °C. Kümme blood stabilate DNA gave a hybridization signal only with the cowdria (Omatjenne) probe.

Of the 129 *A. hebraeum* ticks collected from animals in the field in various regions of South Africa, the majority were obtained from commercial farms or game ranches in the Northern and North West provinces. Only 12% gave amplicons hybridizing to any 16S cowdria genotype probe and one amplicon hybridized to two of these. 16S amplicons from 11 ticks (8.5%) hybridized to the Group II ehrlichia probe (Fig. 1) and no group III ehrlichia was detected.

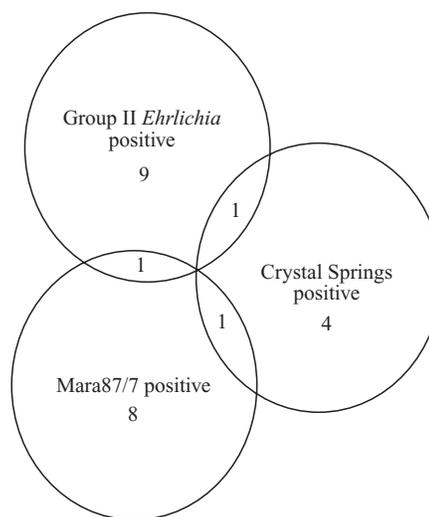


Fig. 1. Diagram showing the number of field ticks hybridizing with 16S probes.

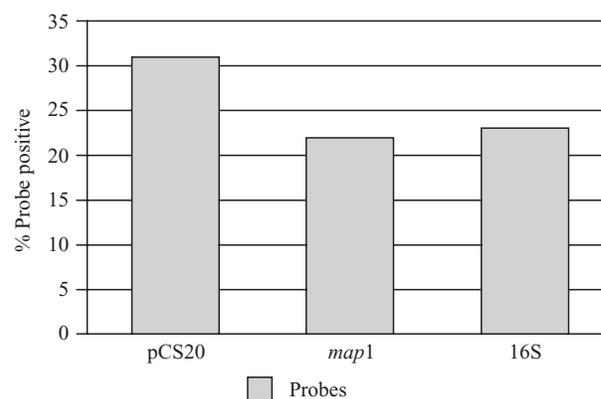


Fig. 2. Laboratory-reared ticks: percentage probe positive.

The most prevalent cowdria 16S genotypes detected in the field samples were Mara 87/7 (10/129) and Crystal Springs (6/129) (Fig. 1).

Table 3. Amplicons from 129 field ticks hybridizing with pCS20, map1 and 16S cowdria genotype probes

	pCS20	map1	16S cowdria genotypes
No. field ticks probe positive	16	10	15
Percentage of total	12.4	7.75	12

The pCS20 probe

This probe gave a positive signal on blots of pCS20 amplicons more frequently in positive control ticks (31%, Fig. 2) and field ticks (13%, Table 3) than either of the other two groups of probes. It did not detect any ehrlichia controls and gave a strong hybridization signal at high stringency with all cowdria-positive control samples except for the Omatjenne genotype. However, when the washing temperature was reduced from 65 to 55 °C, the probe remained bound to the cowdria (Omatjenne) amplicon.

The map1 probe

The probe used in these experiments was an ~ 870 bp fragment cloned from the cowdria (Welgevonden) map1 gene (Table 2). It detected cowdria less frequently in the control ticks (22%, Fig. 2) than the pCS20 probe. The percentage of field tick map1 amplicons hybridizing to this probe was < 8% (Table 3).

Mara 87/7 16S sequence

The near full-length 16S sequence of the cowdria (Mara 87/7) genotype was obtained on both strands. This sequence was submitted to GenBank with accession number AF 069758.

DISCUSSION

The 16S probes

The high percentage of Kwanyanga, Nonile and Welgevonden cowdria 16S amplicons from experimentally infected ticks detected with the Crystal Springs probe indicates that these cowdria isolates all have the same 16S rDNA sequence in the hyper-variable (V1 loop) region. It also suggests that passage of this genotype in *A. hebraeum* is more readily achieved than with some other genotypes. The low number of experimentally infected ticks hybridizing with the cowdria (Omatjenne) and cowdria (Mara 87/7) probes suggest that these two genotypes are less

readily tick-passaged in *A. hebraeum* than the Crystal Springs genotype. Cowdria (Omatjenne) was originally isolated from a single *Hyalomma truncatum* tick from Namibia [19] so the possibility exists that, although *A. hebraeum* will transmit this genotype [19], *H. truncatum* is a more favourable arthropod host.

In this work the Kümme blood stabilate used, and ticks experimentally infected with the Kümme isolate, were positive with only the Omatjenne probe. This differs from previous results [1] in which both Omatjenne and Crystal Springs V1 loop sequences were obtained from Kümme DNA isolated from blood stabilates. As no Crystal Springs V1 loop sequence was detected in the blood stabilate used in this study to infect the sheep upon which the ticks were fed, it is possible that the sheep used to prepare the original batch of stabilate [1] was carrying a sub-clinical cowdria infection of the Crystal Springs genotype.

The 16S oligonucleotide probes are technically demanding to use because, among the different cowdria genotypes, there are few nucleotide sequence differences in the most variable region (the V1 loop) of the rRNA gene. The necessity for end-labelling also means that the probes can carry less label than a random-primed probe so that hybridization signal strength may be low. Stringency washing conditions, particularly washing temperature, are therefore critical. However, these probes are the only ones which give any phylogenetic information.

The pCS20 probe

This appears to be the most sensitive probe for cowdria detection. It is a ~ 1300 base pair (bp) fragment and hence can be labelled to a high specific activity. Our results suggest that the probe is cowdria specific but that some pCS20 polymorphisms may exist in the amplified target. The main disadvantage of this probe is that it gives no phylogenetic information.

The map1 probe

Like the pCS20 probe, this fragment can be labelled to high specific activity. The gene is known to exhibit

extensive polymorphisms between isolates (20) which necessitate the use of relaxed stringency during washing of the blots. Under these relaxed conditions, the probe detected the Group II ehrlichia control (data not shown) indicating a conflict between sensitivity and specificity requirements.

The percentage of field ticks (12%) in which cowdria organisms were detected by all three probe types was higher than the 4.7% infection rate recorded in wild-trapped and unfed adult *A. hebraeum* from the North West province of South Africa [21]. Since our ticks were removed from their mammalian hosts while actively feeding the high percentage of probe positives may indicate the presence of cowdria organisms in the blood meal. Our experimental results therefore give no indication of the direct threat of infection posed to animals in the field. This could best be achieved by collecting fully engorged nymphae and holding them under suitable conditions of temperature and humidity until they moulted, or by trapping wild unfed adult ticks, which requires the use of pheromone/carbon dioxide traps and must be carried out during the period of the year when the ticks are most active (between September and May). Detection of cowdria DNA in adults from both these sources would indicate the potential for infection of the next host animal.

A valuable outcome of these experiments is the indication that the most prevalent cowdria 16S genotypes in the N. and N.W. provinces of South Africa are Mara 87/7 and Crystal Springs. Application of the 16S probes to PCR amplicons from mammalian and arthropod tissues has revealed that any given sample may contain more than one rickettsial genus and/or genotype [1]. In any investigation into the epidemiology of heartwater, it is essential to be able to distinguish those organisms which cause disease from those which may be apathogenic, such as the cowdria (Omatjenne) genotype [3], but which react positively to antigens used in current serological tests. So far, the only way to achieve such discrimination is by the use of the 16S molecular probes and similar studies underway in other heartwater endemic areas will enable us to assess cowdria genotype prevalence.

The pCS20 probe appears to be the most sensitive indicator for cowdria genotypes other than Omatjenne, and it shows no cross-hybridisation with *Ehrlichia* spp. DNA. It is therefore the probe of choice for initial screening for cowdria of large numbers of samples. If phylogenetic information is required the

16S probes must be employed, although they are technically difficult to use. The *map1* probe currently in use in our laboratory is less sensitive and specific than the pCS20 probe and it does not provide any genotype information. Comparison of *map1* sequences from a number of geographically different isolates (20) has shown that the gene exhibits sequence polymorphisms and it may therefore be possible to design *map1* probes which will provide immunotypic information about the distribution of cowdria genotypes in any given area.

Although the three probe types have been used successfully for cowdria detection in DNA from mammals and ticks [1, 4, 14, 22, 23] no direct comparison between the probes has previously been carried out. This study suggests that, in the present state of our epidemiological knowledge of heartwater, any field survey should use all the available probe types.

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