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Prevalence of *Cowdria ruminantium* infection in *Amblyomma hebraeum* ticks from heartwater-endemic areas of Zimbabwe

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SUMMARY

Analysis of the transmission dynamics of *Cowdria ruminantium*, the tick-borne rickettsial agent of heartwater in ruminants, requires accurate measures of infection in vector populations. To obtain these, *Amblyomma hebraeum* ticks were collected at two heartwater-endemic locations in the lowveld and highveld regions of Zimbabwe and assessed for *C. ruminantium* infection with specific polymerase chain reaction (PCR) and DNA probe detection assays. At the lowveld site, 11·2% (50/446) of adult ticks and 8·5% (23/271) of nymphs carried *C. ruminantium*, as detected by PCR. At the highveld site, the prevalence of infection in adult ticks was 10·2% (40/392). DNA probe analysis revealed that most infections at both sites were of low intensity; only 9% and 23% of all nymph and adult tick infections, respectively, were greater than 70000 organisms, the detection limit of the DNA probe. However, the majority (70%) of probe-detectable adult tick infections were high, between 10^7 and 10^9 organisms/tick, while those within nymphs were lower, between 10^5 and 10^6 organisms/tick.

INTRODUCTION

Cowdria ruminantium is an intracellular tick-borne rickettsia causing heartwater (cowdriosis) in domestic and wild ruminants [1–5]. It is transmitted by members of the tick genus *Amblyomma*, principally *A. hebraeum* in southern Africa and *A. variegatum* throughout much of the rest of sub-Saharan Africa and on several islands in the Caribbean [6]. Heartwater is a key economic constraint to livestock productivity in the tropics [7, 8].

Current control of heartwater relies on intensive acaricide treatment of animals, which is cumbersome and costly. The development of new control strategies requires knowledge of the disease epidemiology which has been previously limited by the lack of reliable tests to detect infection in hosts and vectors. In particular, the development of population based control strategies through the use of transmission dynamics models requires field data on the prevalence and intensity of infection in both the adult and nymph instars of the vectors. Previous studies of *C. ruminantium* prevalence in *Amblyomma* spp. have utilized xenodiagnosis by feeding of ticks or by inoculation of homogenized tick material into susceptible small ruminants or mice. These have yielded prevalence estimates in the range of 0·2–4% in adult

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A. variegatum on the Caribbean island of Guadeloupe [9, 10], 1·1–1·2% in adult *A. variegatum* in Senegal [11, 12], and 0–45% in adults and 0–14% in nymphs of *A. hebraeum* from heartwater-endemic areas of South Africa and Zimbabwe [13–16]. The accuracy of these estimates is, however, uncertain because the reliability of xenodiagnostic tests has not been evaluated, small sample sizes were used and, in most cases, the ticks analysed had been feeding on animals for unknown periods of time. These ticks may have acquired or lost infection during feeding and were unlikely to have been representative of the host-seeking tick population. Better estimates of infection prevalence, as well as information on the intensity of infection in field vectors, for which no data are presently available, would substantially improve the accuracy of transmission dynamics models of *C. ruminantium* infection.

Highly sensitive and specific DNA-based detection assays have recently been developed and used to detect *C. ruminantium* in experimentally infected ticks [17–22]. Here we describe the application of these improved diagnostic tests to gain better estimates of the prevalence and intensity of *C. ruminantium* infection in field *Amblyomma* tick populations from heartwater-endemic regions of Zimbabwe.

MATERIALS AND METHODS

Study sites

Field collections of *A. hebraeum* ticks were undertaken on two heartwater-endemic large-scale cattle farms, located within the two main agro-ecological zones of Zimbabwe, highveld and lowveld [23, 24].

Lowveld site

This farm (20° 17′ S, 30° 11′ E; 840 m) lies within Natural Region IVb of the southern lowveld of Zimbabwe, which is characterized as a semi-arid, semi-intensive farming region with low and unreliable total rainfall (450–650 mm) [23]. Vegetation on the farm was mainly mopani (*Colophospermum mopani*) woodland on sandy soils. A herd of 1500 cross-bred cattle (*Bos indicus* × *Bos taurus*) consisting of approx. 350 calves, 800 sub-adults and 350 breeding cows, grazed freely in rotation through paddocks over a total area of 9900 ha. The cattle shared pasture with a breeding herd of approx. 200 Boer breed domestic goats, as well as a large number of wildlife species. Many of these were hosts of *A. hebraeum*, including bushbuck (*Tragephalus scriptus*; approx. 100), duiker (*Cephalophus* spp.; 250), impala (*Aepyceros melampus*; 500), kudu (*Tragephalus strepsiceros*; 300), steenbok (*Raphicerus campestris*; 100), warthog (*Phacochoerus aethiopicus*; 100) and numerous feral, lagomorph and avian hosts [25–27]. Tick control had been strategic for over 10 years and was limited to domestic stock which were spot-treated with a synthetic pyrethroid/amidine acaricidal spray at approx. 3 to 4 week intervals. Mortality due to heartwater in the cattle was rare (less than 1% per annum), despite high levels of *A. hebraeum* tick infestation (unpublished data). This suggested that endemic stability existed, i.e. low morbidity and mortality due to high infection pressure and consequent early infection during the periods of innate and maternally derived immunity [28].

Highveld site

This farm (18° 47′ S, 30° 40′ E; 1350 m) is situated in Natural Region IIIb on the central highveld plateau of Zimbabwe, an intensive crop and livestock producing region receiving 750–1000 mm of reliable rainfall per annum. Vegetation consisted of mainly *Acacia* and *Combretum* woodlands on clay soils, with grassland areas. A cross-bred cattle herd of 260 breeding cows, approx. 500 sub-adults and 200 calves were rotated through paddocks on 2000 ha. A breeding herd of approx. 100 cross-bred domestic goats grazed on one of the paddocks. Small herds of kudu, impala, duiker and warthog shared the paddocks with the cattle, in much lower abundance than on the lowveld site. Feral, lagomorph and avian tick hosts were common. Low intensity tick control had been practiced for 5 years and was restricted to occasional spot treatment of cattle with a synthetic pyrethroid spray. Ticks were not controlled on the goats and wildlife. Heartwater mortality was rare despite the high *A. hebraeum* tick levels (unpublished data), and endemic stability was considered to exist.

Tick collections

Tick collections were performed using sentinel cattle. Ten adult cows were randomly selected from herds maintained on each ranch and all adult *A. hebraeum* ticks were manually removed from these animals and discarded. Thereafter, all newly attached *A. hebraeum* adult male and female ticks were collected daily from the 10 cattle, between 6 and 8 am. Collected adult

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ticks were placed at 4 °C immediately after collection and stored for 3–14 days before analysis.

To collect nymphs, engorged larvae were detached daily from the cattle. The engorged larvae were kept at ambient temperature and relative humidity, and allowed to moult into the nymphal stage. Nymphs were identified as *A. hebraeum* and analysed within 1 month of moult.

Tick collections were conducted between February and May, 1996 at the lowveld site and from July to September, 1996 at the highveld site. At the lowveld site 526 adult ticks and 362 engorged larvae were collected. At the highveld site, 462 adult ticks and 298 engorged larvae were collected.

Isolation of *C. ruminantium* in culture

To confirm *C. ruminantium* infection in the study tick populations, 60 adult ticks (30 males and 30 females) from each site were fed on heartwater-naive sheep which were monitored for clinical signs of heartwater. Autopsies and brain smear examinations [29] were performed on sheep that died to determine the cause of death. Attempts were made to isolate *C. ruminantium* in bovine endothelial cell culture [30, 31] from plasma collected between the second and fourth days of the febrile reaction (rectal temperature > 40.5 °C). To confirm the identity of isolated agents, supernatant (2 ml) from a heavily infected (4+) culture of each isolate was inoculated intravenously into heartwater-naive sheep and *C. ruminantium* infection was confirmed by examination of brain smears prepared from biopsies [32] taken between the second and third day of the febrile reaction, or post mortem. Additionally, DNA of organisms harvested from infected cultures was analysed with the PCR and DNA probe assays, as described below, to further confirm *C. ruminantium* identity.

Prevalence and intensity of infections in ticks

Collected ticks were individually dissected and tested with specific polymerase chain reaction (PCR) and DNA probe assays based on the pCS20 DNA sequence of *C. ruminantium* [17–22]. The PCR assay, which is highly sensitive (detection limit: approx. 200 organisms per tick) but non-quantitative was used to estimate infection prevalence. The less sensitive and semi-quantitative DNA probe assay (detection limit: approx. 70000 organisms) was used to estimate the intensity of infection in each tick.

PCR analysis

DNA was extracted from the combined internal organs of each tick using QIA-amp DNA extraction tissue kits (Qiagen, Germany). Five microlitres (5%) of each adult tick DNA sample and 20 μl (20%) of each nymph sample were utilized as template in 50 μl PCR assays. The PCR assay was based on the primers AB 128 (5′-ACTAGTAGAAATTGCACAATCTAT-3′) and AB 129 (5′-TGATAACTTGGTGCGGGAA-ATCCTT-3′), which amplify a 279 bp fragment from within open reading frame 2 of the 1306 bp pCS20 DNA sequence. Reaction conditions for adult tick PCRs were as described previously [22], except that the primer concentrations were set at 0.3 μM each and the $MgCl_2$ concentration was 2.0 mM. For nymph PCRs, the concentrations were 0.5 μM and 2.0 mM, respectively. Additionally, initial denaturation of sample DNA for all PCRs was done by incubation of the reactions for 1 min at 94 °C in the PCR heating block immediately prior to the first cycle. Controls for each set of PCRs included negative reagent controls (no DNA), sample controls containing uninfected male, female or nymph *A. hebraeum* tick DNA, and positive reagent control reactions spiked with 0.1 ng of *C. ruminantium* DNA (homologous strain). Forty microlitres (80%) of each completed PCR were denatured with 0.4 N NaOH, dot-blotted onto nylon membranes and hybridized with the cloned pCS20 DNA sequence, random-primer labelled (Boehringer–Mannheim, South Africa) with [^{32}P]dCTP (Amersham, UK), as a probe. The hybridized blots were exposed to X-ray film (Kodak Biomax) for 1–7 days and infection within each tick determined by visual examination of hybridization signals on exposed films.

DNA probe analysis

Residual DNA from each tick sample was denatured with 0.4% NaOH, blotted onto nylon membranes and hybridized with the pCS20 DNA probe as above. Negative controls on each blot included DNA extracted from uninfected male, female or nymph *A. hebraeum* ticks. To provide a set of positive control standards, *C. ruminantium* organisms from bovine endothelial cell cultures were purified on percoll gradients and enumerated by staining of an aliquot with acridine orange [33]. Tenfold dilutions of *C.*

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ruminantium, 10^3–10^8/μl, were prepared in phosphate buffered saline. Ten microlitres of each dilution were added separately to individual, dissected tick tissue samples prior to DNA extraction, and these samples were processed normally. A set of these standards, containing DNA extracted from 10^9 to 10^4 organisms, were included on each blot. The 10^9 standard was used because this represented the upper limit of tick infection that has previously been observed in experimental studies [19–22]. The 10^4 standard was used because this is approx. tenfold lower than the detection limit of the DNA probe. Hence, these standards cover the full range of likely tick infection intensities that are detectable by DNA probe. Hybridized blots were exposed to X-ray film for 2–10 days and the level of *C. ruminantium* infection in each tick was estimated by comparison of the intensity of its hybridization with that of the positive standards.

Statistical analysis

Statistical analysis of prevalence of infection in ticks, by instar and site of collection, was by means of ordinary logistic regression using SAS Version 6.12 (Proc Logistic, SAS Institute Inc., Cary, NC, USA). Significance of covariates was assessed by means of change of deviance of nested models.

RESULTS

Isolation *C. ruminantium*

Ticks collected at both study sites transmitted *C. ruminantium* to the recipient sheep, which developed clinical signs of heartwater by 15 days after tick attachment and died after 3–5 days' febrile reactions. *Cowdria ruminantium* infection in both sheep was confirmed by autopsy and brain smear examination, and by isolation of the agent from plasma in culture. The sheep inoculated with infected culture supernatant from these isolates (Finale and Beatrice [34]) also developed heartwater and were positive for *C. ruminantium* infection by brain smears prepared at biopsy and post-mortem. Both the PCR and DNA probe assays detected *C. ruminantium* DNA prepared from these isolates.

Prevalence and intensity of *C. ruminantium* in ticks

Lowveld site

A total of 446 adult ticks, 184 males and 262 females, from the lowveld site were analysed by PCR and DNA probe (Table 1). The prevalence of infection, by PCR, in adult males and females was 8·2 and 13·4%, respectively ($P = 0.09$), yielding a combined prevalence of 11·2% (Table 1). The majority (78%) of tick infections were below the detection limit of the DNA probe. However, the intensity of infection within the 11 DNA probe-positive ticks was high. Eight ticks (73%) carried infections of 10^7–10^9 *C. ruminantium* (Table 1), and the modal level of infection was 10^7 organisms. On the basis of proportion of infections which were DNA probe positive, infected male ticks may have had slightly, but non-significantly higher ($P = 0·13$) burdens of infection than female ticks. The DNA probe detected infection in 22% of PCR-positive ticks, i.e. in 40 and 14% of the infected male and female ticks, respectively.

Two hundred and seventy-one nymphs were available for analysis after moult. The prevalence of infection by PCR in nymphs (8·5%, Table 1) was similar to that of the adult ticks ($P = 0·24$). Only two ticks (9%) were positive by DNA probe and both carried infections in the range of 10^5–10^6 organisms. One of these probe-positive nymphs, bearing 10^5 organisms, was negative by PCR.

Highveld site

Three hundred and ninety-two adult ticks from the highveld site, 237 males and 155 females, were analysed. Although the overall infection prevalence by PCR was 10·2% (Table 1), the prevalence in male ticks (13·1%) was higher ($P < 0·01$) than in female ticks (5·8%), though the proportion of infected ticks carrying DNA probe-detectable infections was similar in both sexes (25·0 *vs.* 20·0% respectively; $P = 0·44$). The intensity of infection in the DNA probe positive adult ticks was high, 70% carried 10^7–10^9 organisms. Two DNA probe positive ticks, one male and one female, each carrying 10^7 organisms, were negative by PCR. Nymphs collected from this site failed to moult successfully and were not analysed for infection.

Overall

The prevalences of infection within male and female ticks at the lowveld site and male ticks at the highveld site were not significantly different ($P = 0·17$), however, the prevalence of infection in female ticks at the highveld site was significantly lower ($P = 0·03$). Nevertheless, when contrasting adult ticks (male + female combined) by site, there was no significant difference in overall prevalence ($P = 0·64$), nor pro-

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Table 1. *Prevalence and intensity of* Cowdria ruminantium *infection in* Amblyomma hebraeum *ticks collected in heartwater endemic areas of the lowveld and highveld of Zimbabwe*

Site	Instar	*n*	PCR % + ve	DNA probe % + ve	Intensity of infection (organisms per tick)					
					10^9	10^8	10^7	10^6	10^5	$< 10^5$
Lowveld	Adult female	262	13·4	1·9	0	0	4	1	0	30
	Adult male	184	8·2	2·7	1	2	1	1	1	9
	Total adults	446	11·2	2·2	1	2	5	2	1	39
	Nymphs	271	8·5	0·7	0	0	0	1	1	21
Highveld	Adult female	155	5·8	1·3	0	0	2	0	0	8
	Adult male	237	13·1	3·4	1	0	4	3	0	24
	Total	392	10·2	2·5	1	0	6	3	0	32

portion of probe-detectable infections ($P = 0{\cdot}57$) between the highveld and lowveld.

DISCUSSION

This study presents the first PCR-based estimates of *C. ruminantium* prevalence in adult and nymph instars of *A. hebraeum*, the major vector of heartwater in southern Africa. These improve on previous estimates based on xenodiagnosis through the use of an assay of known reliability and statistically adequate samples. Our prevalence estimates for adult ticks fall within the wide range found for *A. hebraeum* in heartwater-endemic regions of Zimbabwe (0–45 % [16]) and are higher than those determined previously for *A. variegatum*. Although differences in infection prevalence were noted between adult males and females at both sites, the absolute magnitudes by gender were reversed, such that the overall adult prevalence and intensity of infection by site appeared to be virtually identical. To what the reduced infection prevalence observed in females from the highveld site can be attributed is open to speculation but may include temporal or spatial factors.

The prevalence within nymphs estimated here (8.5 %) is similar to that observed previously (0–14 % [16]) and is high in relation to the adult ticks, particularly as infections acquired during the larva-nymph feed are carried through to the adult stage. This is likely because nymphs were collected as engorged larvae from cattle which were almost certainly infected carriers [28]. Free-living nymphs are likely to have lower infection rates because larvae feed on a wide range of hosts [25, 27], including those not susceptible to *C. ruminantium*. Transovarial transmission of *C. ruminantium* has not been demonstrated in *A. hebreaum*, but has been shown experimentally to be occur in the closely related *A. variegatum* [35]. However, its occurrence is rare and, if present in *A. hebraeum*, it is unlikely to have contributed significantly to infection within the ticks.

Both sites supported multiple hosts for *A. hebraeum*, domestic and wild, which may have been sources of *C. ruminantium* infection. Cattle were probably a dominant source, due to their greater abundance and high tick burdens relative to most wild animals (unpublished observations). Most cattle in endemically stable areas are sub-clinically but persistently infected [28] and infection in the collected ticks is likely to reflect carrier infectivity. The relative contribution of wildlife is difficult to assess as immature *A. hebraeum* feed on a range of wildlife, including rodents, reptiles, birds, lagomorphs, suids and large vertebrate species [25–27], many of which are not known to be susceptible to *C. ruminantium*. Recent comparative studies of tick infection in an purely wildlife area suggest that wildlife may be a substantially smaller reservoir of infection than cattle [36].

This study also provides the first estimates of the intensity of *C. ruminantium* infection in field vector ticks. The majority of infections were below the DNA probe detection limit, but a few infections contained large numbers of rickettsia. It is likely that this was the result of a highly skewed distribution of infection intensities, but as the intensities of infections below 70000 organisms were not measured, this distribution cannot be analysed further. The majority (70 %) of adult ticks at both sites that were DNA probe-positive carried very high levels of infection, similar to those acquired experimentally on clinically-ill hosts, typically 10^7–10^9organisms per tick in 40–100 % of ticks [18–21]. Infection intensities in nymphs were lower; this is probably due simply to differences in size between the two instars. Heavy infections with *C.*

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ruminantium in ticks may have been acquired on the small proportion of animals in endemic areas undergoing primary infection, or on recovered animals undergoing a recrudescence of rickettsemia due, potentially, to super-infection, fluctuations in antigenic sub-types or reduced immunity. Alternatively, heterogeneity of infection intensity may be a feature of ticks fed simultaneously on carriers. The implications of these aspects to heartwater epidemiology have not been determined, although sequelae of infection may be, at least to some extent, determined by the dose of infection, such that the severity of morbidity and proportional mortality increase with size of dose. This effect has been shown to be important for understanding epidemiological patterns [28]. Further studies are needed to quantify the dose/disease severity relationship and its role in determining the transmission dynamics of *C. ruminantium*.

PCR has been used previously to detect natural infections of pathogens in tick and insect vectors [37–39]. Disadvantages of the use of PCR on haematophagous vectors include the risk of detecting non-viable organisms residual from previous bloodmeals or aborted infections. This was unlikely here as *A. hebraeum* takes a single bloodmeal between moults and organisms that fail to establish infection are probably digested during moult. The frequency of PCR-negative, probe-positive samples in this study was low, but not unexpected given the less that 100% reliability of these assays [22].

The use of sentinel cattle for tick collections, while laborious, was necessary because initial attempts to collect free-living stages of ticks with pheromone traps [40] were unsuccessful. However, this method provided epidemiologically relevant specimens, i.e. those ticks that have successfully attached to hosts. The acquisition of detectable infections by ticks from the sentinel animals was improbable due to the low rickettsemia of carrier cattle (which predominate in endemic areas) and short period of attachment (0–24 h) prior to collection. The storage of the ticks at 4 °C immediately after collection would have further restricted multiplication of recently ingested organisms to detectable levels. It is also unlikely that the ticks discharged their entire infections during their short attachment to the sentinel hosts as *A. hebraeum* ticks maintain infection with *C. ruminantium* over several days of feeding [41].

Accurate estimates of tick infection prevalence are essential in the development and validation of models of heartwater disease transmission dynamics [28]. These models can be used to evaluate the effectiveness of different disease control strategies, such as vaccination and acaricide treatment, and assist in the formulation of cost-effective control programmes [42, 43]. This study demonstrates that the PCR assay is an effective tool for determining *C. ruminantium* infection prevalence. Future use of this assay should focus on its application to different *Amblyomma* species and in different ecological, geographic and livestock management systems.

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