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Laboratory reared *Amblyomma hebraeum* and *Amblyomma variegatum* ticks differ in their susceptibility to infection with *Cowdria ruminantium*

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SUMMARY

The susceptibility of laboratory reared Zimbabwean *Amblyomma hebraeum* and *A. variegatum* ticks to infection with geographically distinct *Cowdria ruminantium* strains was investigated by feeding both species simultaneously on individual sheep infected with one of the four strains (Crystal Springs [Zimbabwe]. Ball 3 [South Africa]. Gardel [Guadeloupe] and Nigeria [Nigeria]). *A. hebraeum* ticks demonstrated a high susceptibility to infection with all four *C. ruminantium* strains. In comparison. *A. variegatum* were less susceptible to infection with the Crystal Springs and Ball 3 strains ($P < 0.001$). but showed a similar susceptibility to the Gardel and Nigeria strains. The differences in susceptibility of *A. variegatum* to infection with the four strains of *C. ruminantium* correlated with the origin of these strains. The consistently higher susceptibility of *A. hebraeum* ticks to infection with geographically different *C. ruminantium* strains may be one explanation for the observation that heartwater is a more serious problem where *A. hebraeum* is the vector of the disease.

INTRODUCTION

Heartwater (or cowdriosis) is an economically important tick transmitted disease of livestock prevalent in sub-Saharan Africa and on three Caribbean islands [1–3]. The causative agent *Cowdria ruminantium*, a rickettsia, is transmitted by ticks of the genus *Amblyomma* following a developmental cycle within the tick [3]. Acquisition and transmission of *C. ruminantium* infection can occur trans-stadially or intra-stadially in the different *Amblyomma* tick species and stages [4]. indicating inherent differences in their physiological interactions with these organisms. *A. hebraeum* and *A. variegatum* are recognized as the major vectors of heartwater [3.5]. *A. variegatum* is widespread in sub-Saharan Africa and in eastern Caribbean [1–3]. but it does not occur in most of southern Africa, where *A. hebraeum* is mainly responsible for the transmission of heartwater [3–5]. *A. variegatum* has traditionally been considered the superior vector of heartwater

mainly because of its distribution [3, 4, 6]. However, the observation that the disease causes more severe losses when its transmission is associated with *A. hebraeum*, has led to the belief that *A. hebraeum* may have a greater capacity to acquire and transmit heartwater [5, 7, 8]. In addition, studies on infection rates of field ticks have demonstrated higher rates in *A. hebraeum* than in *A. variegatum* [6, 9–12]. To understand the reasons for these differences, we conducted a study to compare the susceptibility of *A. hebraeum* and *A. variegatum* to infection with four geographically disparate *C. ruminantium* strains.

MATERIALS AND METHODS

C. ruminantium *infections of sheep*

Four 6–8 month old Merino sheep (numbers: 4943, 4946, 4947, 4962) were obtained from the heartwater free Veterinary Research Laboratory Field Station in Mazowe, Zimbabwe. Each sheep was inoculated with 5 mls of infected sheep blood stabilate of one of four *C. ruminantium* strains, which had all been previously tested and shown to cause acute heartwater (data not shown). The strains used were Crystal Springs from Zimbabwe (sheep 4946; [13]), Ball 3 from South Africa (sheep 4943; [14]) Gardel from Gaudeloupe (sheep 4962; [15]), and Nigeria from Nigeria (sheep 4947; [16]). The inoculation dose of 5 mls for each strain was chosen as it resulted in similar pre-patent periods and clinical responses. This allowed us to synchronize the tick feeding on the four sheep.

The rectal temperature of each sheep was recorded daily and on the third day of the febrile reaction (when the rectal temperature was > 40·5 °C) a brain biopsy [17] was performed for confirmation of *C. ruminantium* infection. In case of death, *C. ruminantium* infection was confirmed by post mortem examination and by examination of Giemsa stained brain crush smears [18].

Tick feeding and handling

Two hundred laboratory reared, uninfected nymphs of the Zimbabwean *A. hebraeum* (Sengwe strain from the lowveld of Zimbabwe, grid reference UL 0545) and *A. variegatum* (Trafalgar strain from Kadoma, grid reference QK 9642) were applied daily in separate body bags on each sheep starting on the sixth and ending on the tenth day after infection. The ticks of both species fed for equivalent periods of time and reached repletion either 1 or 2 days before the febrile reaction (i.e. the pre-febrile period,) or during the febrile reaction of each sheep. Ticks of each species were collected daily as they reached repletion and were stored separately at 27 °C and 75 % relative humidity to allow molting to occur.

Analysis of tick infection

Tick infection rates and levels were analysed in those ticks which reached repletion either during the pre-febrile or the febrile period of the four sheep infected with the different *C. ruminantium* strains. Approximately equal numbers of *A. hebraeum* and *A. variegatum* were analysed according to their repletion dates, and wherever possible equal numbers of male and female ticks were analysed.

Ticks were cut open at the posterior end and the organs within extruded with a scalpel blade and placed in an autoclaved eppendorf tube. DNA was extracted collectively from the extruded organs, which comprised essentially of the midgut and salivary glands of each tick [19–21], and dot-blotted onto nylon membranes

(Genescreen, Du Pont). Infection within each tick was detected by hybridization of the *C. ruminantium*-specific radiolabelled pCS20 DNA probe to individual tick DNA samples by methods described previously [20–22]. Positive control Crystal Springs genomic DNA (100, 10, 1, 0·1 nanograms), and negative control DNA from uninfected *A. hebraeum* and *A. variegatum* ticks (one male and one female from each species) were also included on each blot. Tick infection rates were evaluated by visual enumeration of positive hybridizations following autoradiography, and were expressed as a percentage of the total tick numbers analysed. These values were then analysed statistically by application of a 2×2 χ^2-test.

RESULTS

Clinical reactions of sheep

The febrile reaction started on day 13 after infection in all sheep and lasted up to 4 days. Sheep 4947 infected with the Nigeria strain died of heartwater on day 15 post-infection (on the second day of febrile reaction), and this was confirmed by post mortem examination and by positive identification of *C. ruminantium* colonies in the brain endothelium. Brain biopsies on the third day of the febrile reaction confirmed heartwater infection in the remaining sheep.

Comparison of susceptibility of A. hebraeum *and* A. variegatum *ticks to infection with four strains of* C. ruminantium

A. hebraeum ticks demonstrated high infection rates with all four strains of *C. ruminantium* (Table 1; and data for Crystal Springs and Gardel strains shown as a sample of the analysis in Figures 1 and 2). *A. hebraeum* ticks (male and female ticks combined), that reached repletion during the pre-febrile and febrile periods caused by Crystal Springs, Gardel, Ball 3 and Nigeria strains developed infection rates of 80%, 48% 34%, 59% (pre-febrile) and 90%, 79%, 52% and 47% (febrile) respectively. In comparison, *A. variegatum* ticks developed infection rates of 4%. 50%, 0%. 48% (pre-febrile) and 5%, 91%, 0% and 53% (febrile) respectively (Table 1). This data demonstrates that the *A. variegatum* ticks were significantly less susceptible than *A. hebraeum* to infection with the Crystal Springs and Ball 3 strains ($P < 0.001$), but were equally susceptible to infection with the Gardel and Nigeria strains. However, male *A. variegatum* ticks from the febrile stage were more susceptible to infection with the Gardel strain than males of *A. hebraeum* ticks ($P < 0.05$), although the analysis of male and female ticks combined showed no significant difference. With the exception of ticks infected with the Gardel strain ($P < 0.001$), there was no significant difference between the infection rates detected in *A. hebraeum* and *A. variegatum* ticks that reached repletion during the pre-febrile and the febrile periods of infection (Table 1).

The level of infection (based on the size and intensity of the hybridization signal) varied within each tick species, suggesting differential development or uptake of *C. ruminantium* in or by the ticks. Although not quantitated, the level of *C. ruminantium* infection (with Crystal Springs and Gardel strains), appeared to be higher in both tick species that reached repletion during the febrile period in comparison to ticks from the pre-febrile period (Fig. 1 and 2). In contrast, by the same criteria, the infection levels of the two tick species (from the pre-febrile and the febrile period). infected with the Ball 3 and Nigeria strains did not differ (data not shown).

Table 1. *Infection rates in* A. hebraeum *and* A. variegatum *ticks following feeding on sheep infected with one of four* Cowdria ruminantium *strains*

C. ruminantium strains	Infection in A. hebraeum (%)						Infection in A. variegatum (%)					
	Pre-febrile			Febrile			Pre-febrile			Febrile		
	♂	♀	Overall	♂	♀	Overall	♂	♀	Overall	♂	♀	Overall
Crystal Springs (Sheep 4946)	75 (15/20)*	84 (21/24)	80 (44)†	95 (18/19)*	87 (20/23)	90 (42)†	0 (0/4)*	5 (1/19)	4 (23)†	10 (2/21)*	0 (0/21)	5 (42)†
Gardel (Sheep 4962)	38 (9/24)	50 (10/20)	48 (44)	68 (15/22)	90 (18/20)	79 (42)	55 (6/11)	48 (12/25)	50 (36)	100 (21/21)	83 (19/23)	91 (44)
Ball 3 (Sheep 4943)	48 (11/23)	13 (2/15)	34 (38)	54 (22/41)	33 (1/3)	52 (44)	0 (0/20)	0 (0/20)	0 (40)	0 (0/23)	0 (0/5)	0 (28)
Nigeria (Sheep 4947)	52 (11/21)	65 (15/23)	59 (44)	43 (16/37)	67 (4/6)	47 (43)	50 (11/22)	45 (9/20)	48 (42)	52 (12/23)	55 (12/22)	53 (45)

* (15/20) means that 15 out of 20 ticks were positive for *C. ruminantium* infection by DNA probe hybridization.
† The total number of ticks analysed (i.e., males (♂) and females (♀) combined).
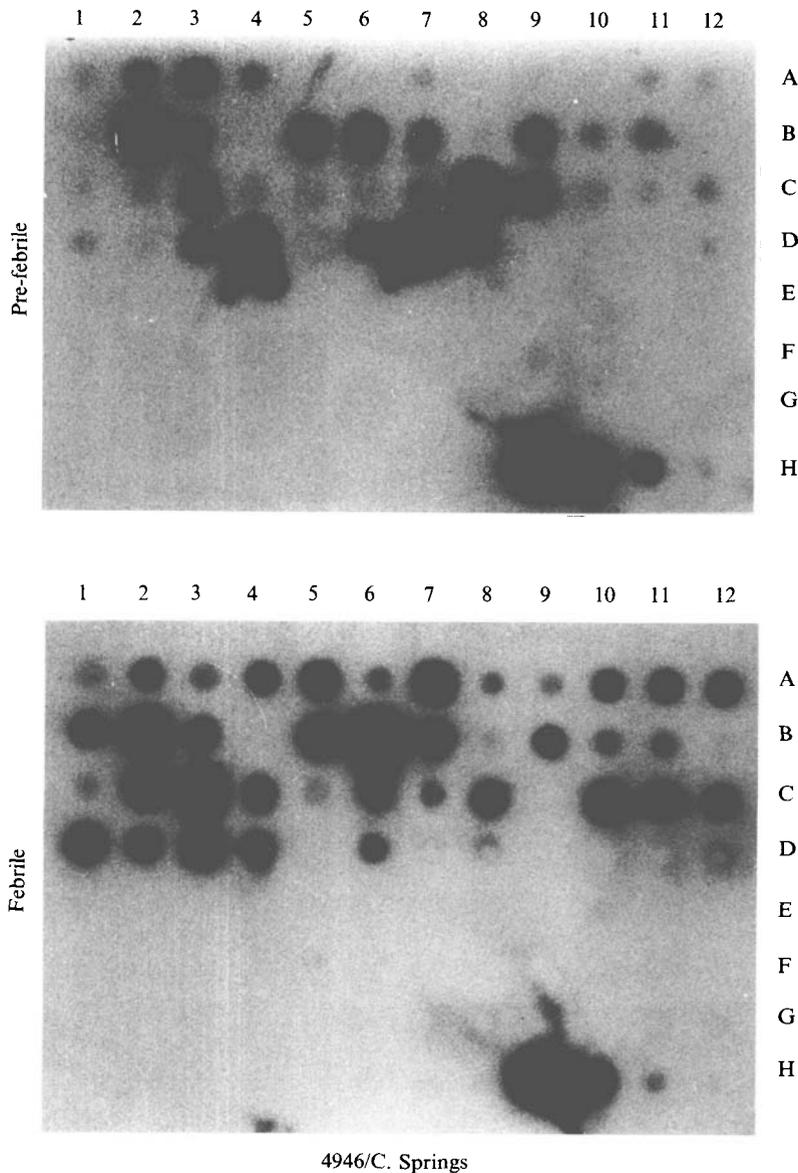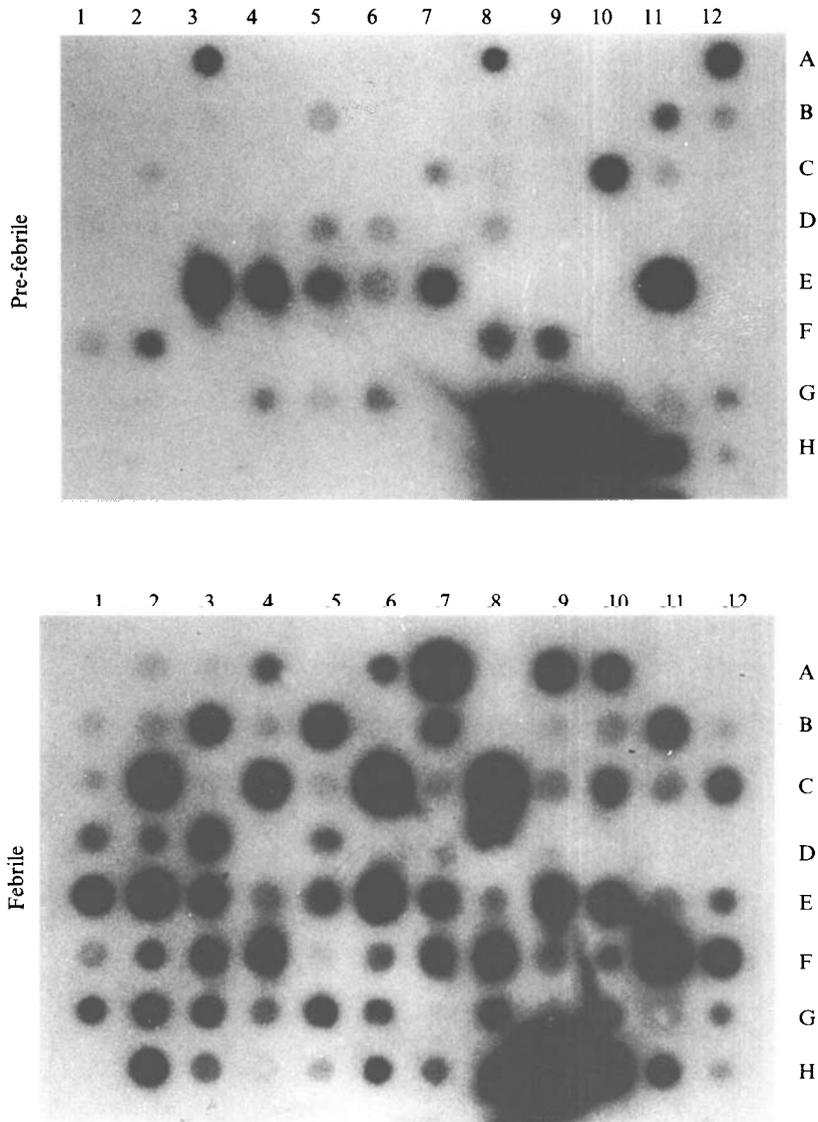
4946/C. Springs

Fig. 1. Hybridization of pCS20 DNA probe to DNA samples form Crystal Springs infected *Amblyomma* ticks fed on sheep 4946. The results of these analyses demonstrate a higher susceptibility of *A. hebraeum* to infection with Crystal Springs strain.

Pre-febrile tricks: Row A lane 1 to Row B lane 8 are infected male and Row B lane 9 to Row D lane 8 are infected female *A. hebraeum* ticks. Row D lanes 9 and 10, 11 and 12 are two uninfected *A. hebraeum* and *A. veriegatum* tick DNA samples, respectively. Row E lane 1 is DNA sample from an infected *A. variegatum* male tick; Row E lane 2 to Row F lane 11 are infected *A. variegatum* female tick samples. Positive control Crystal Springs DNA 100, 10, 1 and 0·1 nanogram samples are in Row H lanes 9 to 12.

Febrile ticks: Row A lane 1 to Row B lane 7 are infected male and Row B lane 8 to Row D lane 6 are infected female *A. hebraeum* tick DNA samples. Row D lanes 9 and 10 and 11 and 12 are uninfected *A. hebraeum* and *A. variegatum* ticks. Row E lane 1 to Row F lane 9 and Row F lane 10 to Row H lane 6 are infected male and female *A. variegatum* ticks samples respectively. Positive control Crystal Springs DNA 100, 10, 1 and 0.1 nanogram samples are in Row H lanes 9–12.

Fig. 2. Hybridization of pCS20 DNA probe to DNA sample from Gradel infected *Amblyomma* ticks fed on sheep 4962. The results of these analysis demonstrate that the Gardel strain of *C. ruminantium* causes high infection rates in both tick species.

Pre-febrile ticks: Rows A and B are infected male and Row C lane 1 to Row D lane 8 are infected female *A. hebraeum* tick samples. Row D lanes 9 and 10. 11 and 12 are uninfected *A. hebraeum* and *A. variegatum*.

Febrile ticks: Row A lane 1 to Row B lane 10 are infected male and Row B lane 11 to Row D lane 6 are infected female *A. hebraeum* tick DNA samples. Row D lanes 9 and 10, 11 and 12 are uninfected *A. hebraeum* and *A. variegatum* ticks respectively. Row E lane 1 to Row F lane 9 are infected male and Row F lane 10 to Row H lane 8 are infected female *A. variegatum* ticks samples respectively. Positive control Crystal Springs DNA 100, 10, 1 and 0·1 nanogram samples are in Row H lanes 9–12.

DISCUSSION

The results of this study provide preliminary evidence on the susceptibility differences between two major species of *Amblyomma* ticks to infection with *C. ruminantium*. This could have important implications on the epidemiology of heartwater if proven that this phenomenon also exists with freshly isolated field ticks. The study demonstrates that laboratory reared *A. hebraeum* ticks were highly susceptible to infection with all four *C. ruminantium* strains of disparate geographic origin, whereas in comparison, the laboratory reared *A. variegatum* ticks are less susceptible to infection with the Crystal Springs and Ball 3 strains, but were of equal susceptibility to the Gardel and Nigeria strains. It is noteworthy that the Gardel and Nigeria strains originate from areas where *A. variegatum* is the major vector of heartwater [3, 6, 9, 23], whereas the Crystal Springs and Ball 3 strains originate from areas where *A. hebraeum* is the primary vector [4, 5].

The differences in susceptibility to infection between the two tick species were not due to differences in their respective feeding periods as the comparisons described here have been made on ticks feeding over the same period and detaching at the same time from the infected sheep. The susceptibility differences between *A. variegatum* and *A. hebraeum* ticks could be explained by their respective physiological compatibilities with the four strains of *C. ruminantium*. *A. variegatum* ticks appear to demonstrate poor compatibility with those *C. ruminantium* strains which do not originate from regions where *A. variegatum* is the major vector of heartwater. In contrast, *A. hebraeum* ticks are physiologically compatible not only with the Crystal Springs and the Ball 3 strains, but also with the Gardel and Nigeria strains. These results indicate a superior vector efficiency of *A. hebraeum*. A similar phenomenon has been described for *Theileria parva* infections in *Rhipicephalus* ticks [24], where the competence of *R. zambeziensis* ticks to transmit buffalo-derived *T. parva* from South Africa or cattle-derived *T. parva* from Zimbabwe was better than that of *R. appendiculatus* [24]. These differences may have important implications on the epidemiology of theileriosis since ticks may be capable of selecting parasite strains in an area. Based on the observations made in this study using the laboratory reared ticks, a similar situation may also exist with *Amblyomma* ticks, although the respective transmissibility of the strains of *C. ruminantium* was not compared in this study.

The phenomenon of physiological adaptation has also been described for *Glossina* flies and infection with two strains of *Trypanosoma congolense*. Two strains of this trypanosome were found to be more infective for flies of one geographical area of Kenya than for flies of another area [25]. Differences in infection were also noted to exist between male and female flies. In the present study, infection rate differences between male and female ticks of the two species infected with any of the *C. ruminantium* strains were not statistically significant.

Studies on *Anaplasma marginale* infections demonstrated that high rickettsemia levels in recovered animals caused higher infection rates in *Dermacenter andersoni* ticks [26]. The *C. ruminantium* infection rates of the ticks that reached repletion during the febrile period were predicted to be higher than those of ticks of the pre-febrile period, because rickettsemia is highest during the febrile period [4, 22, 27], and is believed to cause higher infection rates in ticks [4]. The reason for the lack of difference between the infection rates of ticks of the pre-febrile and febrile

period may have been due to the fact that the rickettsemia levels were adequate during the pre-febrile period to result in high tick infection rates.

Our results demonstrate that the levels of *C. ruminantium* infection in the two tick species varied with the infecting *C. ruminantium* strain. Visually the levels of infection were greater in those ticks that reached repletion during the febrile period of Crystal Springs and Gardel infection, although there appeared to be no difference between the ticks of the pre- and the febrile phases of infection with the Ball 3 and Nigeria strains. This may be explained by the differences in rickettsemia caused by the infecting *C. ruminantium* strain or by individual tick uptake variations or by individual animal differences [22], although the breed and age of sheep used was the same.

Field observations have suggested that *A. hebraeum* may be a more efficient vector of heartwater than *A. variegatum* [5, 7, 8]. This is supported by the data presented here which demonstrates that *A. hebraeum* ticks have a higher susceptibility to a wider range of *C. ruminantium* strains than *A. variegatum*. This may also be one reason for the observed higher infection rates in *A. hebraeum* field ticks in Zimbabwe [12].

The data presented here are a preliminary indication that differences of susceptibility to infection with *C. ruminantium* may also exist in *A. hebraeum* and *A. variegatum* ticks in the field. To examine if the susceptibility differences between the two tick species are universal, further studies need to be conducted which would incorporate more *C. ruminantium* strains and *Amblyomma* ticks (both laboratory reared and from the field) from other areas of Africa and the Caribbean. In addition, a study to compare the transmission of heartwater by these two vector species would be of importance. Such studies would improve our understanding of the epidemiology of heartwater.

ACKNOWLEDGEMENTS

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