

## Detection of *Cowdria ruminantium* by means of a DNA probe, pCS20 in infected bont ticks, *Amblyomma hebraeum*, the major vector of heartwater in Southern Africa

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### SUMMARY

A DNA probe, pCS20, previously described for use in detection of *Cowdria ruminantium* infections in *Amblyomma variegatum* (the principal vector of heartwater) hybridized with *C. ruminantium* DNA in organs of laboratory-infected *A. hebraeum* adult ticks (the major southern African vector of heartwater). The probe hybridized with *C. ruminantium* DNA in 46/49 midguts from male ticks and 26/29 from females, thus indicating infection. Corresponding salivary glands were less heavily infected, but infections were more numerous in glands from males. Infection in ticks was confirmed by transmission of the disease to susceptible goats. The probe did not hybridize with DNA from uninfected ticks or with DNA from a spotted fever group rickettsia commonly associated with *A. hebraeum* in Zimbabwe. The *C. ruminantium* specific pCS20 DNA probe can be applied to determine accurately the infection rates in the two major vectors of heartwater and the risk of exposure of ruminants in endemic areas.

### INTRODUCTION

Heartwater (cowdriosis), the most important rickettsial disease of domestic ruminants in Africa [1], is caused by *Cowdria ruminantium* and transmitted by at least 12 species of *Amblyomma* ticks [2]. Acute heartwater disease, with high

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mortality in susceptible livestock, is common in endemic areas and wild ruminants may also be affected [3, 4].

The distribution of heartwater is not limited to the African mainland, but includes certain associated islands in the Indian Ocean and the Gulf of Guinea [5]. The disease was introduced into the New World over 150 years ago, probably with tick-infested cattle imported into Guadeloupe from Senegal [6, 7]. Heartwater is now endemic on three Caribbean islands and poses a threat to livestock and wildlife of North, Central and South America, where potential vectors are known to exist [8]. The wide distribution of the disease is largely a reflection of the distribution of its principal vector, the tropical bont tick, *A. variegatum* (Fabricius). However, most of southern Africa is free of this tick; here, in areas where heartwater is endemic, transmission is usually by the bont tick, *A. hebraeum* Koch [2].

An understanding of the natural history and epidemiology of heartwater is basic to development of control strategies, and depends in part on the ability to demonstrate the organism in large numbers of suspected vector ticks. Until recently, this was done by either transmission studies [9], or electron and light microscopy [10, 11], but each method has disadvantages [12]. Recently, two nucleic acid probes, pCR9 and pCS20, were developed from genomic DNA libraries of *C. ruminantium* of the Kiswani isolate from Kenya and the Crystal Springs isolate from Zimbabwe, respectively, and shown to be effective at detecting *C. ruminantium* DNA in *A. variegatum* ticks [12]. One of these, pCS20, which is superior to pCR9 in specificity, was used in the present study to determine its applicability to the detection of infection in midguts and salivary glands of adult *A. hebraeum* ticks.

#### MATERIALS AND METHODS

##### *Heartwater infection of sheep and transmission to A. hebraeum ticks*

The ticks used in this study were from a heartwater-free laboratory colony maintained at the Veterinary Research Laboratory, Harare, Zimbabwe. They were fed as larvae on rabbits and held at 27 °C and 80% relative humidity for moulting. Nymphal ticks were divided into two groups and each was confined within a cloth bag glued to the clipped dorsal surface of two Dorper sheep. The first group was placed on sheep No. 208 which, 8 days previously, had been inoculated intravenously (i.v.) with 5 ml of a blood stabilate of the Crystal Springs isolate of *C. ruminantium* [13]. The animal was monitored daily for clinical signs of heartwater which was confirmed by brain-crush smear at death [14] and by autopsy findings. The second group, were used as a control to feed on an uninfected sheep No. 195. The two sheep were housed in separate stalls, each surrounded by a moat. Replete ticks were collected from both sheep after they had detached and were held as described above for moulting.

Approximately 5.5 months after detaching from their hosts as nymphs, the adult ticks were prepared for dissection by surface-sterilization with 70% ethanol. For one experiment, 49 male and 29 female ticks from sheep No. 208 and 30 of each sex from sheep No. 195 were dissected. Viscera were removed in Hanks' balanced salt solution. Salivary glands and midguts were isolated and washed

twice in this solution, transferred to separate 1.5 ml Eppendorf tubes, and frozen at  $-80^{\circ}\text{C}$ .

A second experiment was performed on the remaining ticks of this study after they had been subjected to heat shock, which is known to induce development of certain haemoparasites in tick salivary glands [15]. Following incubation at  $37^{\circ}\text{C}$  for 3 days, midguts and salivary glands were prepared as above from 35 males and 14 females fed as nymphs on sheep No. 208.

To determine whether the emergent adult ticks that had been fed as nymphs on sheep Nos. 208 and 195 had been infected with *C. ruminantium*, samples of 10 ticks of each sex were fed on susceptible Boer goats to transmit heartwater.

#### *Nucleic acid extraction*

This was done by a modification of our published method [12]. Briefly, midguts and salivary glands from each tick were subjected to two cycles of freeze–thawing, after which  $200\ \mu\text{l}$  of buffer ( $0.1\ \text{M}$  Tris-HCl at pH 7.5,  $0.15\ \text{M}$ -NaCl,  $12\ \text{mM}$ -EDTA, 1% SDS) were added to each tube. Ten  $\mu\text{l}$  of a solution of lysozyme dissolved in excess in saline-EDTA ( $0.15\ \text{M}$ -NaCl,  $12\ \text{mM}$ -EDTA) were added and samples were incubated for 30 min at  $37^{\circ}\text{C}$ . The tissues were further digested by addition of  $100\ \mu\text{g}/\text{ml}$  proteinase-K and incubation at  $37^{\circ}\text{C}$  overnight. Undissolved fragments of tissue were pelleted by brief centrifugation in a microcentrifuge (No. 235C, Fisher Sci., Pittsburgh, PA, USA) and removed by suction. The DNA was then extracted by standard methods [16], denatured with  $0.4\ \text{M}$ -NaOH and blotted onto nylon membranes using a Hybridot apparatus (No. 1050MM, Bethesda Research Labs., Gaithersburg, MD, USA). The blotted DNA samples were cross-linked to the membranes with u.v. light and the membranes were allowed to dry before use in hybridization assays.

DNA extracted from *C. ruminantium* (Crystal Springs strain) [17] propagated in bovine vascular endothelial cells [18] was applied to the nylon membranes in three dilutions (100, 10 and 1 ng) as positive controls. DNA was also extracted from a rickettsia Z3-Ah, of the spotted fever group (SFG), and applied to the membranes in three dilutions of 100, 10 and 1 ng. The latter organism is serologically related to *Rickettsia conorii* [19], is commonly found in *A. hebraeum* ticks in Zimbabwe and its DNA was included as a specificity control.

#### *The pCS20 DNA probe*

The development and characteristics of this probe have been described earlier [12, 17]. The probe was prepared for use by digesting the plasmid DNA with *Xba* I and *Kpn* I restriction endonucleases, and the insert (1306 bp long), was isolated from low melting point agarose gels [16]. Gel-purified probe (100 ng) was labelled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  by the random primer extension method (Boehringer-Mannheim Corporation) [16] for hybridization to DNA blots.

#### *Hybridization of pCS20 DNA probe to DNA blots*

The protocol used for the hybridization of the pCS20 DNA probe to DNA blots has been described previously [17]. The DNA-blotted nylon membranes were prehybridized overnight at  $42^{\circ}\text{C}$  in a hybridization buffer containing 10%

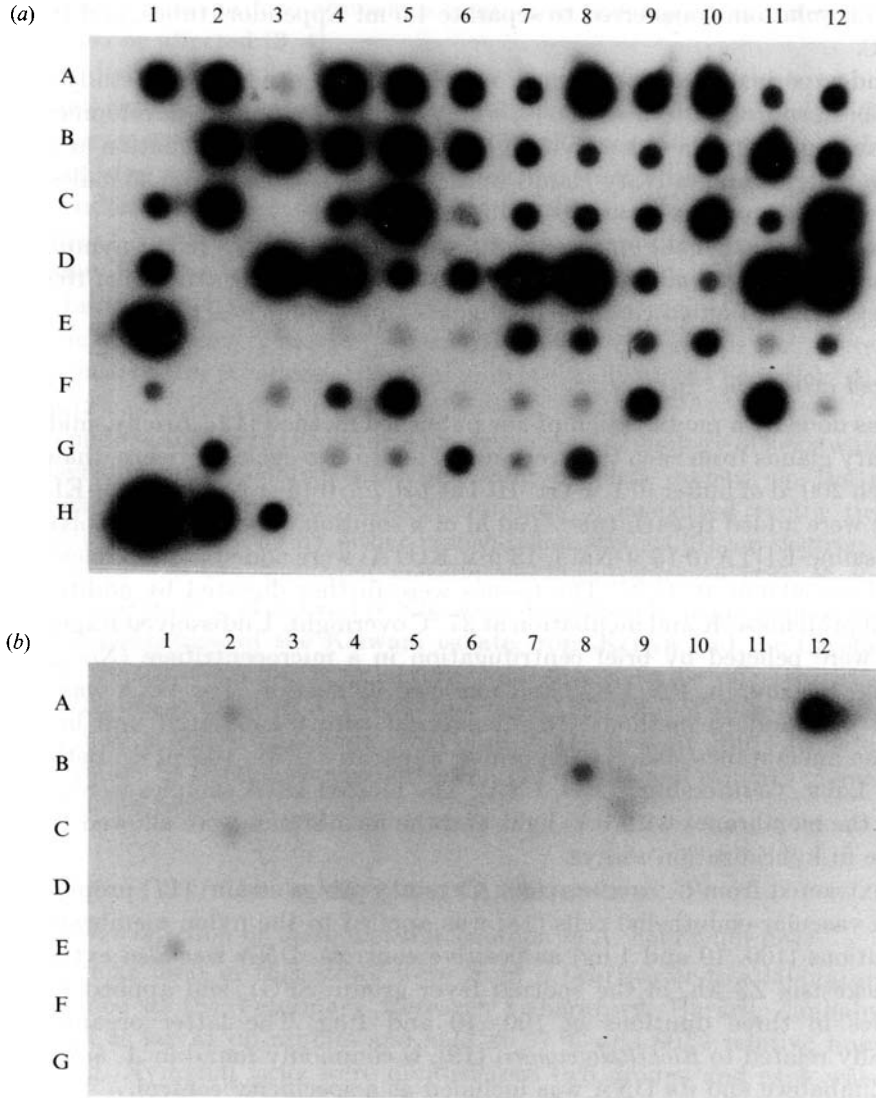


Fig. 1. (a) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 49 male (rows A1-E1) and 29 female midgut DNA samples (rows E3-G8) from infected *A. hebraeum* ticks. Row H1-3 shows Crystal Springs DNA samples (100, 10 and 1 ng), included as positive control samples. (b) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 49 male (rows A1-E1) and 29 female salivary gland DNA samples (rows E3-G8) from infected *A. hebraeum* ticks.

dextran sulphate in formamide, and then hybridized to the [ $\alpha$ - $^{32}\text{P}$ ]dCTP-labelled pCS20 DNA probe for 16 h. at 42 °C. The blots were washed under highly stringent conditions which consisted of four washes at room temperature of 15 min each: 1 wash in  $2 \times \text{SSC}$ ; 1 wash in  $2 \times \text{SSC}$ , 0.1% SDS; 1 wash in  $0.5 \times \text{SSC}$ , 0.1% SDS; 1 wash in  $0.1 \times \text{SSC}$ , 0.1% SDS. A final wash at 60 °C for 30 min was conducted in  $0.1 \times \text{SSC}$ , 1.0% SDS and the membranes were then exposed to X-ray films to record hybridization signals.

Table 1. *Organs of Amblyomma hebraeum adults positive for Cowdria ruminantium by DNA probe\**

Expt†	Midguts				Salivary glands			
	Males	(%)	Females	(%)	Males	(%)	Females	(%)
1	46/49	93·9	26/29	89·7	12/49	24·5	1/29	3·5
2	35/35	100·0	12/14	85·7	14/35‡	40·0	1/14	7·1

\* Ticks had been exposed to *C. ruminantium* as nymphs 5·5 months earlier by feeding on sheep 208 infected with heartwater.

† In experiment 1, ticks were dissected without incubation at elevated temperatures. In experiment 2, ticks were dissected immediately following exposure to 37 °C for 3 days.

‡  $\chi^2 = 2·966$ ;  $P < 0·1$ .

## RESULTS

### *Heartwater infection in sheep and goats and transmission to A. hebraeum ticks*

Sheep No. 208 which was used to infect ticks developed a persistent febrile reaction 11 days after inoculation with the Crystal Springs blood stabilate, and died 4 days later. Autopsy and a brain-crush smear confirmed that this animal died of heartwater. Sheep No. 195 which was uninfected and served as host for the control ticks remained healthy throughout the study. It was later challenged by i.v. inoculation with the Crystal Springs blood stabilate and died from heartwater 15 days after inoculation.

Two goats hosting 10 male or 10 female ticks previously fed on sheep No. 208 became febrile on days 19 and 20, respectively, after ticks were applied. On the third day of fever, the brains of these goats were biopsied and the presence of intracytoplasmic colonies of *C. ruminantium* was demonstrated. A single goat parasitized by 10 adult control ticks of either sex originally fed as nymphs on sheep 195 remained afebrile and healthy.

### *Detection of rickettsial DNA*

The pCS20 DNA probe detected *C. ruminantium* DNA in midguts of 46 of 49 male (93·9%) and 26 of 29 female (89·7%) *A. hebraeum* ticks fed as nymphs 5·5 months previously on heartwater-infected sheep No. 208 (Fig. 1*a*, Table 1). From these same ticks, the number of salivary glands in which *C. ruminantium* infection could be demonstrated was low (Fig. 1*b*, Table 1). Only 12 of 49 salivary glands from males (24·5%) and 1 of 29 from females (3·5%) were positive for *C. ruminantium* DNA. Signal intensities were generally high in midgut samples, corresponding to 1–100 ng Crystal Springs DNA, and lower in those from salivary glands. No hybridization reactions were seen in samples of midguts or salivary glands from ticks fed as nymphs on uninfected sheep No. 195 (Fig. 2*a, b*). The probe also reacted with all three dilutions (100, 10 and 1 ng) of the positive control, Crystal Springs DNA (from culture-produced *C. ruminantium* organisms), but not with any dilutions of DNA from the *R. conorii*-related SFG rickettsia, Z3-Ah (Fig. 3).

In a second experiment to determine if the *C. ruminantium* infection of midguts or salivary glands increased after incubation of live, infected ticks at 37 °C for

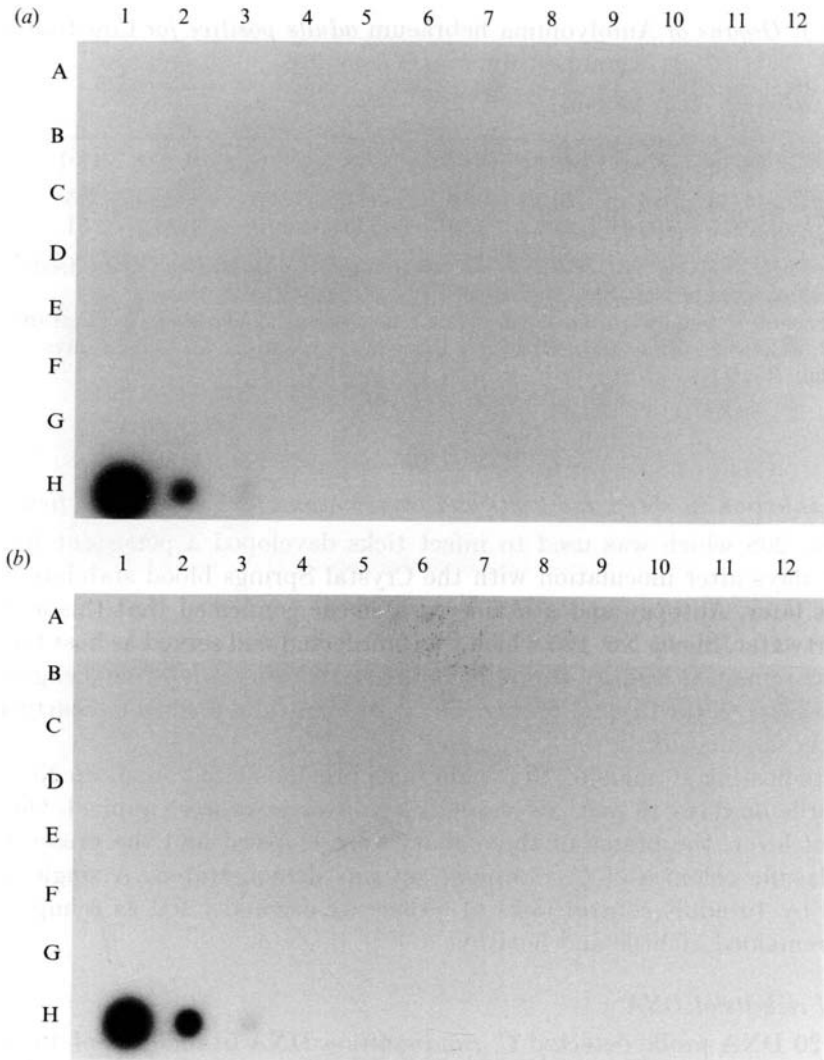


Fig. 2. (a) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 30 male and 30 female midgut DNA samples (row A1–C6 and row E1–G6, respectively) from uninfected *A. hebraeum* ticks. Row H1–3 shows Crystal Springs DNA samples (100, 10 and 1 ng), included as positive controls. (b) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 30 male and 30 female salivary gland DNA samples (row A1–C6 and row E1–G6 respectively) from uninfected *A. hebraeum* ticks. Row H1–3 shows Crystal Springs DNA samples (100, 10 and 1 ng), included as positive controls.

3 days (heat shock), a high infection rate of midguts of both sexes seen before incubation was also detected after the incubation period (Fig. 4*a, b*, Table 1). All 35 midgut samples from males (100%) and 12 of 14 from females (85.7%) were positive. Fourteen of 35 male tick salivary glands (40%), and 1 of 14 female salivary glands (7.1%) were positive as detected by DNA probe hybridization (Fig. 4*a, b*, Table 1). Generally, signal intensities of heat-shocked ticks were similar to those of ticks not subjected to heat shock.

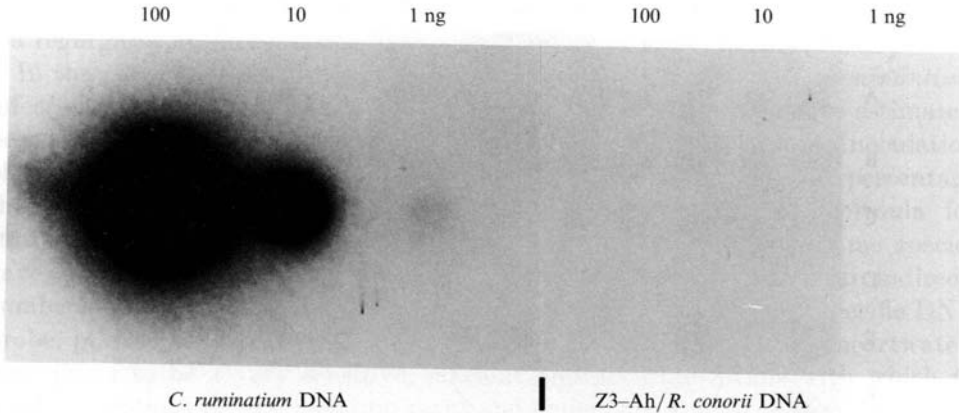


Fig. 3. Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 100, 10 and 1 ng of Crystal Springs DNA and to 100, 10 and 1 ng of the *R. conorii*-related rickettsia Z3-Ah DNA.

#### DISCUSSION

In a previous study [12], we showed that the pCS20 DNA probe was sensitive and specific for *C. ruminantium* DNA and applicable to the detection of *C. ruminantium* infection in *A. variegatum* ticks. In the present study we have shown that the pCS20 DNA probe is also suitable for the detection of *C. ruminantium* infection in *A. hebraeum*, the major vector of heartwater in southern Africa. The probe's suitability for detection of *C. ruminantium* infection in *A. hebraeum* was further shown when it failed to cross-react with uninfected tissues of *A. hebraeum* or with a spotted fever group (SFG) rickettsia [19], which is frequently associated with this tick species in Zimbabwe. Although the infection rates of *A. variegatum* and *A. hebraeum* ticks are not expected to differ, a higher number of infected *A. hebraeum* ticks were detected in this study compared to the numbers of infected *A. variegatum* detected in the previous study [12]. This may be related either to the fact that in the *A. variegatum* study only 1/10 of the tick DNA was blotted for hybridization, whereas in this study all the DNA was blotted to maximize the chances of detection of infection; or to a difference in the ability of the two isolates of *C. ruminantium* (Kiswani and Crystal Springs) to establish infection in the *A. variegatum* and *A. hebraeum* ticks.

A comparison of *C. ruminantium* infection in midguts and salivary glands of untreated or heat-shocked unfed adults of *A. hebraeum* revealed that *C. ruminantium* occurs primarily in the midgut, and that infection of the salivary gland does not always correlate with infection in the midgut. Guts of both sexes were approximately equally infected, but infection rates of salivary glands of males were considerably higher than those of females in both untreated and heat-shocked ticks. It appears that heat shock may have caused an increase in the percentage of males with salivary gland infection ( $\chi^2 = 2.966$ ,  $P < 0.1$ ), although there was no way of accurately determining the infection status of these salivary glands before the treatment. The reason for the difference between males and females in salivary gland infections is unclear, but it compares well with the reported infection rates in males and females of *A. hebraeum* collected in endemic

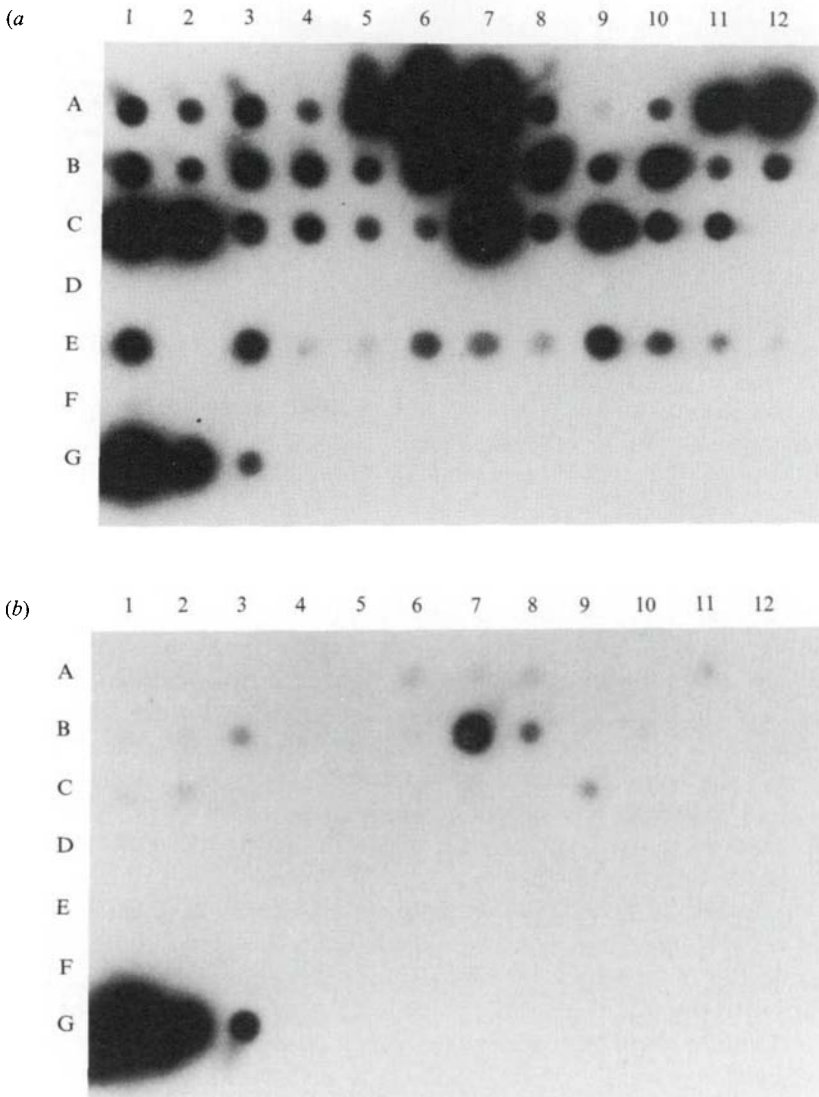


Fig. 4. (a) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 35 male (row A1–C11) and 14 female midgut DNA samples (E1–F2) prepared from infected *A. hebraeum* ticks after incubation at 37 °C for 3 days. Row G1–3 shows Crystal Springs DNA samples, included as positive controls (100, 10 and 1 ng). (b) Hybridization of  $^{32}\text{P}$ -labelled pCS20 DNA probe to 35 male (row A1–C11) and 14 female salivary gland DNA samples (E1–F2) prepared from infected *A. hebraeum* after incubation at 37 °C for 3 days. Row G1–3 shows Crystal Springs DNA samples included as positive controls (100, 10 and 1 ng).

areas of heartwater in Zimbabwe [20], and lends credence to the belief that males serve an important role as reservoirs of *C. ruminantium* [21]. Nevertheless, it remains to be determined whether gut infection will equate with salivary gland infection after ticks have undergone a complete period of feeding on the host. If not, then DNA probing to detect *C. ruminantium* in salivary glands, rather than midguts, might better reflect the actual risk of exposure of animals to heartwater



in endemic areas, if this is the mode of transmission [10] rather than transmission via regurgitation of organisms during the feeding process of ticks [22, 23].

In the past, the lack of direct and precise methods to detect *C. ruminantium* infections in large numbers of suspected vectors has led to disparate estimates, based on indirect methods, of infection rates of ticks in endemic areas. Inoculation of suspensions of field-collected *A. hebraeum* into mice resulted in a low percentage that subsequently developed specific antibodies [24]. Use of a formula for statistical estimation of infection rates in field populations of the same species gave a high degree of infection [20], and this method was also indirect, cumbersome and expensive. Thus, the availability of a sensitive and specific DNA probe, pCS20, for detection of infection in the two major vectors of heartwater, may prove to be a very sensitive, efficient and accurate means with which to resolve questions of tick infection rates and endemic stability of the disease.

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