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DISTINCTIVE STAINING OF COLONIES OF *COWDRIA RUMINANTIUM* IN MIDGUTS OF *AMBLYOMMA HEBRAEUM**

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ABSTRACT

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Mallory's phloxine-methylene blue stain was used to differentiate colonies of *Cowdria ruminantium* in midgut epithelial cells of nymphal *Amblyomma hebraeum* that had been infected as larvae. Gut tissues were collected from nymphs that had fed on a susceptible sheep and were fixed in formol-saline on the day of repletion. Paraffin sections, 3–4 μm thick, were then stained and this rendered colonies and cell nuclei densely blue against a uniformly pink background of tick tissues. Colonies were easily distinguished from nuclei by their specific morphology. This method of parasite visualization may be adapted to field-collected ticks for rapid detection of *C. ruminantium* or to assays of susceptibility of tick populations to various strains of the organism.

INTRODUCTION

The bont tick, *Ablyomma hebraeum* Koch, is the major vector of heartwater (caused by *Cowdria ruminantium*) to ruminants in southern Africa. It can become infected in pre-adult instars and subsequently transmit the organism transstadially and to susceptible hosts. To fully understand the epidemiology of heartwater it is necessary to determine infection rates in field-collected ticks. Also, the production of adequate numbers of organisms from ticks for development of vaccines and diagnostic tests (Bezuidenhout, 1981; 1984) depends upon an understanding of the susceptibility of vector sub-populations to various strains of the organism. Numerous techniques, including animal inoculation, microscopic examination, and serological and biochemical screening, have been used to demonstrate infectious organisms in vectors. However, some of these procedures are poorly sensitive for heartwater, or are costly, time-consuming or unavailable in countries affected by the disease. We report distinctive staining of *C. ruminantium* in paraffin-sections of *A. hebraeum* midgut. This simple technique may be applicable to sampling of field populations of *Cowdria*-infected ticks as well as to assays of infectivity in laboratory-reared forms.

MATERIALS AND METHODS

Tick and rickettsial strains used

A Zimbabwean strain of *Amblyomma hebraeum*, originally collected at Malapati (22° 05′ S, 31° 25′ E) by R. A. I. Norval in 1978 and subsequently reared in the laboratory, was used. Larvae were infected by feeding on a Boer goat inoculated intravenously with 5 mℓ of blood infected with *Cowdria ruminantium*. This blood was obtained from a pyrexic Boer goat that had been inoculated i.v. 17 days previously with 5 mℓ of heartwater blood vaccine (Ball 3 strain) as currently issued by the Veterinary Research Institute, Onderstepoort (Bezuidenhout, 1981). Larvae that engorged and dropped off the host during the period when its temperature was about 40 °C were collected, held at 80 % RH and 27 °C, and allowed to moult. Approximately 2 weeks after moulting, nymphs were fed on a heartwater-susceptible sheep. These nymphs were prepared for sectioning on the day they dropped off.

Giemsa-stained brain-crush smears were made from all ruminants at autopsy to confirm the presence of the organism.

Preparation of specimens

Tick midguts were fixed, after dissecting in phosphate-buffered saline, by transferring the organs to formol/saline solution. Forty-eight hours later these organs were dehydrated, paraffinized and cut into sections 3–4 μm in thickness. After dewaxing and hydration, they were stained by a modification of Mallory's phloxine-methylene blue method as reported by Thomas (1953). Briefly, sections were stained for 2 min in filtered acid phloxine (0,5 g phloxine in 100 mℓ distilled water containing 0,2 % acetic acid), rinsed in warm water for 2 to 3 min, and stained for 1 min in boracic-methylene blue solution (0,25 g each of methylene blue and borax in 100 mℓ distilled water). Sections were partially destained in a 0,2 % solution of acetic acid in distilled water by gently agitating the slide until colour ceased to stream from the section. They were differentiated by immersion for 5 min in each of 2 changes of 95 % ethanol, and similarly dehydrated in 2 changes of absolute ethanol. After clearing for 5 min in each of 2 changes of xylene, sections were mounted in Permount for examination by light microscopy.

RESULTS

At low magnification (100–250 ×), colonies of rickettsiae and cell nuclei in midgut were seen to stain a deep blue against a uniformly pink background of tick elements, host erythrocytes and haemoglobin (Fig. 1, 2). At higher magnifications, individual, homogeneously stained, coccoid organisms could be seen within the colonies and easily distinguished from cell nuclei (Fig. 3). Midgut sections obtained from uninfected nymphal ticks, used as controls, showed no colonies. All ruminants used in this study responded with symptoms typical of heartwater and died. Heartwater was confirmed at autopsy, with characteristic organisms seen in brain-crush smears.

DISCUSSION

Although colonies of heartwater organisms were seen by Cowdry as early as 1925 in Giemsa-stained paraffin sections of tick tissues, histological studies of the organism were, until recently, limited to tissues of the vertebrate host. The development of a tick vaccine for heartwater (Bezuidenhout, 1981), together with the need to obtain purified, tick-derived organisms for serological antigens, prompted studies aimed at detecting heartwater

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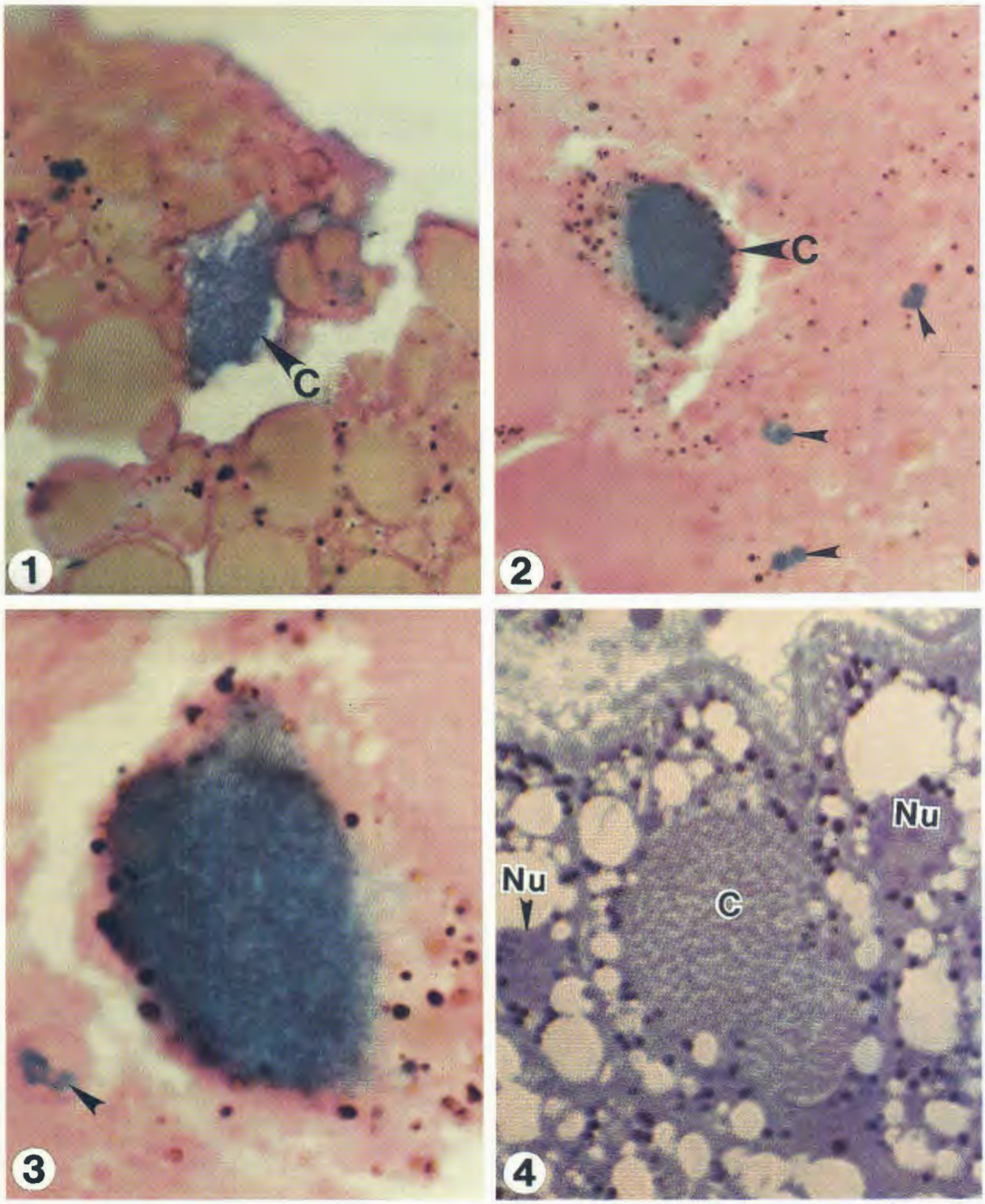

FIG. 1, 2 Colonies of *Cowdria ruminantium* (C) in midgut epithelium of fed, nymphal *Amblyomma hebraeum;* note host-cell nuclei (arrows). Mallory's phloxine-methylene blue stain of paraffin sections, 500 ×.

FIG. 3 Colony of *C. ruminantium* shown in Fig. 2, enlarged (2 500 ×); host-cell nucleus (arrow).

FIG. 4 Colonies of *C. ruminantium* (C). Mallory's monochromatic blue stain of epoxy embedded section (Nu, host-cell nucleus), 2 000 ×. Compare with Fig. 3.

rickettsiae in vector tissues (Bezuidenhout, 1984; Kocan, Morzaria, Voight, Kiarie & Irvin, 1987). These studies utilize epoxy-embedded sections and a variation of Mallory's stain which renders colonies, tissue components, and haemoglobin in various shades of blue (Fig. 4). This monochromatic effect was also noted by Cowdry (1925). Because *Cowdria* micro-organisms within colonies may be confused with pigmented granules and inclusions of tick tissues, electron microscopy may be required to confirm the presence of the parasites. Bezuidenhout (1984) also reported that frozen sections stained with Giemsa, methyl green and pyronin Y, and acidine orange were unsatisfactory.

Our objective was to find a relatively simple and rapid means to identify *Cowdria*-infected ticks for epidemiological studies. Paraffin sections can be produced in most diagnostic laboratories, and the bichromatic rendering of tick midgut by the phloxine-methylene blue stain facilitates quick differentiation of parasites from other structures. Additionally, this method may find application in assays of susceptibility of different vector species and populations to various strains of the heartwater organism.

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