Establishment of *Boophilus microplus* Infected with *Babesia bigemina* by Using *in vitro* Tube Feeding Technique

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(Received 24 October 1997/Accepted 11 December 1997)

ABSTRACT. The *in vitro* tube feeding technique is used to establish a laboratory colony of *Boophilus microplus* infected with *Babesia bigemina*. Pre-fed engorged female ticks offered 2 × 10⁴ and 2 × 10⁵/ml of *B. bigemina* infected bovine red blood cells (RBC) showed sporokineties in the haemolymph smear sample, and positive signals for *B. bigemina* in polymerase chain reaction (PCR). Larvae laid from the ticks offered 2 × 10⁴/ml of *B. bigemina* infected RBC showed evidence for *B. bigemina* infection in microscopic method and PCR. While larvae laid from the ticks offered 2 × 10⁵/ml of *B. bigemina* infected RBC showed positive for *B. bigemina* in only PCR. The females offered 2 × 10⁴/ml *B. bigemina* infected RBC and their larvae did not show positive evidences for *B. bigemina* infection. It is thought that the *in vitro* tube feeding technique can be a convenient method to study the relationship between ticks and tick-borne pathogens. It is also suggested that the superior sensitivity of PCR compared to the microscopic method in detection of *B. bigemina* from the tick sample, especially in larvae. — KEY WORDS: *Babesia bigemina*, *Boophilus microplus*, in *vitro* tube feeding.

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The relationship between *Babesia bigemina* and its vector tick *Boophilus microplus* had been studied by using numbers of cattle for experimental infection [5, 6]. To save the numbers of experimental animals and study the relationship between ticks and tick-borne protozoa more directly, many *in vitro* feeding techniques have been reported [9, 12, 14–16] since the first method had attempted [1]. *B. microplus* can also feed host blood through both membranes [5, 6, 13] and capillary tubes [17]. Thus we tried to establish a laboratory colony of *B. microplus* infected with *B. bigemina* by using one of the *in vitro* feeding techniques.

**MATERIALS AND METHODS**

*B. bigemina* infected bovine red blood cells: *B. bigemina*, Lismore strain, infected bovine red blood cells (RBC) were obtained from an experimentally infected non-splenectomized calf using heparin as the anticoagulant. The number of RBC infected with *B. bigemina* was counted on smears of peripheral blood of the calf [4]. The blood (Packed cell volume (PCV): 25%) was diluted with heparinized blood from a non-*B. bigemina* infected calf (PCV: 41%) to prepare different levels of infectious RBC (2 × 10⁴, 2 × 10⁵, 2 × 10⁶, and 0 infected RBC/ml).

Infection of *B. microplus* with *B. bigemina*: Females of the Yeerongpilly laboratory strain of *B. microplus* were fed on cattle and were picked off when they had reached 4–6 mm in length. Cement material was cleared from the mouthparts before the ticks were used. Six ticks were prepared for each concentration of *B. bigemina* infected RBC, and 0.5 ml of infected blood was offered to each tick using an *in vitro* tube feeding technique [17]. The ticks and tubes were incubated on a warm plate at 35°C for 18 hr in the dark (Fig. 1A). After removing the tubes, ticks were incubated at 28°C with 90% relative humidity. Eggs from each engorged tick were separated into small tubes and incubated under the same condition as adults until larvae were hatched.

Monitors of *B. bigemina* in ticks: Microscopic observation on the haemolymph of females and on smears of the larvae were performed by the method of Mahoney and Mirre [8]. Samples of haemolymph were obtained from each female tick 5 days after *in vitro* feeding. Smears were also made of the larvae hatching from each tube of eggs on the 10th day after hatching. Ten smears were made for the larvae sample from each tube. All preparations were fixed in (Fig. 1B).
methyl alcohol, stained with Giemsa and examined whole smears with a light microscope.

Polymerase chain reaction (PCR) was also used to detect *B. bigemina* specific DNA fragment from tick samples. To isolate DNA from the haemolymph of females and whole body of larvae, a modified method of Gage et al. [3] was used. A leg was removed from each female 5 days after *in vitro* feeding and crushed with a plastic grinder in extraction buffer (10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, Proteinase K 100 µg/ml, RNase 20 µg/ml) then incubated at 50°C for 3 hr. Subsequently, the sample of DNA was isolated using phenol extraction and ethanol precipitation. Each DNA sample was finally suspended in 5 µl of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at -20°C until PCR analysis. DNA was isolated from five larvae from each group on the same day as smears were sampled using the same method as described for female legs. Samples were analyzed with PCR described by Figueroa et al. [2] with slight modification. Oligonucleotide 5’-CGCAACGCCCCAGCCTGGGTCC-3’ and 5’-CC TCGGCCTCAACTCTGAGTCCAAAG-3’ were used for the primers. The following conditions for amplification were used; 200 µM (each) deoxynucleoside triphosphate (dNTP), 4.0 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 µM (each) primer, 2 µl of each DNA sample in a total volume of 99.5 µl. Mineral oil was layered over the top of the mixture and the reaction tubes were incubated at 80°C for 5 min. After the addition of 2.5 units of Taq polymerase (Perkin-Elmer, U.S.A.), the tubes were placed in a Gene-Machine (Bartelt Instruments Pty. Ltd. VIC, Australia) and the reaction was performed under the following temperature profile: 2.5 min of template denaturation at 94°C, 2.5 min of primer annealing at 65°C, and 2.5 min of primer extension at 72°C for a total of 35 cycles. After the amplification reaction, the 10 µl of reaction mixture was added to 2 µl of loading buffer (0.25% BPB, 30% Glycerol, 2 mM EDTA) and electrophoresed on a 2.0% agarose gel containing 0.5 µg/ml ethidium bromide in TAE buffer (40 mM Tris-HCl (pH 7.7), 5 mM sodium acetate, 1 mM EDTA). DNA was visualized with ultraviolet light.

## RESULTS

The efficiency of the *in vitro* feeding was shown in Table 1. Seventy percent (16/23) of ticks began to feed the blood almost immediately after transfer to the *in vitro* feeding system, but rest of them did not feed at all. Starting from a mean, pre-fed female weight of 36.1 mg, ticks fed on bovine blood reached a final mean weight of 169.1 mg (range: 101–270 mg) after 18 hr (Fig. 1B), although the exact volume and numbers of ingested RBC were not determined. Subsequently, 15 females among the 16 laid eggs, however 4 of them laid quite small numbers of eggs.

Results of the detection of *B. bigemina* from haemolymph of females and larvae are summarized in Table 2. In all ticks offered 2×10³/ml *B. bigemina* infected RBC, 5 to 80 sporokinetes were observed in the haemolymph smears (Fig. 2), and strongly positive signals were detected by PCR (Fig. 4A). However, severe mortality occurred on day 5 of oviposition (8 days after engorgement) in those ticks. They stopped laying eggs and changed their body color to dark brown. In all batch of larvae from 4 females offered 2×10³/ml *B. bigemina* infected RBC, sporokinetes or round forms were found in the smear samples (Fig. 3) and positive signals were also detected by PCR from all samples (Fig. 4B). One of the 4 females offered 2×10⁴/ml *B. bigemina* infected RBC showed one sporokinet in the smear sample, while all DNA samples extracted from them gave positive signals in PCR (Fig. 4A). None of larvae laid from the ticks offered 2×10⁴/ml *B. bigemina* infected RBC showed sporokinetes or round forms in the smears samples. However, positive signals were detected by PCR in all batch (Fig. 4B). *B. bigemina* were not detected by either the microscopic method or PCR in the haemolymph of females offered 2×10⁵/ml infected RBC or in the control. Larvae laid from those ticks also showed no evidence of *B. bigemina* infection in both methods.

## DISCUSSION

In the present results, females of *B. microplus* could be fed successfully on bovine blood by *in vitro* feeding system during the last phase of engorgement and they were also

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### Table 1. The efficacy of feeding and oviposition of *Boophilus microplus* engorged by *in vitro* tube feeding technique

<table>
<thead>
<tr>
<th>Concentration of <em>B. bigemina</em> infected RBC offered to ticks</th>
<th>Numbers of ticks</th>
<th>Mean pre-body weight (mg)</th>
<th>Numbers of success feeding</th>
<th>Mean post-body weight (mg)</th>
<th>Numbers of ticks produce eggs</th>
<th>Numbers of batch produce larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×10³/ml</td>
<td>6</td>
<td>36.8</td>
<td>5</td>
<td>196.2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2×10⁴/ml</td>
<td>6</td>
<td>35.7</td>
<td>4</td>
<td>138.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2×10⁵/ml</td>
<td>6</td>
<td>36.2</td>
<td>4</td>
<td>157.8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>35.6</td>
<td>3</td>
<td>179.7</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Total 23 36.1 16 169.1 15 15
infected with *B. bigemina* by the technique. It is suggested that the *in vitro* feeding technique is a useful method to establish a laboratory colony of *B. microplus* infected with *B. bigemina*.

The biggest advantage of the technique is that the numbers of experimental animals can be saved. We used one calf for an experimental infection with *B. bigemina* to supply infected RBC, however, it may saved by using in *vitro* cultivation strain or freeze stabilize strain of *B. bigemina*. The tube feeding technique need pre-fed ticks, so at least one cattle was required to supply the pre-fed female ticks. Using the membrane system, that is another *in vitro* feeding system [5, 6, 9, 12–16], the more numbers

<table>
<thead>
<tr>
<th>Concentration of <em>B. bigemina</em> infected RBC offered to ticks</th>
<th>Numbers of ticks examined</th>
<th>Numbers of positive by Microscope</th>
<th>Numbers of larvae batch examined</th>
<th>Numbers of positive by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 × 10^5/ml</td>
<td>5</td>
<td>5*</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2 × 10^4/ml</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2 × 10^3/ml</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*: Sporokinetes or round forms were observed in the smears of hemolymph of females or scotched larvae.

**Fig. 2.** Many sporokinetes were observed haemolymph of engorged female offered 2 × 10^5/ml of *Babesia bigemina* infected bovine red blood cells. Giemsa stain. × 400.

**Fig. 3.** A round form (arrow) was observed in a larva from a female offered 2 × 10^5/ml of *Babesia bigemina* infected RBC. The later stage of *B. bigemina* in the tick are often difficult to differentiate from surrounding tissue. Giemsa stain. × 400.

**Fig. 4.** (A) The positive signals were detected from all samples of haemolymph from tick offered 2 × 10^5 and 2 × 10^4/ml of *Babesia bigemina* infected RBC (Lane 1–5 and 6–9, respectively). In the haemolymph from ticks offered 2 × 10^3/ml of infected RBC (lane 10–13) and control tick (lane 14–16), *B. bigemina* specific DNA fragments were not detected. Lane 17 shows the result of PCR reaction without genomic DNA. (B) The signals were detected from all samples of larvae from tick offered 2 × 10^5 and 2 × 10^4/ml of *B. bigemina* infected RBC (Lane 1–4 and 5–8, respectively). In the larvae from tick offered 2 × 10^3/ml of infected RBC (lane 9–12) and control tick (lane 13), *B. bigemina* specific DNA fragments were not detected. Lane 14 shows the result of reaction without genomic DNA. Arrows shows positive signals at 223 bp. M: Molecular marker.

*bigemina*. The tube feeding technique need pre-fed ticks, so at least one cattle was required to supply the pre-fed female ticks. Using the membrane system, that is another *in vitro* feeding system [5, 6, 9, 12–16], the more numbers
of experimental cattle could be saved.

The second advantage of the technique is that the relationship between tick and protozoa can be studied quantitatively. In our results, the females offered 2 \times 10^4 and 2 \times 10^3/ml \textit{B. bigemina} infected RBC and their larvae showed positive signals for \textit{B. bigemina} specific fragment in PCR, while ticks offered 2 \times 10^3/ml infected RBC and their larvae did not. It is possible that the minimal \textit{B. bigemina} dose required to infect vector ticks and transmit to next generation would be revealed by the method.

Light microscopic observation of free-living tick stages for sporokinetes or other forms of \textit{B. bigemina} has been used in the past for studying the epidemiology of babesiosis [4, 8, 10]. The method is simple and inexpensive. Tick sporokinetes have a unique shape and are relatively easy to detect and identify with the microscope [8, 10], however, later stages of \textit{B. bigemina} in the tick are often difficult to differentiate from surrounding tick tissue (Fig. 3). That is why we used both microscopic method and PCR to detect the evidence of \textit{B. bigemina} from adult haemolymph and larvae. Because the PCR detect the \textit{B. bigemina} specific DNA fragment [2], we can apply the technique for both merozoites in bovine and sporokinetes in ticks. As for the sensitivity of the PCR, Figueroa et al. [2] reported that it could detect 30 \textit{B. bigemina} infected RBC from 200 \mu l of blood sample. In our experiment, none of larvae laid from the tick offered 2 \times 10^4/ml \textit{B. bigemina} infected RBC showed sporokinetes or round forms in the smear samples, however, positive signals were detected by PCR in all batch. It is suggested that the superior sensitivity of PCR compared to the microscopic method in detection of \textit{B. bigemina} from the tick sample, especially in the lave. As mentioned by Stiller and Coan [11], application of PCR to detect specific DNA fragments of \textit{Babesia} parasites from ticks is potentially useful.

We did not determine if these PCR positive and microscopic negative larvae were infectious to bovine hosts. More studies are required to understand the significance of the larvae infected with \textit{B. bigemina} by using \textit{in vitro} feeding technique.

ACKNOWLEDGMENT. The authors would like to thank T. Vuocolo, D. Waltisbuhl, G. Leach, Dr. C. M. Elvin, Dr. P. Willadsen for valuable comments for Babesia and ticks (All belong to the Division of Tropical Animal Production, CSIRO, Australia).

REFERENCES