

Ability of the Vector Tick *Boophilus microplus* To Acquire and Transmit *Babesia equi* following Feeding on Chronically Infected Horses with Low-Level Parasitemia

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The protozoan parasite *Babesia equi* replicates within erythrocytes. During the acute phase of infection, *B. equi* can reach high levels of parasitemia, resulting in a hemolytic crisis. Horses that recover from the acute phase of the disease remain chronically infected. Subsequent transmission is dependent upon the ability of vector ticks to acquire *B. equi* and, following development and replication, establishment of *B. equi* in the salivary glands. Although restriction of the movement of chronically infected horses with *B. equi* is based on the presumption that ticks can acquire and transmit the parasite at low levels of long-term infection, parasitemia levels during the chronic phase of infection have never been quantified, nor has transmission been demonstrated. To address these epidemiologically significant questions, we established long-term *B. equi* infections (>1 year), measured parasitemia levels over time, and tested whether nymphal *Boophilus microplus* ticks could acquire and, after molting to the adult stage, transmit *B. equi* to naive horses. *B. equi* levels during the chronic phase of infection ranged from $10^{3.3}$ to $10^{6.0}$ /ml of blood, with fluctuation over time within individual horses. *B. microplus* ticks fed on chronically infected horses with mean parasite levels of $10^{5.5} \pm 10^{0.48}$ /ml of blood acquired *B. equi*, with detection of *B. equi* in the salivary glands of 7 to 50% of fed ticks, a range encompassing the percentage of positive ticks that had been identically fed on a horse in the acute phase of infection with high parasitemia levels. Ticks that acquired *B. equi* from chronically infected horses, as well as those fed during the acute phase of infection, successfully transmitted the parasite to naive horses. The results unequivocally demonstrated that chronically infected horses with low-level parasitemia are competent mammalian reservoirs for tick transmission of *B. equi*.

Babesia equi, a protozoan of the phylum Apicomplexa, is biologically transmitted by ixodid ticks and causes acute disease in equids with clinical signs characterized by fever, anemia, icterus, and hemoglobinuria (7, 22). Horses that recover from the acute phase of infection remain chronically infected, apparently for life (22). The significance of the disease is sufficient that positive horses are prohibited from international movement, a requirement that has substantial economic impact (8, 22). This restriction is not limited to horses in the acute phase of infection but extends to chronically infected horses, as detected by serological assays (6, 14, 15, 23). This restriction is based on the presumption that chronically infected horses serve as reservoirs for vector ticks to acquire and subsequently transmit *B. equi*. This presumption, however, is untested.

Following acquisition tick feeding, sexual reproduction of *B. equi* occurs in the midgut of the tick vector, with subsequent development of parasite kinetes that invade salivary glands via the hemolymph (17, 20, 21, 27). Within the tick salivary glands, the developmental process culminates in infectious sporozoites, which can then be transmitted to a susceptible horse upon

feeding (9, 12). Thus, the initial parasitemia in the horse during acquisition feeding may be a critical component of transmission efficacy, as there must be sufficient parasites in the blood to meet the threshold requirement for development within the tick. While there are clearly differences in the level of parasitemia between the acute phase of infection, during which *B. equi* parasitized erythrocytes are abundant when peripheral blood smears are examined microscopically, and the chronic phase of infection, in which levels are below those reliably detected microscopically, the actual levels of parasitemia during the chronic phase of infection are unknown.

In this study, we tested the hypothesis that chronically infected horses are a competent reservoir for tick transmission of *B. equi*, and we specifically addressed three components of transmission. The first was to quantify the levels of *B. equi* in peripheral blood during the chronic phase of infection and to determine whether these levels are static or dynamic over time during infection. The second was to determine the ability of *Boophilus microplus* to acquire *B. equi* by feeding on horses during the chronic phase of infection. *B. microplus* is a natural vector of *B. equi* with a widespread distribution in tropical and subtropical regions where *B. equi* infection is endemic. The third and most important component was to test whether *B. microplus* acquisition fed on chronically infected horses would transmit *B. equi* to susceptible horses. The use of *B. microplus*

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fed on horses in the acute phase of infection, previously shown to transmit *B. equi* (14, 24), provided the positive transmission control needed to definitively address the role of chronically infected horses as reservoirs for tick transmission.

MATERIALS AND METHODS

Animals, pathogen, and tick vector. Fifteen horses (mixed-breed ponies), all geldings between 2.5 and 8 years of age and free of *B. equi* as determined by a nested PCR targeting *ema-1* and serological testing using the equi merozoite antigen 1 (EMA-1) competitive inhibition enzyme-linked immunosorbent assay (14, 16), were utilized in this study. The *B. equi* isolate was obtained in 1976 from a horse in Florida and cryopreserved in liquid nitrogen as a blood stabulate containing 10% dimethyl sulfoxide. A horse with an intact spleen (H2) was intravenously inoculated with 30 ml of Florida isolate *B. equi* first-passage stabulate. This horse was monitored for clinical signs and parasitemia. At a parasitemia level of 0.64%, infected erythrocytes were collected and cryopreserved in liquid nitrogen as a stabulate containing packed erythrocytes 1:1 with a cryopreservative of 20% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) glucose in Puck's saline G, as previously described (15). The La Minta strain of *B. microplis* ticks was maintained at the U.S. Department of Agriculture Agricultural Research Service Animal Disease Research Unit, University of Idaho, Moscow (24). To rear *B. microplis* nymphs for acquisition of *B. equi* from infected horses, approximately 1 g of normal *B. microplis* larvae were fed for 5 days on Holstein-Friesian calves (26). Engorged larvae were removed using microsurgery forceps and incubated for 3 days at 26°C with 94% relative humidity and a 12-h photoperiod to allow molting to the nymphal stage (26).

Determination of *B. equi* levels in the peripheral blood by using real-time PCR. To quantify *B. equi* levels in the peripheral blood of infected horses, a previously reported (26) real-time PCR assay based on the single-copy *B. equi ema-1* gene (13) was used. Briefly, this assay amplifies a 155-bp fragment stretching from nucleotide 115 to 270 (numbering is based on the *ema-1* sequence previously reported), with detection using a PE Applied Biosystems fluorogenic probe that anneals at bp 137 to 161 (26). The cycling conditions and preparation of the standard curve utilizing 10^2 to 10^7 copies of recombinant *ema-1* (*pema-1*) were identical to those previously reported (26). All assays were run in triplicate.

To compare the results of the real-time PCR with parasite levels determined by microscopic examination of Giemsa-stained blood smears, horses H038 and H069 (26) were inoculated with the *B. equi* Florida isolate and levels determined daily during the ascending phase of parasitemia using both methods. When the microscopically determined *B. equi* level was $10^{7.50}$ /ml from one time point, real-time PCR demonstrated a mean of $10^{7.6}$ parasites per ml of blood, indicating agreement between real-time PCR and microscopic examination. This sample was diluted to a level equivalent to $10^{5.6}$ *B. equi* parasites per ml (based on PCR quantification), typical of the chronic phase of infection, and used as an internal standard in all subsequent real-time PCR assays.

To ensure that the sequence of *ema-1* used as the target for the real-time PCR was stable during the chronic phase of infection, full-length *ema-1* was cloned and sequenced from 11 time points over a period of 39 months after infection of horse H057. Genomic DNA from H057 was extracted at each time point and the 816-bp gene amplified as previously described (13). Amplified *ema-1* fragments were cloned into the pCR-4 TOPO vector and competent TOP10 *Escherichia coli* cells transformed (Invitrogen Corporation, Carlsbad, CA). Approximately 10 individual transformed colonies were selected from each PCR, plasmid DNA was isolated, and the presence of inserts confirmed by restriction enzyme EcoRI digestion. The inserts were sequenced in both directions by using a Big Dye Kit and an ABI Prism automated sequencer. Sequencher (Gene Codes Corporation, Ann Arbor, MI) was utilized to assemble and edit *ema-1* sequences. Plasmid clones that contained no changes in the *ema-1* sequence and clones with changes at the real-time PCR primer and probe binding sites were compared to determine if the changes at the primers and probe binding sites altered the quantification of *ema-1* by real-time PCR. Equivalent concentrations of clones were diluted to a known copy number and then quantified by real-time PCR.

The levels of *B. equi* in the peripheral blood during the chronic phase of infection of horses H057, H063, H065, and H066, infected by intravenous inoculation of approximately 8×10^7 parasitized erythrocytes, were determined by real-time PCR. Blood samples from H057 (650 to 720 days postinoculation), H063, H065, and H066 (480 to 550 days postinoculation) were collected in EDTA biweekly and genomic DNA extracted (Gentra Systems, Inc., Minneapolis, MN).

Tick acquisition feeding on acutely infected and chronically infected horses. *B. microplis* nymphs were acquisition fed on horses H059, H072, and H076 for 8

days during the chronic phase of infection. As a positive control, *B. microplis* nymphs were identically fed on splenectomized horse H047 during the ascending phase of parasitemia and used as a comparative measurement for tick infection and subsequent transmission. During each of the 8 days of nymphal acquisition feeding, the levels of *B. equi* in the peripheral blood of all infected horses were quantified by real-time PCR. Engorged *B. microplis* nymphs acquisition fed on *B. equi*-infected horses were removed and incubated for 5 days at 26°C with 94% relative humidity and a 12-h photoperiod to allow molting to adulthood. Freshly molted adult ticks were utilized to determine the ability of adult ticks acquisition fed as nymphs on infected horses to transmit *B. equi* to naive horses.

Tick transmission feeding. *B. microplis* adult ticks infected with *B. equi* as nymphs were transmission fed on naive horses for 8 days. The animal numbers and numbers of adult ticks that attached and fed were as follows: (i) 280 adult ticks fed as nymphs on chronically infected horse H059 were transmission fed on naive horses H072 (140 ticks) and H076 (140 ticks), with subsequent dissection of 127 adult ticks (64 and 63 obtained from H072 and H076, respectively) to identify the percentage of ticks with *B. equi* in the salivary glands; (ii) 233 adult ticks fed as nymphs on H072 during the chronic phase of infection were transmission fed on naive horse H073, with 98 ticks being dissected and examined; (iii) 213 adult ticks fed as nymphs on H076 during the chronic phase of infection were transmission fed on naive horse H068, and 98 ticks were dissected and examined; and (iv) 152 adult ticks fed as nymphs on horse H047 during the ascending phase of parasitemia were transmission fed on naive horses H045 and H064 (76 ticks per animal), with 31 (H045) and 47 (H064) ticks being dissected and examined.

To detect infected salivary glands, genomic DNA was extracted from the salivary gland pairs of individual adult ticks recovered following transmission feeding. Briefly, salivary glands were lysed in 450 μ l of Tris-EDTA and sodium dodecyl sulfate (SDS) with 50 μ l of proteinase K (2 mg/ml) and incubated overnight at 55°C. Following the incubation, 1 μ l of glycogen (10 μ g/ml) was added to the samples. The proteins were precipitated with ammonium acetate. Genomic DNA samples were precipitated in isopropanol, washed in 70% ethanol, and resuspended in Tris-EDTA. Duplex nested PCRs using primers targeting *B. equi ema-1* and *B. microplis α -tubulin*, as previously described (4, 11, 26), were utilized to determine *B. equi* infection in the salivary glands and to control for the presence of tick genomic DNA. To confirm the identity of *B. equi ema-1* amplicons, Southern hybridization was performed using a digoxigenin-labeled 226-bp *ema-1* probe generated from *pema-1*. Following electrophoresis on a 1.5% agarose gel and transfer to a nylon membrane (Hybond; Amersham Biosciences, Piscataway, NJ), the membrane was hybridized with the *ema-1* probe in DIG EASY Hyb (Roche Applied Science, Indianapolis, IN) at 42°C overnight. The membrane was washed four times: three times with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS (twice at room temperature and once at 68°C for 15 min) and once in $0.1 \times$ SSC-0.1% SDS at 68°C for 15 min. Detection of bound probe used alkaline phosphatase-labeled anti-digoxigenin antibody, followed by CDP-Star chemiluminescence (Roche Applied Science, Indianapolis, IN). Tick infection percentages were calculated by dividing the total number of infected ticks detected by the total number of ticks examined.

RESULTS

Determination of *B. equi* levels in peripheral blood by using real-time PCR. The copy number and threshold cycle of the assay correlated with an r^2 value of 0.93 to 0.99 in repeated assays using the standard curve based on known numbers of plasmid copies. In addition, there was close agreement, less than 0.5 log difference, between the number of *B. equi* parasites detected by real-time PCR and microscopic examination of blood smears during the ascending phase of parasitemia (Fig. 1). A sample of this parasitemic blood, diluted to the equivalent of $10^{5.6}$ parasites per ml (based on PCR quantification) to approximate the levels predicted for the chronic phase of infection, was included in all assays as an internal control. There was minimal variation in the results of individual assays (coefficient of variability of 1.2%). The genetic stability of the *ema-1* target sequences during long-term infection was examined by cloning and sequencing the full-length gene from infected horse H057 at 11 time points over a period of 39 months postinfection. The levels of identity among all clones

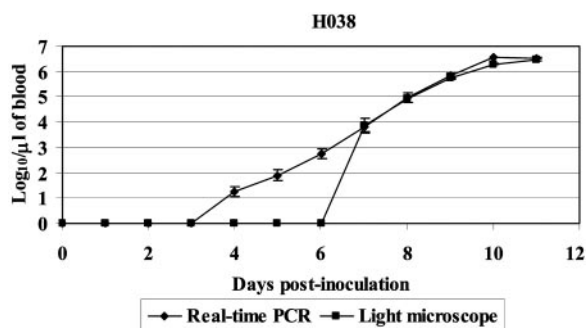


FIG. 1. Comparison of *B. equi* levels in the peripheral blood during the ascending phase of parasitemia detected by real-time PCR and by microscopic examination.

compared to the previously described *B. equi* Florida *ema-1* sequence (11) ranged from 99.7 to 100%. Of the total of 90 sequenced clones, 5 contained single nucleotide substitutions within the real-time PCR forward primer or at the probe binding sites. There were no changes within the reverse primer sequence. Direct comparison of the clones with or without these single changes revealed no differences in the efficiency of amplification and detection (data not shown).

Application of the assay to four chronically infected horses during 10 weeks of evaluation revealed levels that varied from 10^{3.3} to 10^{6.0} parasites per ml of blood. The variation was derived from repeated fluctuations within individual horses, which occurred in all chronically infected horses examined (Fig. 2).

Tick infection percentage in *B. microplus* fed on *B. equi*-infected horses. *B. equi* levels in the peripheral blood of infected horses were determined by real-time PCR during the 8 days of *B. microplus* nymphal acquisition feeding. The levels of *B. equi* within chronically infected horses H059, H072, and H076 ranged from 10^{4.4} to 10^{6.1} (mean parasite level of 10^{5.5} ± 10^{0.48}/ml of blood) at the time of tick feeding (Table 1). In contrast, horse H047 in the acute phase of infection had a mean level of 10^{8.8} parasites per ml, with a range of 10^{4.1} to 10^{9.4}, during tick acquisition feeding (Table 1). The presence of *B. equi* in the tick salivary glands, which is requisite for transmission, was determined following transmission feeding using a duplex nested PCR (Fig. 3a). *B. microplus* *α-tubulin* was amplified from all samples to ensure that *B. equi* *ema-1*-negative salivary glands had amplifiable DNA. The identity of the *B. equi* *ema-1* amplicons was confirmed by using Southern hybridization with a digoxigenin-labeled 226-bp *ema-1* probe generated from *pema-1* (Fig. 3b). *B. microplus* ticks acquisition fed as nymphs on chronically infected horses demonstrated a wide range, 7 to 50%, in the percentage of ticks with positive salivary glands (Table 1). The percentage of infected salivary glands in *B. microplus* ticks acquisition fed as nymphs on a horse during the ascending phase of parasitemia was 22% (Table 1).

Transmission of *B. equi* to naive horses. *B. microplus* adults that were acquisition fed as nymphs on horses in the acute (H047) or chronic (H059, H072, and H076) phase of infection successfully transmitted *B. equi* to naive recipients. Transmission was verified by microscopic detection of *B. equi*-parasitized erythrocytes in Giemsa-stained blood smears, seroconver-

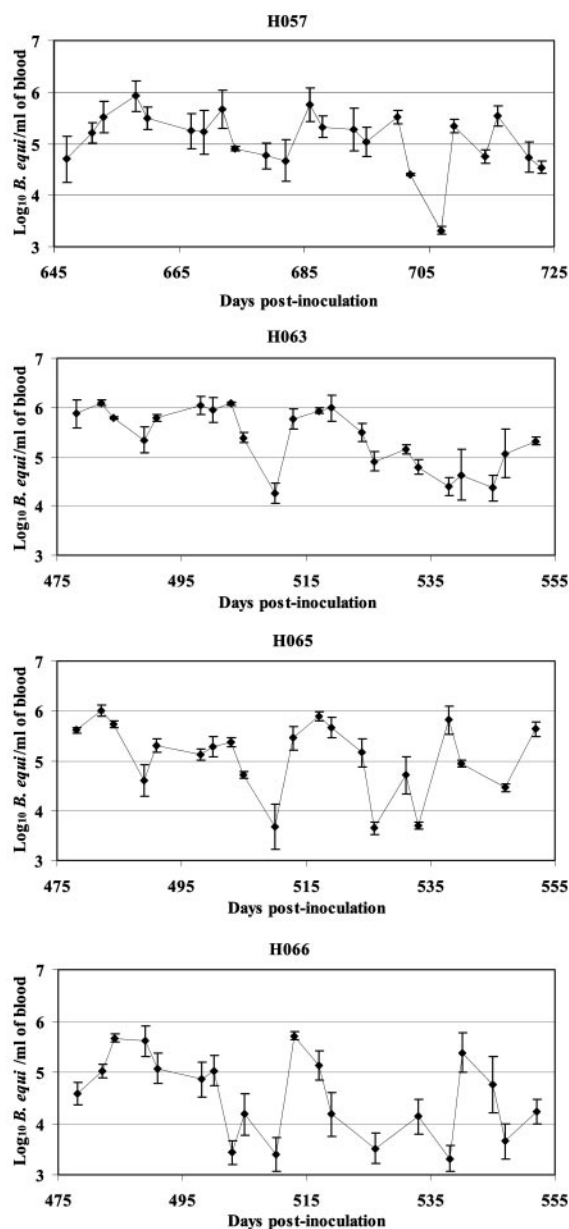


FIG. 2. Determination of *B. equi* levels over time in four chronically infected horses.

sion to competitive inhibition enzyme-linked immunosorbent assay-positive status, and *ema-1* PCR. As ticks acquisition fed on a single horse could be allocated for transmission feeding on more than a single naive horse, the total number of successful transmissions was six (two of two using ticks acquisition fed during the acute phase of parasitemia, four of four using ticks acquisition fed during the chronic phase of infection). Adult *B. microplus* ticks that had been acquisition fed as nymphs on chronically infected horse H059 transmitted *B. equi* to naive horses H072 and H076. After resolution of the acute phase of parasitemia and establishment of chronic infection, H072 and H076 were utilized for nymphal acquisition feeding, and adult ticks acquisition fed as nymphs on these two horses transmitted the parasite to H073 and H068, respectively. As a

TABLE 1. Infection percentages within *B. microplus* adult ticks fed as nymphs on horses in the acute and chronic phases of infection

Infection phase	Horse	<i>B. equi</i> levels (log ₁₀ /ml of blood) in ticks acquisition fed as nymphs	Infection % of ticks transmission fed as adults ^a
Chronic	H076	4.4–5.8	50 (47/94)
Chronic	H059	5.2–5.9	11 (14/127)
Chronic	H072	4.4–6.1	7.1 (7/98)
Acute	H047	4.1–9.4	22 (17/78)

^a The number of positive ticks and the total number examined are in parentheses.

positive control, adult *B. microplus* acquisition fed as nymphs on horse H047 during the ascending phase of parasitemia transmitted *B. equi* to H045 and H064.

DISCUSSION

Transmission studies using *B. microplus* adults acquisition fed as nymphs on horses with long-term infections and defined low levels of *B. equi* parasitemia establish that chronically infected horses, as well as acutely parasitemic horses, serve as mammalian reservoirs for tick transmission of *B. equi*. In contrast to the relatively short duration of the acute phase of parasitemia (22), the lifelong nature of *B. equi* infection in horses provides a continual source for pathogen acquisition by ticks, required for each tick generation as *B. equi* cannot be maintained solely in the tick. Despite a difference of $>10^3$ *B. equi* parasites per ml between peak levels during nymphal feeding in the acute ($10^{9.4}$ /ml) versus the chronic ($10^{5.8}$ to $10^{6.0}$ /ml) phase of infection, there was no difference in the percentage of adult ticks that developed *B. equi* within the salivary gland. This is consistent with other studies of tick transmission of blood-borne pathogens in which, once a threshold level is reached, a further increase in the level of pathogens per milliliter has little or no effect on the percentage of ticks that acquire infection and are able to subsequently transmit the infection (5, 19, 25). Our data indicate that the threshold level for nymphal *B. microplus* to acquire *B. equi* and have development progress to salivary gland colonization following molting to the adult stage is $\leq 10^{5.8}$ *B. equi* parasites per ml. Examination of parasitemia levels over time in chronically

infected horses indicates that at 16% of the time points, levels were $\geq 10^{5.8}$ /ml (Fig. 2). The duration of the chronic phase of infection and the frequency with which horses maintain parasitemia levels at or above the maximal threshold to infect ticks are consistent with chronically infected horses being the most important reservoir for *B. equi* transmission.

The levels of parasitemia fluctuated over time in each of the chronically infected horses, ranging from a low of $10^{3.3}$ to a high of $10^{6.0}$ parasites per ml. The coefficients of variability derived from 20 time points per chronically infected horse for the real-time PCR were 11.1% (H059), 11.6% (H063), 14.4% (H065), and 17.6% (H066). In contrast, the coefficient of variability for the detection of the internal control of $10^{5.8}$ parasites was 1.2%. The differences in the coefficient of variability between the time point assays of blood obtained from the chronically infected horses and the repeated assay of the internal control supports actual fluctuation in parasitemia levels, as opposed to assay-to-assay variation. This variation is also consistent with prior microscopic examination of Giemsa-stained blood smears from chronically infected horses, in which parasitized erythrocytes are observed at infrequent intervals, very likely corresponding to the peaks identified by quantitative PCR (Fig. 2). Fluctuation in the levels of parasitized erythrocytes during the chronic phase of infection is common with pathogens in the genera *Babesia* and *Plasmodium* (1, 3). In both *Babesia bovis* and *Plasmodium falciparum*, this fluctuation reflects intertwined processes of antigenic variation and cytoadhesion-sequestration (2, 10, 18). Having demonstrated significant fluctuation in the *B. equi* levels during the chronic phase of infection, the testing of specific hypotheses regarding the basis of this variation is a logical next step.

The number of infected ticks required to transmit *B. equi* is unknown. Although it was not a primary goal of this study to address this variable, examination of the number of fed ticks with *B. equi* in the salivary gland at the time of transmission provides some new information that may guide future studies, both in the field and in experimental transmission. The minimal number of ticks with detectable *B. equi* in the salivary gland at the time of successful transmission feeding was 4; 4/64 nymphs fed on H059 and transmission fed on H072 had positive salivary glands. Similar numbers of positive ticks were associated with positive transmission to H073 (7/98 positive ticks that had been acquisition fed on H072), H045 (8/31 positive ticks that had been acquisition fed on H047), H064 (9/47 positive ticks that had been acquisition fed on H047), and H072 (10/63 positive ticks that had been acquisition fed on H059). Thus, it appears that only a few positive ticks are required for transmission under the conditions described here. However, there remain many unknowns that may influence the association between PCR-positive ticks and transmission, including variables in the number, viability, and infectivity of sporozoites within the salivary gland, pathogen and tick strain differences, and duration of tick feeding in the field, that require additional investigation.

Currently, the international movement of equids is restricted in many countries, including the United States, to animals shown to be serologically negative for *B. equi*, with the goal of preventing the entry of chronically infected horses. The demonstration here that chronically infected horses commonly maintain parasitemia levels at or above the maximal threshold

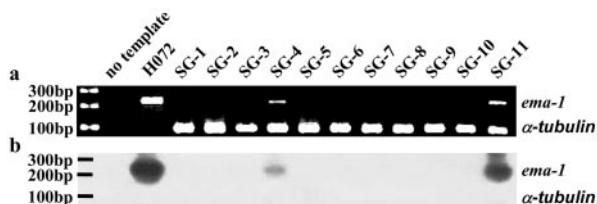


FIG. 3. (a) Detection of *B. equi*-positive salivary glands from individual *B. microplus* adults fed as nymphs on a chronically infected horse. (b) Confirmation of amplicon identity of positive *B. equi* salivary glands from a duplex nested PCR with a digoxigenin-labeled *ema-1* probe. SG1 to SG11, DNA amplified from salivary glands of individual ticks; H072, DNA amplified from *B. equi* Florida isolate-infected erythrocytes as a positive control. The predicted amplicon sizes for *B. equi ema-1* and *B. microplus alpha-tubulin* are indicated in the right margin. Molecular size markers are in the left margin.

for ticks to acquire *B. equi* and that small numbers of infected ticks are capable of transmission supports this restriction on movement into areas where competent tick vectors are present or may become established.

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