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Borreliacidal activity of saliva of the tick *Amblyomma americanum*

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Abstract. *Amblyomma americanum* (Linneaus) (Acari: Ixodidae), an important tick vector of human and animal disease, is not a competent vector of the bacterial agent of Lyme disease, *Borrelia burgdorferi*, although its range overlaps the geographical distribution of Lyme disease within the United States. A possible mechanism that could prevent acquisition of *B. burgdorferi* spirochetes from infected hosts is the toxic effect of *A. americanum* saliva on *B. burgdorferi*. The data presented here indicate that after 24 and 48 h of exposure to *A. americanum* saliva, significantly fewer *B. burgdorferi* were alive compared to treatment controls as assessed by spirochete motility under dark-field microscopy and resistance to the dead stain, propidium iodide. After 48 h, fewer than 13% of saliva-exposed *B. burgdorferi* were alive. In contrast, significantly more *B. burgdorferi* exposed to *Ixodes scapularis* (Acari: Ixodidae) saliva survived after 24 or 48 h compared to *A. americanum* saliva or treatment controls.

Key words. *Amblyomma americanum*, *Ixodes scapularis*, borreliacidal effect, salivary glands, salivation, tick saliva.

Introduction

Amblyomma americanum (Linnaeus), the lone star tick, is the primary vector of *Ehrlichia chaffeensis*, the bacterial agent of human monocytic ehrlichiosis (Anderson *et al.*, 1993). This aggressive human-biting tick species is also thought to vector other potential pathogens including *Ehrlichia ewingii*, *Rickettsia rickettsii*, *R. amblyommii*, *Borrelia lonestari*, *Francisella tularensis* and *Coxiella burnetii* (Calhoun, 1954; Burgdorfer, 1975; Barbour *et al.*, 1996; Murphy *et al.*, 1998; Burkot *et al.*, 2001; Childs & Paddock, 2003). *Amblyomma americanum* ticks feed for 4–14 days (Sauer & Hair, 1972) on a wide variety of mammalian, reptilian and avian hosts (Cooley & Kohls, 1944; Clymer *et al.*, 1970). Some reservoir hosts are also commonly infested by *Ixodes scapularis* Say, the tick vector of *Borrelia burgdorferi*, the agent of Lyme disease in the United States (Magnarelli *et al.*, 1986; Oliver *et al.*, 1999; Clark *et al.*, 2001). *Amblyomma americanum* is found throughout geographical regions that overlap areas of human Lyme disease occurrence, perennially in southern latitudes and seasonally in northern latitudes (Bishopp & Trembley, 1945; Anderson & Magnarelli, 1980; Hair & Bowman, 1986; Ginsberg *et al.*, 1991; Luckhart *et al.*, 1991; Keirans & Lacombe, 1998). Despite the confluence in geographical distribution and host species, there is no evidence that *A. americanum* transmits *B. burgdorferi* (Piesman & Happ, 1997).

During *A. americanum*, *I. scapularis* and other Ixodid tick feeding, saliva is continuously secreted into the host (McMullen & Sauer, 1978; Coons *et al.*, 1986; Sonenshine, 1991). Ixodid saliva is known to have a broad range of haemostatic and immunomodulatory effects in mammalian hosts (Ribeiro, 1995; Wikel, 1999). Anecdotal reports indicate significant inflammation and irritation at the site of

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A. americanum feeding on humans (Armstrong *et al.*, 2001), and evidence for a haemolytic saliva factor (Zhu *et al.*, 1997) suggests that *A. americanum* saliva could have a broadly cytotoxic effect.

Arthropod vector refractoriness to a pathogen can be classified into three categories: (1) a lack of acquisition; (2) an inability to maintain growth of the pathogen; or (3) an inability to transmit the pathogen. Spirochetes have been detected in 0.2–15.6% of field-collected *A. americanum* (Magnarelli *et al.*, 1986; Schulze *et al.*, 1986; Levine *et al.*, 1991; Luckhart *et al.*, 1991; Teltow *et al.*, 1991; Luckhart *et al.*, 1992; Feir *et al.*, 1994; Rawlings & Teltow, 1994; Sonenshine *et al.*, 1995; Ouellette *et al.*, 1997; Stromdahl *et al.*, 2001). However, many of the reports of spirochete-positive *A. americanum* predate the discovery of the spirochete *B. lonestari*, or use methods that would not be able to discriminate between the two spirochete species, *B. burgdorferi* and *B. lonestari* (Barbour *et al.*, 1996). Although as many as 25% of experimentally exposed *A. americanum* larvae acquired spirochetes during infectious feedings, nearly all of these larvae were spirochete-negative before moulting to the nymphal stage (Piesman & Sinsky, 1988; Mather & Mather, 1990; Ryder *et al.*, 1992; Piesman & Happ, 1997). There is only one report of trans-stadial maintenance of infection in *A. americanum* nymphs (Ryder *et al.*, 1992) and one report of spirochete isolates from three pools of *A. americanum* adults (Teltow *et al.*, 1991). Moreover, there has been no successful transmission of *B. burgdorferi* between infected and naïve hosts by *A. americanum* (Mukolwe *et al.*, 1992; Ryder *et al.*, 1992; Oliver *et al.*, 1993; Sanders & Oliver, 1995; Piesman & Happ, 1997). It was hypothesized that a cytotoxic effect of *A. americanum* saliva might be one mechanism preventing the acquisition of *B. burgdorferi* spirochetes from an infected reservoir host. In the current study, the survival of *B. burgdorferi* exposed to *A. americanum* saliva was measured. To document that this is a species-specific effect in a refractory vector, the survival of *B. burgdorferi* exposed to saliva of *I. scapularis* was also measured.

Materials and methods

Tick infestation and saliva collection

Colony-produced, pathogen-free *A. americanum* adults were purchased from the Oklahoma State University Tick Rearing Facility (Stillwater, Oklahoma). Colony-maintained *I. scapularis* adults originated from the Lyme Disease Vector Laboratory of the CDC (Fort Collins, Colorado). Adult female New Zealand rabbits (Western Oregon Rabbit Co., Philomath, Oregon) were infested with 20–30 pairs of *A. americanum* or *I. scapularis* adults, as described previously (Piesman *et al.*, 1991). During infestation, ticks were confined within fabric bags completely enclosing each ear of the rabbit. Tick engorgement was monitored daily, and adult females were removed from rabbit ears at the beginning of the rapid engorgement phase, typically 11–13 days post-infestation for *A. americanum* or 5–7 days post-infestation for *I. scapularis*. All animal procedures were reviewed and approved by the CDC Institutional Animal Care and Use Committee (AUP no. 00-08-010 RAB).

Near-replete ticks were prepared for saliva collection as described previously (Ribeiro *et al.*, 2004). Briefly, each immobilized tick had its mouthparts fitted with a finely drawn capillary tube and 5 μL of 5% pilocarpine in methanol was applied topically to the dorsal scutum. Salivating ticks were maintained for 1–2 h in a humid chamber at 35°C; collected saliva was pooled on ice and stored at −70°C until use. One μL aliquots of saliva from each pool were tested by an HPLC-MS/MS method to determine a pg to mg range of pilocarpine concentrations (Ribeiro *et al.*, 2004) and this value was utilized to determine control standards for subsequent borreliacidal assays.

Spirochete culture

Frozen stocks of low-passage B31 *B. burgdorferi* isolates (Shelter Island, New York) were reconstituted in BSK-H culture medium and maintained at 35°C to log phase (Piesman, 1993). Spirochete cultures were then diluted with BSK-H to a density of 4.7×10^7 viable spirochetes per mL as counted in a Petroff–Hauser chamber under dark-field microscopy, and distributed in 15 μL aliquots into 0.7 mL tubes for a total of 7.05×10^5 spirochetes per tube. A mean starting concentration of 155 live spirochetes per field was assessed prior to the addition of specific treatments. Each 15 μL aliquot of spirochetes in BSK-H was then treated with either 15 μL of sterile PBS, 15 μL of 1.33 mg/mL pilocarpine (based on the mid-range of pilocarpine found in previous analysis of tick saliva, Ribeiro *et al.*, 2004) in sterile PBS, or 15 μL of tick saliva, and incubated at 35°C. A total of 10 cultures were prepared with each treatment (PBS, pilocarpine control, tick saliva) for each tick species; 10 μL samples taken from each culture were then assessed for spirochete survival at 0, 24 and 48 h. Time points for spirochete survival assessment were chosen based on previous observations of post-feeding decreases in spirochete-positive ticks (Ryder *et al.*, 1992).

Spirochete survival assessment

Spirochete survival was evaluated by enumeration of spirochetes stained with the live/dead *Bac*Light Viability Kit (Molecular Probes, Eugene, Oregon). Briefly, fluorescence stains SYTO 9 (live stain) and propidium iodide (dead stain) were combined at a 1 : 100 dilution in sterile PBS (Invitrogen, Grand Island, New York). A total of 0.5 μL of this solution was then mixed with 10 μL from each spirochete culture. This staining concentration was chosen in preliminary experiments for optimal discrimination of spirochetes using fluorescent microscopy. Stained cultures were suspended on a standard glass slide under a 22×22 mm coverslip and viewed at 100× magnification

through FITC and rhodamine filters. Live and dead spirochetes were counted from 10 randomly chosen high-power fields per slide.

Statistics

Mean counts of live spirochetes made at 24 and 48 h were compared using an over-dispersed Poisson generalized linear mixed model (Wolfinger & O'Connell, 1993). Fixed effects for time and treatment (*A. americanum* saliva, *I. scapularis* saliva, pilocarpine, and PBS) and their interaction were included, as were the random effects of separate trials and trial by time interaction, to accommodate potentially differential effects with each trial. Models were compared using the likelihood ratio chi-squared test to evaluate both fixed effects and covariance parameters for statistical significance, with $P < 0.05$ considered statistically significant. Final comparisons of treatment effects were made using the parsimonious model thus derived and computations were performed using the glimmix macro in SAS v9.1 (SAS Institute, Cary, North Carolina).

Results and discussion

To evaluate the effect of tick saliva from *I. scapularis* and *A. americanum* on *B. burgdorferi*, a fluorescence method was used to quantify spirochete viability. With this method, live spirochetes, staining green (Figs 1A and B), could be readily visualized and distinguished from injured and dead spirochetes, which stained red (Figs 1C and D). The uptake of live and dead stains observed after saliva or control treatments of spirochete cultures was consistent with the preliminary dark-field microscopy observations of treatment-induced differences in spirochete appearance and motility. It was not possible to quantify the total number of dead spirochetes at 24 and 48 h consistently due to disintegration of cells in more severe treatments (data not shown); therefore, only live spirochete numbers were used for analysis. This analysis (Fig. 2) indicates that treatment with *A. americanum* saliva significantly reduced the average number of live spirochetes at both 24 h (mean = 39.6) and 48 h (mean = 20.1) compared with both pilocarpine at 24 and 48 h (82.1/58.2) and PBS at 24 and 48 h (133/112) (all $P < 0.001$). Furthermore, average counts for *A. americanum* saliva were also significantly lower than average counts for *I. scapularis* treatment at 24 and 48 h (100.2/75.0, $P < 0.001$ for both time points). In contrast, *I. scapularis* did not yield significantly different spirochete counts than pilocarpine at both time points, while demonstrating a significant killing effect compared to PBS at 24 and 48 h ($P < 0.001$ for both). Finally, differences in the borreliacidal activity of *A. americanum* and *I. scapularis* saliva could not be accounted for by differences in pH after saliva preparation; both tick salivas were basic, with pH measuring between 9.5 and 10.0 (data not shown).

Numerous vector-competency studies have demonstrated that *A. americanum* is unable to maintain or transmit *B. burgdorferi*. Although *A. americanum* can acquire

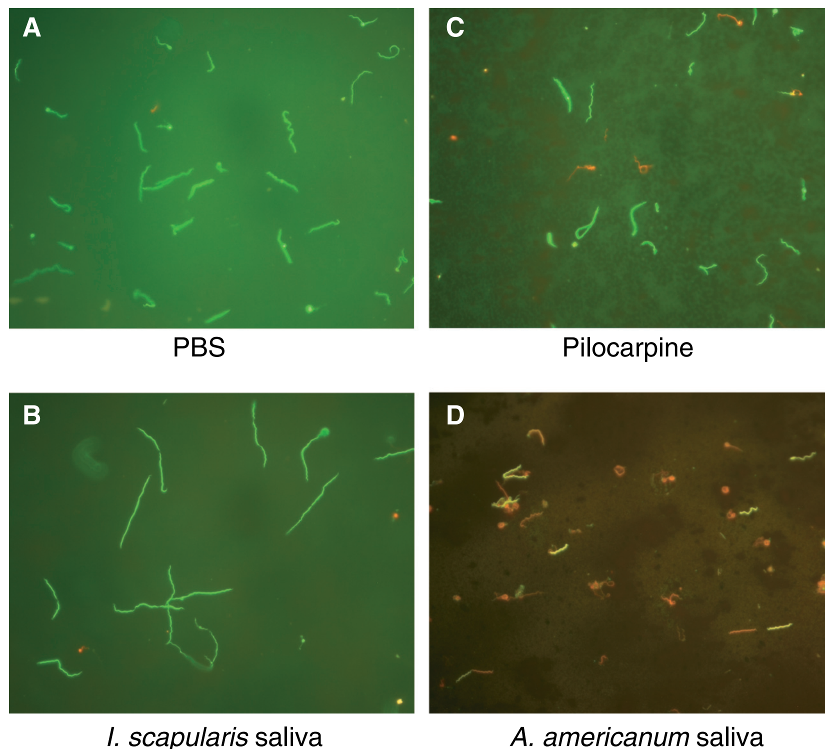

Fig. 1. Fluorescent microscopy indicating live (SYTO9, green) and dead (propidium iodide, red) staining as a measure of *B. burgdorferi* survival 48 h after exposure to specific *in vitro* treatments (100×) as described in the Materials and Methods. (A) PBS, (B) *I. scapularis* saliva, (C) Pilocarpine, (D) *A. americanum* saliva.

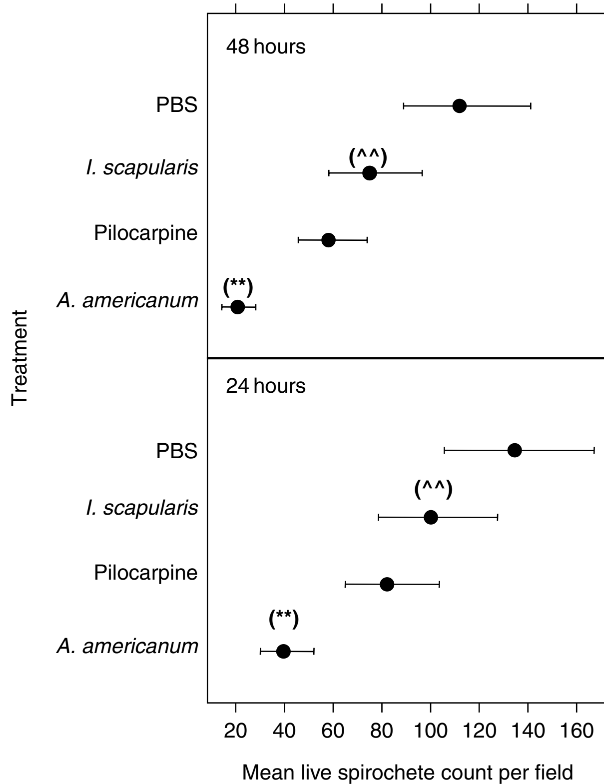

Fig. 2. (A) Mean live *B. burgdorferi* counts per 10 high powered (100×) fields and estimated 95% confidence intervals after exposure to *A. americanum* saliva, pilocarpine, *I. scapularis* saliva, or PBS at 24 and 48 h post-exposure. Means were computed using the model as described in the Materials and Methods. Error bars indicate the computed confidence intervals. ** and ^^ indicate a significant difference in viable spirochete numbers when comparing *A. americanum* saliva to pilocarpine controls and to *I. scapularis* saliva ($P < 0.001$, for both, Chi-squared test). Note the confidence intervals are asymmetric due to the logarithmic nature of the Poisson model used.

B. burgdorferi by experimental feeding, the number of spirochetes detectable in *A. americanum* rapidly decreases post-feeding, as 4.6-fold fewer *A. americanum* than *I. scapularis* larvae are spirochete-positive 48 h after taking an infective bloodmeal (Ryder *et al.*, 1992). The number of *A. americanum* larvae with detectable spirochetes decreases to zero within 10 days post-feeding, and remains so for the duration of the larval stage (Piesman & Sinsky, 1988). The results of the studies presented here suggest that the significant borreliacidal activity of *A. americanum* saliva may be a possible mechanism for the lack of acquisition of *B. burgdorferi* and transmission *in vivo*. The cytotoxic effect of *A. americanum* saliva in reducing spirochete numbers (Fig. 2) could readily reduce the dose of spirochetes acquired during feeding on a highly spirochetemic host. Likewise, saliva injected into the host by feeding *A. americanum* ticks (McMullen & Sauer, 1978) might reduce spirochete numbers at the cutaneous bite site. Moreover, saliva re-ingested with host fluids could gradually cause spirochete mortality as the bloodmeal is processed within the tick midgut (Coons *et al.*, 1986). Why *A. americanum* saliva would have a cytotoxic effect on the spirochete *B. burgdorferi* and not a *Borrelia* spp. associated with *A. americanum*, *B. lonestari* (Barbour *et al.*, 1996) needs to be investigated further. With the advent of new techniques to culture *B. lonestari* (Varela *et al.*, 2004) these comparative studies can now be attempted.

The effect of *A. americanum* saliva on *B. burgdorferi* spirochetes is in significant contrast to the effect of *I. scapularis* saliva (Figs 1B and 2). *Ixodes scapularis* saliva did not have a borreliacidal effect greater than the pilocarpine control treatment, and appears not to be an obstacle in maintaining *I. scapularis* vector competency. In addition to the anticoagulation and immunomodulatory activities of *I. scapularis* saliva in the mammalian host (Ribeiro *et al.*, 1985), studies have suggested the presence of a factor in *I. scapularis* saliva that is permissive for enhancement of *B. burgdorferi* dissemination *in vivo* (Zeidner *et al.*, 2002). It has been hypothesized that cofeeding of *Dermacentor variabilis* with *I. scapularis* can increase *D. variabilis* infection rates (Piesman & Happ, 1997). Finally, viable *B. burgdorferi* have been collected directly from pilocarpine-stimulated collections of *I. scapularis* saliva (Ribeiro *et al.*, 1987; Ewing *et al.*, 1994).

The current study has demonstrated a cytotoxic effect of pilocarpine on *B. burgdorferi*. Pilocarpine is known to have a physiological effect on mammalian cells in quantities as low as 500 μm (Arzt *et al.*, 1989), and has been detected in saliva collected from *A. americanum* and *I. scapularis* (Ribeiro *et al.*, 2004). Therefore, as noted by Ribeiro *et al.* (2004), the concentration of pilocarpine should be determined in every sample of saliva destined for *in vitro* testing on living cells, and compensated for with appropriate controls or by the addition of an atropine inhibitor (Arzt *et al.*, 1989). In this study, *A. americanum* saliva had a significantly greater borreliacidal effect on spirochetes than exposure to both background levels of pilocarpine or *I. scapularis* saliva (Fig. 2). The difference in borreliacidal activity might be the result of a specific molecule or compound present in the saliva of *A. americanum* but not *I. scapularis* ticks. Proteins are abundant in the saliva of these two tick vectors (Madden *et al.*, 2002; Valenzuela *et al.*, 2002). A direct comparison of saliva from these vector species by SDS-PAGE gel revealed at least two protein differences in the 200–90 kDaA range and four distinct differences within the 34–20 kDaA range (data not shown). Isolation and identification of a borreliacidal factor from *A. americanum* saliva could lead to the development of antispirochete compounds for prophylactic treatment of *B. burgdorferi* exposure, or to the molecular engineering of transgenic, *B. burgdorferi*-refractory *I. scapularis* ticks to study mechanisms of vector competency.

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