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Minimal Duration of Tick Attachment Sufficient for Transmission of Infectious *Rickettsia rickettsii* (Rickettsiales: Rickettsiaceae) by Its Primary Vector *Dermacentor variabilis* (Acari: Ixodidae): Duration of Rickettsial Reactivation in the Vector Revisited

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Abstract

It has been reported that starving ticks do not transmit spotted fever group *Rickettsia* immediately upon attachment because pathogenic bacteria exist in a dormant, uninfectious state and require time for 'reactivation' before transmission to a susceptible host. To clarify the length of reactivation period, we exposed guinea pigs to bites of *Rickettsia rickettsii*-infected *Dermacentor variabilis* (Say) and allowed ticks to remain attached for predetermined time periods from 0 to 48 h. Following removal of attached ticks, salivary glands were immediately tested by PCR, while guinea pigs were observed for 10–12 d post-exposure. Guinea pigs in a control group were subcutaneously inoculated with salivary glands from unfed *D. variabilis* from the same cohort. In a parallel experiment, skin at the location of tick bite was also excised at the time of tick removal to ascertain dissemination of pathogen from the inoculation site. Animals in every exposure group developed clinical and pathological signs of infection. The severity of rickettsial infection in animals increased with the length of tick attachment, but even attachments for less than 8 h resulted in clinically identifiable infection in some guinea pigs. Guinea pigs inoculated with salivary glands from unfed ticks also became severely ill. Results of our study indicate that *R. rickettsii* residing in salivary glands of unfed questing ticks does not necessarily require a period of reactivation to precede the salivary transmission and ticks can transmit infectious *Rickettsia* virtually as soon as they attach to the host.

Keywords

Rickettsia rickettsii; transmission dynamics; reactivation; grace period

Approximately 40 species of ticks (Ixodida) have been recorded to parasitize humans in the United States (Merten and Durden 2000) and at least 11 transmit viral, bacterial, or protozoan agents pathogenic to humans (https://www.cdc.gov/ticks/diseases/). People are exposed to ticks and tick-borne pathogens during occupational and recreational activities, but also within the premises of their residence (Demma et al. 2005). Avoidance of tick

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habitats, use of acaricides and tick repellents, and prompt removal of attached ticks are recommended prophylactic measures that reduce the risk of tick-borne infections. Prompt removal of ticks is essential in minimizing the duration of attachment, which can lower the chances for successful pathogen transmission by infected ticks (Piesman et al. 1987, Breuner et al. 2017).

Most tick-borne pathogens are not merely contaminating tick mouthparts ready for immediate mechanical transmission, but reside within the vector's internal tissues and are inoculated with tick saliva. The time between insertion of tick mouthparts into the skin and the delivery of an infectious agent via the salivary route is often called the 'grace period' because removal of an attached tick prior to the actual delivery of a pathogen protects the host from being infected (Katavolos et al. 1998).

The length of grace periods diverges widely between different pathogens, largely depending on their physical location (survival niche) within the tick-vector and whether they need to undergo physiological, phenotypical, or life-stage changes prior to becoming infectious for susceptible vertebrate hosts. For example, multiple studies have demonstrated that the agent of Lyme disease, *Borrelia burgdorferi* s.s., is rarely transmitted by infected *Ixodes scapularis* within the first 48 h of attachment (Piesman et al. 1987, 1991), whereas closely related *Borrelia afzelii* can be transmitted after approximately 12–24 h (Crippa et al. 2002). This grace period is required for the spirochetes to change expression of outer surface proteins and to migrate from the midgut to the salivary glands (Schwan et al. 1995; de Silva et al. 1996; Hefty et al. 2001, 2002). Likewise, *Babesia microti*—a protozoan parasite with a complex intrinsic life cycle—was not visualized in salivary glands of infected *I. scapularis* until after 48 h of attachment (Piesman et al. 1986). These fairly long feeding periods required for pathogen development and translocation within the vector provide a window of opportunity—grace period—for detection and removal of attached ticks before tick-to-host transmission can happen, thus precluding human infection with respective agents.

In contrast, pathogens that reside in the salivary glands of ticks prior to attachment, if present in a virulent state, can be delivered into the host skin more quickly, resulting in much shorter grace periods. For example, *Borrelia turicatae* colonizes the salivary glands of its vector *Ornithodoros turicata* prior to feeding and is ready for inoculation into the mammalian host within seconds of tick attachment (Boyle et al. 2014). Likewise, tick-borne flaviviruses are present in large quantities within salivary glands of unfed ticks and can be injected with the very first portion of tick saliva—the cement (Chunikhin et al. 1988, Alekseev et al. 1996). Ebel and Kramer (2004) reported that essentially no grace period exists between tick attachment and Powassan virus transmission as ticks efficiently transmitted virus to naive mice after as few as 15 min of attachment (Ebel and Kramer 2004).

Similarly to the tick-borne viruses, spotted fever group (SFG) *Rickettsia* spp. develop generalized infection within the body of their vectors including the salivary glands (Burgdorfer and Brinton 1975, Hayes and Burgdorfer 1979, McDade and Newhouse 1986, Santos et al. 2002, Milhano et al. 2014). Yet, it has been generally accepted that flat/starving ticks do not transmit SFG rickettsiae immediately upon attachment because pathogenic

bacteria supposedly exist in a dormant or noninfectious state and require time for reactivation before they can be transmitted to a susceptible host. In the1920s, Spencer and Parker reported that inoculation of guinea pigs with a suspension prepared of flat *Dermacentor andersoni* collected from vegetation regularly resulted in development of immunity against *R. rickettsii* but not clinical infection, whereas inoculation of recently engorged (partially or fully) ticks from the same collection sites invariably resulted in clinical infection and death (Spencer and Parker 1923, Spencer 1929, Parker et al. 1933).

Consequently, Spencer and Parker suggested that acquisition of blood by infected ticks for at least 48 h is necessary to 'reactivate' the pathogen inside the tick and transfer it from the noninfectious to the infective state. It should be pointed out that in those original studies transmission was defined as demonstration of lethal illness in laboratory animals and methods for differentiation between isolates or even species of SFG rickettsiae were limited. By now, it is well established that the severity of rickettsial infection varies widely among different isolates of the same pathogen species as well as between species, strains, and age groups of animal models (Price 1954, McDade and Newhouse 1986, Levin et al. 2014). Unfortunately, since those first reports almost 100 years ago, there has been no confirmation of the delay in rickettsial transmission by the primary North American vectors of *R. rickettsii*—*Dermacentor* spp.—in a natural setting, or reassessment of such delay using modern molecular methods.

The primary goal of this study was to assess the minimal duration of attachment by a *R. rickettsii*-infected *Dermacentor variabilis* necessary for inoculation of viable infectious pathogen using clinical, pathological, and molecular data. We also evaluated the dynamics of rickettsial load in tick salivary glands and dissemination of the pathogen from inoculation site during the first 48 h of tick attachment. The infectious dose (ID_{50}) of *R. rickettsii* sufficient for causing clinical illness in a host differs greatly between rickettsial strains and species of vertebrate hosts (Ormsbee et al. 1978). It is also unknown how much *R. rickettsii* is being injected by a tick at any time during feeding. Therefore, our study focused on the evidence of rickettsial replication and dissemination in the model host rather than on measuring the amount of rickettsial cells injected by individual ticks. In order to elucidate the minimal duration of attachment and, consequently, the length of reactivation period, we exposed guinea pigs to bites of individual *R. rickettsii*-infected *D. variabilis* and allowed ticks to remain attached for predetermined periods of time from 0 to 48 h.

Methods

The study was undertaken at a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All procedures and husbandry were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, eighth edition. All procedures of this study were pre-approved by the Centers for Disease Control Institutional Animal Care and Use Committee (IACUC).

Ticks

Infected female *D. variabilis* used in this study were derived from a *R. rickettsii*-infected colony (3-d generation) maintained at the CDC Medical Entomology Laboratory by feeding on infected guinea pigs (*Cavia porcellus*) and specific pathogen-free (SPF) New Zealand white rabbits (*Oryctolagus cuniculus*) as previously described (Levin and Schumacher 2016, Schumacher et al. 2016). An isolate of *R. rickettsii* (BSF-Di6) used in this study was originally acquired from an opossum (*Didelphys virginiana*) caught in Virginia. This isolate is pathogenic in domestic dogs and guinea pigs (Levin et al. 2014, 2016). Between feedings, ticks are held in environmental incubators at 22°C, 90–98% relative humidity, and 16-h day/8-h night light cycle.

The uninfected male ticks were derived from a SPF colony of *D. variabilis* maintained separately by feeding exclusively on naive New Zealand white rabbits. Absence of other *Rickettsia* spp. in both colonies has been assured in every generation by PCR-testing samples of larvae, nymphs, and adults. Both colonies were also confirmed free of known tick-borne *Anaplasma*, *Borrelia*, or *Ehrlichia* species.

Model Animals

Pathogen-free and tick-naive male Hartley guinea pigs 8- to 12-wk-old were used as model animals. For tick infestation, each animal was fitted with a plastic capsule fashioned from the barrel of a 6 ml syringe. A 15 mm length of the syringe barrel with the flange was cut and glued to the shaven skin of a guinea pig's dorsum. The rubber seal was removed from the syringe plunger and used to plug the open end of the capsule preventing tick escape.

Study Design

In order to stimulate prompt attachment of infected females, a single uninfected male tick was placed in a feeding capsule attached to each guinea pig. Three days later, a single female *D. variabilis* from the *R. rickettsii*-infected colony was added into each capsule. A preliminary evaluation of this method revealed that when individual unfed female ticks were placed into capsules with individual feeding males, approximately 40% (5 of 12) of females attached to guinea pigs within 30 min after placement and approximately 90% (11 of 12) were attached within 60 min after placement, whereas the remaining female remained unattached for the following 12 h. Hence, it was determined that the majority of unfed female ticks completed their initial attachment within 60 min after placement into a feeding capsule and this time point was designated as the duration of attachment ≤0.5 h.

Ticks were removed from subsets of guinea pigs at designated attachment intervals ≤0.5, 4, 8, 12, 16, 20, 24, 32, 40, and 48 h. Immediately upon removal from guinea pigs, female ticks were dissected and salivary glands of each individual tick were removed to be tested by PCR to assess the presence and quantity of *R. rickettsii* DNA. For comparison, the quantity of rickettsiae in hemolymph of the same individual ticks was measured by testing two amputated legs per tick. Guinea pigs, on which female ticks did not attach or tested negative for rickettsial DNA, were removed from the study. The remaining animals were kept under clinical observation as described below.

To assess differences in rickettsial loads between unfed and feeding ticks, 18 flat *D. variabilis* females from the infected colony were dissected on the day of tick placement on guinea pigs. As above, salivary glands and two legs from each individual tick were tested by PCR.

Two parallel experiments were conducted following the same timetable. In the first, only the ticks were removed from guinea pigs and the skin at the site of tick attachment was left intact allowing the pathogen inoculated by ticks to proliferate and disseminate naturally. In the second experiment, the immediate site of the female tick attachment was excised simultaneously with the tick removal using sterile disposable 2-mm biopsy punch (Robbins Instruments, Chatham, NJ). At the same time, three additional 2-mm skin biopsies were taken at distances 10, 20, and 30 mm from the bite-site. This allowed an assessment of the time frame after the initial attachment when *R. rickettsii* is capable of spreading from the site of inoculation.

An additional group of nine guinea pigs was inoculated subcutaneously with salivary glands of unfed *R. rickettsii*-infected *D. variabilis.* To prepare an inoculum for each individual guinea pig, salivary glands dissected from two individual female ticks were re-suspended in 200 µl of sterile phosphate-buffered saline and macerated by needle homogenization. Hundred microliters of the resulting suspension was inoculated intradermally into the shaven skin between guinea pig's shoulder blades using a 27–29 gauge needle.

All guinea pigs were monitored daily for clinical signs of infection including fever (defined as body temperature ≥39.7°C), scrotal edema, and dermatitis in ears and foot pads (Walker et al. 1977). Ear-skin biopsies were collected from experimental guinea pigs every 2–3 d using sterile 2-mm-diameter ear punches (Kent Scientific Corporation, Torrington, CT). Animals were euthanized at 10–12 d post-infestation via CO_2 inhalation. During necropsy, typical pathological signs of rickettsial infection were recorded including discolored necrotic lesions of the liver, prominent congestion and erythema of the testes, lung petechiation, and splenomegaly. Splenomegaly is defined as the weight of spleen exceeding 0.15% of the total body weight at the time of necropsy (Garcia-Carrillo 1977). Tissue samples of skin, liver, spleen, bladder, testis (with epididymis), lung, and heart were collected at the time of euthanasia. Samples were stored at −20°C until tested by PCR for the presence of rickettsial DNA.

PCR Assays

DNA extraction and PCR procedures were carried out in separate facilities to prevent contamination. DNA was extracted from tick and tissue samples using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA) according to manufacturer's protocols with a final elution volume of 100 µl.

A two-tiered PCR testing was employed to verify that no *Rickettsia* spp. other than *R. rickettsii* was present in any of the samples. All tick and animal tissue samples were initially tested using a broad range *pan-Rickettsia* assay targeting the 23S rRNA gene. Samples positive in the *pan-Rickettsia* screening were then tested using a *R. rickettsii*-specific assay amplifying the gene encoding hypothetical protein A1G_04230 (Kato et al. 2013). Both

assays have a limit of detection of eight to nine genome copies per reaction with 95% reproducibility (Kato et al. 2013). The threshold CT discrepancies between these two assays were minimal (less than three cycles) for all PCR-positive samples, confirming that *R. rickettsii* was the only species present in the experimental samples.

All PCR reactions were run in duplicate. If only one of the duplicate aliquots tested positive, the sample was retested with an additional duplicate run for confirmation. Two negative (distilled water) and two positive (*R. rickettsii* genomic DNA) samples were included in each run.

The amount of rickettsial DNA in each sample was determined by quantitative PCR assays described below, which included standard serial dilutions (10^1–10^6) of an *R. rickettsii* plasmid (Blue Heron Biotechnology LLC, Bothell, WA). Each PCR reaction used 5 µl (5%) of the DNA sample as a template, and the number of rickettsiae was calculated per total sample. The sensitivity threshold for detection of rickettsiae in this study was expected to be 160–180 DNA copies per sample based on the reported limit of detection of eight to nine genome copies per PCR reaction with 5% of the total sample being used for each reaction.

In order to negate potential variations between individual ticks in the size of dissected salivary glands or the amount of hemolymph in legs, quantities of rickettsial DNA in tick samples were normalized to the amount of tick DNA. Tick DNA in each sample was quantified using primers DvarlTS2F and D. variITS2R and a FAM probe, amplifying a 155 bp region of the *ITS2* rRNA gene of *D. variabilis*, as described by Shone et al. (2006). Both the rickettsial *A1G_04230* gene and the tick *ITS2* are single-copy DNA regions, thus allowing quantification of rickettsiae as *A1G_04230* copy numbers per *ITS2* copy numbers.

Statistical Analysis

To compare quantities of *R. rickettsii* in samples of tick hemolymph and salivary glands collected at different time points, a ratio of rickettsial A*1G_04230* gene copy number to the tick *ITS2* copy number was calculated for each sample. Pathogen titers in tick samples were expressed as the number of rickettsial genomes per 10,000 copies of tick DNA.

GraphPad statistical software PRISM v.8 for Windows (GraphPad Software, San Diego, CA) was used to analyze quantitative data. Two-tailed analysis of variance (ANOVA) and Mann–Whitney tests were used to assess differences in rickettsial titers, relative spleen size, and body temperature following tick attachment. Pearson regression coefficient was calculated to examine potential correlation between titers of rickettsiae in salivary glands and hemolymph. For all assessments, a P value ≤0.05 was considered statistically significant.

Results

Quantification of *R. rickettsii* in Ticks

Among both unfed and feeding ticks, *R. rickettsii* was detected in the salivary glands of every tick with a PCR-positive hemolymph sample and vice versa. The total copy numbers of rickettsial DNA in samples from individual ticks varied from approximately 100 to almost 10,000,000 per pair of tick legs, or to tens of millions per pair of salivary glands in both

unfed and feeding ticks. This variability could be influenced by the size of dissected salivary glands or by the volume of the hemolymph in a pair of cut legs, both of which are known to fluctuate in a feeding tick.

Titers of rickettsial DNA in individual ticks varied from approximately 0.004 to 300–500 per 10,000 tick cells widely overlapping between different time points in both the hemolymph and salivary grands (Fig. 1A). Interestingly, titers of rickettsia in salivary glands and in the hemolymph were largely unrelated (Pearson $r = 0.3248$; 95% CI 0.1709–0.4633; $P < 0.0001$)—ticks with high titers of rickettsiae in the hemolymph at any time point could have much or little pathogen in the salivary glands and vice versa (Fig. 1B). Although titers of rickettsiae in salivary glands of individual ticks fluctuated widely at any time point, the trend of the mean demonstrates that the load of *R. rickettsii* in salivary gland of feeding ticks did not increase continuously through the first 48 h of feeding, but rather oscillated up and down at approximately 12- to 20-h intervals (Fig. 1C).

Rickettsial Infection in Model Animals

Following exposure to individual infected ticks attached for predetermined periods of time, some of the guinea pigs developed a range of clinical signs including fever, scrotal edema, as well as vasculitis or desquamating dermatitis of ears and footpads (Fig. 2A). Combinations of recognizable clinical signs of rickettsial infection, including fever and scrotal edema, were observed in the 93% (25 of 27) guinea pigs where infected ticks remained attached for longer than 8 h. On the other hand, vasculitis in ears and footpads was evident even in animals exposed to ticks for only short periods of time. Four out of six guinea pigs developed fever when ticks were allowed to feed for 8 h (Fig. 2A). The height of fever generally corresponded with the duration of tick attachment. While animals exposed for 8 h developed only mild fever, tick attachment of 12 h or longer resulted in high fever (above 40.5°C) in the majority of exposed animals (Fig. 3A).

Surprisingly, guinea pigs needle-inoculated with salivary glands of unfed ticks also became severely ill, developing the full range of classical clinical sigs including high fever, scrotal edema, and severe vasculitis of ears and desquamating dermatitis on footpads. Indeed, fever in these animals was as high as in those where ticks remained attached for more than 16 h (Figs. 2A and 3A).

Internal pathology in individual guinea pigs varied even wider than clinical signs. In this case, variability is increased by the fact that necropsy observations present a snapshot of dynamic processes at the moment of euthanasia. However, the pathological examination conducted at 10–12 d post-exposure regularly identified changes in multiple organs including liver, spleen, testes, urinary bladder, and lungs of guinea pigs from all exposure groups, including those where ticks were attached for 4 h or less. The overall score of illness severity calculated as the total number of individual clinical and pathological manifestations generally increased with the duration of attachment (Fig. 2A). While guinea pigs exposed to infected ticks for 8 h or less became only mildly ill (illness severity score 1–3), longer durations of tick feeding resulted in severe illness (illness severity score 4–7) in 17 of 27 animals. The higher severity of illness also coincided with the frequency of detection of generalized rickettsial infection by PCR in skin biopsies and tissue samples. Again, both

prominent pathological changes and the overall scores of illness severity observed in guinea pigs inoculated with salivary glands of unfed ticks were comparable with those exposed to longest periods of tick attachment (Fig. 2A).

Dissemination of *R. rickettsii* From the Site of Tick Bite

ln a parallel experiment, we not only removed the attached ticks but also excised the site of tick attachment by taking a 2-mm skin biopsy. This allowed us to assess the timeframe when the tick-inoculated rickettsiae are capable of disseminating from the site of inoculation. In guinea pigs where both the infected ticks and the bite-site were removed almost immediately (within 30 min) after attachment, animals remained healthy throughout the study period displaying neither fever, scrotal edema, nor vasculitis. However, removal of the bite-site at 4 h or later after tick attachment did not seem to alleviate any of the clinical signs of rickettsial infection with the majority of animals becoming clinically ill (Fig. 2B). The height of fever in guinea pigs where removal of the bite-site was delayed by 4 h or longer was comparable to animals with the same length of tick attachment where the bite-site was not excised at all (Fig. 3B).

Similarly, extraction of the bite-site was effective in averting development of pathological signs of rickettsial infection only if done within the first half hour of tick attachment. In guinea pigs where the bite-site was excised 4 h after tick attachment or later, pathological observations included liver necrosis, erythematous testes, petechiae and congestion in lungs, and/or splenomegaly (Fig. 2B). Disseminated infection was also confirmed by detection of rickettsial DNA in ear-skin samples or internal organs in majority of guinea pigs if the bite-site remained in place for 4 h or longer after tick attachment (Fig. 2B).

When excising the bite-site, we simultaneously took serial biopsies at 10, 20, and 30 mm linearly away from the bite—in total four biopsies per animal. Altogether, 180 skin biopsies were collected from 45 guinea pigs at and from the defined distances from the attachment site of an infected tick. Of those 180, *R. rickettsii* DNA was identified in 40 samples representing 27 individual guinea pigs. No significant differences were detected in the quantity of rickettsia in skin samples collected at the point of tick attachment and at 10–30 mm away from that point (Fig. 4). Out of the 45 bite-site biopsies, rickettsial DNA was detected in 18 samples (40%) even though every tick was infected with *R. rickettsii* and most animals later developed generalized infection. Where detected, rickettsial copy number within the bite-site displayed gradual upward trend from 0 to 48 h of tick feeding ($Y = 211.9*X - 144.4$; $R^2 = 0.3381$; P = 0.0114). *Rickettsia rickettsii* DNA was also detected in measurable quantities at distances of 10–30 mm away from the point of tick bite starting as early as 4 h after attachment (Fig. 4). The expected gradual concentric spread of the pathogen through the skin could not be observed in this study as *R. rickettsii* was found in biopsies taken at all distant points (10, 20, and 30 mm) as early as 4 h of tick feeding (Fig. 4).

Discussion

Prevention and case prognosis of tick-borne infections requires knowledge about dynamics of pathogen transmission from infected vector to host, including the minimal length of

attachment necessary for delivery of an infectious agent. Tick-to-host transmission of a pathogen depends, among other factors, on the amount of agent carried by a tick, its location within the tick before feeding, the prerequisite period of intrinsic replication and/or physiological changes, and, of course, the length of tick attachment. In general, the longer a tick remains attached, the higher the likelihood that an ID of a pathogen is transmitted (https://www.cdc.gov/lyme/removal/index.html). In many cases, pathogen transmission and the consequent host infection can be prevented if a biting tick is removed within the grace period before it has time to inject the infectious agent.

Almost 100 years ago, Spencer and Parker (Spencer and Parker 1923) noticed that when they inoculated guinea pigs with homogenates of flat ticks that were refrigerated for several months after being collected from vegetation, animals would develop only mild or asymptomatic infection. Conversely, if ticks were allowed to feed for 48 h or incubated at 37°C for 72 h prior to inoculation into naive guinea pigs, 25–50% of animals succumbed to severe illness (Spencer and Parker 1923, 1930). Consequently, it was concluded that a period of 24–48 h is required for ‘reactivation’— transformation of bacteria within the vector from a noninfectious (dormant) to the infectious state.

Since then, Hayes and Burgdorfer (1979) described reversible structural modifications linked to physiological changes in a feeding tick, which in their view correlated with the restoration of pathogenicity and virulence. Galletti et al. (2013, 2016) found that the transcriptional profile of *Rickettsia* changes in response to acquisition of bloodmeal by its vector or even to a rise in ambient temperature. In particular, transcription of virulence genes is upregulated in rickettsiae residing within tick salivary glands, apparently preparing the bacteria for invasion of a vertebrate host. These studies showed how *Rickettsia* prepares for the drastic change of its environment—for a switch from survival mode in an invertebrate vector with limited immune capabilities to active attack mode in a vertebrate host, where it has to evade and overwhelm a sophisticated combination of cellular and humoral immune responses.

What these studies have not addressed is whether these structural and physiological changes absolutely must precede transmission of *Rickettsia* to the host, or if the pathogen can adapt to the new environment during or even after its injection into the host’s skin. Therefore, the main goal of our study was to assess the length of the grace period for *R. rickettsii*—the time between tick attachment and delivery of infectious pathogen to a fully susceptible host. Keeping in mind that susceptibility to rickettsial infection and the ID_{50} in guinea pigs can be different from those in humans, we necessarily focused on the evidence of rickettsial viability and infectivity in the model animal at the time of transmission and not on the amount of the pathogen injected by ticks.

Because people and animals are normally bitten by a single infected tick at a time, we exposed model animals in our study to individual infected ticks for approximation of natural processes. To capture transmission events where quantities of inoculated pathogen may be insufficient for causing clinically recognizable illness in model animals, we supplemented clinical and pathological observations with testing ear-skin biopsies and internal organs by PCR.

In the current study, most of individual *R. rickettsii*-infected ticks transmitted the pathogen to naive guinea pigs as was proven by detection of rickettsial DNA in animal tissues as well as observation of clinical and pathological signs of infection. Signs of rickettsial infection in guinea pigs are notoriously variable between individual animals. A guinea pig that develops high fever or scrotal edema after a tick bite may or may not display liver damage or splenomegaly at the time of necropsy and vice versa. Yet, effective rickettsial transmission was demonstrated by clinical, pathological, and/or micro-biological observations in all groups of animals including some of those where ticks were allowed to remain attached for only up to 30 min. Remarkably, guinea pigs inoculated with salivary glands of unfed ticks were among the most severely ill animals.

The total number of clinical and pathological manifestations in individual guinea pigs, as well as the frequency of detection of generalized rickettsial infection by PCR in skin biopsies and tissue samples, generally increased with the duration of tick bite, although even brief tick attachments resulted in milder infection in some of the guinea pigs. This suggests that the longer ticks were attached the more bacteria they were able to pump into the host. Our observations correlate with a study by Saraiva and colleagues showing that guinea pigs exposed to *R. rickettsii*-infected *Amblyomma aureolatum* for less than 10 h did not become clinically ill, whereas guinea pigs exposed for longer periods of time developed clinical signs of infection including fever and scrotal edema (Saraiva et al. 2014). Unfortunately, authors of that study relied solely on clinical data and did not assess the timing of rickettsial transmission with molecular methods. Both our observations and those by Saraiva et al. are in agreement with a 90-yr-old observation by R.R. Spencer of correlation between the duration of tick attachment and the odds of a severe clinical infection in humans (Spencer 1929).

We detected *R. rickettsii* in salivary glands of every infected *D. variabilis* female tested prior to acquisition of the blood meal. Hence, there is a potential for bacteria being injected into the host skin with the very first portions of tick saliva. In the process of attaching themselves to the host ticks secrete a so-called cement cone, which secures tick's mouthparts in the skin and prevents the parasite from being easily dislodged. The primary secretion of cement has been reported within 5–30 min after penetration of the host's skin (Gregson 1960, Kemp et al. 1982, Alekseev et al. 1995). Microscopic observations of attachment and feeding processes in *D. andersoni* revealed that female ticks secrete the cement within 10 min after penetrating the host epidermis (Gregson 1960).

Yet, the presence of bacteria in the salivary glands of a vector by itself does not necessarily mean that it can immediately be infectious to vertebrate hosts. In a meticulous study by Piesman (1995), mice inoculated with salivary glands of B*. burgdorferi*-infected *I. scapularis* did not become infected unless ticks had fed for at least 60 h prior to dissection, even though live spirochetes were present in salivary glands of approximately 19% of ticks before the feeding (Piesman 1995). Likewise, some of the European strains of B*. burgdorferi* s.l. have been detected in tick salivary glands prior to the blood meal, but tick feeding for 12–24 h was apparently necessary before mice became infected (Sertour et al. 2018). However, in the current study, guinea pigs inoculated with salivary glands dissected from unfed ticks became infected and severely ill indicating that *R. rickettsii* residing in the salivary glands of hungry

D. variabilis ticks can undergo the necessary structural and molecular alterations during or even after being inoculated into the host. At least in the case of Dl-6 isolate of *R. rickettsii* transmitted by laboratory-reared *D. variabilis*, reactivation processes do not have to precede the event of salivary transmission. Observation of clinical signs and detection of rickettsial DNA in guinea pigs exposed to tick bites for less than an hour confirms that viable and infectious *R. rickettsii* can be inoculated by infected ticks with the very first portions of tick saliva.

The amount of the pathogen injected by ticks into the host skin could not be determined. Out of the 180 skin biopsies collected from 45 guinea pigs at and around the attachment site of an infected tick, presence of *R. rickettsii* was identified in 40 samples. The copy number of rickettsial DNA in the positive samples ranged from 122 to 122,700 with 21 (52.5%) of the positive samples containing <1,000 copies. Among 45 skin biopsies collected at the tick bite-sites, rickettsial DNA was detected in only 18 samples (40%) even though most ($n = 35$) animals later developed generalized infection confirmed by PCR-positive skin and tissue samples. Considering that the threshold of rickettsial detection was expected to be 160–180 DNA copies per sample, it is likely that some of the skin samples did contain rickettsial DNA, but in the amounts below the limits of detection.

It is apparent that *R. rickettsii* injected into the host skin by a tick does not remain confined to the site of inoculation for long. Crippa and colleagues have shown that if the tick and tissue surrounding the tick bite-site are removed before the pathogen disseminates in the skin, infection with *B. burgdorferi* and *B. afzelii* may be prevented (Crippa et al. 2002). In our study, extraction of the skin with bite-site itself prevented rickettsial infection in model animals only if performed immediately (within 30 min) after tick attachment. In guinea pigs where extraction of the bite-site was delayed for at least 4 h, neither clinical, pathological, nor molecular indications of rickettsial infection differed from those where only ticks were removed but not the skin at the bite-site. Moreover, rickettsial DNA was detected in the skin of guinea pigs at least as far as 30 mm from the point of tick bite within 4 h from attachment (Fig. 4). Taken together, these observations demonstrate that *R. rickettsii* injected by a tick immediately after attachment is fully capable of escaping the site of inoculation and begins to spread through the skin of a vertebrate host shortly—within 4 h—after inoculation. Whether this dissemination is due to active (actin-based motility) or passive (by infected leukocytes) mechanisms or both remains to be ascertained.

Titers of *R. rickettsii* in salivary glands of unfed *D. variabilis* ranged from 0.003 to 300 per 10,000 copies of tick ITS2 DNA sequence and were comparable to those in the hemolymph samples. Titers in both the salivary glands and the hemolymph of feeding ticks varied within a similar 100,000-fold range at all measured time points. Interestingly, amounts of rickettsiae in salivary glands of individual ticks did not correlate with those in corresponding hemolymph samples (Fig. 1B). A study in the distribution of *Rickettsia amblyommatis* in feeding *Amblyomma americanum* ticks also found no apparent correlation between relative densities of bacteria in different tissues, although samples from only three ticks per time point were analyzed (Zanetti et al. 2008). This indicates that the quantity of *R. rickettsii* in tick salivary glands at a given time point reflects rickettsial proliferation and depletion within the salivary tissue itself rather than simply influx of infected hemocytes. This

conclusion of *R. rickettsii* proliferation within salivary glands independently of the hemolymph corresponds with the increase in numbers of *Rickettsia conorii* within different cell types of salivary acini between flat and engorged *Rhipicephalus sanguineus* as observed with electron microscopy (Santos et al. 2002). Likewise, *Rickettsia slovaca* has been reported to multiply in all cell types of salivary acini of *Dermacentor marginatus* (Diehl et al. 1980). Notably, *R. conorii* had been observed not only inside various cell types in salivary glands of unfed *Rh. sanguineus* ticks but even extracellularly—free in the lumen of salivary ducts (Santos et al. 2002). Therefore, it has been hypothesized that rickettsiae are able to multiply in salivary gland cells and disseminate into the salivary ducts before the initiation of blood feeding, and that rickettsiae present in the salivary ducts lumen can be transmitted to the vertebrate host as soon as salivation begins, during the very early period of blood feeding (Santos et al. 2002).

Contrary to our expectation, we did not observe a continuous gradual accumulation of *R. rickettsii* within the salivary glands through the first 48 h of tick feeding (Fig. 1C). Instead, the rickettsial titers appeared to rise and fall. In ixodid females, salivary acini are known to undergo dramatic increase in the overall mass during tick feeding, but without any change in cell number (Binnington 1978, Fawcett et al. 1986). This means that the number of tick DNA copies within salivary glands should also remain constant throughout the feeding. If so, an increase in the titer of rickettsial DNA demonstrates proliferation of bacteria within live functioning salivary acini. Conversely, a decrease of that titer most likely is a result of *Rickettsia* being evacuated (ejected) from salivary glands. We hypothesize that up and down fluctuations of *R. rickettsii* quantities within salivary glands of feeding *D. variabilis* reflect the dynamic balance between processes of rickettsial proliferation and evacuation with saliva. Markedly, the first noteworthy evacuation of rickettsiae from the salivary glands appears to take place within 4 h after tick attachment (Fig. 1C).

This, of course, does not mean that every bite by a *R. rickettsii*-infected tick must instantly result in a disease as the initial dose of pathogen may not be sufficient to cause clinical illness. However, if a tick is allowed to continue feeding, the total amount of tick-injected bacteria increases and so does the probability of illness in the host. The average ID_{50} for humans exposed to *R. rickettsii* via intradermal inoculation is estimated at 23 organisms with the 95% CI of 1–89 (Tamrakar and Haas 2011). However, it is likely to vary between different isolates of the pathogen, just as the susceptibility to infection changes for people with different health background and immuno-logical status. Considering that four out of six guinea pigs became febrile after being exposed to tick feeding for 8 h, the ID_{50} for guinea pigs in our study was reached between 4 and 8 h after tick attachment. Whether this timeframe can be extrapolated to humans or to other isolates of *R. rickettsii* remains to be ascertained.

In conclusion, this study did not replicate results of the earlier observations where *R. rickettsii*-infected *D. andersoni* appeared incapable of causing infection in guinea pigs unless and until they fed for 36–48 h. Instead, the current study has demonstrated that the DI-6 isolate of *R. rickettsii* was present in salivary glands of unfed *D. variabilis* ticks in an apparently fully infectious state. Transmission of the pathogen to susceptible hosts took place without a delay as initial doses of the rickettsiae were injected into the host skin

almost immediately after tick attachment with the very first portions of tick saliva. Additional doses of rickettsiae continued to be injected by a feeding tick as the pathogen multiplied within the salivary glands and evacuated with saliva. This continuous inoculation of rickettsiae into the skin by feeding ticks caused the severity of illness in model animals to increase with the duration of tick attachment. Once inoculated, *R. rickettsii* began spreading through the skin fairly quickly and within 4 h could be detected at least as far as 30 mm from point of tick bite. Together, these observations demonstrate that at least this isolate of *R. rickettsii* circulating in the eastern United States does not require a long period of 'reactivation' within its natural vector *D. variabilis* prior to becoming fully infectious for vertebrate hosts.

It has been pointed out that contrary to the best studied North American isolates of B. *burgdorferi* s.s. requiring 48–72 h before transmission can occur, some of the European strains of B. *afzelii*, *Borrelia bavariensis*, and *Borrelia garinii* may be transmitted within 12–24 h of tick attachment. Similarly, results of individual observations in dynamics of tick-host transmission involving specific isolates of *Rickettsia* sp. and different tick species may not be generalizable for diverse isolates and vectors of *R. rickettsii* throughout the Western Hemisphere, let alone for other species of SFG rickettsiae. However, results of our study indicate that at least some *R. rickettsii* isolates are present in the infectious state within salivary glands of questing unfed ticks and can be transmitted to the host within less than an hour of a tick attachment. This stresses the paramount importance of preventing tick attachment in the first place and removing ticks as soon as possible in order to prevent rickettsial transmission.

Centers for Disease Control and Prevention recommends prompt removal of attached ticks as the best prevention strategy for prevention of tick-borne rickettsial diseases (Biggs et al. 2016). Our results clearly demonstrate that removing a tick as soon as possible is critical because longer periods of attachment considerably increase both the amount of pathogen inoculated by a feeding tick and the probability of clinical infection in the host. Therefore, timely tick-checks increase the likelihood of finding and removing ticks before they can transmit an infectious agent. Considering that *R. rickettsii* transmission can happen shortly after tick attachment with a very short or even nonexistent grace period, thorough tick-checks as frequently as hourly may be recommended for prevention of rickettsial infection.

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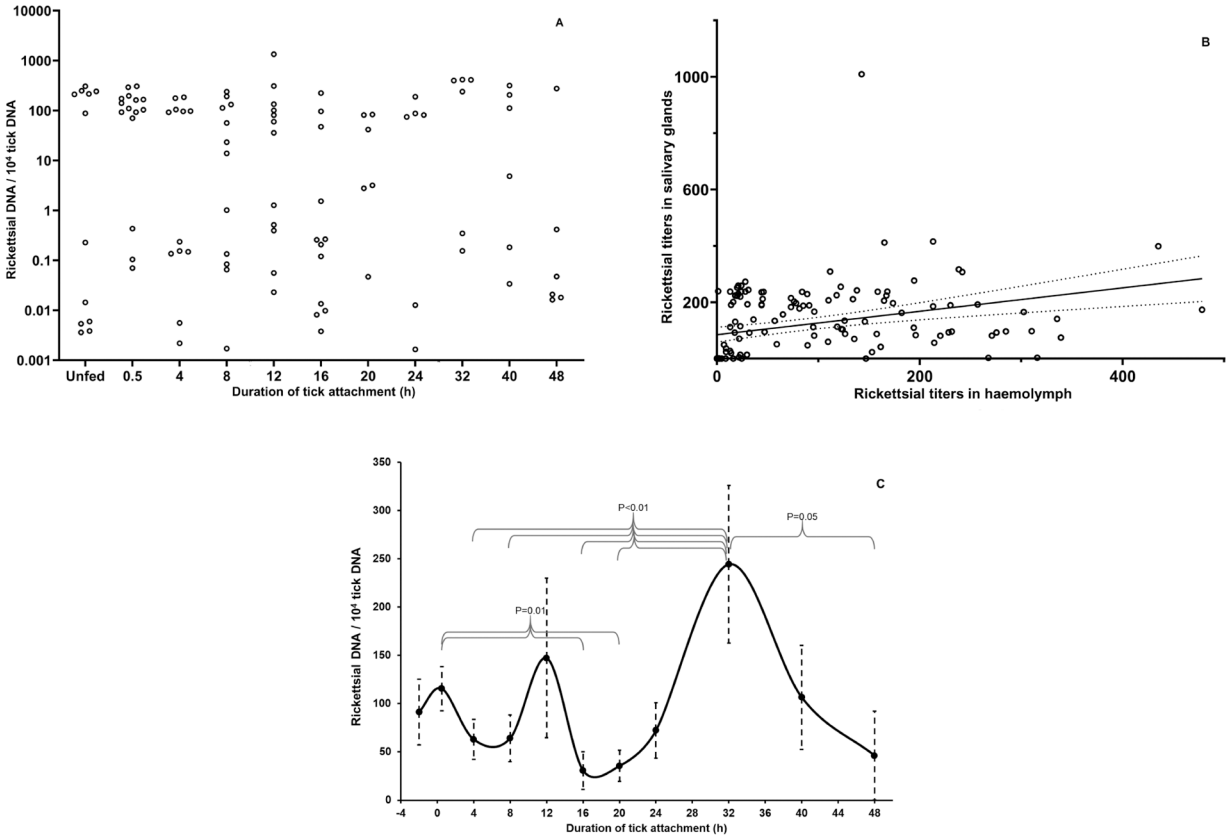

Fig. 1.

Titers of *R. rickettsii* (no. of rickettsial DNA copies per 10,000 copies of tick DNA) in salivary glands of unfed and feeding female *D. variabilis* ticks: (A) variability of rickettsial titers in salivary glands of individual ticks prior to feeding and during the initial 48 hours of tick attachment; (B) rickettsial titers in salivary glands of individual ticks in comparison to the corresponding hemolymph samples. Line – linear regression + 95% CI: *r*Pearson = 0.3248 (95% CI 0.1709–0.4633), $P < 0.0001$; (C) mean (±SE) titers of *R. rickettsii* in tick salivary glands; brackets identify time points with significantly different (*P*ANOVA) means.

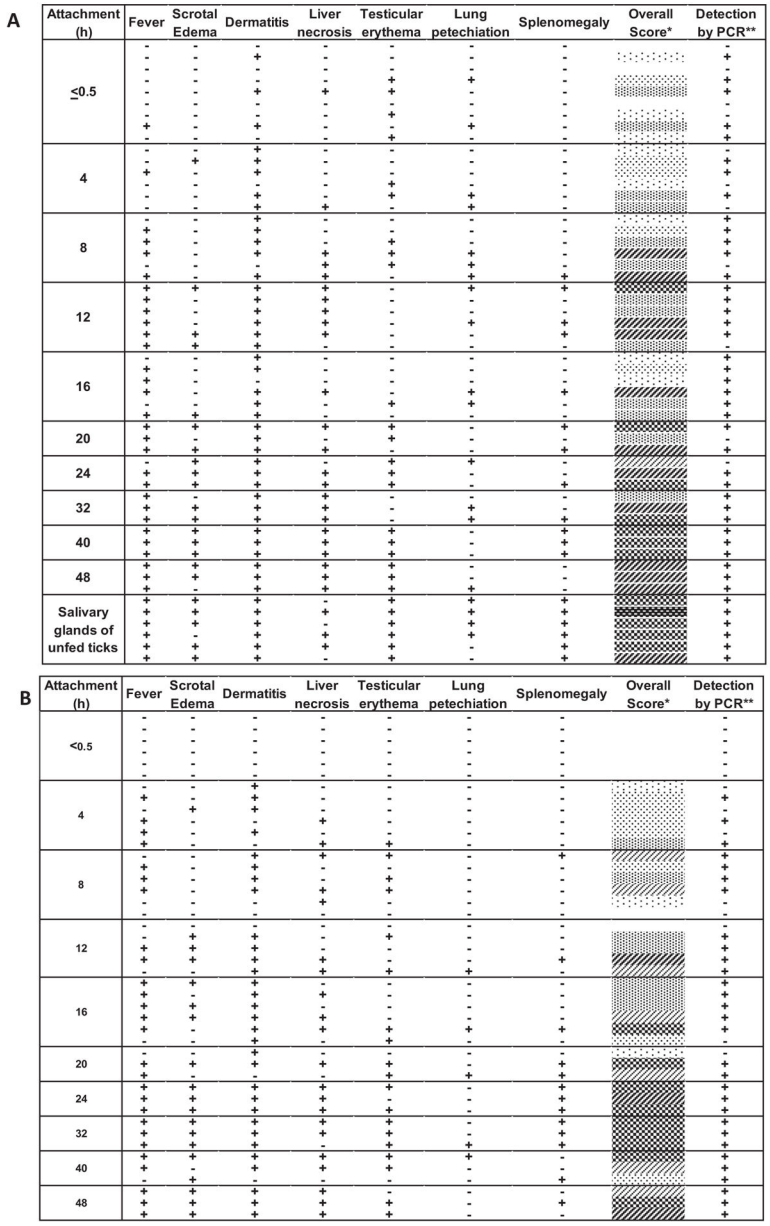

Fig. 2.

Summary of clinical and pathological signs of rickettsial infection in guinea pigs exposed to individual *R. rickettsii*-infected *D. variabilis* ticks for preset time intervals or needle-inoculated with salivary glands of unfed ticks. (A) Bite-sites (inoculation site) remained intact after tick removal; (B) bite-sites were extricated simultaneously with tick removal. *Overall score = the total number of observed clinical and pathological signs: 0–□; 1–▨; 2–▨; 3–▨; 4–▨; 5–▨; 6–▨; 7–■. **Detection of rickettsial DNA in ear-skin biopsies and/or internal tissues signifies dissemination of *R. rickettsii* from the site of tick bite or needle-inoculation.

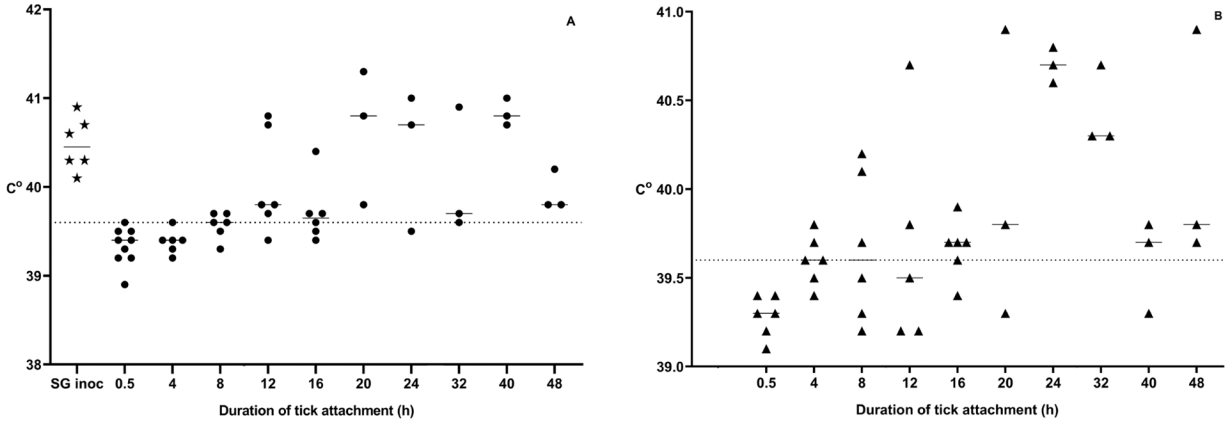

Fig. 3.

Peak temperature (— median) in guinea pigs following attachment of *R. rickettsii*-infected *D. variabilis* female ticks for preset periods of time, or subcutaneous inoculation (black stars) of salivary glands from unfed ticks: (A) bite-site intact (black circles); (B) bite-site is removed with the ticks (black triangle). Dotted line: fever threshold.

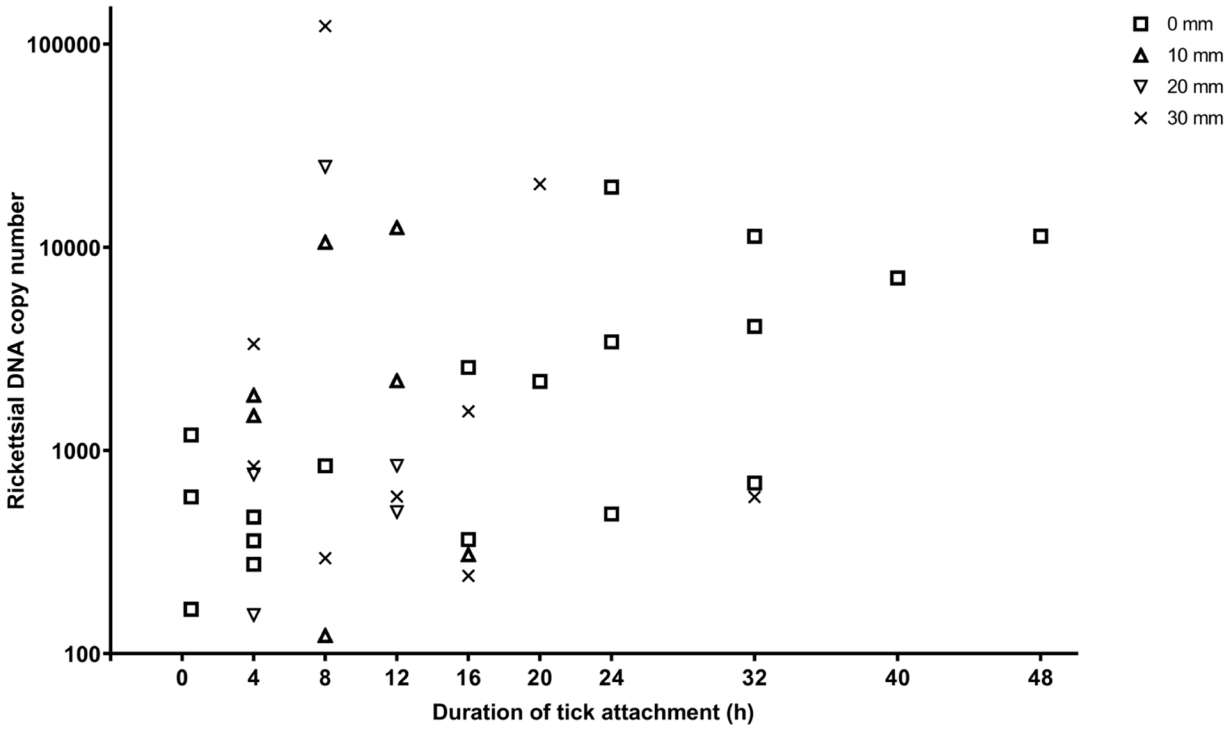

Fig. 4.

Rickettsia rickettsii DNA copy number of detected in individual 2-mm skin biopsies taken at the site of tick bite (0 mm) and at the distances of 10, 20, and 30 mm simultaneously with removal of feeding tick at predetermined time intervals after attachment.